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PATHOLOGY OF THE HEAD AND NECK: A RETROSPECTIVE APPRAISAL

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Published work submitted to the University of Pretoria for the degree of

Doctor of Science in Odontology (Oral Pathology)

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DECLARATION

I, the undersigned, declare that the work contained in this presentation of publications is my own original work, as set forth in the statements which precede the published articles, and has not previously in its entirety or in part been submitted at any University for a degree.

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WFP VAN HEERDEN

I certify that on the ..14.. day of ...July.... 2003, Willem Francois Petrus van Heerden signed this declaration in my presence.

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DEDICATION

To Marlene, Cherese and Bernice

INTRODUCTION

The author graduated as a dentist (BChD) from the University of Pretoria in 1981 where after he completed his two-year compulsory national military service as a dental officer with the rank of lieutenant. He was stationed at the Maphuta-L-Malatji hospital, a rural hospital in the vicinity of Phalaborwa in the Limpopo province. It was during this period that his interest in Oral Pathology was stimulated. He was exposed to a wide spectrum of tumours and conditions of the head and neck and was fortunate to assist in many surgical procedures. After an eighteen-month period in general private practice in London, UK, he was appointed as registrar in Oral Pathology at the University of Pretoria. This four-year specialization course had laid the foundation for his future academic career. His training in Anatomical Pathology was under the expert guidance of Profs Ian Simson and Leonora Dreyer who were responsible for installing a foundation and passion for histopathology. The author completed his Oral Pathology training under the auspices of Prof At Ligthelm, the then head of Department of Oral Pathology and Oral Biology. He was also fortunate to be exposed to the Oral Pathology training programme at the University of the Witwatersrand under the supervision of Prof Mario Altini. The author completed his MChD degree (*cum laude*) in 1988 and was appointed as specialist/senior lecturer in the Department of Oral Pathology at the Medical University of Southern Africa (Medunsa). He worked at Medunsa with Prof Erich Raubenheimer for 8 years where after he was appointed as head of the Department of Oral Pathology and Oral

Biology, at his Alma Mater, The University of Pretoria. He obtained his PhD degree in 1998.

Oral pathology is the speciality of dentistry and pathology that deals with the nature, identification, and management of diseases affecting the oral and maxillofacial regions. It is a science that investigates the causes, processes and effects of these diseases. The practice of oral pathology includes research, diagnosis of diseases using clinical, radiographic, microscopic, biochemical or other examinations, and management of patients. Oral pathology is one the six specialities officially recognised by the Health Professions Council of South Africa.

Diagnostic histopathology is based on morphological features of the tissues examined. These entail the categorization of cells in the tissue, their morphology as well as their growth pattern. Apart from the standard haematoxylin and eosin staining, the use of histochemical and immunohistochemical techniques are widely used and are still the cornerstones of the diagnostic histopathological process. The rapid advancement in science and technology, especially in the field of molecular techniques, has contributed significantly to our understanding of the disease processes. The use of non-isotopic *in situ* hybridisation (NISH) to detect the presence of possible infective agents, the availability of high resolution DNA flow cytometry for ploidy analyses as well as the numerous applications of the polymerase chain reaction (PCR) are all accessible

techniques that have been used. These developments are reflected in the research profile of the author.

Participation in multi-disciplinary research programmes involving national or international collaboration is an important evaluation criterion in the allocation of research grants. This and the complexity of advanced techniques are reflected in the well-established fact that most research papers nowadays have multiple authors. Joint authorship from one department is also commonplace when such a department has more than one research interest.

The author was fortunate to be associated with outstanding researchers throughout his career. The enthusiasm and guidance received from Prof Erich Raubenheimer have played a valuable role in developing and stimulating his research interest. Their close collaboration is reflected in the number of co-authored publications. Similar collaborations with world authorities in specific fields have also lead to the successful completion of multidisciplinary research projects. The roles of Prof Joerg Hemmer, head of the division of Tumour Biology at the University of Ulm, Germany, with regard to high-resolution flow cytometry and Prof Estrelita van Rensburg, head of the Department of Medical Virology, University of Stellenbosch, with regard to molecular and viral associated research, need to be mentioned.

The papers are grouped into three sections. The first section deals with several studies on squamous cell carcinoma of the head and neck. It includes studies on the clinical presentation of oral cancer, the role of viruses in the development of oral squamous cell carcinoma, the potential of DNA flow cytometry in the management of this disease and the role of certain tumour suppressor genes in the oral carcinogenesis process. Section 2 includes several studies on tooth composition as well as the presentation of selected tumours and cysts of the jaws. Section 3 consists of studies on saliva and salivary gland neoplasms.

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- Professor Erich Raubenheimer, a colleague and friend, who introduced me to all the aspects of research and with whom I had the privilege of working and co-authored numerous scientific papers.
- Professors Joerg Hemmer and Estrelita van Rensburg for their friendship and valuable contribution towards the development of my academic career.
- My promoter Professor At Ligthelm under whom I qualified as an Oral Pathologist, for his advice and guidance throughout my academic career as well as in the preparation of this presentation.
- All my colleagues throughout my academic career, especially the very competent laboratory staff that I worked with.

SUMMARY

This presentation consists of 52 selected publications that have appeared in national and international peer reviewed journals over the last 14 years (1989 – 2003). The presentation consists of the following headings: Studies on squamous cell carcinoma of the head and neck; dental hard tissues, tumours and cysts of the jaws and saliva and salivary gland neoplasms.

Squamous cell carcinoma of the head and neck

Twenty-three publications are presented in this section. The clinical and histological diagnostic criteria of oral cancer and premalignant lesions, especially aimed at the general practitioner, are discussed. Studies on the role of the Epstein-Barr and human papillomavirus in the pathogenesis of various squamous cell carcinomas of the head and neck region and different patient groups are included. The importance of DNA ploidy in oral carcinoma obtained with high-resolution flow cytometry is described. Valuable information can be obtained with this technique even when using archival paraffin-embedded tissues. Molecular studies, including gene sequencing and protein expression of the p53 suppressor gene as well as the *FHIT* gene, a candidate suppressor gene, were reported. This includes the evaluation of novel antibodies used for Fhit protein expression in oral squamous cell carcinomas.

Dental hard tissues and tumours and cysts of the jaws

In this section, 19 publications are presented. Two publications described the inorganic composition of dentine. The suitability of the sealing ability of a tri-cure glass ionomer material as a retrograde root filling material is illustrated. A unique case of permanent tooth germ injury is reported. A unusual case of amelogenesis imperfecta with multiple impactions associated with tumourous fibrous proliferations is described while a case of papillomavirus infection in an ameloblastoma is documented. The remaining publications include newly described entities as well as exceptional presentations of jaw tumours and cysts. The majority of these publications documented tumours and cysts in a Black African population and described the features seen in neglected and late presentation of these tumours.

Saliva and salivary gland neoplasms

Ten publications are presented in this section that include a study of immunoglobulin concentrations in saliva of multiple myeloma patients as well as the role of salivary glands in vertebrates. The remaining publications describe several aspects of salivary gland neoplasms. These include epidemiological data, diagnostic criteria, the role of DNA flow cytometry and unusual case presentations.

CONTENTS

LIST OF PUBLISHED SUBMISSIONS

SECTION 1: STUDIES ON SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

STUDIES ON SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Declaration

Twenty-three publications are submitted in this section. Study (1) was initiated by Ligthelm. I was responsible for case selections while all the co-authors participated in the preparation of the manuscript. I initiated studies (2 and 3) and prepared the manuscripts for publication. Study (4), a review on the role of Langerhans cells and Human papillomavirus infection in aerodigestive squamous cell carcinoma was initiated by van Rensburg. I was involved in the literature retrieval and review and participated in preparation of the manuscript. I initiated studies (5 and 6), was responsible for case selection and provided all the histological material. Raubenheimer and I were responsible for interpretation of the histological slides and participated in preparation of the manuscript. I initiated study (7) with equal participation of Postma in the design of the study. The data collection and statistical interpretation were performed by Postma. We equally contributed towards preparation of the manuscript. I initiated studies (8 and 9) with valuable assistance from van Rensburg and Engelbrecht in the study design. I was responsible for case selection and provided all the material while Raubenheimer and I were responsible for evaluation of the histological slides. I initiated study (10) and did the study design although interpretation of the data was mainly done by co-authors van der Hoven and Swart. Study (11) was initiated by van Rensburg as an extension of

study (10), using the same cases. I initiated studies (12 and 13) and was involved in case selection, ploidy interpretation and did the grading in collaboration with Raubenheimer. Studies (14, 15 and 16) were initiated by Hemmer. I was involved in preparation of the manuscripts. I initiated and designed study (17), did the ploidy analyses and histological interpretations and was responsible for the manuscript preparation. I initiated studies (18 and 19) with equal participation of van Rensburg in the study design. Engelbrecht and van Rensburg were responsible for the molecular analyses while I did the case selection, DNA extraction and immunohistochemical analysis. I took responsibility of the manuscript preparation for study (19). Study (20) was initiated by co-author van Heerden. I was responsible for the study design, histological evaluation of the special stains and participated in the manuscript preparation. I initiated studies (21, 22 and 23) with valuable participation by Huebner in the study design. I was responsible for case selections, immunohistochemical analyses and manuscript preparations.

Abstract

The early detection of premalignant and early intraoral squamous cell carcinoma lesions are important to reduce the mortality and morbidity of oral squamous cell carcinoma (OSCC). This is especially relevant in South Africa where OSCC is one of the most common malignancies. The purpose of study (1) was to describe the clinical and histological diagnostic criteria necessary for the early diagnosis of premalignant lesions and

OSCC. The role of stomatoscopy, toluidine blue staining and exfoliative cytology were discussed. It was however concluded that histological examination was still the method of choice to assess the nature of OSCC and its precursors. The purpose of study (2) was to give an overview on the epidemiology, aetiology and clinical presentation of OSCC and premalignant lesions and conditions in South Africa. This study was aimed towards the general medical practitioner. Study (3) focussed on the role of the general dental practitioner in the prevention and early diagnosis of OSCC. It discussed the identification of high-risk patients, recognition and diagnostic criteria of premalignant lesions and conditions as well as the management principles relevant to the general dental practitioner.

Human papillomavirus (HPV) infection is implicated in squamous cell carcinogenesis. The possible role of HPV in head and neck squamous cell carcinogenesis was the topic of several studies. Oesophageal carcinoma is the most common malignancy in Black males in South Africa. Study (4) was a review about the role of HPV and Langerhans cells (LCs) in the development and behaviour of oesophageal and laryngeal carcinomas. Oesophageal carcinoma shows a remarkable geographical distribution with a high incidence in South African Black males. Conflicting results on HPV infection suggest a role for other environmental factors *e.g.* fungal infestation of corn, alcohol and tobacco and/or vitamin and trace element deficiencies in the carcinogenesis process. A common factor for all high-risk areas is a low socio-economic status of the affected population. HPV DNA, especially HPV 16, has been detected in laryngeal carcinomas,

especially verrucous carcinomas. Patients with a marked infiltration of LCs in both oesophageal and laryngeal carcinoma have a better survival rate than those with a low density of LCs.

The presence of HPV DNA in OSCC was determined in study (5). *In situ* hybridisation with radiolabelled probes for HPV-6, 11, 16 and 18 along with immunohistochemistry for HPV common viral antigen was used on paraffin blocks from 66 cases of OSCC. It was the largest series of OSCC to be investigated for HPV DNA to that date. Radiolabelled, instead of biotinylated probes were used because of their reported superior sensitivity. HPV-18 DNA was detected by *in situ* hybridisation in only one case. This low prevalence was most likely due to the method not being sensitive enough to detect low viral copy numbers. Alternatively, the possibility that the transformed tumour cells contained altered viral DNA not detectable by the probes used or that other HPV types might have been involved, could also explain the low prevalence.

Study (6) was a follow-up of the previous study in order to utilise the sensitive polymerase chain reaction (PCR) technique to detect HPV DNA in OSCC. One hundred and forty six cases of OSCC were included in this study. It was the largest study of its kind to that date. A segment of the E6 region of HPV 6, 11, 16 and 18 was amplified using type specific primers. Southern blotting was used to confirm the PCR results. The case selection included OSCC in young patients as well as OSCC containing normal epithelium. Only two cases showed HPV DNA (HPV 11 and 16

respectively). The sensitivity of the technique was such that one copy of control plasmid DNA could be detected. The suitability of the specimens for DNA amplification was evaluated with human β-globin primers in all cases. It was concluded that the HPV types investigated were probably not important in the development of OSCC in the population sample studied. It however subsequently came to light that DNA extraction from formalin-fixed paraffin-embedded tissue for HPV DNA detection was in all likelihood responsible for the low positivity rate in the sample studied.

Cancer of the cervix is the most common malignancy of Black, Coloured and Asian women in South Africa. Infection with oncogenic HPV types is the most important risk factor in its aetiology. An epidemiological study (7) was undertaken to determine if a correlation could be found between the incidence of cervical SCC and OSCC in females and OSCC in males in South Africa. The raw data for the ten-year period 1986 to 1995 were obtained from the National Cancer Institute. The study demonstrated several strong correlations between the incidences of cervical SSC and OSCC in the Black and Coloured populations, supporting the concept of systemic susceptibility and infection through a common agent, such as HPV, to be involved in the carcinogenesis process.

Epstein-Barr virus (EBV) is a double stranded virus and causes widespread infection. It is closely associated with Burkitt's lymphoma, nasopharyngeal carcinoma and a link to other epithelial tumours has been suggested. Study (8) was undertaken to determine the presence of EBV

DNA in OSCC using the PCR technique with primers for the *Bam* HI W-fragment of the EBV genome. The presence of EBV DNA in the OSCC groups was not dependant on the presence of histologically normal adjacent epithelium and its presence in the OSCC groups was lower compared to the control group. Over 90% of the adult population worldwide is infected with EBV implying the presence of viral DNA. EBV was considered to be merely a passenger when neoplastic change occurs in a latently infected epithelial cell.

Study (9) was a follow-up of the previous study to determine the presence of EBV DNA in OSCC in patients from two age categories and to evaluate the possible role of EBV as an aetiological agent in carcinogenesis in young patients. EBV DNA was demonstrated in 24% of the OSCCs in both the group younger than 40 years and in the older patient group suggesting that the prevalence of EBV in OSCC was not influenced by the age of the patient.

No data was available on the prevalence and incidence of nasopharyngeal carcinoma (NPC) or its association with EBV in South Africa. The purpose of study (10) was to determine the prevalence of EBV in the different types of NPCs. *In situ* hybridisation using probes to detect expression of the smaller nuclear RNAs (EBER-1 and EBER-2) was used. Use of the more sensitive PCR technique was not applicable due to the close association of the tumour cells with surrounding lymphoid tissue. All the non-keratinising NPCs, both the differentiated and undifferentiated groups

yielded EBV signals in the tumour cells indicating an important role in its pathogenesis while none of the squamous cell NPCs showed any positivity. No positive signals were detected in the dysplastic epithelium found adjacent to positive primary tumours, suggesting a late role for EBV in the carcinogenesis process of NPCs.

Study (11) was a follow-up on the previous study to determine the subtype distribution of EBV DNA in South African NPCs. This was done by amplifying and sequencing the EBNA-2A and EBER regions of the EBV genome. The results demonstrated that the consensus genotype was A/B. Type A virus strains dominated with the EBNA-2 analysis while the EBER region showed a distinct combination of mutations belonging to type B, indicating that the EBV strains had arisen by recombination between viral types A and B. The impact of the HIV epidemic on EBV strain evolution and recombination should be investigated. This was the first study to type EBV strains from Southern Africa.

The search for the ultimate grading system for OSCC is an ongoing process. The different histopathological grading systems are based on phenotypical features of tumour cells and associated cell populations. Any biological data of tumour cells should ideally be incorporated into a grading system. Study (12) was performed to determine the inter-observer reproducibility of the invasive cell grading method on OSCC and to correlate this with the DNA ploidy status of the tumours. This grading method was reproducible but no correlation was found between the

grading results and ploidy status. The energy source of the flow cytometer used for ploidy analysis was an Argon ion laser and therefore propidium iodide had to be used to stain the nuclei. This did not allow for high resolution DNA flow cytometry and could have been responsible to possible "false diploid" classification of tumours.

Study (13) was an extension of the previous study to investigate a possible correlation between the DNA ploidy status, LC population in the tumour and adjacent epithelium and invasive cell grading method. No such a correlation could be demonstrated.

The advantages of high-resolution flow cytometry were demonstrated in the following studies (14-17). The flow cytometer used was equipped with a high-pressure mercury lamp allowing the use of more sensitive DNA staining with DAPI (4'6 diamidino 2 phenylindole) or ethidium bromide. This improved sensitivity was reflected by the low coefficient of variation (cv) of the measurements compared to those obtained from standard flow cytometers. This allows for the detection of tumour cells with small DNA abnormalities. Study (14), done on 386 consecutive patients with OSCC showed that DNA flow cytometry could be used to screen primary biopsies of OSCC to determine the relative risk of metastases. Lymph node metastases at admission were present in 18% of patients with diploid primary tumours compared to 52% of those with aneuploid tumours. A delay between aneuploidy formation and expression of the metastatic phenotype was postulated to explain the lack of cervical lymph node

involvement in all the aneuploid carcinomas. The 5-year survival rate of 90% in the diploid N0 group suggested that tumour progression could be prevented by local surgery alone if treated before the emergence of aneuploid cell lines. In contrast, a 5-year survival rate of only 52% was found in the aneuploid N0 patients.

A follow-up study (15) on 348 patients with OSCC who underwent radical surgery established a definite correlation between DNA ploidy of the primary tumour and the risk of local recurrence development. Recurrences were found in 9% of patients with diploid tumours compared to 46% of those with aneuploid primary tumours over the same period. Clinical staging and histological grading of the primary tumour did not have an effect on this finding. This study provided support that cytogenetic events responsible for aneuploidy formation from diploid progenitor cells are linked to the development of tumour cell populations with the potential to metastasise.

Study (16) evaluated the ploidy status of 93 primary OSCC and their subsequent recurrences. Thirteen of the primary tumours were diploid of which five recurred with aneuploid cell lines. All the recurrences of the 80 aneuploid tumours had aneuploid DNA content with a different DNA content aberration observed in 21 cases. The 5-year overall survival rate of patients who underwent radical surgery of diploid recurrent tumour was 87% while three of the 5 patients who developed aneuploid recurrences from diploid primary tumours died of cancer within 22 months. Only 31% of

those with aneuploid recurrence were 5-year survivors. This study confirmed the importance of a complete resection during initial treatment and the treatment of local recurrences before the emergence of aneuploid cell lines.

In a retrospective study (17) using archival material, DNA flow cytometry was the only parameter that could be used to predict the presence of regional metastases. This was possible regardless of the shortcomings of using paraffin-embedded blocks instead of fresh tissue. DNA flow cytometry is not used by clinicians to its full potential due to the conflicting results that have been reported in numerous studies regarding the role of ploidy in OSCC. By far the majority of studies on DNA content in OSCC were performed using flow cytometers not dedicated for DNA analysis. This has resulted in relative high cv's with resultant false diploid classification in many cases. The flow cytometer in the Department of Oral Pathology an Oral Biology is the only dedicated DNA unit in South Africa.

The p53 mutation profile of OSCC from a Black population sample in South Africa was determined in study (18). Exons 5-9 of the p53 gene were amplified. Mutations were identified in 23.6% (13/55) of the tumours. The majority were single base pair substitutions and 2 were deletions. Two novel mutations were identified. The hot spot region at codons 238-248 for p53 mutations was not prominent in this study, but rather the region between codons 272 to 292. There also appeared to be a geographical

distribution in the exons affected. The importance of the p53 gene in oral carcinogenesis in a local population sample was established.

Study (19) is a follow up on the previous study. The p53 mutation profile was compared with p53 protein and PCNA expression. No association between p53 protein expression and p53 gene mutation could be established. Overexpressed p53 cannot be described as mutated protein and no conclusions can be made on the presence of p53 gene mutations based on the immunohistochemical evaluation of the p53 protein alone. A possible difference between PCNA and p53 expression was suggested but the difference was not statistically significant.

Special circumstances occasionally necessitate the utilisation of a rapid processing technique. These circumstances usually involve malignancies. Study (20) was undertaken to evaluate the rapid acetone processing technique used in the department pertaining to histotechnical quality and reliability of histochemical and immunohistochemical staining techniques. The staining intensity of the rapidly processed sections was similar or superior to that of routine processed sections showing that it can be used with confidence in histopathology laboratories. It is unfortunately a labour intensive method and therefore not suitable as a routine procedure.

The expression of the fragile histidine triad (Fhit) protein in OSCC and adjacent dysplastic and normal epithelium was evaluated in study (21). This was the first study to evaluate Fhit expression using

immunohistochemistry. Normal epithelium showed a strong expression of Fhit protein while a reduction or loss of expression was found in 66% of the OSCC. Loss of expression was also present in the atypical cells in epithelial dysplasia. This study suggested that *FHIT* gene inactivation plays a role in oral carcinogenesis.

Study (22) was done to correlate the pattern of Fhit protein expression in OSCC with detectable abnormalities of the *FHIT* gene. RT-PCR was used because the *FHIT* genomic locus is very large while the coding region is small. Immunohistochemistry for the Fhit protein was negative in all cases of abnormal RT-PCR results. The *FHIT* gene is inactivated by deletion rather than mutation. The complexity of these alterations in studies has suggested that protein detection through immunohistochemistry may be the best method to assess the involvement of Fhit in malignancies.

Study (23) was a follow-up to compare the different antisera against Fhit protein and to evaluate the staining pattern in different epithelial disease states. Two of the antisera used were commercially available. This study showed that all three antisera could be used for evaluation of Fhit expression in OSCC although different staining characteristics were observed in non-neoplastic epithelial states.

STUDIES ON DENTAL HARD TISSUES AND TUMOURS AND CYSTS OF THE JAWS

Declaration

Nineteen articles are submitted in this section. Study (1) was initiated by Weber. I was involved with the management of the patient and participated in preparation of the manuscript. I initiated studies (2 and 3), participated in the clinical and histological evaluation of the material and was responsible for preparation of the manuscripts. Study (4) was initiated by Pretorius. I took part in the study design, did the histological, morphometrical and statistical analyses and participated in preparation of the manuscript. Studies (5 and 6) were initiated by Raubenheimer. I took part in the manuscript preparation of the manuscripts. I initiated study (7), participated in the clinico-pathological evaluation of the cases and was responsible for preparation of the manuscript. Study (8) was initiated by Raubenheimer. I participated in the histological evaluation and in preparation of the manuscript. Study (9) was initiated by Weber. Our department supplied two cases. I participated in the histological interpretation of the cases and preparation of the manuscript. Studies (10 and 11) were initiated by Raubenheimer. I took part in reviewing the histopathology of all cases and in the preparation of both manuscripts. I initiated studies (12 and 13), took part in the histological evaluation of the cases and were responsible for preparation of the manuscripts. Studies (14-16) were initiated by Raubenheimer. I was involved in evaluating and

reviewing of the cystic lesions and participated in preparation of the manuscripts. I initiated studies (17 and 18), participated in the histological interpretation of the cases and was responsible for preparation of the manuscripts. I initiated study (19), participated in the study design and preparation of the manuscript.

Abstract

Study (1) reported an unusual case of injury to a permanent incisor tooth following trauma to the deciduous predecessor. The patient presented with a soft tissue tag extending from the gingiva, entering the pulp chamber in the middle third of the crown of a maxillary incisor. The possible chain of events leading to this unique presentation was discussed.

Study (2) presented the clinicopathological features of two patients with rough hypoplastic amelogenesis imperfecta presenting with multiple impacted teeth. The impactions were associated with pericoronal odontogenic fibromas of the WHO type. These fibromas were considered to be the main reason for the impactions in both cases and were regarded as hamartomatous growths from follicular origin.

Study (3) reported a case of regional odontodysplasia associated with a soft tissue tumour. Histological examination of the teeth showed hypoplastic dentine with a prominent predentine layer and numerous interglobular masses. The associated tumour had a hamartomatous

appearance consisting of odontogenic epithelial islands associated with amorphous calcifications in a cellular fibrous stroma.

Study (4) was performed to evaluate the sealing ability of a tricure glass ionomer material (Vitremer) as an apical sealant after apicectomy and to compare it with amalgam. Micro leakage was determined according to the extent of dye penetration measured by an image analyses system on ground sections of prepared teeth. The extent of dye penetration was significantly less in the glass ionomer group compared to the amalgam group. These results were obtained with "bulk" placement of the glass ionomer to make the study more clinically relevant. It was concluded that Vitremer could be recommended for routine clinical use as an apical sealant.

The amino acid composition of dentine in permanent human teeth was determined using ion-exchange chromatography in study (5) in order to compare the results with previously published data obtained from different analytical methods. Nineteen amino acids were detected, including small quantities of asparagine and 1-methylhistidine which have not previously been documented in human dentine. This finding and other quantifiable differences with previous reports might have been the result of different analytical methods, but might also reflect dietary and other regional factors that might influence dentinogenesis.

Study (6) is a follow-up of the previous study to compare the inorganic contents of opaque and translucent dentine. A significant difference in the fluoride, magnesium and zinc contents were found.

The clinicopathological features of eight cases of giant ossifying fibromas were reported in study (7). All cases had a prominent fibrous component. The resorption of mineralised tissue is indicative of altered cellular differentiation and proliferative activities in large ossifying fibromas. Focal areas of aneurysmal bone cyst formation were identified in the majority of cases.

Study (8) reported two cases of adenomatoid odontogenic tumour, both measuring in excess of 7 cm. The progressive growth and cortical perforation in these two cases substantiated the view that it is a benign neoplasm rather than a hamartoma.

Three cases of diffuse peripheral odontogenic fibromas were described in study (9). The diffuse involvement of the gingiva in all three cases supported the likelihood that the peripheral odontogenic fibromas have a hamartomatous origin rather than being a true neoplastic lesion. The one case, which was associated with ocular and skin lesions, could be part of a yet undescribed syndrome.

A rare case of a peripheral dentinogenic ghost cell tumour was reported in study (10). The lesion was present on the mandibular right alveolar ridge

and presented as a broad based polypoid lesion. The lesion was excised and no recurrence was detected after a six-year follow-up period.

The clinicopathologic diversity of ameloblastomas was described in a study (11) of 108 such tumours. An association with ameloblastic fibroma, adenomatoid odontogenic tumour and aneurysmal bone cyst formation in some cases was illustrated. Ameloblastomas with melanocytes and mucous metaplasia were also found. These changes emphasized the differentiation potential of neoplastic odontogenic epithelium.

A peculiar presentation of an intrabony ameloblastoma was reported in study (12). Histological examination of a multicystic mandibular ameloblastoma revealed a papillomatous lesion resembling a verruca vulgaris in one cyst. HPV type 18 DNA was identified in this lesion using radiolabelled *in situ* hybridisation. The absence of HPV DNA in other epithelial areas of the ameloblastoma was suggestive of a secondary infection although the mode of infection could not be established.

The clinicopathological features of unicystic ameloblastomas were reported in study (13). Most lesions were located in the mandible and were frequently associated with impacted teeth, root resorption and tooth displacement. The value of thorough histological evaluation of the cyst wall to determine the appropriate sub-classification was emphasised.

An extensive review of cystic jaw lesions in a German population sample was reported in study (14). Although a large percentage of the patients were military personnel with a bias towards young males, unicystic ameloblastomas presented one and a half decades later than is generally reported. Radicular and residual cysts were as expected the most common cystic lesion.

Recent new developments in the classification of odontogenic cysts of the jaws were described in study (15). The significance of a correct diagnosis as well as the importance of communication between the clinician and histopathologist in the diagnostic process was emphasised.

In study (16), the clinical and radiographic features of a series of unilocular lesions resembling dentigerous cysts were correlated with the microscopic diagnosis. A significant number was found to be unicystic ameloblastomas or odontogenic keratocysts. The importance of histological evaluation of all pericoronal cystic lesions was underlined.

The clinicopathological features of two cases of glandular odontogenic cysts were reported in study (17). The electron microscopy characteristics were the first to be reported in these types of cysts. It demonstrated a process comparable to apoptosis in the superficial eosinophilic cuboidal cells of the epithelial lining.

Study (18) reported the largest pigmented neuroectodermal tumour of infancy yet. These tumours are usually 1-3 cm in size but the excised tumour in this case measured 18 cm in diameter. The tumour had a normal histological appearance and no malignant change could be detected. No recurrence was found.

The prevalence of Epstein-Barr virus in Burkitt's lymphoma in a South African population sample was investigated in study (19). This was the first study involving a South African sample of its kind. *In situ* hybridisation for EBERs found EBV DNA to be present in 50% of the BLs. No difference was observed in the positivity and proliferation index between the oral-maxillofacial and the non-facial group of BL supporting the view that EBV is no longer thought to be the sole cause of BL, but is still accepted as a co-factor in the pathogenesis of this neoplasm.

STUDIES ON SALIVA AND SALIVARY GLAND NEOPLASMS

Declaration

Ten publications are submitted in this section. Study (1) was initiated by Raubenheimer. I was involved in the fieldwork obtaining some of the material and participated in preparation of the manuscript. Study (2) was initiated by Raubenheimer while I performed the statistical analysis and participated in preparation of the manuscript. I initiated study (3), took part in reviewing the material and participated in preparation of the manuscript. I initiated studies (4 and 5), was responsible for the study design and participated in the interpretation of data and manuscript preparation. Raubenheimer initiated study (6) while I participated in preparation of the manuscript. Study (7) was initiated by Hemmer while I took part in preparation of the manuscript. I initiated study (8), was responsible for the study design, interpretation of the histological slides and manuscript preparation. I initiated study (9), participated in the case selections and reviews and was responsible for the manuscript preparation. Study (10) was initiated by Raubenheimer; I participated in the manuscript preparation.

Abstract

The function of saliva and the role of salivary glands in a variety of vertebrates were discussed in study (1). The adaptation of salivary glands to suit the range of environments of the different vertebrates was emphasised.

Multiple myeloma (MM) is typed according to the circulating monoclonal immunoglobulin and or light chain type produced by the neoplastic plasma cells. Immune suppression due to a decrease of circulating normal immunoglobulins is a serious complication of MM. Study (2) demonstrated that the patients with MM had significantly increased concentrations of the specific immunoglobulin related to the type of MM in their saliva compared to the control group. It further showed that the salivary IgA concentration in non-IgA MM and salivary IgG concentration in non-IgG MM patients were within normal range despite a significant decrease in circulating normal immunoglobulins in these patients. This lack of suppression of normal salivary immunoglobulin concentrations in patients suffering MM was supported by the lack of clinical evidence of an opportunistic infection in the oral cavities in any of the MM patients.

The purpose of study (3) was to determine the relative frequency and distribution of intraoral salivary gland neoplasms in a black African population taking newly described entities into account. The majority of tumours (52%) were malignant. Polymorphous low-grade adenocarcinoma was found to be the most common intraoral malignancy in contrast to mucoepidermoid carcinoma reported in the majority of other studies. It was

suggested that these differences were probably related to criteria used for diagnosis of polymorphous low-grade adenocarcinoma. The malignant tumours were also found to occur at a significantly older age than benign tumours.

The diagnosis of salivary gland neoplasms can be problematic, especially when small biopsy specimens are submitted for histopathologic interpretation. The evaluation of the nucleolar organizer region associated proteins (AgNOR) staining technique as an additional microscopic criterion to benefit the diagnostic process was evaluated in study (4). Although the difference between the mean AgNOR count per nucleus between benign and malignant tumours and between polymorphous low-grade adenocarcinoma and adenoid cystic adenocarcinoma were highly significant, the presence overlapping AgNOR count between various tumours prohibited the use of this technique as an absolute criterion in establishing a final diagnosis. It could however be used as a diagnostic aid in differentiating between salivary gland neoplasms.

Study (5) was a follow-up on the previous study to correlate the AgNOR counts in salivary gland tumours with the proliferation index and DNA ploidy status as determined by a standard flow cytometer. Although a positive correlation between the AgNOR count and proliferation index was found, it was not statistically significant. Only three tumours (3/33) showed aneuploid DNA content. The low number of aneuploidy tumours was most

likely the result of paraffin-embedded blocks for obtaining a cell suspension to use on a non-dedicated DNA flow cytometer.

The role of DNA ploidy analyses, histochemistry and immunohistochemistry in the diagnosis of epithelial salivary gland neoplasms was reviewed in study (6). The utilisation of fine needle aspiration and frozen sections for the establishment of a diagnosis were discouraged. It was concluded that although special investigations may contribute, the diagnosis still relied mainly on the growth pattern and cytological features of a tumour.

Cytogenetic studies have proposed that three groups of epithelial cells with different karyotypic patterns exist in Warthin's tumour of salivary glands, suggesting the existence of etiologically different subsets of this tumour. Study (7) was undertaken to evaluate the role of high-resolution DNA flow cytometry to the cytogenetic analysis of Warthin's tumour. All 28 cases of Warthin's tumour consisted of flow cytometrically diploid cells. The mean cv was 1.31% indicating the sensitivity of the DNA analyses. This study did not support the hypothesis of existence of cytogenetically distinctive subgroups of Warthin's tumour.

The histogenesis of Warthin's tumour is controversial. The heterotopic theory suggests entrapped salivary gland epithelium in associated lymph nodes while the immune theory postulates a lymphocytic response to epithelial changes. Study (8) was a follow-up on the previous study to

evaluate a proposed role of EBV in the pathogenesis of Warthin's tumour. Using *in situ* hybridisation, no signals using EBER1/2 probes could be detected in the epithelium of 20 cases of Warthin's tumour or adjacent normal salivary gland tissue. Individual positive lymphocytes were present in 7 cases. Although this study did not prove any theory regarding the histogenesis of Warthin's tumour to be correct, it demonstrated that EBV was not involved in the pathogenesis of Warthin's tumour as had been suggested by other studies.

Study (9) reported 5 cases of intraoral salivary duct carcinoma (SDC), a high-grade malignancy usually encountered in the parotid gland. This was the largest series of these tumours reported to date. The histological features were similar to those SDC originating from the major salivary glands. The immunohistochemical profile confirmed a ductal origin while four tumours displayed DNA aneuploidy suggesting aggressive behaviour and poor prognosis. Due to the rarity of intraoral SDC, the diagnostic criteria and differential diagnoses were discussed in detail.

Several publications had regarded the presence of tyrosine-rich crystalloids as a unique microscopic feature of pleomorphic adenomas. Study (10) reported the presence of tyrosine-rich crystalloids in a well-documented case of polymorphous low-grade adenocarcinoma with obvious important diagnostic implications. It was speculated that the presence of tyrosine-rich crystalloids in polymorphous low-grade adenocarcinomas might imply a level of differentiation closer to that of

pleomorphic adenomas than more malignant tumours of salivary gland origin.

Diagnosis of oral precancer and cancer

A. J. LIGTHELM, A. WEBER, P. J. VAN NIEKERK, W. F. P. VAN HEERDEN

Summary

The early diagnosis of oral cancer and precancer is important because oral cancer is usually well advanced at the time of diagnosis, having spread to regional lymph nodes in a substantial percentage of cases. The earlier the diagnosis is made, the better the prognosis.

Several diagnostic methods that are used, such as stomatoscopy, toluidine blue staining, oral exfoliative cytology, histopathological examination and other more advanced diagnostic procedures, are discussed.

Oral squamous cell carcinoma (oral cancer) is usually well advanced at the time of diagnosis,[1] having spread to regional lymph nodes in a substantial percentage of cases.[2,3]

It must be realised, however, that apart from the easy accessibility of the oral cavity, oral squamous cell carcinoma is readily discernible in its early symptomatic stages, long before ulceration, bleeding, induration and lymphadenopathy are present.[1] Since oral squamous cell carcinoma originates from the oral mucous membrane, superficial or surface changes in colour and texture are always detectable in the precursor or precancerous stages.

Clinical diagnosis

A fundamental principle in early diagnosis is that any changes observed in the oral mucous membrane should be considered suspicious, especially those in high-risk sites (lateral borders of the tongue and floor of the mouth) and in high-risk patients (smokers and consumers of alcohol, especially over the age of 40 years).

All dental patients should receive a thorough evaluation, including a complete medical and dental history as well as physical examination of the mouth and neck.[4]

If a lesion with clinical features described elsewhere in this supplement is detected, the following steps should be taken: (*i*) remove all possible sources of irritation, e.g. ill-fitting dentures, sharp-cusped teeth, smoking or other habits; (*ii*) re-evaluate the lesion after 8 - 10 days of symptomatic or conservative treatment; (*iii*) if the lesion has not resolved or improved during this period, biopsy and histological examination must be performed; and (*iv*) when a patient presents with a lesion which has been clinically diagnosed as advanced carcinoma, a biopsy specimen should be taken immediately.[4]

Other means of clinical screening should be considered in high-risk patients in whom initial oral examination does not reveal clinically detectable surface changes. These additional diagnostic procedures can also be applied to support the clinical diagnosis[5] with or without surgery. The methods include stomatoscopy, toluidine blue staining and oral exfoliative cytology.

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Stomatoscopy

This diagnostic aid comprises direct observation of the oral mucosa using a special optical system whereby morphological changes of the oral mucosa are magnified and thus more readily evaluated. Although this method might be of some value in the early diagnosis of oral squamous cell carcinoma, it is not widely used.[5]

Toluidine blue staining

Toluidine blue is a topical agent which is widely used in medicine. It is also used to identify malignant changes of the oral mucosa, which it stains more intensely than the surrounding normal areas because of its affinity for DNA and RNA, levels of which are increased in malignant cells, enabling the clinician to detect a potentially malignant lesion at an early stage. It can be used as a mouth rinse or applied to a localised lesion.

If a lesion is found using the rinse technique, all possible aetiological factors should be eliminated and the area stained again after 2 weeks by topical application. A biopsy is mandatory if the lesion is still present.

Benign ulcers can cause false-positive dye uptake, but display a well-defined marginal uptake in contrast to the diffuse marginal patterns seen in dysplastic or malignant lesions.[6]

In a study of 235 persistent mucosal lesions, Mashberg[7] found 6,7% of results to be false negative and 8,5% to be false positive. Despite these results, the toluidine blue method has been shown to have good results if used in association with exfoliative cytology and histopathological examination. High-risk patients form the target population that may benefit most from toluidine blue staining, since the minimal mucosal alterations of early cancer may be delineated by this method.[8]

Toluidine blue staining may also be used to select a biopsy site in order to obtain the most representative tissue for examination.

If general medical and dental practitioners would use this simple technique routinely in high-risk patients, many more cases of cancer would be diagnosed in the early stages. This would not only increase survival but would reduce post-treatment morbidity.

Exfoliative cytology

This technique is widely used for detecting malignant lesions in both the oral cavity and the uterine cervix. The morphological characteristics of exfoliated superficial cells are examined when the cells are stained according to the method of Papanicolaou and Traut.[9]

The practical value of exfoliative cytology in diagnosing malignant lesions is widely accepted (Fig. 1). Its use in diagnosing malignant transformation in leucoplakias is limited, however, since the superficial keratinised cells impede the emergence of the deeper dysplastic cells.

False-negative results in hyperkeratotic lesions were observed in 62% of cases in the study of Dabelsteen *et al.*[10] Exfoliative

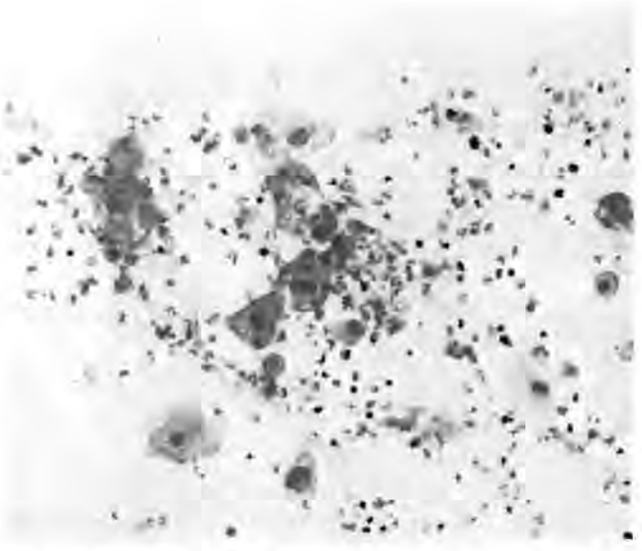

Fig. 1. Cytological smear of an oral lesion displaying variation in size and shape of the epithelial cells, nuclear hyperchromatism and pleomorphism, which are suggestive of malignancy (H and E x 126).

cytology is much more efficient in erosive erythroplakias and other lesions than in leucoplakias.

Folsom *et al.*[11] stated that exfoliative cytology is a useful diagnostic adjunct in the evaluation of visible oral lesions when used in conjunction with other diagnostic methods. Its limitations should be recognised but its value should never be under-estimated, especially in high-risk patients.

Radiography and computed tomography

Plain radiography is of limited value in the early diagnosis or investigation of oral cancer. Radiographic change is apparent only after at least 50% of the calcified component of bone is lost.[12]

In patients with radiographic evidence of squamous cell carcinoma the general radiographic appearance is that of a radiolucent lesion with indistinct margins, displaying no sclerotic reaction.[13]

Computed tomography is of great benefit in the investigation of head and neck tumours, but its value for intra-oral tumours is limited.[12]

Histological diagnosis

Clinical identification of epithelial dysplasia and early squamous cell carcinoma is virtually impossible owing to the varied appearance of these lesions and confusion with benign lesions of similar appearance.[6]

The exact nature of the histopathological changes in lesions detected during clinical evaluation can only be confirmed by histological examination of biopsy material. These changes can range from dysplasia to carcinoma *in situ*, squamous cell carcinoma or verrucous carcinoma.

Biopsy procedures

When a biopsy is performed, certain principles and techniques should always be followed to establish a reliable diagnosis.

1. Drugs should not be applied to the lesion before biopsy, since they can alter the staining characteristics of the tissue and complicate the diagnosis.

2. Local anaesthetic should be infiltrated around the periphery of the lesion rather than directly into it. Direct penetration of a needle into the tumour may also cause seeding of tumour cells along the needle tract.[4]

3. The tissue specimen should be removed in such a way that both normal and abnormal tissue are included for histological examination. The specimen must be deep enough to determine the degree of invasion of the tumour in the submucosa. In order to enhance healing of the biopsy site the incision line should, if possible, be parallel with the blood vessels and major nerves as well as the lines of stress in the tissue.[4]

4. Excessive handling of the tissue during its removal may cause distortion and surgical trauma that can complicate the examination and diagnosis. A suture should be placed into the normal surrounding tissue to be removed and not into the lesion itself. The biopsy specimen can then be lifted out by traction on the suture.

5. After the specimen is freed from the underlying tissue, it must immediately be placed into a bottle containing enough 10% buffered formalin to cover it.

6. Biopsy specimens of the oral mucosa are often thin and it is therefore recommended that they be laid flat on a piece of card or filter paper before placing in the fixative. This avoids curling and distortion of the specimen.

7. Once a specimen is removed and placed in a fixative, it must be sent to an oral pathologist without delay.

8. A good clinical history is as important as the biopsy specimen itself. The following information must be included: (*i*) age, race and sex of the patient; (*ii*) duration of the oral lesion/s; (*iii*) tempo of growth; (*iv*) symptoms; (*v*) previous treatment; (*vi*) other oral lesions; (*vii*) palpable lymph nodes; and (*viii*) aetiological factors such as smoking, use of alcohol and exposure to sun.

9. The lesion must always be described in detail. This plays an important role in its final evaluation. The following information should be included: (*i*) position of the lesion; (*ii*) size; (*iii*) colour; (*iv*) configuration; (*v*) consistency; and (*vi*) mobility or fixation of the lesion in relation to the surrounding tissue.

10. X-ray plates, where applicable, should always by included.

11. A lesion smaller than 1 cm in diameter should be excised in its entirety for histological examination. An incisional biopsy is performed on lesions larger than 1 cm and the specimen should be taken from the area that is clinically the most suspicious. Necrotic areas should be avoided because they tend to complicate the diagnosis.

12. Punch and aspiration biopsies can also be carried out in the oral cavity. Punch biopsies are not reliable because only a small specimen is obtained, which may not be typical of the lesion as a whole. Aspiration biopsies are performed by aspirating the tissue through a large-gauge needle. Only a limited amount of tissue can be obtained and more often than not it is badly distorted, making a definite diagnosis extremely difficult. Under certain circumstances, however, this is the only means of obtaining a biopsy specimen.

Histopathological examination

Epithelial dysplasia and carcinoma in situ

Epithelial dysplasia denotes changes of the epithelium which suggest a possible development of malignancy, whereas carcinoma *in situ* indicates definite development of malignancy.[14]

In the past, the terms 'epithelial dysplasia', 'epithelial atypia' and 'dyskeratosis' have often been used synonymously. However, the individual cellular changes should be referred to

as 'atypia' and the general disturbance in the epithelium as 'dysplasia'. The World Health Organisation Collaborating Reference Centre for Oral Precancerous Lesions[15] mentions the following changes as possibly occurring in epithelial dysplasia: (*i*) loss of polarity of the basal cells; (*ii*) the presence of more than one layer of cells with a basaloid appearance; (*iii*) an increased nuclear/cytoplasmic ratio; (*iv*) drop-shaped rete processes; (*v*) irregular epithelial stratification; (*vi*) an increased number of normal mitotic figures — a few abnormal mitoses may also be present; (*vii*) the presence of mitotic figures in the superficial half of the epithelium; (*viii*) cellular pleomorphism; (*ix*) nuclear hyperchromatism; (*x*) enlarged nucleoli; (*xi*) reduction of cellular cohesion; and (*xii*) keratinisation of single cells or groups of cells in the spinous cell layer.

Not all of these changes will necessarily be present in the epithelium. The histological diagnosis of dysplasia is established when two or more of the changes are present together.[14]

According to the number/ratio of changes in dysplasia is classified as mild, moderate or severe (Figs 2, 3 and 4). Dysplasia in the precancerous uterine cervix is described as cervical intra-epithelial neoplasia (CIN I, CIN II and CIN III). These changes are similar to the dysplastic changes seen in mild, moderate and severe dysplasia, respectively, in the oral mucosa.

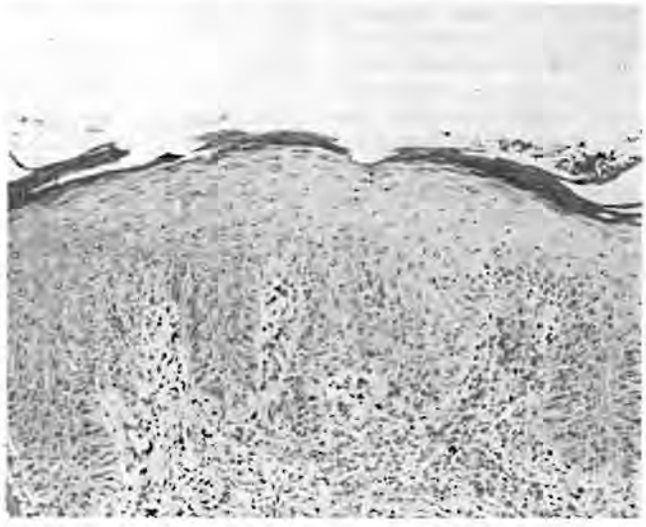

Fig. 2. Slight epithelial dysplasia. Drop-shaped rete ridges, irregular epithelial stratification and hyperkeratosis are illustrated (H and E x 200).

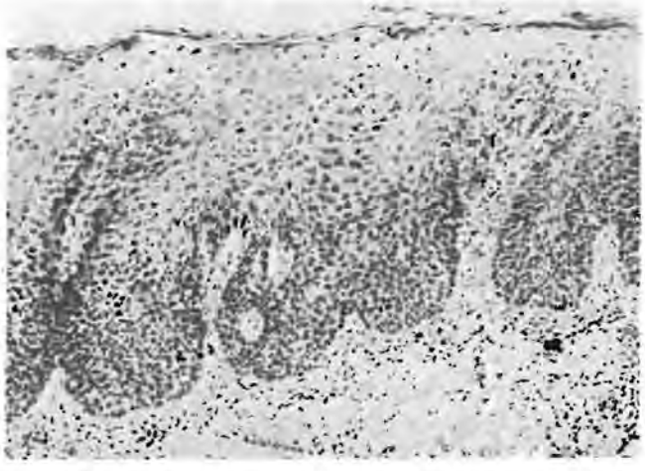

Fig. 3. Moderate epithelial dysplasia, characterised by an increased nuclear cytoplasmic ratio, drop-shaped rete ridges, cellular pleomorphism and nuclear hyperchromatism (H and E x 80).

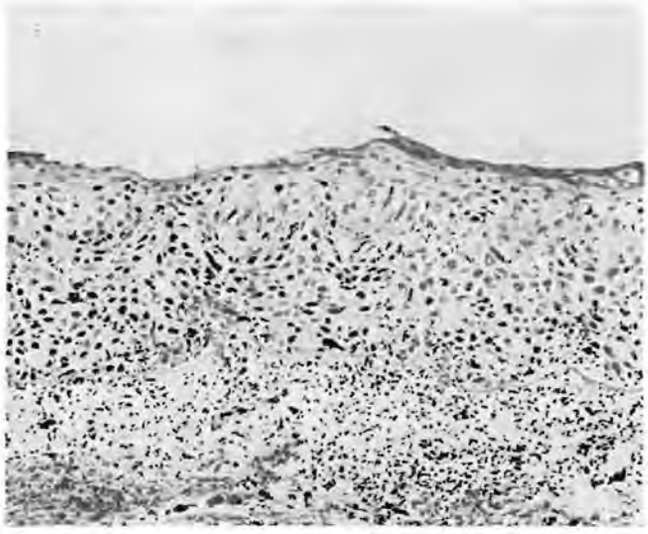

Fig. 4. Carcinoma *in situ* (severe epithelial dysplasia). The epithelial cells exhibit all the features of malignant cells, without infiltration into the underlying connective tissue (H and E x 80).

Severe grades of epithelial dysplasia may merge into the lesion known as carcinoma *in situ* (Fig. 4), in which the whole thickness of the epithelium is involved.[15] Carcinoma *in situ* is considered a histopathological rather than a clinical entity, since a number of clinical diseases may display the characteristics of carcinoma *in situ*.[16] Whether the histological distinction between severe dysplasia and carcinoma *in situ* is of practical value in the case of oral mucosa remains unresolved.[15]

The degree of dysplasia, as in the case of the uterine cervix, is linked to the degree of probability of the development of malignancy and therefore has definite therapeutic and prognostic implications.

The prevalence of epithelial dysplasia has been found to be between 3,7% and 28,8% in oral leucoplakia.[5] Between 3,2% and 8,1% of clinical leucoplakia was found to be squamous cell carcinoma on examination of a first biopsy specimen.[17] Shafer and Waldron[18] found squamous cell carcinoma in 51% of erythroplakias and carcinoma *in situ* in 40%.

Squamous cell carcinoma

Squamous cell carcinoma is characterised by atypical neoplastic cells which are located throughout the epithelium as well as beyond the basement membrane in the submucosa.[4]

According to the histopathological appearance, squamous cell carcinoma may be classified as well differentiated, poorly differentiated or anaplastic. The degree of differentiation will determine the nature of the total treatment plan and will influence prognosis.

Well-differentiated squamous cell carcinoma displays invasion into the underlying connective tissue and is usually localised. The cells are easily discernible as epithelial cells, while abnormal cell forms are rarely seen (Fig. 5). Large amounts of keratin are formed within the tumour. A chronic inflammatory reaction is always present in the submucosa and is restricted to the base of the tumour (Fig. 6). The tumour rarely infiltrates bone.

Poorly differentiated squamous cell carcinoma displays extensive cellular pleomorphism, together with a marked absence of keratin. An extensive inflammatory infiltration appears in the submucosa underlying the tumour. Bone infiltration may be present.

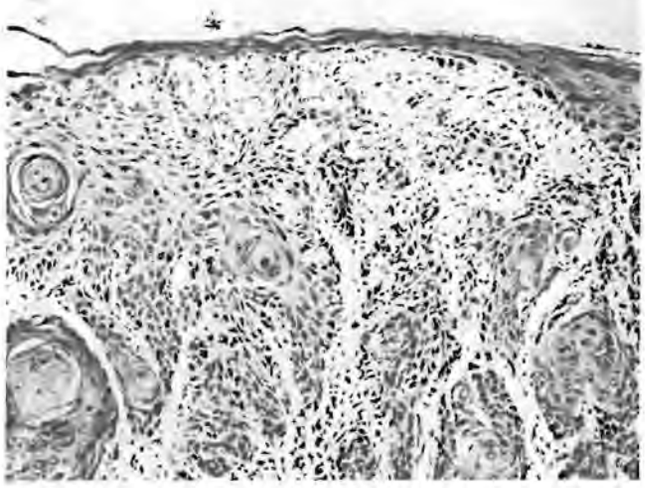

Fig. 5. Well-differentiated squamous cell carcinoma. Invasion of the submucosa and abundant keratinisation are present (H and E x 126).

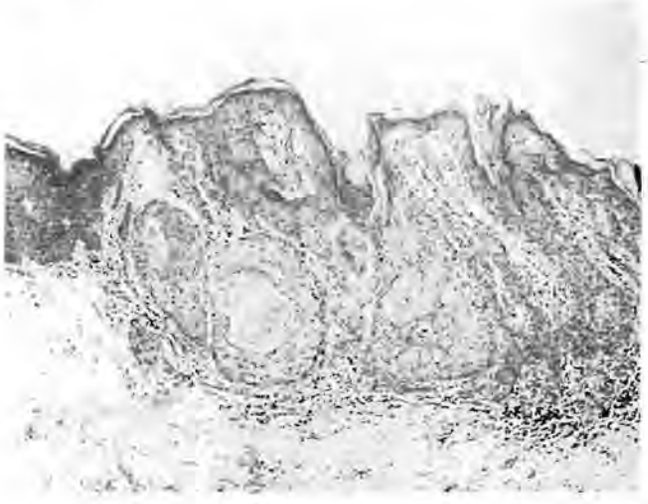

Fig. 6. Well-differentiated squamous cell carcinoma. There is localised invasion into the underlying submucosa. A chronic inflammatory reaction is present at the base of the tumour (H and E x 126).

In highly anaplastic squamous cell carcinoma, where the epithelial origin of the tumour cells is not readily apparent, spindle cells can be present. Bone infiltration is common and distant metastases occur.

Histological grading

Broders[19] originally used grades 1-4 as a means of indicating the percentage of normal differentiation of the tumour cells. Although the WHO[20] agreed that any system of histological grading is to some extent subjective, they suggested an arbitrary subdivision into three grades. The guiding features are those indicative of proliferation and differentiation. Shklar[21] also used a grading system with emphasis on the importance of proliferation and differentiation of the tumour.

Histological grading of malignancy in squamous cell carcinoma of the oral cavity is one of the variables which may be used to predict prognosis. Treatment modalities can therefore be better selected according to the biological behaviour of the tumour.[22]

Advanced diagnostic procedures

The histological diagnosis of oral squamous cell carcinoma does not usually present a major problem.[23] However, carcinomas differ in biological behaviour, resulting in different clinical manifestations and subjective histological grading. Advanced methods for the evaluation of oral precancerous lesions and carcinoma could contribute to the better understanding and management of these lesions. Several techniques have recently been implemented in order to find a reliable indicator of the biological potential of precancerous lesions and carcinomas.[23] These methods are based on morphological or on functional evaluation of the lesions. The morphological methods include histochemical and immunohistochemical procedures, *in situ* hybridisation and electron microscopy. Methods such as cellular proliferation studies, DNA histograms and analysis of the immune status of the patient are included in the group of functional methods.[23]

Although the above methods have promising aspects, at present there is no alternative to histological examination in assessing the nature of oral squamous cell carcinoma and its precursors.[23]

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Oral cancer

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Oral cancer, strictly speaking, includes all malignancies of the oral cavity. For the purpose of this article, however, it refers to squamous cell carcinoma of the oral mucosa.

The prevalence of oral cancer varies greatly among the different geographical regions worldwide. In the United States it accounts for about 4% of all cancers diagnosed, while the figure is 50 to 70% for the Indian subcontinent. The incidence generally appears to have stabilised in developed countries while there is growing evidence that the incidence of oral cancer is increasing in developing countries.[1]

During the period 1990 to 1992, an average of 1336 new cases of intraoral cancers per year has been reported in South Africa. Oral cancer ranked fifth behind cancer of the prostate, oesophagus, lung and colon in males in South Africa. Together with cancers of the pharynx, these malignancies were the fourth most common in white South African males and the second most common in black males. In both black and coloured males, oral cancer represents 6 to 7% of all cancers and ranked fourth and fifth in their respective population groups, whereas it constitutes 2,3% (eighth position) of all cancers in white males and 3,4% (tenth position) in Asian males. These figures, obtained from the National Cancer Registry of South Africa,[2] include only histologically and cytologically diagnosed cancers and thus underreport true prevalence of oral cancer, as a significant number of these are diagnosed clinically.

Despite recent advances in treatment modalities like surgery, chemotherapy and radiotherapy, no improvement in the mortality and morbidity rates of oral cancer has been reported over the last 50 years. Oral cancer has a five-year survival rate of about 54%, one of the worst of all major cancers.[3]

Clinical features and diagnostic considerations

Because oral cancer is usually not diagnosed in its early stages, less than half of all cancer patients are cured. This is an alarming state of affairs since the majority of oral cancers can be prevented by eliminating the well-documented, most important aetiological factors, i.e. tobacco and alcohol. Furthermore, no specialised techniques are required to examine the oral cavity for the presence of premalignant lesions or early oral cancers. Prevention and early detection of oral cancer can be achieved by identifying high-risk patients, recognising premalignant lesions and the early detection of cancerous lesions.

High risk patients

About 90% of all oral cancers are found in persons older than 40 years with an average age at the time of diagnosis of about 60. The *ageing process* influences proto-oncogenes and suppressor genes, causing cellular dysregulation through alterations in the cell growth and suppressor proteins. The primary aetiological agents of oral cancer are *tobacco* products and the heavy use of *alcohol*, especially in combination.

More than 90% of oral cancer patients have a history of tobacco use. N-nitrosamines are the compounds thought to be the major carcinogenic agents in tobacco. Analytical studies have shown that all forms of tobacco use increase the risk of oral cancer. Tobacco chewing together with betel nuts, (with or without other additives) frequently practised in the Indian subcontinent, is responsible for the high incidence rate of oral cancers in that region.

After stopping smoking for ten or more years, no excess risk is found and this provides great opportunities for the prevention and control of oral cancer.

Other aetiological factors are also implicated in oral cancer. Iron metabolism is important in maintaining the health of the mucosa and many disease states are associated with iron deficiency. There is strong evidence that chronic sideropaenia is linked to an increase in oral cancer development.

Infections may also contribute to oral carcinogenesis. Chronic candidiasis is associated with some premalignant lesions while human papillomavirus DNA has been found in oral cancer cells.[4]

Premalignant lesions and conditions

White and red lesions of the oral mucosa are the most common premalignant clinical lesions. A premalignant lesion is defined as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart.[5] Although premalignant lesions do not precede all oral cancers, the presence of these lesions presents an opportunity for preventive action. The most common lesion is termed *leukoplakia*. (This term must not be confused with the recently described entity of *oral hairy leukoplakia*, which has no premalignant potential, is caused by the Epstein-Barr virus and is found in immunocompromised patients.) Leukoplakia is defined as 'a whitish patch or plaque that cannot be characterised clinically as any other disease and which is not associated with any physical or chemical causative agent except the use of tobacco'.[5] This is a clinical term which carries no histological connotation (see Fig. 1). *Erythroplakia*, the other important premalignant lesion, has a similar definition except that it is described as a red lesion (see Fig. 2).

Leukoplakias may vary in appearance from homogeneous to nodular and to a

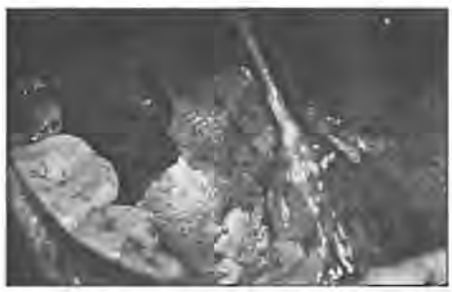

Fig. 1. *Leukoplakia lesion in the floor of mouth. Histologic examination showed a severe epithelial dysplasia.*

Fig. 2. *Erythroplakia on the soft palate in a 55 year-old patient with a long history of tobacco use. The histological diagnosis was a moderately differentiated squamous cell carcinoma.*

combination with erythroplakia. Although tobacco use increases the risk for oral cancer, it has been found that patients with oral leukoplakia who are non-smokers, have a higher risk of malignant change. This is difficult to explain, but it is speculated that in the absence of tobacco as a causative agent, more dangerous initiating or potentiating factors may be present.
The majority of pre-malignant conditions are associated with atrophy of the oral epithelium. These include sideropaenic dysphagia, oral submucous fibrosis and the still controversial erosive oral lichen planus.

Early detection of cancerous lesions

Although easily detected and often cured in its early stages, most oral cancers are advanced at the time of diagnosis. Early cancer lesions are usually painless and may appear as small apparently harmless areas of induration, erosion or keratosis, often deceiving the unsuspecting clinician into a false sense of security. It may also present with the clinical features of a pre-malignant lesion (leukoplakia or erythroplakia). Patients usually seek consultation after developing persistent pain, most commonly from a non-specific ulcer or irritation in the mouth.
Advanced oral cancer lesions usually present as painless ulcers with indurated rolled margins (see Fig. 3). Fixation to underlying tissues is present and regional lymphadenopathy may be seen.

Management principles

The clinical significance of these premalignant lesions is that all must be biopsied to determine the possible presence of malignant change. The malignant transformation rate of homogeneous leukoplakia is about 6%, while the rate for proliferative leukoplakia is reported to be as high as 70%. The histologic diagnosis of erythroplakia is usually either a severe epithelial dysplasia or already being a squamous cell carcinoma. This higher risk associated with red lesions implies that with combination lesions (erythroleukoplakia), the red areas should always be included in the biopsy site. The toluidine blue staining method can be used in patients with extensive leukoplakia without red areas to select sites for biopsy.[6] This technique consists of painting the lesion with a 1% aqueous solution of toluidine blue and then, after ten seconds, decolourising it with 1% acetic acid solution. The sites that retain the dye are then included in the biopsy.
These premalignant lesions are usually painless and the majority of patients are unaware of their presence. It is therefore essential to perform regular examinations of the oral cavity in especially high-risk patients. Screening can be made more efficient by inspecting the high-risk sites where 90% of all oral cancers arise: the floor of the mouth, the ventrolateral aspect of the tongue and the soft palate complex. It has been shown that high-risk individuals visit their physicians more frequently than

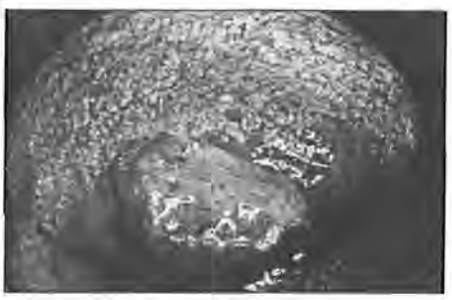

Fig. 3. *Squamous cell carcinoma on the lateral border of the tongue. Note the rolled indurated margins.*

they visit their dentists. An examination of the oral cavity should be part of every examination at the physician's office.

Pharmacological agents available for treatment

The role of pharmacological agents in the prevention and treatment of premalignant lesions is still under investigation. Primary treatment of oral leukoplakia and prevention of secondary primary lesions in patients with treated oral cancer have been studied in several randomised, placebo-controlled chemoprevention trials with high-dose isotretinoin.[7] Variable reductions in the occurrence of oral leukoplakia and dysplasia have also been demonstrated in trials using beta-carotene.

Avoiding treatment errors

Any suspicious oral mucosal lesion, including any ulcer not healing within two weeks, must be biopsied. The biopsy should be sufficiently large to include enough abnormal and clinically normal tissue to give the pathologist a chance to make a diagnosis without requesting additional material. An excisional biopsy should be avoided unless the lesion is very small since it is unlikely that the margin would have been wide enough if the lesion was found to be malignant. It may further destroy clinical evidence of the site and character of the lesion, which are important facts for the surgeon or radiotherapist.

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Further reading

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The role of the dentist in the prevention and early diagnosis of oral cancer

Keywords: Oral cancer, prevention, early detection

Clinical relevance

Oral cancer is a life-threatening problem in most countries, especially in developing countries. The severity of oral cancer in South Africa is demonstrated by the fact that it is one of the most common malignancies in South African males.[1] This fact is not often appreciated. One of the reasons may be that oral cancer is not registered as a single entity in the local cancer registry, but separated into cancers originating from the mouth, tongue or gums.[1]

Early detection of oral cancer allows for a five-year survival rate of 90%. However, most oral cancers are diagnosed at an advanced stage with a five-year survival rate of about 20%, which is one of the worst prognoses of all major cancers. This is a tragic situation because no specialised techniques are required to examine the oral cavity for the presence of premalignant lesions or early oral cancers. Prevention and early diagnosis are therefore of the utmost importance.

Identifying high-risk patients, recognising premalignant lesions and the early detection of cancerous lesions is the prime responsibility of the dentist.

High risk patients

The most common factor contributing to the development of oral cancer is age. About 90% of all oral cancers are found in persons older than 40 years, averaging 60 years at the time of diagnosis. The ageing process influences proto-oncogenes and suppressor genes, resulting in cellular dysregulation through alterations in the cell growth and suppressor proteins. The primary aetiological agents of oral cancer are tobacco products, heavy use of alcohol, and especially the combination of tobacco and heavy alcohol use.[2] More than 90% of oral cancer patients give a history of tobacco use. Tobacco contains nicotine and other alkaloids. N-nitrosamines are the compounds that are considered to be the major carcinogenic agents in tobacco. All forms of tobacco use are implicated, including smokeless tobacco (chewing tobacco and snuff dipping).[3] Tobacco chewing, together with betel nuts, (with or without other additives) frequently practised on the Indian subcontinent, is responsible for the high incidence rate of oral cancers in that region.

The risk of oral cancer among non-alcohol drinkers increases with the amount of tobacco smoked, while the risk among non-smokers increases with the level of alcohol intake.[2] Among those who both smoke and drink alcohol, the risk seems to increase in multiplicative fashion. The substantial fall in the incidence of precancerous lesions and reduced risk for the development of oral cancer after cessation of tobacco use is well documented and provides great opportunities for the prevention and control of oral cancer.[4]

Other aetiological factors are also implicated in oral cancer. Iron metabolism is important in maintaining the health of the mucosa and many disease states are associated with iron deficiency. There is strong evidence to suggest that chronic sideropenia is linked to an increase in oral cancer development. Infections, especially viruses, may also be a contributing factor in oral carcinogenesis. Human papillomavirus DNA has been found in oral cancer cells.[5] The possible implications thereof are currently under investigation by a number of centres.

Premalignant lesions and conditions

White and red lesions of the oral mucosa are the most common premalignant clinical lesions. A premalignant lesion is defined as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart.

Although premalignant lesions don't precede all oral cancers, the presence of these lesions presents an opportunity for preventive action. The most common lesion is termed leukoplakia. (This term must not be confused with the recently described entity of *oral hairy leukoplakia*, which has no premalignant potential, is caused by the Epstein-Barr virus and found in immunocompromised patients).
Leukoplakia (Fig. 1) is defined as a whitish patch or plaque that cannot be characterised clinically as any other disease and which is not associated with any physical or chemical causative agent apart from the use of tobacco.[6] This is a clinical term that carries no histological connotation.
Erythroplakia, the other important premalignant lesion, has a similar definition, although it is described as a red lesion (Fig. 2).

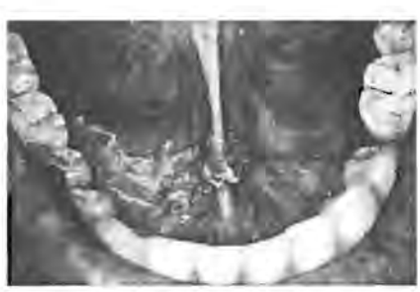

Fig. 1. Leukoplakia on the floor of mouth.

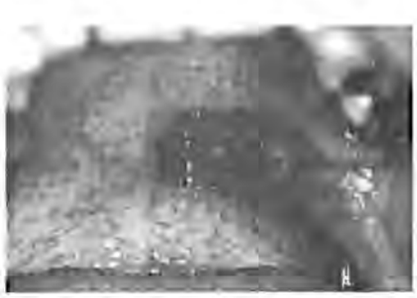

Fig. 2. Erythroplakia on the soft palate.

Leukoplakias may vary in appearance from homogeneous to nodular (Fig. 3), or may be found in various combinations with erythroplakia. Although tobacco use increases the risk for oral cancer, it has been found that patients with oral leukoplakia who are non-smokers, have a higher risk of malignant change. This is difficult to explain, but it is speculated that it may be associated with an inherited genetic susceptibility.

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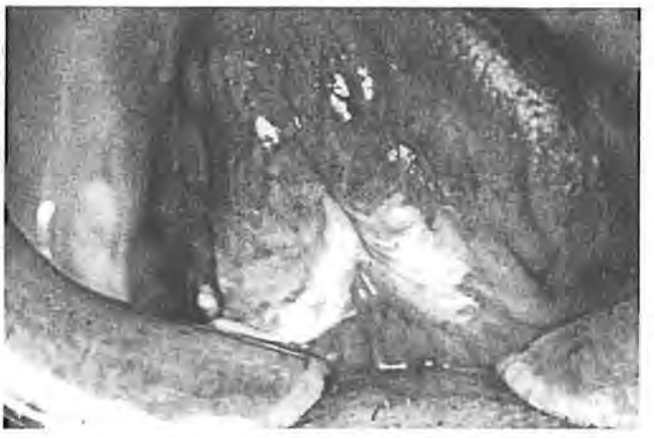

Fig. 3. Irregular/nodular leukoplakia on the ventral aspect of the tongue.

These premalignant lesions are usually painless and the majority of patients are unaware of their presence. It is therefore of the utmost importance to perform regular examinations of the oral cavity in especially high-risk patients. Screening can be made more efficient by inspecting the high-risk sites where 90% of all oral cancers arise: the floor of mouth, the ventrolateral aspect of the tongue and the soft palate complex.

Premalignant conditions are defined as generalised states associated with a significantly increased risk of cancer. The majority of these conditions are associated with atrophy of the oral epithelium. These include sideropenic dysphagia, oral submucous fibrosis and the still-controversial erosive oral lichen planus.

Early detection of cancerous lesions

Early cancerous lesions are usually painless and may appear as small, apparently harmless, areas of induration, erosion or keratosis, often deceiving the unsuspecting clinician into a false sense of security (Fig. 4). It may also present with the clinical features of a premalignant lesion (leukoplakia or erythroplakia). Patients usually seek consultation only after developing persistent pain, most commonly from a non-specific ulcer or irritation in the mouth.

The advanced oral cancerous lesion usually consists of a painless ulcer with indurated rolled margins. In most cases the ulcer has a characteristic appearance, which may serve as a diagnostic aid[7] (Fig. 5). It is fixed to underlying tissues and regional lymphadenopathy may be seen.

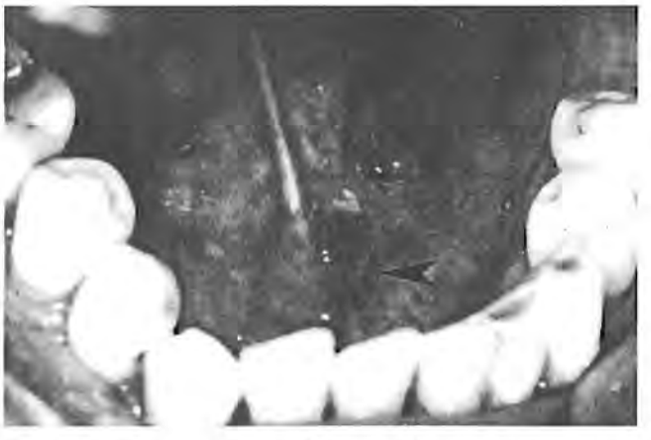

Fig. 4. Small leukoplakia on the floor of mouth. Biopsy (site indicated by arrow) showed an early invasive squamous cell carcinoma.

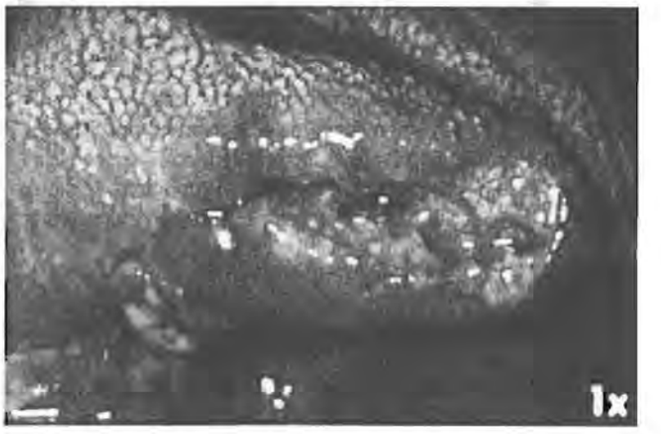

Fig. 5. An advanced oral squamous cell carcinoma with indurated rolled margins.

Management principles

The clinical significance of premalignant lesions is that all must be biopsied to determine the possible presence of malignant change. The malignant transformation rate of homogeneous leukoplakia is about 6%, while the rate for proliferative verrucous leukoplakia is reported to be as high as 70%.[8] The histological diagnosis of erythroplakia is usually either a severe epithelial dysplasia or already a squamous cell carcinoma. This higher risk associated with the red lesions implies that in the case of a combination lesion (erythroleukoplakia), the red areas should always be included in the biopsy site (Fig. 6).

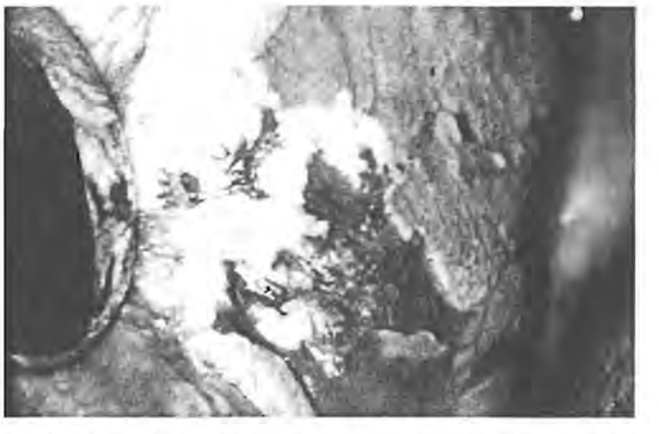

Fig. 6. Erythroleukoplakia on the buccal mucosa. The biopsy should always include a red area.

Any suspicious-looking oral mucosal lesion, including any ulcer not healing within two weeks after conservative treatment, must be biopsied. The biopsy should be sufficiently large to include enough abnormal and clinically normal tissue, enabling the pathologist to make a diagnosis without requesting additional material. The applicable biopsy techniques include an excision biopsy, incision biopsy or cytological curettage.

Excision biopsy

The excision biopsy is used to remove the entire lesion, together with normal tissue (Fig. 7). This procedure must be devised in such a way that it becomes a curative intervention. The lesion must therefore be eliminated in its entirety, i.e. with its incised borders and normal tissue. Where there is a possibility of the presence of a malignant neoplasm, an additional border of at least 2 - 3 mm healthy tissue also 2 - 3 mm deep, must be removed for histological examination. Excision biopsies are mostly used where the clinical diagnosis suspects the presence of benign conditions. It should only be used when the lesion is very small, since it is unlikely that the additional margin removed, will be wide enough if the lesion is found to be malignant. It may also destroy clinical evidence of the site and character of the lesion, which are important facts for the surgeon or radiotherapist.

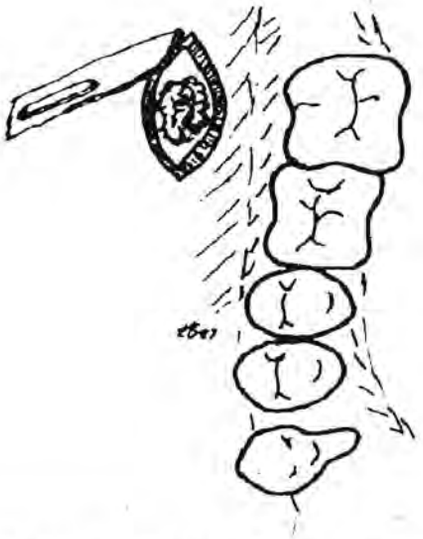

Fig. 7. Schematic drawing of an excision biopsy.

Incision biopsy

With this biopsy technique, only a small portion of the lesion, together with a portion of the surrounding normal tissue, is removed (Fig. 8). This part should be incised in such a way that approximately half of the biopsy material is situated in tumour tissue and the other half in normal tissue. The incision biopsy is used for most larger benign, as well as malignant conditions.

The surgical technique

- Anaesthetise without injecting directly into the lesion.
- Select a representative specimen of all the tissues that appear to be pathological, and also the parts that appear to be normal. However, be aware of surrounding anatomical structures.
- Disinfect the region thoroughly with minimum disturbance of the lesion; avoid disinfecting materials with colouring agents.
- Avoid areas where excessive bleeding and possible necrosis might occur.
- When performing a skin biopsy, always incise according to the Langer lines.

Cytological curettage

The suspected epithelium and/or ulcer must be curetted by scraping the area and placing the material received on a glass slide. The curetted cells must be fixed and then sent for histopathologic examination. It must also be remembered that there is a possibility

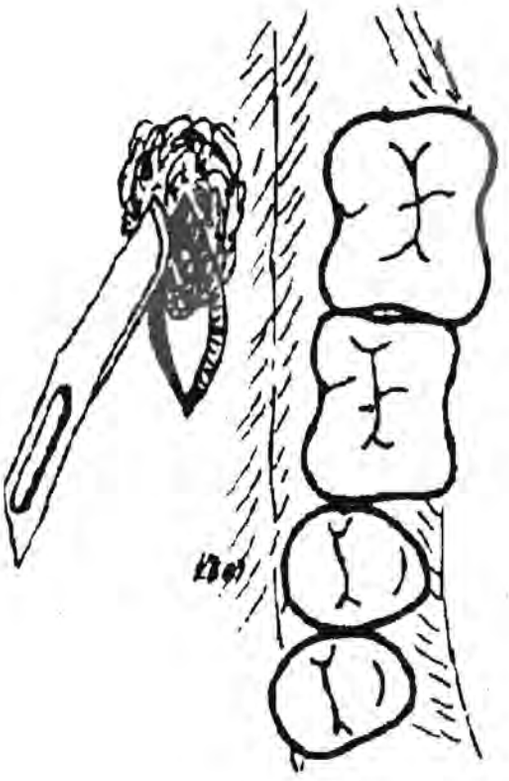

Fig. 8. Schematic drawing of an incision biopsy.

that a false negative result could be reported. This method is only used for erythroplakias or ulcerated tumours where taking a biopsy is complicated or contra-indicated. It should never be used on leukoplakias.

Conclusion

We as dentists should not be seen as only caretakers of teeth and periodontal tissues in need of preservation or repair, but as true health care professionals, caring for the total health of our patients. A first important step will be to fulfil our responsibility towards the prevention and early diagnosis of oral cancer. We can begin this task by the meticulous examination of the oral mucosa for the existence of premalignant lesions or early carcinomas, particularly of most vulnerable areas (floor of mouth, ventrolateral aspect of the tongue and soft palate complex) in especially high-risk patients (all over the age of 40 and all smokers).

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Langerhans Cells and Human Papillomaviruses in Oesophageal and Laryngeal Carcinomas

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Abstract. *Human papillomavirus (HPV) infection is implicated in squamous cell carcinogenesis. Oesophageal carcinoma has a high incidence in certain geographical regions and, using different methods of detection, HPVs have been found in these tumours. HPV 6 and 11 are frequently detected in laryngeal papillomas, benign lesions which rarely become malignant. HPVs have been detected in squamous carcinoma of the larynx but more frequently, especially HPV 16, in verrucous carcinomas a distinct variant of squamous carcinoma. An increased density of Langerhans cells, important in immunosurveillance in squamous epithelium, has been associated with a more favourable patient prognosis in laryngeal and other carcinomas.*

Langerhans cells (LCs) are bone marrow derived dendritic cells (1) located in stratified squamous epithelium, dermal connective tissue, dermal lymphatic channels and lymph nodes (2). LCs and their immature precursor cells are also defined as T-zone histiocytes (3) but differ from the monocyte-macrophage series by their lack of nonspecific cross-reacting antigen and lysozyme. Functionally LCs are antigen presenting cells (4) and are ideally located in squamous epithelium for a role in immunosurveillance.

Epidermal carcinomas (basal cell and squamous cell types), in contrast to other malignancies, generally have a limited tendency to metastasise and early diagnosis and treatment results in a good prognosis. This less aggressive behaviour raises the question as to whether local mechanisms such as LCs and lymphocytes play a protective role in inhibiting the spread of neoplastic cells. It has been demonstrated that an increase in the number of LCs can be correlated with a more favourable prognosis in cancers of the nasopharynx (5), oral cavity (6), stomach (7), lung (8), thyroid (9) and in T-cell lymphomas (10).

Various risk factors have been identified which may be important in the development of head and neck squamous carcinomas *eg.* smoking, alcohol intake (11,12), multiple micronutrient deficiencies (13), poor oral hygiene (14) and infection with human papillomaviruses (HPVs) (15). HPVs have a predilection for squamous epithelium where they induce benign proliferative lesions such as warts on the skin, and papillomas and condylomas on mucosal surfaces (16). HPV infection, especially HPV 16, 18 and 33, has been implicated in squamous cell carcinogenesis because of its ability to immortalise human epithelial cells after transfection (17, 18). In tumours viral DNA is frequently integrated into the cellular genome. This integration interrupts certain open reading frames (ORFs) of the viral genome with a loss of DNA sequences. Two ORFs, E6 and E7, however, are consistently retained and expressed in tumours and tumour cell lines and are considered to be important in the development and maintenance of the malignant phenotype (19). Viral integration can either activate or inactivate cellular genes, resulting in a failure of host-cell control of persisting viral genes (20). HPV 16 and 18 have been found integrated in the vicinity of c-*myc* resulting in elevated levels of c-*myc* RNA (21), but the viral integration sites vary considerably so that c-*myc* activation cannot be regarded as a consistent mechanism.

HPV 16 is the type most frequently detected in anogenital carcinomas. HPV DNA is present in up to 90% of cervical carcinomas (22) and has also been found in cervical metastatic lymph node lesions (23). Several cervical carcinoma cell lines harbour integrated HPV sequences, *e.g.* Hela, SiHa, CaSki, C-41, MS 751, and ME 180 (24-28). HPV 5, and less frequently HPV 8, 14, 17, 20 and 47 can be found in skin carcinomas on sun exposed areas in patients with epidermodysplasia verruciformis (EV), a rare genetic disorder (29-31). In immunosuppressed patients there is a significant increase in cutaneous and mucosal malignancies. HPV 5 or HPV 5-related HPVs were characterised in skin

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carcinomas of immunosuppressed patients (32), while HPV 16/18 DNA is more frequently detected in cervical intraepithelial neoplasia in women with renal allografts than in controls (33). HPVs have also been detected in carcinomas of the tongue (34) and lung (35-37).

Oesophageal carcinoma shows a striking geograpical distribution with a remarkably high incidence in certain regions of China, (14) Iran (38) and South Africa (39). Oesophageal cancer also occurs three times more often in blacks than in whites in the USA. The incidence and mortality from this tumour has increased rapidly over the past 25 years (40), a trend also observed in South Africa (29). A well described animal model exists for the development of oesophageal carcinoma. In cattle bovine papillomavirus causes alimentary papillomas, which undergo malignant change when the animals feed on bracken fern *(Pteridium aquilinum)* (41). Various studies aimed at establishing the importance of HPV infection in human oesophageal carcinoma have been performed. Two investigations carried out in South Africa described HPV epithelial changes adjacent to carcinomas in 30% (42) and 65% (43) of cases respectively. HPV antigen could be demonstrated in Japanese and Chinese patients using immunocytochemistry in carcinomatous (13%; 19%) as well as in adjacent uninvolved epithelium (13%;23%) (44). HPV sequences were detected in 22/51 (43,1%) oesophageal squamous carcinomas from China using *in situ* hybridisation (45). In a South African study, polymerase chain reaction (PCR) was used on 14 carcinomas with a 71% positivity rate for HPV DNA (46). The latter study also included a control group where HPV was detected in 6/41 (15%) normal oesophageal mucosal specimens. In an investigation involving more control groups (47), 5/12 oesophageal carcinoma patients had HPV infection while none of 17 controls exposed to alcohol and tobacco, and only one out of seven non-exposed controls had HPV oesophageal infection. Negative results were obtained by Loke *et al* on 37 oesophageal carcinomas from Hong Kong applying DNA slot blot analysis and *in situ* hybridisation (48). Using PCR, Kijabu *et al* (49) evaluated 13 carcinomas from California but no HPV could be detected.

The extraordinary variation in the geographical localisation of oesophageal squamous carcinoma and HPV infection suggests that other environmental factors are important, *e.g.* fungal infestation of corn, ingested opium residues, alcohol and tobacco, vitamin and trace element deficiencies (13,14). One factor all high-risk areas have in common is the low socio-economic status of the population. This provides a basis for different carcinogens possibly involved in the multi-step process of tumour development. Morris and Pierce postulated that the primary pathway to oesophageal carcinoma consists of an aberration in the relationship between LCs and the keratinocytes secondary to a persistent HPV infection (50). This may then lead to neoplastic transformation when the mucosa is exposed to one or more co-carcinogenic factors (50).

The prognosis of patients with oesophageal cancer is poor and is dependent on the size of the tumour, histologic type, depth of penetration, lymph node involvement, intraepithelial spread, DNA distribution pattern (51,52) and LC infiltration (53). Patients with a marked infiltration of LCs survived longer than those where infiltration was slight (53). The density of LCs can therefore serve as an indication of host defence against the carcinoma.

Laryngeal carcinoma was traditionally a disease which predominantly affected men in the sixth and seventh decades of life and who also had a history of excessive smoking and alcohol abuse (54). However, in the past decade laryngeal carcinoma appears to have increased in younger patients and women (55).

HPV 6 and 11 DNA is regularly found in juvenile and adult onset laryngeal papillomas (56,57). These tumours rarely convert into malignant neoplasms, except when associated with irradiation treatment (58) or in severe papillomatosis of long standing duration with signs of spread throughout the respiratory tract (59,60). Attempts to evaluate the role of HPV in laryngeal squamous carcinogenesis led to the cloning and characterization of HPV 30 from an invasive laryngeal carcinoma (61). HPV 16 or 16-related sequences have been detected in all verrucous laryngeal carcinomas investigated to date (62-64) and Scheurlen *et al* found integrated and episomal HPV 16 genome in one of 36 laryngeal carcinomas (65). In a series of 116 laryngeal squamous carcinomas from Finland, analysed by *in situ* hybridisation, 13% were found to be positive for HPV DNA (66). In a South African study we detected HPV 7 in one of 10 laryngeal carcinomas using *in situ* hybridisation (67). Results from PCR studies varies from 75% (16 tumours) (68) to 90% (30 tumours) (69), while Dickens *et al* (70) could not detect DNA in 16 nasopharyngeal carcinomas from Hong Kong. Bradford *et al* (71) evaluated 22 cell lines from head and neck squamous carcinomas using Southern blot hybridisation and detected HPV-specific signals in two of 22 cell lines. One was HPV 31 positive and the other hybridised with both HPV type 18 and 31. Five tumour cell lines were further tested with PCR, of which two were positive: one for HPV 16 and the other for HPV 52. A much lower PCR positivity was reported by Ogura *et al* (72). In one of 11 pharyngeal carcinomas (9%) and three of 28 laryngeal carcinomas (11%), both HPV 16 and 18 were detected.

Prognostic studies have been done involving LCs. Intratumoral and peritumoral infiltration of LC was investigated in 88 patients with laryngeal squamous carcinoma (73). Patients with high or intermediate density of LC had a better survival rate than those with a low density of LCs. Another study evaluating 49 specimens of nasopharyngeal carcinoma also showed an increased survival rate for patients with dense infiltration of T-zone histiocytes compared to those without such infiltration (5).

A study done on cervical intra-epithelial neoplasia to investigate the relation between HPV subtypes and LCs revealed an apparent decrease of LCs with moderate to high

copy numbers of HPV 16. The reduction was even more significant for HPV 18 even at low copy numbers (74). The depletion of LCs may be a function of both HPV subtypes and copy number. It may be possible that a progressive immunological failure on the afferent limb of the T cell response results from the increased presence of a particular HPV subtype. Alternatively, a local immunodeficiency may precede HPV infection, facilitating the infection and subsequent cell transformation by viral gene products.

Unfortunately, no studies have so far been conducted on the presence of specific HPV subtypes and numbers of LCs in oesophageal and laryngeal carcinomas. In one investigation of LC counts in a few laryngeal and oesophageal carcinomas, no significant differences between HPV positive and negative specimens were revealed (van Rensburg, unpublished results).

LCs have also been shown to decrease in the uterine cervix of smokers, possibly indicating reduced immuno-surveillance and increased risk of malignant change (75). A study comparing LCs numbers in lingual epithelium of heavy smokers found a significantly higher count than in moderate and non-smokers. No differences in LC numbers were noted in relation to alcohol consumption, age and sex (76). The mechanisms of local immune regulation in oral and cervical epithelium appear to be different since it has been hypothesised that tobacco compromises the local immune defences in female smokers causing a reduction in number of local LCs. Smoking, however, is associated with an increased incidence of squamous cell carcinoma at both anatomical locations.

Both HPV and LCs appear to be implicated in the development and behaviour of oesophageal and laryngeal carcinomas. The possibility of an interdependent relationship between HPV and LCs in carcinogenesis needs to be investigated further.

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Detection of human papillomavirus DNA with *in situ* hybridisation in oral squamous carcinoma in a rural black population

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Intra-oral carcinoma is the third most common malignancy among men in developing countries, and carries a high mortality rate, particularly in Africa, where patients often present initially with lesions at an advanced stage. The present study was undertaken to determine the prevalence of human papillomavirus (HPV) DNA in oral squamous carcinoma in the west of the Northern Transvaal, an area where a large number of new cases has been diagnosed over the past few years. Paraffin blocks from 66 cases (51 men, 15 women; mean age 58,7 years) of oral squamous carcinoma were randomly selected. Blocks contained samples of both tumour and adjacent normal epithelium. The presence of HPV antigen was established by means of immunocytochemistry and HPV DNA by *in situ* hybridisation with radiolabelled probes for HPV-6, 11, 16 and 18. Immunocytochemistry for viral antigen was negative in all the specimens. HPV-18 was detected in normal epithelium adjacent to the tumour in one case only. It appears from our study that HPV is of limited importance in oral squamous cell carcinogenesis in the population studied.

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Human papillomaviruses (HPVs) have a predilection for squamous epithelium where they induce benign proliferative lesions such as warts on the skin, and papillomas and condylomas on mucosal surfaces.[1] Certain types, especially HPV-16, 18 and 33, have been implicated in squamous cell carcinogenesis because of their ability to immortalise human epithelial cells after transfection.[2,3] HPV-16 and 18 are strongly associated with high-grade premalignant lesions and anogenital carcinomas.[4] Oral HPV infections have not been studied to the same degree as those of the genital tract, but are known to be associated with a variety of oral lesions in man, including papillomas, focal epithelial hyperplasia, hyperkeratotic lesions, lichen planus and leukoplakia.[5] In view of the obvious oncogenic potential of some HPVs and the close similarity between the oral and genital mucosa, the possibility that certain HPV types may play a causative role in oral cancer does not seem too remote. Certain HPV types have been detected in benign oral lesions, as well as in oral squamous carcinoma.[6]

Studies originating from different geographical areas show a variation in HPV positivity ranging from 0% to 76,4%.[7-11] This difference in positivity is due to different populations studied and different methods used in HPV DNA detection.

According to the Federation Dentaire International, intra-oral cancer, of which squamous cell carcinoma accounts for the majority of cases, is the third most common malignant disease among men in developing countries.[12] The mortality rate remains unacceptably high, particularly in Africa, where advanced lesions are common on presentation. Although oral squamous carcinoma rarely occurs before the age of 50 years, Fleming *et al.*[13] found a significant difference in age distribution between black and white South African men: 33,4% of the blacks were below the age of 50 years, as opposed to 15,6% of white men. The authors suggest that the black group may have been exposed to carcinogenic agents at an earlier age than the white group.

Our study was undertaken to determine the prevalence of HPV DNA in oral squamous carcinoma at Ga-Rankuwa Hospital, a referral centre for the western Northern Transvaal, an area in which a high number of new cases has been diagnosed over the past few years.

Material and methods

Formalin-fixed paraffin embedded blocks from 66 patients (51 men, 15 women, mean age 58,7 years) with oral squamous carcinoma were randomly selected from the archives of the Department of Oral Pathology at MEDUNSA. These blocks contained samples of both tumour and adjacent normal epithelium. Sections 5 µm thick were cut and used for light microscopy and *in situ* hybridisation. All sections were evaluated for epithelial changes consistent with HPV infection. These include verrucous hyperplasia with hyperparakeratosis and the presence of koilocytes.

Immunocytochemistry

All sections were examined for the presence of HPV structural proteins (group-specific papillomavirus capsid antigens) with an ABC immunoperoxidase kit (Lipshaw Corporation, Detroit).

HPV DNA in situ *hybridisation (ISH)*

All sections were mounted on 3-aminopropyltriethoxysilane-coated slides,[14] deparaffinised and rehydrated by sequential

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immersion into xylene and ethanol and digested with proteinase K (0,05 mg/ml) to expose the fixed target DNA. After post-fixation in 4% paraformaldehyde, slides were prehybridised for 30 minutes at 52°C, prior to the application of the denatured probe solution. DNA probes for HPV-6, 11, 16 and 18, cloned in pBR322, were used. These were kindly provided by Dr E.-M. de Villiers of the Human Papillomavirus Reference Center, DKFZ, Heidelberg, Germany. The probes were labelled with ^{32}p dCTP according to the multiprime DNA labelling system (Amersham, UK). Hybridisation was allowed to take place at 52°C for 16 hours in a humidified chamber. There were two post-hybridisation washes in a 2 x SSPE/50% formamide solution and one in 50% formamide, 0,1% SDS, 2 x SSC; each wash lasted 1 hour at 37°C. Slides were dehydrated through graded ethanol containing 0,3M NH acetate and then dipped in LM-1 emulsion (Amersham, UK). After a 4-day exposure time at 4°C, slides were developed (Ilford, Ciba Geigy), rinsed briefly in water and fixed for 5 minutes in Hypam fixative (Ilford). Sections were counterstained with haematoxylin and eosin before mounting.

Assay sensitivity was confirmed with two known positive control slides (one of cervical intra-epithelial neoplasia and the second, a carcinoma of the vulva positive for HPV-6 and 16 respectively). Assay specificity was confirmed by hybridisation of plasmid vector pBR322 on all sections investigated.

Results

In none of the biopsies were HPV-associated morphological changes found in the normal epithelium adjacent to the carcinoma. HPV-antigen expression could also not be demonstrated by means of the ABC immunoperoxidase technique in any of the tumour sections or in the adjacent normal epithelium.

HPV-18 DNA was detected by *in situ* hybridisation in the normal epithelium adjacent to the carcinoma in a single case (Fig. 1).

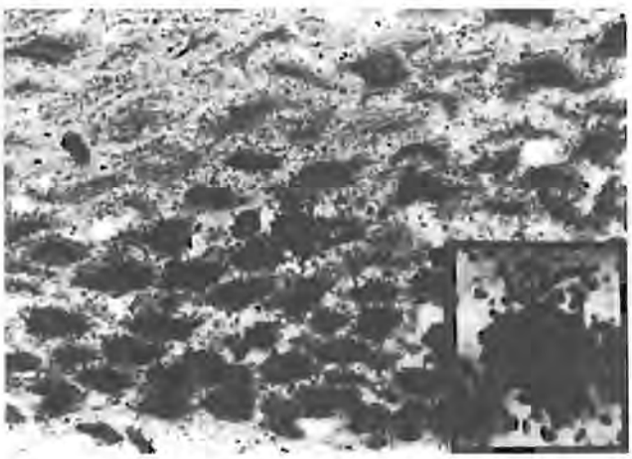

Fig. 1. Micrograph showing the presence of HPV-18 DNA as condensations of black-silver grains superimposed on the nucleus (x 200), with inset of high-power detail of positive cell (x 400).

Discussion

The oral mucosa is continuously exposed to minor trauma, micro-organisms and chemical factors such as tobacco and alcohol, which may act synergistically with HPV, leading to the development of carcinoma.[15,16]

This study determined the prevalence of HPV infection in tumours and adjacent normal epithelium in cases of oral squamous carcinoma. The detection system used was selected because *in situ* hybridisation is useful for retrospective biopsy screening, as it permits detection, typing and localisation of HPV DNA in paraffin-embedded tissues that are readily available in a department of pathology. Radiolabelled probes, instead of biotinylated ones, were used because of their superior sensitivity.[9]

Viral antigens were not demonstrated in any of our cases by immunocytochemistry. The use of immunocytochemical methods is seriously limited by the fact that HPV antigens are only expressed in productive infections. Therefore, false-negative results may be obtained in infections where viral structural proteins are not likely to be expressed, as in cancers, because viral expression is inversely related to the degree of neoplasia.[17]

HPV-18 DNA was found in the normal oral mucosa adjacent to the tumour in only 1 of the 66 cases investigated. Other studies of oral carcinoma found a different pattern with HPV positivity within the tumour but a lower detection rate in the adjacent normal mucosa.[11,18,19]

HPV detection in oral carcinoma in studies from different geographical regions show a great variation. An American study evaluating 10 squamous carcinomas detected HPV in a single case with ISH and polymerase chain reaction (PCR),[7] while two other American studies could not detect HPV in any of their specimens with ISH alone.[8,9] Tsuchiya *et al.*[10] from Japan detected three positives from 30 samples using ISH while 76,4% of oral carcinomas in a Taiwan study were positive for HPV-16 according to the Southern blot technique.[11] In the last study mentioned, a high incidence of betel quid chewing and smoking was found in the patients studied.

The low prevalence of HPV in our collection can be explained in several ways: (*i*) the lesions studied may not have a viral aetiology; (*ii*) the possibility in the lesions studied of the occurrence of HPV genomic sequences other than the ones used by us as probes, cannot be ruled out; (*iii*) transformed cells may contain altered viral DNA not detectable by the probes used; and (*iv*) the method is not sensitive enough to detect low viral copy numbers. The ISH technique is highly sensitive in cases where individual nuclei contain a high copy number of the target DNA but often fails to detect cases in which subgenomic fragments of the viral DNA have been incorporated into the host genome.[7,20] The extremely sensitive PCR may demonstrate a higher positivity rate than ISH. In a study by Watts *et al.*,[21] ISH detected HPV in 60% of the oral squamous carcinoma cases examined while PCR could detect it in 90%. The same degree of increased sensitivity was not found in two other studies where results show a high degree of correlation between the two methods used.[7,19] Although we did not use PCR in the present study, we feel that the outcome would not have been significantly different, given that only 1 case was found positive by means of ISH.

The present study, in which 66 cases of oral squamous cell carcinoma were reviewed retrospectively, is the largest series to have been investigated so far. It appears that in these cases of squamous cell carcinoma, HPV-associated oral infection occurs only rarely in the black population of the western Northern Transvaal.

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Assessment of quality of life by clinicians — experience of a practical method in lung cancer patients

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Objective. To evaluate a practical method (brief scale) of assessing the quality of life in patients with lung cancer.

Design. To compare the scores obtained by means of the brief scale with those obtained on formal tests. The brief scale consists of an Outlook score (measuring psychological status) and a Support score (measuring psychosocial support). The formal tests were the Hospital Anxiety and Depression Scale (HADS) for psychological status, and the Rotterdam Symptom Checklist (RSCL) and Spitzer QL-Index for quality-of-life assessment.

Setting. Lung cancer follow-up clinic, Groote Schuur Hospital.

Participants. A total of 40 patients selected by random sample.

Main outcome measures. The correlation between the brief scale and standard formal tests.

Results. The HADS indicated that psychological morbidity was present in 30% of patients. Both the RSCL and the Spitzer QL-Index indicated a significantly poor quality of life in 25% of patients. The Outlook score correlated with both psychological status and quality of life. The Support score correlated with psychological status but not with the assessment of quality of life. It did, however, correlate with the independent evaluation of social support in the Spitzer QL-Index.

Conclusions. The brief scale is a cost-effective and useful tool for quality of life assessment in the clinical management of patients with lung and other cancers.

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The practice of medicine is concerned not only with prolongation of the survival of patients, but also with their quality of life. However, although subjective assessment of the factors affecting quality of life may form part of the clinician's management of a patient with lung cancer, it may

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Human Papillomavirus DNA in Oral Squamous Cell Carcinomas from an African Population Sample

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Abstract. *Background. The incidence of oral squamous cell carcinoma (OSCC) is on the increase in developing countries. Materials and Methods. Formalin fixed paraffin embedded blocks of OSCCs from a Black South African population sample of peri-urban and rural origin were selected as follows: Group 1 - 57 OSCCs with a mean age of 59 years; Group 2 - 43 OSCCs all cases younger than 40 years; Group 3 - 46 OSCCs with blocks containing only tumour tissue without any normal epithelium and Group 4, a control group of 38 non-neoplastic epithelial lesions. Type specific primers were used in a standard PCR to amplify a segment of the E6 region of HPV 6, 11, 16 and 18. Results. HPV 11 and 16 DNA were found in one sample each from groups 1 and 2 respectively. Conclusion. HPV is not an etiologic factor in the development of OSCC in the population studied.*

The incidence of oral squamous cell carcinoma (OSCC), the most common malignancy of the oral cavity, is on the increase globally, especially in developing countries (1). OSCC varies from a low prevalence of 1-2% of all cancers in Japan and Western Europe to more than 45% in parts of Asia, implying major geographical differences in risk factors (1). The age standardised incidence rates per 100 000 for OSCC in the Black population in South Africa are 8.84 for males and 1.91 for females as obtained from the National Cancer Registry of 1989 (2). OSCC is the fourth most common malignancy among black males (2). OSCC of the oral cavity has traditionally been attributed to use of tobacco and alcohol (3), but recent work suggests that viral factors such as the human papillomaviruses (HPVs) may contribute to the etiology of these malignant neoplasms (4).

The association of HPV infection with the development of anogenital squamous carcinomas has been firmly established (5). Although the role of HPV in the etiology of OSCC remains speculative, most of the major criteria necessary for it to be considered oncogenic, are met for example; the integration of the viral genome into the cellular genome; the *in vitro* transformation and immortalisation of cells by HPV and the detection of RNA transcripts of the principal transforming genes E6 and E7 in some squamous cell neoplasms (6). In view of the evidence for HPV association in malignant neoplasia of squamous epithelial origin, we investigated the possibility that these viruses might also contribute etiologically to oral squamous cell carcinogenesis in a group of Black Africans from a rural and peri-urban origin from the North Western part of South Africa.

Materials and Methods

Patients. Formalin fixed paraffin embedded blocks of OSCC were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa, (Medunsa). The sections were screened to confirm the diagnoses, whereafter 146 blocks were selected and divided into 3 groups. Group 1: Fifty-seven blocks containing OSCC with a fragment of normal appearing overlying or adjacent epithelium. The mean age of this group was 58.8 ± 15.4 years. Forty-six were male. Group 2: Forty-three blocks containing OSCC tissue with a fragment of normal appearing overlying or adjacent epithelium from patients 40 years of age or younger. The mean age of the group was 32.4 ± 8.7 years. Thirty-two were male. Group 3: Forty-six blocks containing OSCC tissue only with no normal appearing epithelium in the block. The mean age of this group was 55.2 ± 7.1 years and consisted of 36 males. Group 4: A control group consisting of 38 non-neoplastic intraoral lesions were included. These were non-viral associated lesions, the majority being

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fibrous epuli and fibro-epithelial polyps. The epithelium in these cases was normal.

Preparation of cell lysates. Two 10 µm sections were cut from each block, using a new disposable blade after each block. To evaluate the possibility of contamination at this stage, 10 µm sections of a block containing normal brain tissue were cut after every five tumour blocks and also included for evaluation. All sections were placed in separate microfuge tubes. Cell lysates were prepared as follows: sections were dewaxed, washed with ethanol and digested with 400 µl of lysis buffer containing proteinase K (7). The samples were then heated to inactivate the proteinase K, centrifuged to pellet the debris and stored at -20°C until use. Ten µl of the supernatants were analysed by polymerase chain reaction (PCR).

PCR. Type specific primers were used to amplify a segment of the E6 open reading frame (ORF) of HPV 6, 11, 16 and 18 (8). The primers amplified 134-239 base pair regions of the E6 ORFs (Table I). Thermal cycling was carried out using a Techne PHC-2 water cooled machine. The hot start method was done before adding enzyme (9). Forty amplification cycles were used: denaturing of DNA (94°C; 1,5 minutes), annealing of primers (45°C, 1,5 minutes) and extension of the annealed primers (72°C, 3 minutes). Reaction mixtures for the amplification contained: 10 µl of cell lysate; 200 µM of each nucleotide; 0,4 µM of each primer; 1,5 mM $MgCl_2$; 1 U of Taq DNA polymerase (Promega Corporation, Madison WI, USA); 10 µl of Promega 10 × buffer and made up to a final volume of 100 µl. Each reaction mixture was covered with two drops of mineral oil.

Reagent controls contained sterile water instead of template DNA. To standardise our PCR reactions, plasmids containing HPV 6b, 11, 16 and 18 genomes (kindly supplied by E-M de Villiers, Deutsches Krebsforschungzentrum, Heidelberg) were used as positive controls. To obtain a quantitative estimate of the sensitivity of our PCR method, we made 10-fold serial dilutions of the four plasmids in 0,3 µg/µl salmon sperm DNA. Dilutions containing 1, 10, 100 and a 1000 copies of plasmid/µl were included in each run. After the last round of amplification 10 µl of the reaction was electrophoresed on ethidium bromide stained agarose gels and evaluated under ultraviolet light. Before HPV amplifications were done, all the specimens were subjected to amplification using human ß-globin primers (10) to evaluate their suitability for DNA amplification.

Southern blot hybridisation. Southern blotting was used to confirm the PCR results. Type specific oligoprobes as listed in Table I were used and labelled using the DIG DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Hybridisation was carried out at 55°C overnight for HPV 6, 11 and 16 and at 45°C for HPV 18 in 6 × SSC, 5 × Denhardt's solution, 0,2% SDS and 100 µg/ml salmon sperm DNA. The blots were washed at high stringency using 2 × SSC plus 0,1% SDS for 5 minutes at 55°C, then twice with 0,2 × SSC and 0,1% SDS at 55°C first for 5 and then for 15 minutes. The hybridisation signal was detected with the DIG DNA detection kit (Boehringer Mannheim, Mannheim, Germany). ß-globin PCR results were hybridised using the dot blot method (10).

Table I. *Oligonucleotide primers and probes for PCR with HPVs.*

	Nucleotide sequence	Amplimer length (base pair)
	Primer	
HPV 6-1	CACCTAAAGGTCCTGTTTCG	
HPV 6-2	CGGTTTGTGACACAGGTAGC	183
HPV 11-1	GTTGCTTAGAACTGCAAGGG	
HPV 11-2	CGGCTTGTGACACAGGTAAC	134
HPV 16-1	ACAGTTACTGCGACGTGAGG	
HPV 16-2	TTTGTTCAGGACACAGTGGC	239
HPV 18-1	TATACCGCATGCTGCATGCC	
HPV 18-2	ACGGTTTCTGGCACCGCAGG	157
	Probe	
HPV 6	AGGCGGCTATCCATATGCAG	
HPV 11	GCTGCATATGCACCTACAGT	
HPV 16	GAGATGGGAATCCATATGCT	
HPV 18	TTCAGACTCTGTGTATGGAG	

Note. PCR, polymerase chain reaction; HPV, human papillomavirus.

Results

All the samples were amplified with the ß-globin primers (results not shown). All the brain samples, placed randomly between the study samples as well as the reagent controls, were negative on PCR. The sensitivity of each type specific PCR was such that one copy of plasmid DNA could be detected in each reaction (Figure 1). HPV 6 and 18 were not demonstrated in any of the samples tested, HPV 11 DNA was found in one sample from group 1 (1.8%) and HPV 16 DNA in one sample from group 2 (2.3%). Thus a total of 2 samples out of 146 cases (groups A-C) tested positive (1.4%). Figures 1 and 2 show the gel electrophoresis and dot blot hybridisation results respectively. No HPV DNA could be detected in any of the control group samples.

Discussion

The mucosal epithelium of the oral cavity is histologically and embryologically similar to that of the genital tract and is continuously exposed to various environmental factors such as irritants and micro-organisms. Thus it is likely that there are parallels in the oral cavity with respect to the capacity of HPV to participate in malignant transformation.

A prevalence of 1.4% was found in our study when we added up the positives of all three groups that we investigated. This is the most extensive study to date, but also the PCR study with the lowest prevalence rate so far. Other studies evaluating OSCC using PCR, reported a HPV DNA prevalence from 8 to 90% (Table II). The inconsistencies in the results of the different studies make it difficult to interpret the relevance of HPV in the development of oral squamous carcinomas. The differences might be attributed to several factors: (i) Various groups used PCR primers designed to amplify different regions of the HPV genome. (ii) Certain authors used only one block of tumour tissue for each patient, whereas at least two blocks of tissue were studied by Woods *et al* (18) in the majority of their patients, and they achieved a 78% positivety. (iii) In our study we could detect one copy of control plasmid DNA but the sensitivity of the PCRs used in other studies were not quantified by their

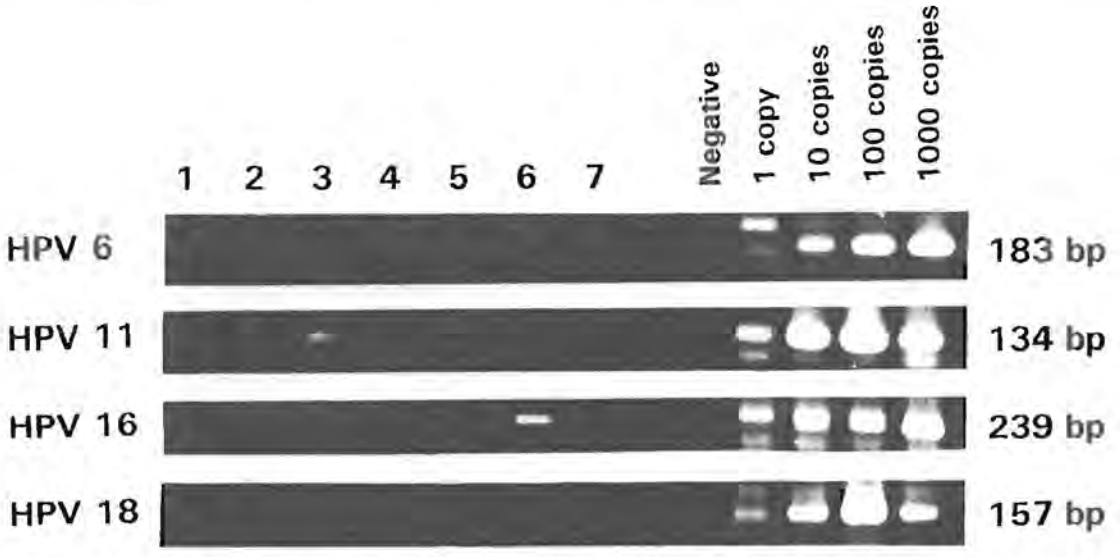

Figure 1. *Gel electrophoresis. Numbers 1-7 represent seven patients selected from groups 1-3. Patients 3 and 6 originated from groups 1 and 2 respectively. The negative reagent control included for each HPV type and the plasmid controls containing 1, 10, 100 and 1000 copies are indicated. The sizes of the PCR fragments are indicated on the right hand side.*

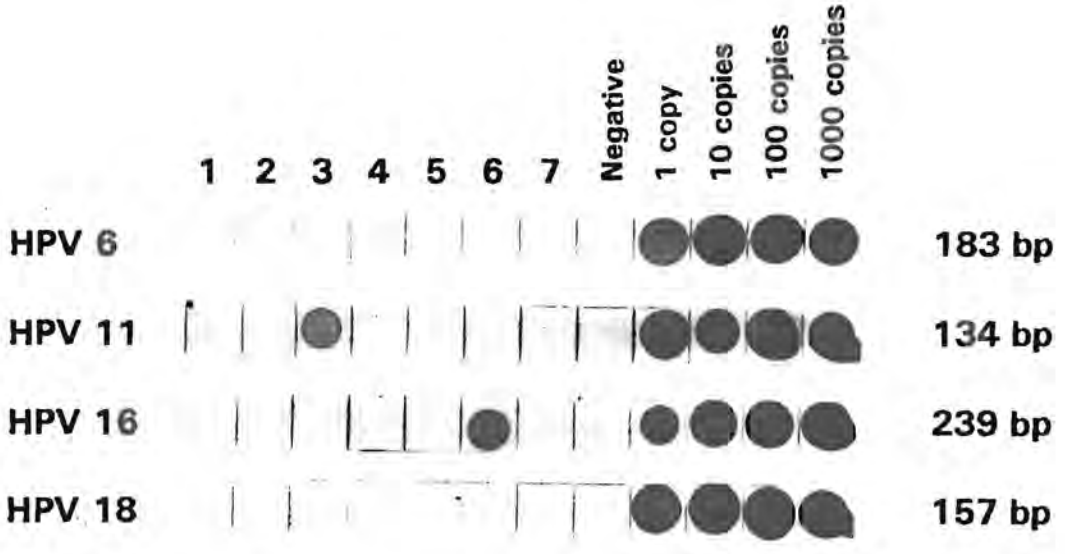

Figure 2. *Dot blot hybridisation. Numbers 1-7 represent the patients selected, as in figure 1. Negative controls, positive controls and PCR fragment sizes are shown.*

authors. (iv) Geographic locations have influenced the prevalence among different regions tested (13, 15, 16, 20,21). Ethnic differences among population groups are essentially contained within geographical areas. This is the first study conducted on a mainly rural and peri-urban African population and may be an important contributing factor to the low prevalence rate.

This study also investigated whether age had a significant bearing on the prevalence of HPV DNA. OSCC occurs over a wide age range with a peak in the sixth and seventh decades. Flemming *et al* (22) however found a significant age distribution between Black and White male patients. They found that 33,4% of Black male patients with OSCC were below the age of 50 years, compared to 15,6% of White males. This difference may be attributed to exposure to a carcinogenic agent at a young age. We arbitrarily defined patients younger than 40 years as "young" and our group 2 would therefore fall into this category. The results of study showed that age was not an important factor in our population sample.

Table II. *Summary of studies detecting HPV DNA in oral squamous cell carcinomas using PCR.*

Reference	Year of study	HPV positive / Number tested (%)	HPV Type Detected
Kiyabu *et al* (11)	1989	5/15 (33)	16/18
Maitland *et al* (12)	1989	?/?(50)	16
Palefsky *et al* (13)	1991	8/25 (32)	6/16/?
		0/10 (0)*	
Shroyer and Greer (14)	1991	1 /10 (10)	16
Watts *et al* (15)	1991	27/30 (90)	16/18
Yeudall & Campo (16)	1991	3/39 (8)	4/16/18
		0/25 (0)*	
Holladay and Gerald (17)	1993	7/39 (18)	11 /16/18
		1/6 (17)*	
Woods *et al* (18)	1993	14/18 (78)	6/11 /16/18
		6/9/ (67)*	
Ostwald *et al* (19)	1994	16/26 (62)	16/18
		1/97 (1)*	
Present study	1995	2/146 (1.4)	11 /16
		0/38 (0)*	

* Indicate normal oral mucosa.

The prevalence of HPV DNA in normal oral mucosa also varied between 0-67% in the PCR studies listed. Three of the six studies listed (including our own) did not detect HPV DNA in normal tissue. In the other three studies (17-19), the prevalence in normal mucosa was always lower than in the carcinoma groups. Differences may be explained by indications that HPVs do not have a predilection for a specific intraoral anatomical site, but that focal infection may occur at any place in the mouth (23, 24). Thus, a higher HPV detection rate may be possible if material is collected from different regions of the mouth.

To conclude, only HPV 6, 11, 16 and 18 were evaluated, because they are the most common types selected for detection by other investigators. HPV 16 and 18 specifically, have been implicated in squamous cell carcinogenesis. Our study shows that these HPV types were not important in the development of OSCC in the population sample studied.

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Is the Human Papillomavirus a Mutual Aetiological Agent in Oral and Cervical Squamous Cell Carcinoma?

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Epidemiological study

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Abstract. *Background: Oncogenic HPV-types are the most important risk factor in cervical SSC and have also been implicated in the aetiology of OSSC. This evidence of infection at different anatomical sites suggests systemic susceptibility that implies that different expressions of disease should more or less correlate over long periods of time. Materials and Methods: This was undertaken to establish whether any correlation could be found between the incidence of cervical SCC and OSCC in females and OSSC in males in South Africa, over a ten-year period, 1986 to 1995. Results: Several moderate to strong correlations, which ranged from significant ($p<0.05$) to highly significant ($p<0.01$), between the incidence of cervical SSC and OSSC in the Black and Coloured populations, and OSSC in the White male population, were found. Conclusion: These results support the idea of systemic susceptibility, and infection through a common agent such as HPV, contributing to the cause of SSC.*

Squamous cell carcinoma (SCC) is the most common malignancy of the oral cavity, and its incidence is on the increase globally, especially in developing countries(1). The prevalence of this malignancy varies from 1-2% of all cancers in Japan and Western Countries to more than 45% in parts of Asia, implying major geographical differences in risk factors(1). Oral cancer is also a problem in South Africa and is one of the most common malignancies in males(2). Although the role of tobacco and alcohol consumption in the pathogenesis of oral squamous cell carcinoma (OSCC) is well established, several studies have evaluated the prevalence of human papillomavirus (HPV) DNA in SCC of the head and neck. The reported prevalences have varied from less than 10% to 100%, depending on the detection methods and the types of tissue examined(3-7). Using meta-analysis, Miller and Johnstone concluded that HPV is an important risk factor for OSCC(8). There is furthermore evidence that women with HPV associated SCC of the head and neck region have a 13-fold greater than average incidence of cervical atypia(9). This evidence of HPV infection at different anatomic sites suggests a systemic susceptibility to HPV infection(9).

Cancer of the cervix is the most common malignancy in women in developing countries. This is also true for South Africa where cervical cancer is the most common malignancy of Black, Coloured and Asian women. Infection with oncogenic HPV types is the most important risk factor in its aetiology. The important HPV types linked to cervical carcinoma world-wide are HPV-16, HPV-18, HPV-45, HPV-31 and HPV-33(10).

Systemic susceptibility, leading to infection at different anatomical sites of similar nature by agents with similar traits, implies that the different expressions of disease should more or less correlate over long periods of time, provided that host factors and environmental factors do not have radical inhibiting or exacerbating influences. This study was therefore undertaken to establish whether any correlation could be found between the incidence of cervical SCC and OSCC in females and males in South Africa, over a ten-year period, 1986 to 1995.

Material and Methods

In South Africa, pathological laboratories from both the private- and public sector report histologically verified cancers to the National Cancer Registry (NCR), which serves as central source for cancer data(2). The NCR, firstly, classifies cancers by organ site, utilising the Systematic Nomenclature of Medicine's (SNOMED), two-digit topographic code-system, and secondly, classifies the morphological tumour utilising the ICD-O-1, five-digit code-system(11). The NCR also provides demographic variables such as age, population group and gender.

Raw data for the period 1986 to 1995 were obtained from the NCR. The SNOMED-codes: mouth="51", tongue="53" and gum="54", were used to identify cancer in the oral cavity. Cancer of the cervix was expressed utilising the SNOMED-code "63". ICD-O-1 codes utilised to identify squamous cell carcinoma are exhibited in Table I(11). Statistics South Africa recommends that the population data obtained from Census 1996(12) be used as baseline for population counts, and subsequently provided formulas to make projections to determine mid-year populations(13).

To determine whether an epidemiological relation exists between cervical SCC and OSCC, the incidence of cervical and OSCC, expressed in age standardised incidence rates (ASIRs), registered during the ten-year period (1986-1995), were correlated using the Pearson correlation test. A probability of <0.05 was considered to be significant, and $p \leq 0.01$ as highly significant.

Results

The ASIR of OSCC and cervical SCC in the different groups is shown in Table II. Several significant correlations were detected (Table III).

The incidence of OSSC in Back females correlated moderately, and highly significantly, with cervical SSC in Black and Coloured females over the ten-year period.

Furthermore, the incidence of OSSC in black females correlated moderately, but significantly, with the incidence of OSSC in Coloured females, and highly significantly with Black and Coloured males. Similar trends were found for the incidence of OSSC in Coloured females, however, significant correlation with the incidence of OSSC in White and an Asian male was illustrated over and above.

Moderate to strong correlations, which were highly significant, were also found between the incidence of cervical SSC in Black females and the incidence of OSSC in Coloured females, Black, Coloured and White males. The incidence of cervical SSC in Black females also correlated moderately and highly significantly with cervical SSC in Coloured females. Similar trends were found for the incidence of cervical SSC in Coloured females.

Finally, the incidence of OSSC in Black, White and Coloured males correlated moderately, and highly significantly with each other, while OSSC and cervical SSC in White and Asian females did not show any significant correlations.

Discussion

Data obtained from the NRC have limitations. Not all cancers are histologically verified, and therefore a degree of under-reporting exists(2). Furthermore, the demographic data is sometimes incomplete and inaccurate. For example, frequent under-reporting of variables such as population group and gender necessitates alternative measures to improve data quality. Surnames are often used to determine or update population group, which builds in a degree of measurement bias, especially amongst the White and Coloured populations groups of South Africa. The NCR also uses a computer algorithm to change unknown and wrongly reported sexes where it is definitely known that the cancer can only occur in one particular sex, for example cervix carcinoma. Inevitably, this is not possible with all types of cancers. Multiple population-based cancer registries (PBCR) are considered of more value compared with centralised cancer registries such as the NCR(2). Despite these limitations the data may still be useful to establish trends in the incidence of different cancers in South Africa.

Furthermore, the methods provided by Statistics South Africa, to project demographic counts are more or less accurate for periods of five years into the past or future, which in this case allows an accurate estimation of population counts as far back as 1991(13). As no alternative was available, previously utilised population counts of 1987 were utilised to project the 1988 to 1990 Black population counts, as well as the Black population counts for 1986(14). However the 1987 population counts for Whites, Coloured and Asians seemed to be overestimates, and therefore unrealistic. The above-mentioned methods(13) were therefore used to make projections back to 1986, which most probably lead to under-estimations in population counts. However, slight to moderate adjustments in the population growth rates did not alter age standardised incidence rates significantly.

Bearing in mind these limitations, the results of this study showed that the incidence of OSSC and cervical SSC in Blacks and Coloureds, and OSSC in White males correlated significantly over the ten-year period 1986 to 1995, possibly indicating a common cause. HPV's have frequently been implicated as the major role-player in the aetiology of cervical cancer(10,15,16). In contrast, smoking and alcohol-use have strongly been implicated in the aetiology of OSSC. However, OSSC also occurs in the absence of these traditional risk factors, indicating a role for other factors(17). Therefore, a possibility exist that HPV may be a commonality in the aetiology of OSSC and cervical SSC in Blacks and Coloureds, and OSSC in White males in South Africa.

The higher socio-economic status of White females, and their subsequent better access to healthcare and prevention programmes, in the form of exfoliative smears, could explain the fact that the incidence of cervical SSC in White females did not show any significant correlation(18). In comparison, Asian females are known for their traditional habits such as betel use, which might have exacerbated the incidence of OSSC(19). However, the fact that the incidence of SSC in White females and Asians did not correlate with SSC in other population groups may also indicate different genetic predisposition.

In conclusion, this study supports the view of the possibility of systemic infection by an agent such as HPV, especially in Blacks, Coloureds and White males. However, further

research is required to verify the commonality in aetiology between OSSC and cervical SSC, probably in the form of identification of HPV in the oral mucosa or OSCC.

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Table I. *ICD-O-1 codes used to identify squamous cell carcinoma*

Code	Neoplasm Morphology
80103	Carcinoma NOS Epithelial tumour, malignant
80113	Epithelioma, malignant Epithelioma, NOS
80203	Undifferentiated carcinoma NOS
80213	Anaplastic carcinoma, NOS
80323	Spindle cell carcinoma
80413	Small cell carcinoma, NOS Round cell carcinoma
80513	Verrucous carcinoma
80523	Papillary squamous cell carcinoma
80702	Squamous cell carcinoma in situ NOS etc.
80703	Squamous cell carcinoma NOS etc.
80713	Squamous cell carcinoma, keratinizing NOS
80743	Squamous cell carcinoma, spindle cell
80753	Squamous cell carcinoma, adenoid type
80763	Squamous cell carcinoma, microinvasive

Table II. *ASIRs of OSCC and cervical SCC in the different population groups (1986-1995).*

	Black Female OSSC	Black Female CSSC	Coloured Female OSSC	Coloured Female CSSC	White Female OSSC	White Female CSSC	Asian Female OSSC	Asian Female CSSC	Black Male OSSC	Coloured Male OSSC	White Male OSSC	Asian Male OSSC
1986	1.78	33.44	1.96	24.75	4.31	9.61	3.51	12.81	10.01	12.98	9.49	2.54
1987	1.86	30.74	1.81	19.56	3.12	9.17	4.33	6.06	10.77	9.86	8.32	2.47
1988	2.16	42.08	2.97	26.32	3.48	12.27	6.09	16.18	13.50	18.61	12.42	3.92
1989	2.33	51.01	5.19	30.00	5.48	11.46	9.34	14.01	13.76	23.76	11.44	8.56
1990	1.76	45.00	3.92	20.44	4.60	15.78	6.80	16.82	13.60	14.70	11.52	3.71
1991	1.76	36.77	3.88	31.17	4.20	13.32	6.79	29.60	11.01	14.59	11.83	6.59
1992	1.51	33.00	2.59	24.76	3.68	17.89	3.91	14.27	11.14	17.78	10.73	2.28
1993	1.34	33.17	2.56	19.52	1.86	13.71	12.71	13.31	10.75	11.52	11.32	3.37
1994	0.74	21.63	1.36	8.41	3.92	13.02	6.01	14.77	6.15	6.11	8.44	5.90
1995	1.21	18.64	1.98	10.63	4.36	14.22	8.05	12.63	5.76	8.34	7.35	3.81

Table III. *Significant correlations between the ASIRs of the different groups (1986 -1995).*

	Black Female OSSC	Black Female CSSC	Coloured Female OSSC	Coloured Female CSSC	White Female OSSC	White Female CSSC	Asian Female OSSC	Asian Female CSSC	Black Male OSSC	Coloured Male OSSC	White Male OSSC	Asian Male OSSC
Black Female OSSC		**r=0.84 p<0.01**	r=0.69 p<0.05	**r=0.83 p<0.01**					**r=0.85 p<0.01**	**r=0.82 p<0.01**		
Black Female CSSC			**r=0.87 p=0.001**	**r=0.79 p<0.01**					**r=0.95 p<0.001**	**r=0.87 p<0.001**	**r=0.82 p<0.01**	
Coloured Female OSSC				r=0.71 p<0.05					**r=0.74 p=0.01**	**r=0.82 p<0.01**	r=0.72 p<0.05	r=0.63 p<0.05
Coloured Female CSSC									**r=0.80 p<0.01**	**r=0.83 p<0.01**	**r=0.76 p=0.01**	
White Female OSSC												
White Female CSSC												
Asian Female OSSC												
Asian Female CSSC												
Black Male OSSC										**r=83 p<0.01**	**r=84 p<0.01**	
Coloured Male OSSC											**r=0.75 p=0.01**	
White Male OSSC												
Asian Male OSSC												

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Detection of EBV DNA in Oral Squamous Cell Carcinomas in a Black African Population Sample

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Abstract. *The purpose of this study was to determine the presence of Epstein-Barr virus (EBV) DNA in oral squamous cell carcinoma (OSCC) patients from a Black African population. Formalin fixed paraffin embedded blocks of OSCC of two randomly selected groups were investigated. Group 1 consisting of 57 blocks containing OSCC with a fragment of normal appearing adjacent/overlying epithelium. Group 2 consisted of 48 blocks containing only OSCC tissue without any normal appearing epithelium. The control group consisted of 38 non-malignant, non-viral associated lesions. A standard polymerase chain reaction (PCR) was used to amplify the Bam HI W-fragment using a nested primer set. EBV DNA was demonstrated in 14/57 (25 %) blocks from Group 1, in 13/48 (27 %) blocks from Group 2 and in 16/38 (42 %) blocks from the control group. No evidence for a direct role of EBV in the process of malignant transformation of intraoral epithelial cells was found in this study.*

Squamous cell carcinoma is the most common malignancy of the oral cavity. Despite the relative easy accessibility of the oral mucosa to detect premalignant lesions, recent studies suggest a global increase in the incidence of oral squamous cell carcinoma (OSCC) (1). The age standardised incidence rate for OSCC in the Black population in South Africa is 8.25 for males and 2.3 for females as obtained from the National Cancer Registry of 1988 (2). OSCC in South Africa is the fourth most prevalent malignancy among black males and eleventh for females.

Epstein Barr virus is a double stranded DNA virus. It causes widespread infection and was found to be the aetiologic agent of infectious mononucleosis (3) as well as being closely associated with Burkitt's lymphoma (4), nasopharyngeal carcinoma (5) and EBV-induced disorders in immunodeficient patients (6). An association of other epithelial tumours with EBV has been suggested recently on the basis of molecular biological techniques. The presence of EBV DNA in tonsillar carcinomas (7), gastric carcinomas (8, 9) epithelial thymic carcinoma (10), undifferentiated salivary gland carcinoma (11-13) and lung carcinoma (14, 1 5) has been reported.

This study was undertaken to determine the presence of EBV DNA in OSCC patients from a subgroup of Black Africans from the North Western Transvaal and to evaluate the possible role of EBV as an aetiologic agent in the carcinogenesis process. PCR was chosen for evaluating our samples because it is the most sensitive diagnostic tool available. The sensitivity was optimised by adjusting the reaction to detect 1 gene copy per reaction mix.

Materials and Methods

Patients. Formalin fixed paraffin embedded blocks of OSCC were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa, (MEDUNSA). The sections were randomly screened to confirm the diagnoses and two groups were selected. Group 1: Fifty-seven blocks containing OSCC with a fragment of normal appearing/overlying epithelium. The mean age of this group was 55.8± 15.4 years. Forty-six were male. Group 2: Forty-eight blocks containing OSCC tissue without any normal appearing epithelium. The mean age of this group was 55.2 ± 7.1 years. Thirty-six were male. Group 3: A control group consisting of 38 non-neoplastic intraoral lesions were included. These were non-viral associated lesions, the majority being fibrous epuli and fibro-epithelial polyps.

Preparation of cell lysates. Two 10 µm sections were cut from each block, using a new disposable blade after each block. To evaluate the possibility of contamination at this stage, 10 µm sections of a block containing normal brain tissue were cut after every five tumor blocks and also included for evaluation. All sections were placed in separate microfuge tubes. Cell lysates were prepared as follows: sections were dewaxed, washed with ethanol and digested with 400 µl of lysis buffer containing proteinase K (16). The samples were then heated to inactivate the proteinase K, centrifuged to pellet the debris and stored at -20°C until use. Ten µl of the supernatants were analysed by polymerase chain reaction (PCR).

PCR. Oligonucleotide primers (Figure 1) corresponded to sequences in

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Key Words: EBV, PCR, oral squamous cell carcinoma.

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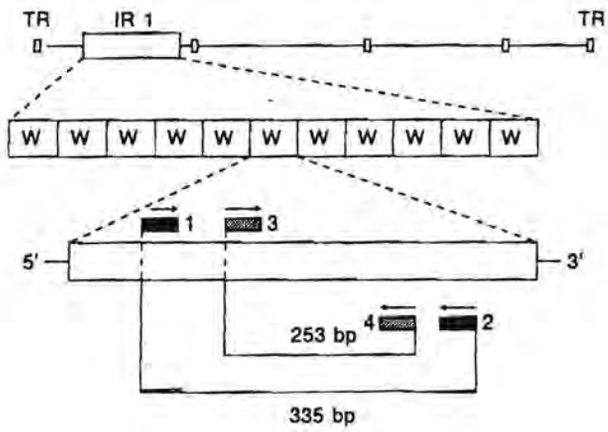

OLIGONUCLEOTIDE SEQUENCES

1 CTTTAAAACTCTAAAAATCAAAACTTTAGA
2 ACCAGAAATAGCTGCAGGACCACTTTATAC
3 AATGGGCGCCATTTTGT
4 TCCCTAGAACTGACAATT

Figure 1. *Sequences and positions of oligonucleotide primers (1-4) for Bam HI W-fragment of EBV genome with positions of terminal repeats (TR) and internal repeat 1 (IR1) indicated.*

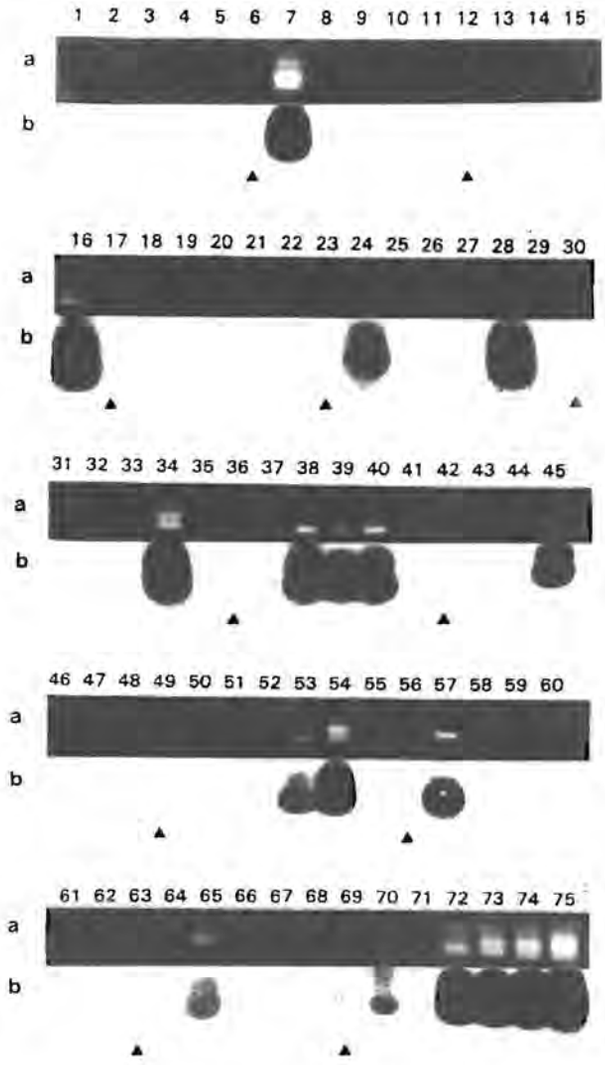

Figure 2. *Carcinomas with normal epithelium. Numbers (10, 11) and (47, 68) were patients tested in duplicate; (71): negative control; (72, 73, 74, 75) represent 1, 10, 100 and 1000 genome copies respectively; (a): gel electrophoresis; (b): hybridization; (▲): brain samples.*

the internal repeat fragment (*Bam* HI W-fragment) of the EBV genome. This fragment is reiterated ten times per genome (17). These primers were selected because the reiterated structure of the target sequence would theoretically enhance the sensitivity of detection (18). Thermal cycling was carried out using a Techne PHC-2 water cooled machine. All specimens were subjected to two rounds of PCR amplification, first with the outer and subsequently with the inner primer sets. The hot start method was done before adding enzyme (19). Forty amplification cycles were used as follows: denaturing of DNA (94°C; 1.5 minutes), annealing of primers (60°C; 1.5 minutes) and extension of the annealed primers (72°C; 3 minutes). Reaction mixtures for the amplification contained: 10 µl of cell lysate; 200 µM of each nucleotide; 0.4 µM of each primer; 1 U of Taq DNA polymerase (Promega Corporation, Madison WI, USA); 10 µl of Promega 10 x buffer and made up to a final volume of 100 µl. Each reaction mixture was covered with two drops of mineral oil. Template DNA for the second amplification round consisted of 10 µl of reaction product from the first round. All other reagents for the second round of amplification were used in the same concentrations as the first round.

Reagent controls contained sterile water instead of template DNA. To standardise our PCR reactions, the EBV *Bam* HI W-fragment cloned in the pACYC 184 plasmid (courtesy of D. Neumann-Haefelin, Freiburg, Germany) was used as the positive control. To obtain a quantitative estimate of the sensitivity of our PCR method, we made 10-fold serial dilutions of the plasmid in 1µg/µl salmon sperm DNA. The dilutions containing 1, 10, 100 and a 1 000 copies of plasmid/µl were included in each run. After the last round of amplification, 10 µl of the reaction product was electrophoresed on ethidium bromide stained agarose gels and evaluated under ultraviolet light.

Southern blot hybridisation. Southern blotting was used to confirm the PCR results. The *Bam* HI W-fragment was used as a probe and labelled using the DIG DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Hybridisation was carried out at 55°C overnight in 6 x SSC, 5 x Denhardt's solution, 0.2% SDS and 100µg/ml salmon sperm DNA. The blots were washed at high stringency using 2 x SSC plus 0.1% SDS for 5 minutes at 65°C, then twice with 0.2 x SSC and 0.1% SDS at 65°C first for 5 and then for 15 minutes. The hybridisation signal was detected with the DIG DNA detection kit (Boehringer Mannheim, Mannheim, Germany).

Results

All the brain samples, placed randomly between the study samples were negative on PCR. The sensitivity was such that 1 copy of plasmid DNA could be detected in each reaction. EBV DNA was demonstrated in 14/57 (25%) blocks containing carcinoma and epithelium (Figure 2), in 13/48 (27%) containing only carcinoma (Figure 3), and in 16/38 (42%) blocks from the control group (Figure 4).

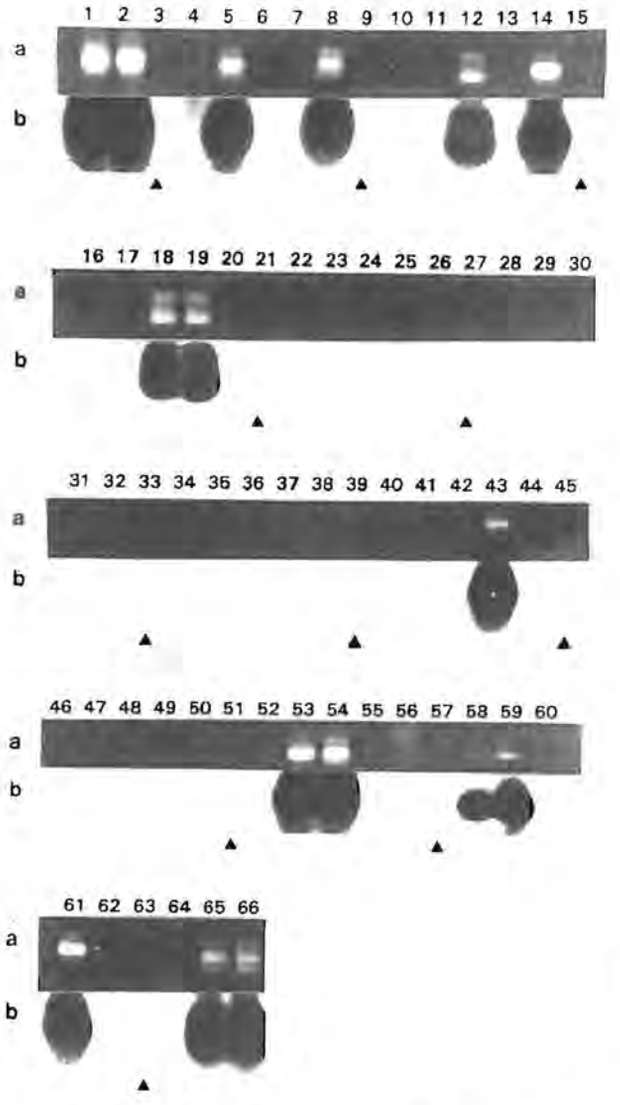

Figure 3. *Carcinomas without normal epithelium. Numbers (28, 32) and (58, 59) were patients tested in duplicate; (64): negative control; (65, 66, 1, 2) represent 1, 10, 100 and 1 000 genome copies respectively; (a): gel electrophoresis; (b): hybridization; (▲): brain samples.*

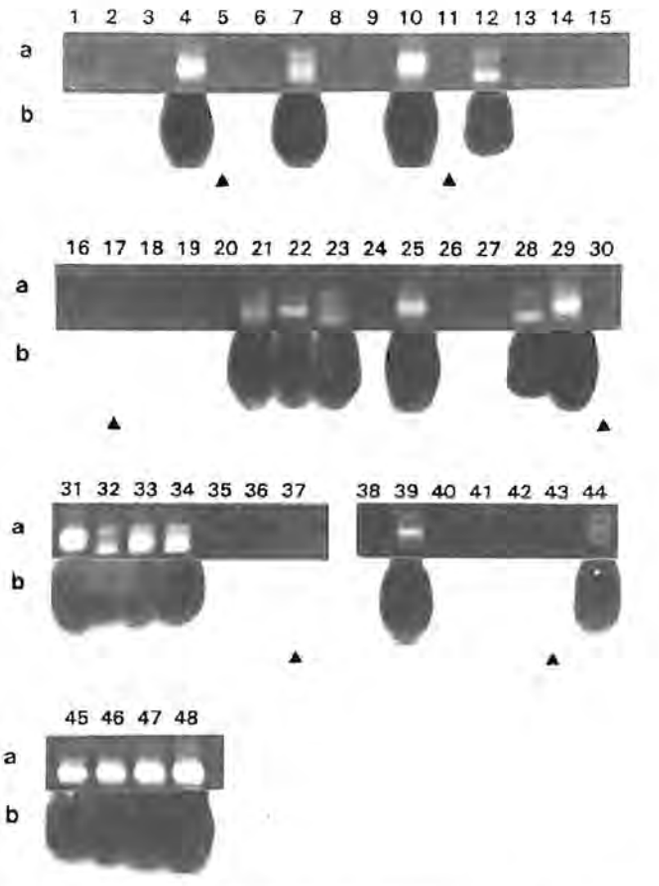

Figure 4. *Control group. (45, 46, 47, 48) represent 1, 10, 100 and 1 000 genome copies respectively; (a): gel electrophoresis; (b): hybridization; (▲): brain samples.*

Discussion

Over 90% of the adult population worldwide is infected with EBV implicating the presence of viral DNA in all of them. Evidence exists that EBV persistence occurs in B lymphocytes, but the importance of epithelial cell infection is uncertain (20). Persistent, low-grade replication and spreading of virus has been demonstrated in squamous epithelial cells of the oropharynx (21), uterine cervix (22) and male genital tract (23). Talacko *et al* (24) using *in situ* hybridisation, suggested that the EBV genome is not present in normal oral stratified squamous epithelium of immunocompetent individuals. Mao *et al* (25) was the first to demonstrate EBV DNA in the oral epithelium of healthy individuals. They showed a 25% positivity using PCR. Although Madinier *et al* (26) could not detect EBV DNA in extraperidontal mucosal specimens, 4/10 gingival biopsies were found positive by Southern blotting. EBV DNA could be demonstrated by means of PCR in 70% of nasopharyngeal tissue obtained from healthy individuals (27).

The 42% EBV DNA found in our control group was between that of other studies mentioned here. The difference may be due to several factors: (i) The selection of specific primer pairs for the PCR may influence the outcome, eg. the *Bam* HI W region is reiterated more than 10 times and this should provide a more readily detected sequence than a single copy viral gene. (ii) The anatomical site from where the biopsies were taken. A higher positivety was found in the nasopharynx (27) than at other anatomical sites (eg. gingiva) (26). (iii) The geographical area from where the patients originate. All the patients in our study were blacks from rural areas, which may be a contributing factor.

The demonstration of EBV DNA in the two carcinoma groups with and without epithelium was almost the same (25% vs 27%), implying that the presence of normal overlying/adjacent epithelium did not influence the number of positives in

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these two groups. This may be due to several factors: (i) the first carcinoma group consisted mainly of tumor cells with only a small fragment of normal epithelium compared with that of the control group; (ii) latent EBV DNA is not present in all oral epithelial cells and, (iii) only $^{1}/40$ of the cell lysate was used for the PCR, thus minimising the contribution of normal epithelial cells.

The EBV DNA positivity was lower in the two carcinoma groups compared with that of the control group. It therefore seems more likely that EBV is merely a passenger when neoplastic change occurs in a latently infected epithelial cell, although a possible role for EBV in the multistep process of squamous cell carcinogenesis cannot be excluded.

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The Suitability of Paraffin-embedded Material to Predict Metastatic Potential of Oral Squamous Cell Carcinoma

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Abstract. *Background: The presence of regional metastasis in oral squamous cell carcinoma (OSCC) is an important prognostic factor. This study was undertaken to identify histological features and biological markers from paraffin-embedded primary OSCC that may predict the presence of regional metastases. Materials and Methods: Fifty-three en-bloc primary OSCC resections were divided into two groups, 26 with lymph node metastases and 27 without metastases. The pattern of infiltration, presence of vascular or perineural infiltration and tumour necrosis were evaluated while expression of p53, p21 and Rb were assessed in the two groups. DNA ploidy status was also determined with a flow cytometer. Results: The presence of DNA aneuploidy was found to be the only statistically significant predictor of regional metastases. Seventy-seven per cent of the primary OSCC with lymph node metastases showed DNA aneuploidy. Conclusion: DNA flow cytometry obtained from archival material could be used as a parameter to predict regional metastases.*

The TNM staging system for patients with oral squamous cell carcinoma (OSCC) is widely used to facilitate treatment planning and to predict behaviour and prognosis. Patients with advanced disease have the worst prognosis while the presence of regional nodal metastasis is considered to be the most important prognostic factor (1, 2). Although T3-4 OSCC's of the tongue and floor of mouth have a higher incidence of nodal metastasis than do T1-2 carcinomas (2, 3), the ability of OSCC to metastasise is not always related to clinical staging (4). Evaluation of the primary tumour for features to predict metastatic potential is important because regional metastases are only detected with an accuracy of around 70% using clinical examination (5). Although modern imaging techniques may improve the detection of overt metastases, even the most sophisticated methods may not disclose occult metastases (6).

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Key Words: Metastases, oral squamous cell carcinoma, oral cancer, flow cytometry, paraffin-embedded material, ploidy.

Conflicting results have been reported when using histological grading and expression of a variety of biological markers to predict lymph node metastasis of head and neck squamous cell carcinomas (4, 7-9). The significance of flow cytometric DNA content in OSCC is well documented. Aneuploidy has been correlated with metastatic spread (10, 11) and shown to be an independent prognostic factor (12, 13). Significant differences are found between flow cytometric data obtained from analyses of paraffin-embedded material *versus* fresh tissue (14) and therefore the source of the material should always be taken into account when comparing results.

The concept of tumour cell heterogeneity with regard to the metastatic potential is widely accepted (15). It is at present not possible to divide the cells in a primary tumour into those with metastatic potential and those without this potential, although completion of the human genome sequence would facilitate studies on possible genetic alterations (16). This retrospective study was undertaken to identify biological markers in paraffin-embedded primary OSCC that might predict the presence of regional metastases.

Materials and Methods

En-bloc tumour and lymph node resections of primary OSCC from the tongue or floor of mouth were retrieved from the files of the departments of Anatomical Pathology and Oral Pathology, University of Pretoria. Fifty-three cases were selected and divided into two groups based on the presence or absence of metastatic tumour in the regional lymph nodes. Twenty-six cases had metastatic tumour in one or more lymph nodes while no metastases could histologically be demonstrated in 27 cases.

All the H&E sections of the primary tumour were reviewed and certain grading features were assessed. These included: the presence of vascular invasion, presence of neural or perineural infiltration, presence of eosinophils in the surrounding inflammatory infiltrate, presence of tumour necrosis and presence of individual tumour cell infiltration in the deep invading margins.

Tumour sections and positive lymph nodes were also evaluated for immunohistochemical expression of p53 (DO-7, DAKO Corporation, Carpinteria, CA 93013 USA; prediluted), $p21^{WAF1/Cip1}$ (Clone SX118, DAKO Corporation, Produktionsvey 42, DK-2600 Glostrup, Denmark; 1:50 dilution) and retinoblastoma tumour-suppressor gene (Rb) (DAKO Corporation, Produktionsvey 42, DK-2600 Glostrup, Denmark; 1:25 dilution). The staining techniques were performed according to the manufacturer's instructions. Antigen retrieval was performed with a microwave pressure cooker in citric acid buffer (pH 7.6) in all cases. For

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Table I. *Histomorphological features used to access metastatic potential of OSCC.*

Grading features	Tumours with metastases (n=26)	Tumours without metastases (n=27)
individual cell infiltration	24	21
presence of eosinophils	17	16
tumour necrosis	7	7
vascular invasion	2	1
neural/perineural spread	7	6

Table II. *Immunohistochemical detection of protein expression in OSCC.*

Antibody		Tumours with metastases (n=26)		Tumours without metastases (n=27)	
p53	-	6		9	
	(+)	3	38%	1	44%
	+	1		2	
	++	5	62%	7	56%
	+++	11		8	
Rb	-	2		2	
	(+)	1	19%	2	18%
	+	2		1	
	++	12	81%	17	82%
	+++	9		5	
p21	-	2		7	
	(+)	6	77%	3	77%
	+	12		11	
	++	6	23%	6	23%
	+++	0		0	

all antibodies, only nuclear staining was regarded as positive. The following scoring system was used for the immunohistochemistry staining: negative - no positive cells; (+) - scattered positive cells; + - less than 10% positive cells; ++ - between 10 and 50% positive cells; +++ - more than 50% positive cells.

Sections from the same tumour block used for immunohistochemical analyses were used for DNA ploidy analysis. The sections were prepared according to the modified Hedley method (17). In short, four to six 40 μm sections were cut, wrapped in 50 μm nylon mesh, placed in a histocasssette and manually dewaxed and hydrated to distilled water. The sections were left in distilled water overnight where after they were digested in subtilisin Carlsberg solution at 37°C for 120 minutes. The cell suspension was then stained with DAPI (4'6 diamidino 2 phenylindole) (Research Organics, Cleveland, OH, USA). Flow cytometry was carried out using a PAS II flow cytometer equipped with a high-pressure 100W mercury lamp (Partec, Münster, Germany). DNA histograms of at least 10,000 cells were plotted. The diploid cell population was used as an internal reference standard for the identification of aneuploid clones.

All the primary tumours were evaluated without knowing the metastatic status of the tumours. The factors evaluated were related to the nodal status in 2X2 contingency tables. The Chi square with Yates correction and Fischer's exact tests were used for the analysis of the categorical data. Correlations with a p <0.05 were considered statistically significant.

Table III. *Flow cytometric analysis of DNA ploidy status of OSCC.*

Ploidy status	Tumours with metastases (n=26)	Tumours without metastases (n=27)
Aneuploid	20 (77%)	12 (44%)
Diploid	6 (23%)	15 (56%)

Results

The grading features of the primary tumours are reflected in Table I. These differences were not statistically significant. No correlation was found between the expression or lack of expression of p53 (p=0.87), Rb (p= 1) and p21 (p=0.80) in the primary tumour and the presence of lymph node metastases. Neither was a correlation apparent for any of these markers if the cases were divided in two groups: one group with more than 10% positive staining cells (++ and +++) and the other group with less than 10% positive staining cells. The immunohistochemical results are summarised in Table II. Virtually the same expression of the three antibodies used was also found when the primary tumours cells were compared to its metastatic tumour cells.

Twenty of the primary tumours with metastases were aneuploid while the majority of the group without metastases were diploid (Table III). These differences were highly significant (p=0.033). The coefficient of variation (CV) ranged between 2.8% and 6% with a mean of 4.4% (SD = 0.7).

Discussion

Grading features are often considered to be unreliable due to interobserver and intraobserver variability although this can be minimised when using well-defined criteria (18, 19). A relation between growth pattern and regional metastases has been described (20, 21) but could not be confirmed in the present study or by others (7). Contradictory results regarding the prognostic value of an eosinophilic infiltrate adjacent to the infiltrating tumour islands have also been reported.

Although a few studies link a favourable prognosis to an eosinophilic infiltrate (22, 23), a correlation between lymph node metastases and stromal eosinophylia could not be demonstrated in previous studies (23, 24) and this study. Vascular and perineural infiltration are well-documented to be important predictors of metastases in OSCC (25-27) but this could not be confirmed in our study.

The overexpression of p53 in head and neck carcinomas is well-established. p53 protein alteration is considered an early event that is maintained during tumour progression and metastases (28). p53 has been widely used as a marker to predict metastatic potential with conflicting results. Expression of p53 has been reported to be associated with lymph node metastases (29) while others, including the present study, found no association (30).

p21 arrest the cell cycle in G1-or S-phase by forming a complex with cyclin A (or B or D or E), cdk2 (cyclin-dependent kinase) and PCNA (31). This complex inhibits the kinase activity of the cdk's and for that reason plays a role in keeping cells in a non-mitotic state. There is evidence that p53 regulates p21 expression directly, although recent studies have suggested that p53 independent pathways may also lead to p21 expression (32). Although no difference in p21 expression could be detected in the two groups in the present study, the expression also appears to be lower than reported in the literature (32-34) Comparison with published reports was difficult due the different criteria used to define positivity. No significant relationship could be demonstrated in our study between p53 and p21 expression supporting the co-existence of p53-independent pathways.

The meaning of expressed Rb is unclear. It could present functional protein, mutated protein or a combination of both. Phosphorylation of pRb during the G1-phase of the cell cycle allows for the transition from G1-to S-phase (35). pRb also has a possible role in the regulation of apoptosis. Using a panel of immunohistochemical markers in laryngeal carcinomas to predict nodal metastases, Rb was the only nuclear factor that showed a positive correlation with nodal metastases (7), although this could not be confirmed by others (36) or the current study.

Inconsistency in results based on immunohistochemistry exists. The scoring categories for immunohistochemical analysis were chosen subjectively without any biological criteria. It is well-known that most tumours express heterogeneity in markers or chromosomal aberrations in different areas due to clonal variation (37). A small subclone may therefore develop alterations reflected in the immunohistochemical profile that lead to metastasis without affecting the scoring of the primary tumour as a whole. In this case, it would be expected that metastatic tumour cells would show a similar staining pattern with the subclone responsible for the metastasis. The fact that expression of the three antibodies in the primary tumour and its metastases were similar, suggested that these alterations were early events in carcinogenesis. Reliable and reproducible results and interpretations of immunohistochemical markers are essential before it can be used on a large scale as a prognostic marker. The need expressed for collaboration to standardize the use of immunohistochemical markers for specific applications (38) are supported.

DNA aneuploidy in the primary tumour was found to be the only statistically significant marker of regional metastases in this study. Although the occasional presence of metastatic disease in diploid OSCC cannot be excluded and should be accepted, it is likely that the majority of the diploid tumours in the positive nodal group were false diploid due to the inability to detect tumour cell populations with only small deviations of their DNA content from normal diploid cells (39). This inability to detect tumour cell populations with small DNA abnormalities is further compromised because of the use of formalin-fixed paraffin-embedded blocks (14). This is most likely a major contributing factor in the conflicting findings reported about the prognostic value of DNA flow cytometry in OSCC. Numerous studies on DNA flow cytometry in OSCC do not reveal the CVs of the measurements and were predominantly done on paraffin-embedded material (40-42). The quality of DNA flow cytometric analyses is measured by its CV. A mean CV of 4.4% was obtained in the present study while most studies described CVs higher than 5% when using archival material (11, 43). The ability to use the more sensitive DAPI staining procedure (12) had contributed towards the relatively lower CV obtained for paraffin-embedded material.

It is well-documented that the development of aneuploid clones contributes to the metastatic development of OSCC (10). It was possible that aneuploid clones in the 12 aneuploid tumours without metastases had not yet disseminated to the regional lymph nodes at the time of surgery or that the metastatic lesions were too small to be detected by routine histological examination (6).

Tumour metastases is a highly intricate and dynamic process consisting of a number of chronological steps based on several interactions between tumour cells and host tissue (44). High-resolution flow cytometry using fresh tumour tissue has been shown to be an independent prognostic factor in OSCC (12). The results of this study indicated that the metastatic potential of OSCC could largely be predicted with the use of DNA flow cytometry, even with the shortcomings of using paraffin-embedded blocks instead of fresh tissue.

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RESEARCH ARTICLE

Detection of p53 Gene Mutations in Oral Squamous Cell Carcinomas of a Black African Population Sample

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Mutations in the p53 gene have been reported in head and neck carcinomas. We determined the p53 mutation profile in 55 oral squamous cell carcinomas (OSCCs) from a black African population sample. DNA from all the patients were investigated using PCR amplification of the p53 gene (exons 5–9), followed by heteroduplex single-stranded conformational polymorphism (HEX-SSCP) analysis on the PCR products. Direct sequencing was performed on cases where mutations were identified. The results showed mutations in 13 of 55 (23.6%) tumours. Eleven of 13 (85%) were single base pair substitutions (9 transitions and 2 transversions), and 2 were deletions. Two novel mutations were identified: a large 63-base pair deletion, and a single base pair substitution. The mutations in our study occurred outside the head and neck tumour hot spot region (codons 238–248). Hum Mutat 11:39–44, 1998.

KEY WORDS: p53; mutations; oral carcinoma; African

INTRODUCTION: DETERMINING P53 MUTATION PROFILE

Approximately 3% of all malignancies arise in the oral cavity (Silverman and Grosky, 1990). Recent studies suggest a global increase in the incidence of oral squamous cell carcinoma (OSCC), especially in developing countries where it is the third most common malignant disease among men (Johnson, 1991). The age standardised incidence rate of oral and pharyngeal cancer in the black population of South Africa is 14.2 for males and 2.6 for females, as obtained from the National Cancer Registry of 1990/1 (Sitas et al., 1996). Historically, there was a strong predilection for the occurrence of OSCC in men, but today, in most parts of the world, the male: female ratio is 2 : 1 (Silverman and Grosky, 1990). OSCC in South Africa is the fourth most prevalent malignancy among black males and the eleventh for females.

OSCC occurs over a wide age range, with a peak incidence in the sixth and seventh decades. A significant different age distribution was found between black and white male patients, where 33.4% of black male patients were below the age of 50 years compared to 15.6% of white males (Flemming et al., 1982). This difference may be the result of exposure to carcinogenic agents at a young age. Conflicting findings regarding tobacco use, a well-documented carcinogenic agent, have been reported in young OSCC patients. Lower tobacco use among young cancer patients was present to site-matched and stage-matched older patients (Cusamo and Persky, 1988; Schantz et al., 1988), whereas Lipkin et al., (1985) found a high exposure to tobacco and alcohol in their young head and neck cancer patients.

The p53 protein was first discovered in association with the SV40 large T-antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). The p53 suppressor gene, located on the short arm of chromosome 17, is 20 kilobases long and encodes

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for a 53-kd phosphoprotein consisting of 393 amino acids (Lamb and Crawford, 1986; Malkin, 1993). The gene consists of 11 exons, interrupted by 10 introns, of which the first comprises approximately half the gene in the human genome (Levine and Momand, 1990; Soussi et al., 1990). It was initially thought to be an oncogene, but later evidence demonstrated that p53 is a tumour-suppressor gene. About 60% of cancers in humans have mutations in the p53 gene, which takes the form of a missense mutation plus a selection for a reduction to homozygosity and a complete loss of the wild-type alleles (Levine et al., 1991).

Epidermal cells with mutations in one copy of the p53 gene are slightly resistant to death by apoptosis (Ziegler et al., 1994). In addition to an inability to suppress cell division, many mutants express a gain of function that actively promotes the tumorgenic potential of cells lacking endogenous p53 protein. Missense mutations can generate mutant p53 protein, which is more stable than wild-type p53 protein and can sequester normal protein into inactive oligomeric complexes rendering the normal p53 inactive (Vogelstein and Kinzler, 1992; Zambetti and Levine, 1993). Mutant p53 protein can act as an oncogene and can transform rodent cells in cooperation with the *ras* gene (Eliyahu et al., 1984; Jenkins et al., 1985).

Mutations in the p53 gene, or increased expression of the p53 protein have also been reported in head and neck carcinomas (Hollstein et al., 1990; Sakai et al., 1992; Somers et al., 1992; Boyle et al., 1993; Caamano et al., 1993; Shin et al., 1994). The objective of our study was to determine the p53 mutation profile in 55 OSCCs from a black African population sample from the North Western Transvaal.

MATERIALS AND METHODS

Patients

Formalin-fixed, paraffin-embedded blocks of OSCCs were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa (MEDUNSA). Fifty-five blocks containing OSCC were selected. The mean age of the group was 50.8 ± 15.9 years; 45 were male and 10 were female.

Preparation of Cell Lysates

Cell lysates were prepared according to the method described by van Rensburg et al. (1996). All specimens were subjected to amplification using human β-globin primers (Saiki et al., 1986) to evaluate their suitability for DNA amplification.

Nested PCR Amplification of the p53 Gene (exons 5–9)

The genomic region containing p53 coding exon 5 to exon 9, together with flanking intron sequences, were amplified in three separate fragments, adapted from manufacturers' instructions (p53 genomic DNA screening module Catalog # 1610, Ambion, Austin, TX): fragment # 1, exons 5–6; fragment # 2, exon 7, and fragment # 3, exons 8–9. A list of the nucleotide sequences of each primer and the sizes of the amplified PCR products are shown in Table 1. Briefly, thermal cycling was carried out using a Perkin-Elmer (Norwalk, CT) GeneAmp® PCR system 9600 cycler. Forty amplification cycles were used: denaturing of DNA (94°C; 1 min); annealing of primers (55°C; 1 min), and extension of the annealed primers (72°C; 1.5 min). PCR was performed using the following reagents: 5 µl of cell lysate; 200 µM of each nucleotide; 250 nM of each primer (Ambion); 1 U of *Taq* DNA polymerase (Promega, Madison, WI); 5 µl of Promega 10x buffer and 1.5 mM $MgCl_2$ made up in a final volume of 50 µl.

Template DNA for the nested amplification round of each exon consisted of 3–7 µl of reaction product from the first round. Each exon 5–9 was amplified separately using primers previously described (Eeles et al., 1993). Briefly, thermal cycling was carried out using a Perkin-Elmer GeneAmp® PCR system 9600 cycler. Forty amplification cycles were used: denaturing of DNA (94°C; 1 min); annealing of primers (55°C; 1 min), and extension of the annealed primers (72°C, 1.5 min). PCR was performed using the following reagents: either 5 µl of first round PCR

TABLE 1. Sequences of p53 Genomic Primers With Expected Sizes of the PCR Products

Primer	p53 sequence	Size of PRC product
#1-S	CCTGAGGTGTAGACGCCAACTCTCT[a]	659 bp
#1-AS	ACTTTGCACATCTCATGGGGTTAT[a]	
#2-S	GGCCTCCCCTGCTTGCCA[a]	405 bp
#2-AS	CTCCAGCTCCAGGAGGTG[a]	
#3-S	AAGGGTGGTTGGGAGTAGA[a]	464 bp
#3-AS	ACGGCATTTTGAGTGTTAGAC[a]	
exon 5a	ATCTGTTCACTTGTGCCCTG[b]	308 bp
exon 5b	ATCAGTGAGGAATCAGAGGG[b]	
exon 6a	GCCTCTGATTCCTCACTGAT[b]	202 bp
exon 6b	GGAGGGCCACTGACAACCA[b]	
exon 7a	CTTGCCACAGGTCTCCCCAA[b]	236 bp
exon 7b	AGGGGTCAGCGGCAAGCAGA[b]	
exon 8a	TTCCTTACTGCCTCTTGCTT[b]	238 bp
exon 8b	TGAATCTGAGGCATAACTGC[b]	
exon 9a	GCAGTTATGCCTCAGATTCA[b]	161 bp
exon 9b	ACTTTCCACTTGATAAGAGG[b]	

[a]Ambion, Austin, TX
[b]Eeles et al., 1993.

products (fragment #1 to amplify exons 5 and 6), or 3 µl of first round PCR products (fragment #2, to amplify exon 7), or 7 µl of first round PCR products (fragment # 3, to amplify exons 8 and 9); 200 µM of each nucleotide; 0.4 µM of each primer; 1 U of *Taq* DNA polymerase (Promega); 10 µl of Promega 10x buffer and 1.5 mM $MgCl_2$ made up in a final volume of 100 µl. Two units of *Taq* polymerase were used in reactions to amplify exons 8 and 9. Reagent controls contained sterile water instead of sample DNA.

Aliquots of 8 µl of the amplification reaction products were analysed by electrophoresis through 3% LE agarose gels (FMC Products, Rockland ME), stained with ethidium bromide and evaluated by UV transillumination as described above.

Heteroduplex Single-stranded Conformational Polymorphism (SSCP) Analysis

DNA fragments of exons 5–9 were amplified as described above. Heteroduplex-SSCP analysis of these fragments were done to observe mutations, essentially as described with a few modifications (Kotze et al., 1995). Briefly, 10 µl of each PCR product was mixed with an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) containing 0.5 X TBE or 1.5 X TBE for either glycerol or urea containing gels, denatured at 95°C for 2–5 min and immediately placed on ice. The samples were loaded on a 24 cm vertical 1.5 mm thick (Hoefer Scientific Instruments, San Francisco, CA) 10% polyacrylamide gels with 1% cross-linking and run overnight at both room temperature and 12°C at 180 to 250 V (Bio-Rad Laboratories, Richmond, CA) depending on the size of the DNA fragments. Gels were supplemented with 7.5% and 5% urea and glycerol, respectively, stained for 10 min in a solution of 0.6 X TBE containing 1 µg/ml of ethidium bromide, and destained for 10 min in water before being evaluated by UV transillumination as described before.

Direct Sequencing

Sequencing of PCR products was performed for exons 5–9 to confirm the presence of mutations that had been detected by heteroduplex-SSCP. The primers used for sequencing was the same as for the nested PCR (Eeles et al., 1993), and the Sequenase PCR product sequencing kit (USB Catalog US70170, supplied by Amersham, Buckinghamshire, UK, life science) was used. The enzymes shrimp alkaline phosphatase and exonuclease 1 were used to remove unwanted materials from the PCR products and inactivated each at 80°C for 15 min after a 15-min incubation step at 37°C. The template was denatured and annealed to the sequencing primer using a heating step (99°C for 3 min) and a snap-cooling procedure on ice at 0°C. This is followed by using normal sequencing protocols including a labelling and termination step. Chain-termination sequencing is achieved with this kit using Sequenase™ Version 2.0 DNA polymerase. Manganese is added to improve the band uniformity and the ability to read sequences close to the priming site. Standard denaturing gel electrophoresis was done (Sambrook et al., 1989) using a glycerol tolerant gel buffer containing taurine (Amersham).

Sequence Analysis

Sequences were visualised by autoradiography, read from both directions, and analysis was done with the Genepro V5.0 software program (Riverside Scientific Enterprises, Bainbridge Island, WA).

RESULTS

Using a combination of SSCP and direct genomic sequencing, we examined exons 5–9 of the p53 gene in 55 OSCCs. DNA fragments showing an electrophoretic mobility shift were identified as positive for p53 gene mutations. They were subsequently selected for DNA sequencing to confirm the SSCP results and to characterise the types and locations of mutations. We scanned the GenBank (Bilofsky and Burks, 1988) data bank for sequence similarities, using GenBank accession number X54156. Mutations of the gene were identified in 13 of 55 (23.6%) tumours (Table 2). One of the 13 mutations was a two nucleotide deletion, the second was a large, novel deletion of 63 base pairs (Fig. 1), and the other 11 (85%) were single base pair substitutions (9 transitions and 2 transversions). One of the single base pair substitutions was

TABLE 2. p53 mutation summary in 13 OSCCs

Patient no.	Exon	Nucleotide change	Codon change
B14	5	C454→T	Pro152→Ser
A70	5	C459→T	Silent (153)
C2	5	G507→A	Met169→Ile
C33	5	G527→T	Cys176→Phe
C34	6	T584→C	Ile195→Thr
A67	6	del625-626	Frameshift
A48	6	A659→G	Tyr220→Cys
B43	7	C689→T	Thr230→Ile
C28	8	C817→T	Arg273→Cys
B34	8	C832→T	Pro278→Ser
C59	8	A874→T	Lys292→Stop
B5	8	C916→T	Arg306→Stop
B4	8	del814-876	del272-292

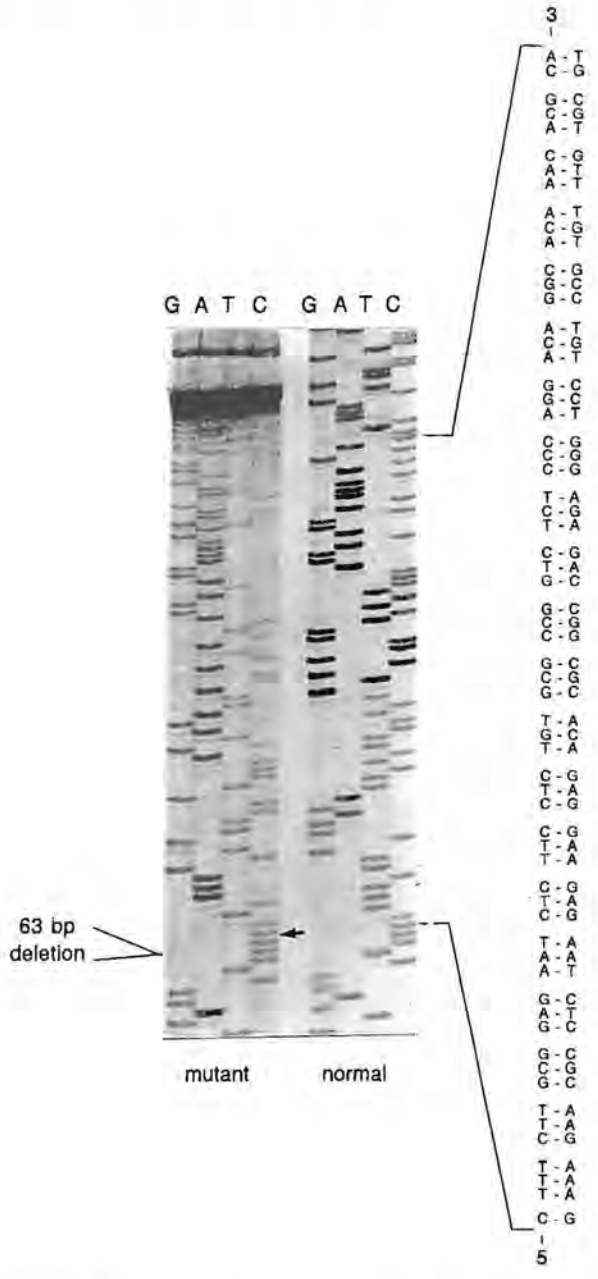

FIGURE 1. **Sequence analysis of a part of the p53 exon 8 gene from patient B4, showing the 63bp deletion.**

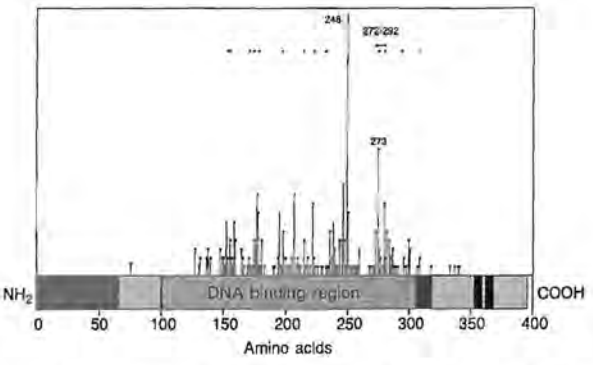

FIGURE 2. **Frequency of p53 mutations found in head and neck tumours (bottom) compared to mutations found in our study (top). The p53 molecule is schematically represented showing the DNA binding region and relative positions of the amino acid codons. Hot spot areas are evident at amino acid sites 248 and 273.**

also a novel mutation when compared to the p53 Database (De Vries et al., 1996; Hollstein et al., 1996). It was an A to T transversion at nucleotide 874. Of the previously described single base pair mutations, one was a silent mutation, two resulted in premature stop codon formation, and the other 8 in amino acid changes. Six of the 9 transitions were C to T changes, one was a G to A change, and one each was T to C, and A to G, respectively. The transversions were G to T, and A to T in one tumour each, respectively. All the mutations in this study occurred in different codons, except in the region of the large deletion, where three other mutations occurred as well. In total, four mutations occurred in exon 5, three in exon 6, one in exon 7, five in exon 8, and none in exon 9. In Figure 2, the mutations found in our study are compared to the frequency of p53 mutations in other head and neck tumours (Hollstein et al., 1996).

DISCUSSION

Approximately 45% of invasive head and neck squamous cell carcinomas have a mutated p53 gene (Koch et al., 1994). Most of the mutations in the p53 gene in general are missense mutations, causing a change in an amino acid, and a probable increase in stability of the protein (Harris, 1993). In a recent review, where 115 head and neck cancer samples were studied, 47% of the mutations were found to be transitions, 37% transversions, and 16% frameshifts, the majority of these being small insertions or deletions of 1–20 base pairs (Brachman, 1994). In our study, 69% were transitions, 15% were transversions, and 15% frameshifts. Two were novel mutations.

Two mutations, at codons 292 and 306, were nonsense mutations, and one mutation at codon 153 was a silent mutation. Of the missense mutations, seven were transitions, and one was a transversion. The most common transition was C to T in six of the nine (67%) cases. There seems to be distinct differences in the mutations reported from different geographical regions, as 50% of p53 mutations in Japan had G to T transitions (Sakai and Tsuchida, 1992), whereas 63% of head and neck cancers in the United States showed G to T transversions (Somers et al., 1992). Two of our tumours (14%) had deletions; one of them was also a frame shift mutation. This is in keeping with other studies, where frame shift mutations are

less commonly seen (Cotran et al., 1989). The novel deletion occurred at codons 272 to 292, which constitutes a 21 amino acid (63 base pair) deletion in the DNA binding region of the p53 protein, which is situated between amino acid 91 and 309 (Bargonetti et al., 1993). The large deletion in this area may cause the mutant p53 protein to loose its DNA binding capacity, with the cells expressing the mutation showing a selective growth advantage.

The p53 mutations in head and neck tumours seem to have a hot spot region at codons 238–248 as summarized by Hollstein et al. (1996). This region was not prominent in our study, but rather the region between 272 to 292 where the large deletion occurred and where three other point mutations occurred, i.e., condons 273, 278, and 292. There also appear to be a geographic distribution in the exons affected. In our study, as well as in a Japanese study (Sakai and Tsuchida, 1992), the majority of mutations were found in exons 5 and 8; exon 4 was the most frequent location for mutations in the United Kingdom (Field et al., 1993) and exon 7 in the United States (Somers et al., 1992).

To conclude, the number of OSCCs in our study with p53 mutations confirmed the importance of this gene in oral carcinogenesis. The unique distribution of the mutatons in the population sample studied suggested a different response to etiological agents. This may be linked to genetic factors that alter the molecular steps leading to cancer.

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ANTICANCER RESEARCH *18*: 237-240 (1998)

Correlation Between p53 Gene Mutation, p53 Protein Labeling and PCNA Expression in Oral Squamous Cell Carcinomas

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Abstract. *Background: The prevalence of oral squamous cell carcinoma (OSCC) among the Black community in South Africa is unacceptably high. The association between p53 protein, and PCNA overexpression and the presence of p53 gene mutations was evaluated. Materials and Methods: One hundred and ten formalin-fixed, paraffin-embedded blocks of OSCC were selected for immunohistochemical studies for p53 protein and PCNA expression using the DO-7 and PC10 monoclonal antibodies, respectively. DNA was extracted from fifty-five blocks and exons 5 to 9 of the p53 gene were amplified with nested primers, thereafter sequencing was performed to confirm the presence of mutations detected by single stranded conformational polymorphism. Results: Fifty-six cases (51%) showed p53 expression, while fourteen mutations (25%) were detected. A significant difference was found between the PCNA index in p53 positive and p53 negative tumors while the mean PCNA index for the tumors with p53 mutations was not significantly different from the tumors without mutations. Conclusions: No association between p53 protein overexpression and p53 gene mutations could be demonstrated.*

Squamous cell carcinoma is the most common malignancy of the oral cavity. Despite the relatively easy accessibility of the oral mucosa to detect premalignant lesions, recent studies suggest a global increase in the incidence of oral squamous cell carcinoma. This increase is especially evident in developing countries (1). The prevalence of oral squamous cell carcinoma (OSCC) in the Black community in South Africa is high. In the period 1990-1991, an average of 1363 new intraoral cancers per year were reported in South Africa, resulting in an average incidence of 3.4% of all diagnosed cancers, excluding basal cell carcinomas and squamous cell carcinomas of the skin (2). In combination with cancers of the pharynx, these malignancies were the 4th most common in South African males and the 2nd most common in South African Black males.

In an attempt to contribute towards the understanding of OSCC in the Black community, p53 gene mutations and their correlation with p53 protein and PCNA expression were evaluated.

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Key Words: p53, PCNA, oral cancer.

Materials and Methods

Patients. Formalin fixed paraffin embedded blocks of Black patients diagnosed with OSCC were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa, MEDUNSA. These consisted of material from resection specimens as well as biopsies. The sections were screened to confirm the diagnosis.

Immunohistochemistry. One hundred and ten cases were selected for immunohistochemical analyses. The mean age of the patients was 50.1 ± 16.6 years. Seventy-three patients were male and 27 female. A 4 µm section of each block was cut and picked up using amino-alkyl saline-coated glass slides. Sections were deparaffinised, hydrated with graded alcohol and brought to distilled water. The sections were placed in a jar filled with 10 mM citrate buffer, pH 6.0. For p53 antibody labeling, the samples were treated in a microwave oven for 2 × 5 minutes. The PCNA slides were immediately incubated at 37°C with 3% H_2O_2 and washed with PBS for 5 minutes as were the p53 slides following microwave antigen retrieval. The sections were then incubated at 37°C for 15 minutes with a p53 protein-specific DO-7 monoclonal antibody (Dako, Southern Cross, Cape Town, South Africa) and a PCNA protein-specific PC10 antibody (Dako, Southern Cross, Cape Town, South Africa). A working dilution of 1:100 with PBS was used for both antibodies. The sections were incubated in the Link antibody from the Steptavidin ABC Complex kit (Dako, Southern Cross, Cape Town, South Africa) for 10 minutes and washed in PBS for 5 minutes. The sections were incubated in Streptavidin for 10 minutes, washed again in PBS for 5 minutes and were then incubated for 20 minutes in a substrate solution at 37°C. The slides were counterstained in Mayers haemotoxylin for 1 minute, and blued for 5 minutes in running water.

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Negative controls consisted of the replacement of the primary antibody with normal mouse serum while positive controls consisted of known positive tumors.

Only nuclear p53 and PCNA staining was regarded as positive. Tumors were defined as p53 positive when more than 30% of the nuclei showed DO-7 labeling. When in doubt, 1000 tumor cells were evaluated at 400× magnification with the aid of an eyepiece graticule.

PCNA activity was quantified by assessing the staining in 1000 cells in areas within each section showing maximum reactivity for PCNA. All nuclei found to exhibit fine to coarse granular staining at ×400 magnification were rated as positive for PCNA. The number of positive cells was expressed as a percentage of the total number counted to give a PCNA index. An eyepiece graticule was used to ensure that all cells were evaluated once only. The Student's t-Test for uncorrelated data was used to compare the means of the PCNA index between the p53 positive and negative tumors

p53 gene analysis. Only fifty-five blocks of the cases used for immunohistochemistry were selected for analysis of exons 5 to 9 of the p53 gene. This was done to reduce costs. Cases were included for mutation analysis if all the exons could be amplified. Mutations were identified by heteroduplex single-stranded conformational polymorphism and confirmed by direct sequencing. The detailed methodology followed has been reported previously (3). The correlation between the presence of DNA mutations in any of the exons and the presence of p53 overexpression was determined using the χ^2-Test with Yates correction, while the significance of the mean PCNA counts between the tumors with mutations and those without was determined using the studentfs t-Test.

Results

The p53 protein expression was observed as a granular nuclear stain. Some tumor cells also expressed cytoplasmic p53 protein labeling. Cytoplasmic staining in this study was present in the minority of cases, and then in all instances associated with nuclear staining. The pattern of p53-positive nuclei in tumor sections varied from diffuse positive, to focal positive areas, to individual cell positivity. PCNA expression was also confined to the nuclei. The PCNA labeling index of p53-positive tumors was significantly higher (Student's t-Test, $p=0.0008$) than in the p53-negative group (Table I).

Mutations of the p53 gene were identified in 14 of 55 (25,5%) tumors (Table II). Two of the mutations resulted in premature stop codon formation. One of these showed no protein overexpression while the other was positive for p53 protein. Details of the mutation profile recorded in this study have been reported elsewhere (3). Only nine of 31 tumors exhibiting p53 protein overexpression showed mutations of the p53 gene. Five of 24 p53-negative tumors had mutations of the corresponding p53 gene. There was no statistically significant correlation between p53 protein expression and p53 mutation ($\chi^2=0.45$, $p=0.50$).

Discussion

Only nuclear staining was considered as a positive immunoreaction for p53 in this study. The cytoplasmic staining found has several explanations. Mutated p53 protein may have accumulated in the cytoplasm because transport to the nucleus was defective due to the altered amino acid sequences. Mutated p53 may also have formed stabilized complexes with wild-type p53 which kept it in the cytoplasm (4). Cross reactivity between the p53 antibodies and cytoplasmic cytokeratins has also been described (5)

Table I. *p53 protein expression and PCNA labeling indices for p53-positive and -negative OSCC patients.*

	n	PCNA index
p53+	56 (51%)	65.8 ± 14
p53-	54 (49%)	55.8 ± 16
Total		61.1 ± 16

The p53-negative tumors presumably contained wild-type p53 at levels too low to detect immunohistochemically. The negativity might also reflect the loss of both p53 alleles or the presence of non sense mutations leading to termination of protein synthesis.

PCNA immunoreactivity does not always correlate with other indices of proliferation (6). This is due to the long half-life of PCNA resulting in persistent staining in cells that have recently left the cell cycle (7). PCNA may also be expressed in association with DNA repair rather than proliferation (8). Quantitative studies of cell proliferation in solid tumors have disadvantages in that only a static moment in the cycle of cellular kinetics is evaluated (9). Techniques to determine cellular kinetics are available, such as the assessment of S-phase fractions using DNA flow cytometry (10). This was modified by estimating bromodeoxyuridine incorporation in DNA synthesis, assessed simultaneously with the S-phase fraction through bivarate flow cytometry (11).

The PCNA count was statistically higher in the tumors expressing p53 protein compared to the p53-negative tumors. Immunohistochemical detection of p53 protein may be due to a mutated p53 gene with intervention in its growth-inhibitory capacity resulting in increased tumor cell proliferation. The altered form of the mutated protein can interact directly or indirectly with growth related genes activating their expression (12). It is postulated that the conformational change associated with the mutated p53 protein can affect its binding to the PCNA promoter, releasing its repressor effect and inducing PCNA transcription (12). It may also be due to accumulation of wild-type p53 induced by DNA damage or specific viral protein binding. These observations are compatible with the functions of p53 as an inducer of DNA repair and of PCNA as a DNA repair protein. Tumors without detectable p53, with a high PCNA index, can also contain cells with a non sense mutation in the p53 gene. No definite conclusions can therefore be drawn on p53 and tumor cell proliferation based the on immunohistochemical data only.

Numerous studies on the relationship between p53 and

Table II. *p53 mutations (3) with p53 protein overexpression profile of the OSCC.*

Exon	Base change	Nucleotide position	Type of mutation	Codon position	Amino acid change	p53 protein expression
5	C>T	459	transition	153	silent	positive
5	C>T	454	transition	152	Pro>Ser	negative
5	G>A	507	transition	169	Met>Ile	negative
5	G>A	514	transition	172	Val>Ile	negative
5	G>T	527	transversion	176	Cys>Phe	positive
6	A>G	659	transition	220	Tyr>Cys	positive
6	del 2	625-6	frameshift	212	Phe del	negative
6	T>C	584	transition	195	Ile>Phe	positive
7	C>T	689	transition	230	Thr>Ile	positive
8	C>T	916	transition	306	Arg>stop	positive
8	del 63	814-876	frameshift	272-292	del	positive
8	C>T	832	transition	278	Pro>Ser	positive
8	C>T	817	trarfsition	273	Arg>Cys	positive
8	A>T	874	transversion	292	Lys>stop	negative

PCNA expression have been conducted. Most of the authors found a positive correlation between p53 and PCNA expression (13, 14, 15). A major disadvantage of immunohistochemical techniques is that comparisons between different proteins cannot be drawn on a cell to cell basis. Double staining techniques are a possibility, but not when a different technique, *e.g.* antigen retrieval, is necessary for one of the antibodies used. Direct comparisons between cells expressing p53 and PCNA were therefore not possible.

Only 9 (64%) of the 14 tumors with p53 mutations showed p53 protein overexpression. Two of the mutations resulted in premature stop codon formation, one of which showed no protein overexpression with immunohistochemistry. The other tumor with a nonsense mutation was positive for p53 protein. The overexpression seen in this patient might have been because only one allele was affected by the mutation and that the wild-type protein was stabilized by protein to protein or protein to gene interaction (16). The other tumors with overexpressed p53 without detectable mutations were probably the result of stabilized protein. It may, however, also be possible that mutations were present in other exons not examined or in the introns.

Tumors with nonsense mutations can be expected to be p53-negative with immunohistochemistry, if p53 protein complex formation is not considered. Only two of the detected mutations in this study were nonsense in nature. The tumors with missense mutations without p53 overexpression could be samples where the mutation resulted in an ephemeral protein or changes in the epitope recognized by the antibody.

The PCNA gene is regulated in a complex manner and its overexpression may be a result of increased PCNA mRNA stability induced by growth factors, regardless of the p53 status (17). This study suggested a possible association between p53 and PCNA based on the immunohistochemical findings. The difference between the PCNA index in tumors with p53 mutations and those without was statistically not significant. This would suggest that overexpressed p53 may influence the PCNA status and not the mutation of the p53 gene alone. The fact that p53 overexpression is not necessarily the result of a mutated gene has been discussed previously.

In conclusion, no association between p53 protein overexpression and p53 gene mutation could be demonstrated in this study. Overexpressed p53 can therefore not be described as a mutated protein, and no conclusions can be made on the presence of p53 gene mutations based on immunohistochemical evaluation of the p53 protein alone. Although an association between p53 protein overexpression and the PCNA expression was demonstrated, it could not be demonstrated with p53 gene analysis. As a result of the

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limitations of immunohistochemistry techniques, the unknown factors involved made the results regarding an association between the two protein expressions inconclusive.

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Rapid Acetone Tissue Processing: An Economical Alternative

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Abstract
This report describes the use of acetone for the rapid processing of small biopsy specimens. Two tissue blocks of approximately equal size and thickness were prepared from a palatine tonsil, submandibular salivary gland and squamous cell carcinoma (SCC) of the tongue. Three blocks, one from each tissue, were routinely processed while the other three blocks were acetone processed and impregnated with paraffin wax. Four (m sections were subjected to different histochemical and immunohistochemical stains. All the stains were performed according to prescribed procedures and evaluated light microscopically by two oral pathologists who were blinded to the labelling of the sections from the rapid processed tissue as well as the staining intensity of all the routine and immunohistochemical stains applied compared equal or superior to the sections of the routine processed tissue.

Introduction

Special circumstances necessitate the availability of a rapid processing technique in a histopathology laboratory. Large throughput diagnostic laboratories are concerned with the speed of fixation and processing as well as the costs. Various rapid processing techniques, such as frozen sections and heat and vacuum processing are available that yield histotechnical results that are comparable to routinely processed tissue. An increasingly important aspect of fixation and processing is the preservation of tissue antigens.

In the past few years there have been several advances in rapid fixation and processing methods by microwave (MW) irradiation[1-4]. MW irradiation however, yields artifacts such as lysis of red blood cells due to irradiation of fresh, unfixed tissue[1]. Furthermore, overexposure to MW's results in smudging and pyknosis of nuclei and intense cytoplasmic eosinophilia, whereas underexposed tissues fail to take up stains adequately[1]. Most of the published results of MW procedures and current usage relate to adapted domestic ovens, probably due to the high cost of laboratory dedicated MW's.

Acetone is a known dehydrate in processing techniques, but prolonged exposure causes hardening of tissue[5]. It has, however been shown that fixation in cold-acetone may be preferable to formalin fixation for immunohistochemical detection of certain antigens[6].

This study evaluated rapid acetone processing pertaining to histotechnical quality of sections, preservation of cellular and nuclear morphology, staining intensity and reliability of immunohistochemical analyses.

Material and Methods

Two tissue blocks of approximately equal size and thickness were prepared from each of a palatine tonsil, submandibular salivary gland and squamous cell carcinoma (SCC) of the tongue. Three blocks, one from each tissue, were routinely processed and the other three blocks were acetone processed by dehydration in two changes of fresh acetone for 30 minutes each. As over-dehydration with acetone leads to tissue brittleness, tissue processing was monitored by gentle squeezing with a pair of forceps in order to detect tissue firmness that indicates adequate dehydration. The blocks were then directly impregnated with paraffin wax for 30 minutes and 45 minutes respectively. Impregnation in the second paraffin wax bath was aided with a vacuum unit of the Shandon Citadel processor (Labotec, Midrand, SA). No clearing agent was used with the rapid acetone processing technique.

The routine overnight processing consisted of dehydration with 50%, 70%, 90%, 96% and absolute alcohol. Clearing was done in two changes of xylene followed by impregnation with two changes of paraffin wax. Four (m sections from the six blocks were secretly labelled in order to differentiate the two processing techniques, and subjected to the histological stains listed (Table 1). Sections for immunohistochemical staining were mounted on 3-aminopropyltriethoxysilane coated slides. A standard Haematoxylin & Eosin (H&E) stain, a variety of special staining and immunohistochemical techniques were performed on the sections that were incubated at 54°C. All the investigations were done on both the routine and rapid processed tissue for comparison.

The special stains included a Periodic Acid Schiff (PAS), PAS-diastase and Southgate's mucicarmine on sections from the submandibular salivary gland, while the Masson trichrome technique of Bancroft & Stevens' was performed on the tongue SCC.

TABLE 1
Histological stains used with staining intensity and tissue brittleness evaluation

Stain	Staining intensity		Tissue brittleness	
	Routine processing	Acetone processing	Routine processing	Acetone processing
Palatine tonsil				
H & E	strong	strong	absent	mild
Anti-LCA	strong	strong	absent	absent
Anti-B-cell (CD20)	strong	strong	absent	absent
Anti-Ki-67	weak	strong	absent	absent
Salivary gland				
H & E	strong	strong	absent	absent
PAS	strong	strong	absent	absent
PAS-d	strong	strong	absent	absent
Southgate's mucicarmine	strong	strong	absent	absent
Anti-SMA antigen	moderate	strong	absent	absent
Anti-S100 antigen	moderate	strong	absent	absent
Tongue with SCC				
H & E	moderate	strong	absent	absent
Masson-trichrome	strong	strong	absent	mild
Anti-Cytokeratin antigen	strong	strong	absent	mild

The immunohistochemical stains evaluated were an anti-cytokeratin antigen (HMW, 34BE12) on the tongue SCC, anti-leukocyte common antigen (LCA) (2B11 & PD7/26), the proliferation marker Ki-67 and a B-cell marker (CD20) on the palatine tonsil, and anti-alpha smooth muscle actin (SMA)(1A4) and anti-S100 alpha specific protein on the submandibular salivary gland sections. Dako (DAKO CORPORATION, Carpenteria CA) prediluted antibodies were used for the investigations.

In all the immunohistochemistry procedures, except with SMA antigens, unmasking of the antigens were done in a domestic microwave oven using a pressure cooker. All the sections, including the SMA were treated with 5% hydrogen peroxide at 37°C for 5 min. to quench endogenous peroxidase activity. Slides were incubated with the primary antibodies for 20 min at 37°C. DAKO LSAB 2 Peroxidase kit (DAKO CORPORATION, Carpenteria CA) was used to detect the antibodies. S100 antibodies were detected using the Immunotech Universal immunostaining kit (Coulter Co. BP177-13276 Marseille Cedex 9-France). AEC substrate was the chromogen of choice. Sections were counterstained with Haematoxylin and mounted with Dako Faramount aqueous mounting media.

The tissue sections were evaluated light microscopically for intensity of staining and tissue brittleness. Staining intensity was subjectively recorded as weak, moderate and strong. Tissue brittleness was subjectively recorded as absent, mild, moderate or severe. Two oral pathologists who were blinded to the labelling of the sections performed all evaluations independently and on consensus basis.

Results

Routine processing was completed after 12 hours compared to 2½ hours of acetone processing. Minimal shrinkage of the tissue was observed with both the routine and acetone processed tissue blocks. Four (m sections of good histochemical quality were easily obtained from the acetone processed tissue blocks and the cellular and nuclear morphology were well preserved (Figs 1, 2). The tissue antigens were also well preserved with rapid acetone processing and heat induced epitope retrieval (HIER) was successfully carried out on these tissue sections. No false positive or false negative staining was observed. Acetone processing yielded improved (stronger) anti-Ki-67 staining of the tonsil, anti-SMA and anti-S100 staining of the salivary gland and H&E of the tongue with SCC. The other stains yielded staining

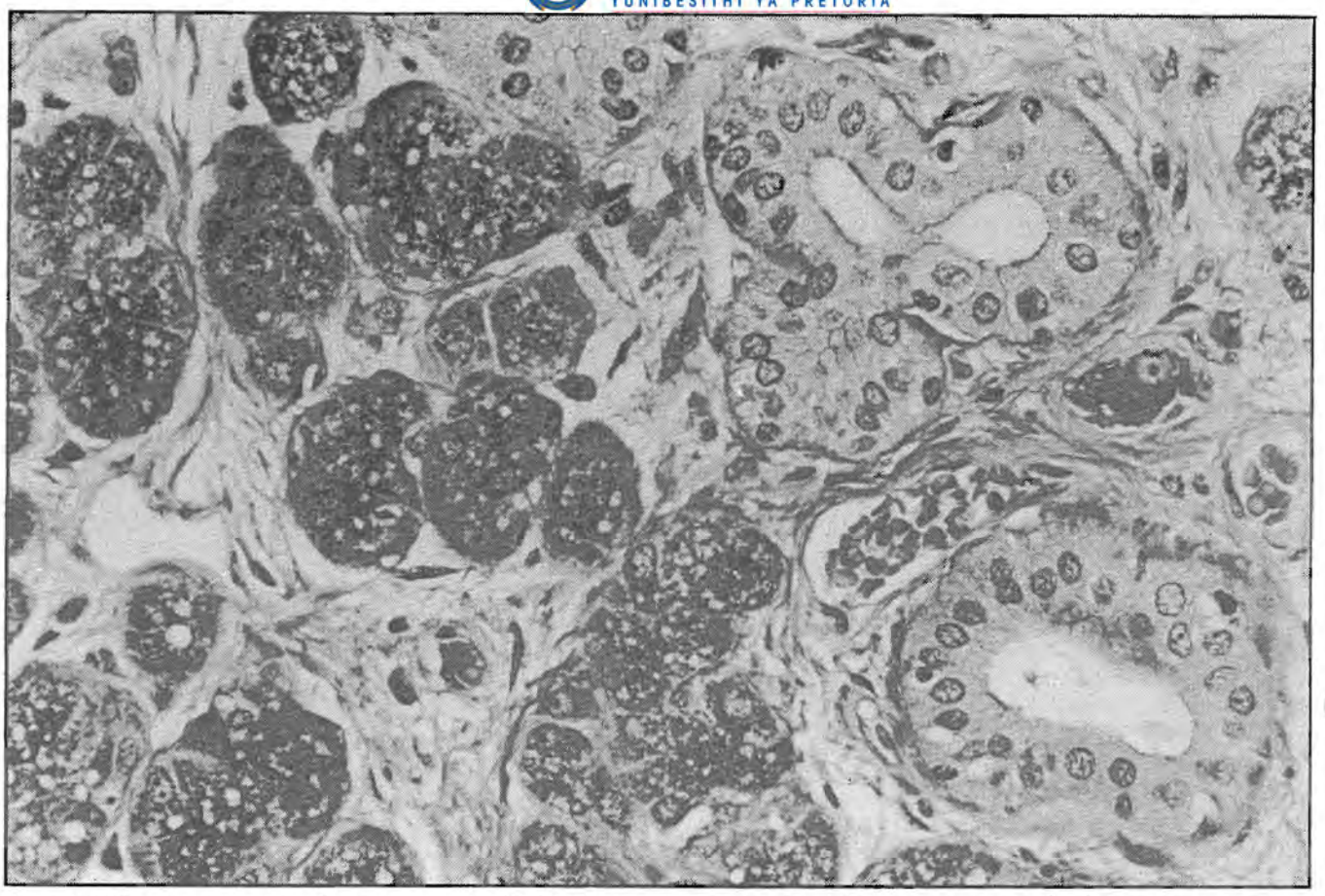

Figure 1: H&E staining of a routinely processed submandibular gland section showing striated ducts, serious acini and small vascular vessels (original magnification × 125).

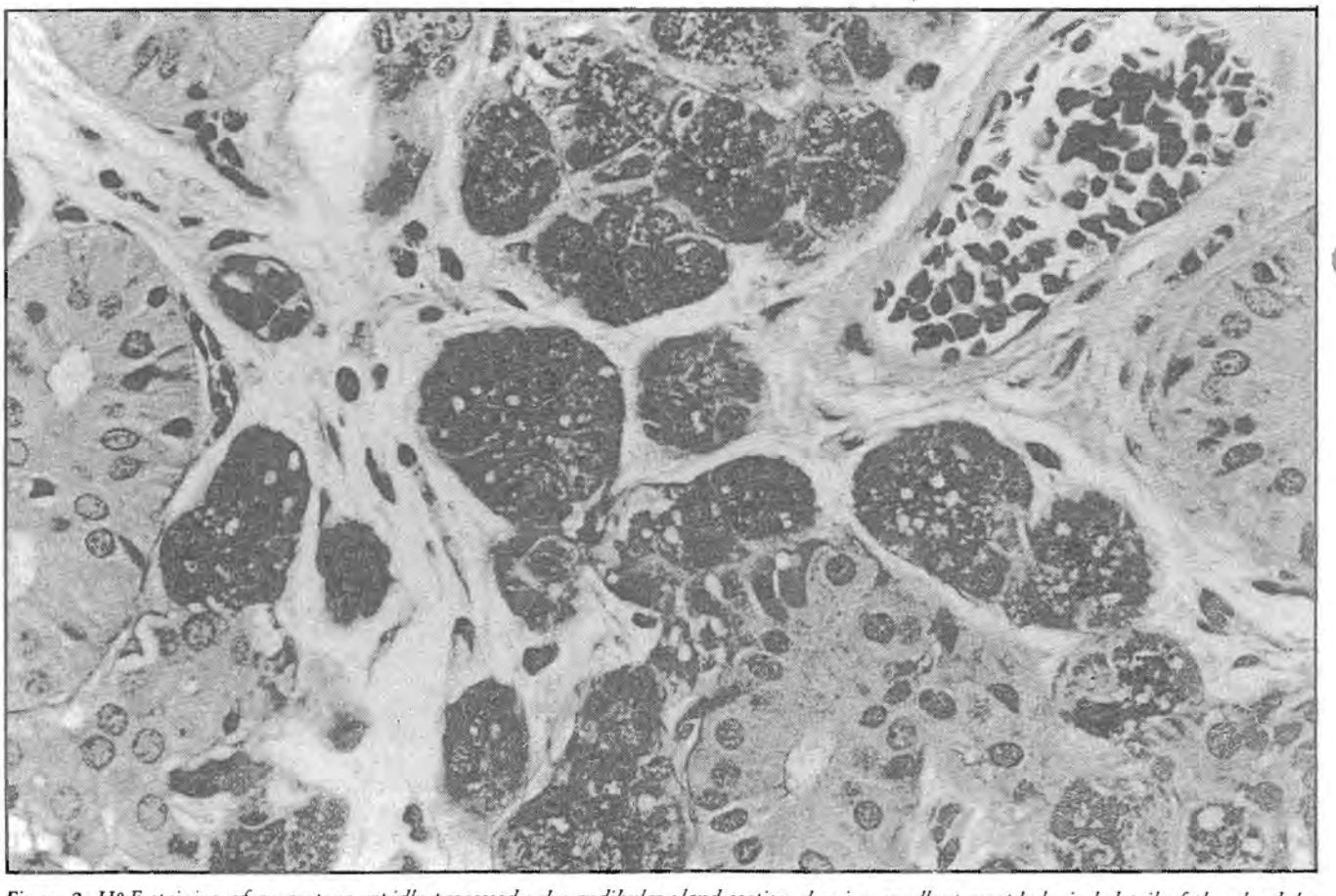

Figure 2: H&E staining of an acetone rapidly processed submandibular gland section showing excellent morphological detail of the glandular structures (original magnification × 125).

intensity similar to the routine processed tissue. Mild tissue brittleness was observed in the H&E section of the palatine tonsil, the Masson trichrome and cytokeratin stained sections of the SCC (Table 1).

Discussion

The use of MW ovens for rapid fixation and tissue processing has increased over the past few years. The standard domestic MW oven is not ideal for this purpose and certain adjustments need to be incorporated in the oven to render it more suitable. Leong suggests that a "stirrer" in the roof of a domestic MW oven should be present to prevent uneven heating and that a large output MW oven (1000 W) is preferable in order to provide a more even field of irradiation[4] This however has financial implications. When a standard domestic MW oven is the only available option in a laboratory, rapid tissue processing with acetone is an economical alternative to adjusting the oven.

No clearing agent was used with the rapid processing technique because the paraffin wax could impregnate the tissue blocks completely without having to remove the acetone with a clearing agent. This saved considerable time.

Tissue processing with acetone has to be monitored manually as processing time may vary according to tissue thickness and tissue type. This requires some practice and is somewhat labour intensive, especially if large numbers of blocks are processed simultaneously. As one becomes more familiar with the technique and tissue types this problem will be eliminated. When single cases are rapid processed the abovementioned yields no problem as the blocks are easily monitored.

The mild tissue brittleness that was observed in three of the twelve rapid processed sections could be due to mild over-dehydration as these sections were probably prepared from the surfaces of the tissue blocks which had been exposed to acetone for a longer period, compared to sections from deeper in the blocks. Minor reductions in the dehydration time of acetone rapid processing should therefore eliminate tissue brittleness. Further investigation is required to prove these statements.

Biopsy specimens are ideal to rapid process because of their size. Routine-size tissue blocks of 10 × 10 × 2mm can also be successfully rapid processed with acetone, as long as the tissue block thickness does not exceed 2mm. Incomplete processing of tissue blocks resulted in the dispersion of sections on the water bath. This can be rectified in the same way as incomplete routinely processed blocks. Acetone processed sections are more likely to wash off the slides when antigen retrieval for immunohistochemistry is performed in a MW. When ample tissue is available this problem can be overcome by rapid processing of half of the tissue and routine processing of the other half. This will ensure that one can obtain sections from the routine processed block that will not wash off the slides during the HIER step. In this study, however, no problem was encountered with washing off of sections.

In our laboratory, acetone processing has been successfully employed as the only rapid processing method for urgent biopsy specimens since 1998. This technique yields thin sections with good cellular and nuclear morphology. There is no difference in the shrinkage of tissue blocks compared to the routine processed tissue blocks. The artifacts encountered with MW irradiation as previously discussed were absent. No signs of smudging or pyknosis of cell nuclei were observed. The staining intensity of the sections was similar or superior to that of the routine processed sections, but as it is a labour intestive method, it is not suitable for a routine procedure. Rapid acetone processing is a simple, rapid and economical technique that can be used with confidence in histopathology laboratories.

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DISCREPANCIES IN HUMAN SERUM ALBUMIN CONCENTRATIONS AS DETERMINED BY COLORIMETRIC, ELECTROPHORETIC AND NEPHELOMETRIC METHODS

(Continued from page 319)

Materials and Methods

1. Colorimetric methods — Albumin concentrations in serum samples utilising BCG and BCP dye were estimated with a Beckman Synchron CX7 Clinical System. (Beckman Coulter Inc., Brea, California).

2. Electrophoretic methods — Cellulose Acetate electrophoresis was performed on a Helena Laboratories (HE) System. (Helena Laboratories, Beaumont, Texas). CZE was performed using a Paragon CZE 2000 System. (Beckman Coulter Inc.). The albumin concentrations in the serum samples were calculated as a percentage of the total protein concentration.

3. Nephelometric methods — Serum albumin concentrations were determined with a Beckman Array 360 System (ARRAY). (Beckman Coulter Inc.) and a Behring Nephelometer-Analyzer (BNA) (Behring Werke AG Diagnostica, Marburg).

Results

Serum albumin concentrations in ten patients, as determined by the different methods, are listed in Table 1. (A/G — Albumin: Globulin ratio for the two electrophoretic methods respectively).

Discussion

From this study it is clear that the BCG dye binding method is not specific for albumin but also binds globulins although with less intensity compared to albumin. The cellulose acetate electrophoretic method in which the proteins are stained (Helena Laboratories) also overestimates albumin. The BCP dye binding method is in line with the CZE electrophoretic and the nephelometric methods.

Conclusion

Although significant differences in human serum albumin concentrations between these methods may exist, the colorimetric method utilising bromcresol purple (BCP) dye is today the most suitable and practical method for routine applications. The capillary zone (CZE) electrophoretic method is superior to cellulose acetate electrophoresis which requires staining of proteins to quantitate albumin. In general, nephelometry is the most specific method for human serum albumin quantitation and is suitable for quantification of albumin in solutions with a low protein concentration. However, nephelometry is expensive to use and not ideal for routine applications. It is therefore not surprising that the BCG reagent has become obsolete as the BCP reagent has now become the method of choice for routine applications.

Acknowledgements

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Immunohistochemical evaluation of Fhit protein expression in oral squamous cell carcinomas

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van Heerden WFP, Swart TJP, van Heerden MB, van Rensburg EJ, Engelbrecht S, Dreyer L, Huebner K: Immunohistochemical evaluation of Fhit protein expression in oral squamous cell carcinomas. J Oral Pathol Med 1999; 28: 433–7.

The expression of Fhit (fragile histidine triad) protein in oral squamous cell carcinoma (OSCC) and adjacent oral epithelium was evaluated by immunohistochemistry on formalin-fixed paraffin-embedded blocks of 32 cases of OSCC. Rabbit polyclonal anti-GST-Fhit antiserum at 1:600 was used, after antigen enhancement in a microwave pressure cooker, in a saturated lead thiocyanate solution. This antiserum has been shown specifically to detect human Fhit by immunohistochemistry at dilutions up to 1:10,000. The Fhit protein expression was evaluated using both the intensity and extent of staining. Normal stratified squamous epithelium showed strong positivity, especially in the stratum spinosum and areas of keratinisation. Basal and parabasal cells were negative or expressed low levels of Fhit relative to the squamous epithelium. Mild and moderate epithelial dysplasia showed Fhit expression in the superficial layers, while Fhit expression was absent from severely dysplastic lesions. A reduction or loss of Fhit expression was found in 21 (66%) of the OSCC. The alterations in Fhit protein expression in OSCC, and not in normal tissues, are consistent with the proposal that Fhit inactivation plays a role in oral carcinogenesis.

Key words: chromosome 3; Fhit; immunohistochemistry; oral cancer; tumour suppressor genes

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The incidence of oral squamous cell carcinoma (OSCC) appears to be on the increase in developing countries (1). During the period 1990–1992, an average of 1336 new intraoral cancers per year was reported in South Africa, resulting in a frequency of 3.4% of all diagnosed cancers, excluding basal cell carcinomas and squamous cell carcinomas of the skin (2, 3). Oral cancer ranked fifth behind cancer of the prostate, oesophagus, lung and colon in males in South Africa. In combination with cancers of the pharynx, these malignancies were the fourth most common in South African males and the second most common in Black males (2, 3).

The *FHIT* (fragile histidine triad) gene was isolated from chromosome band 3p14.2 (4). The gene is encoded by 10 exons in a 1.1-kb transcript encompassing at least 1 Mb of genomic DNA (5). The protein has 147 amino acids and shows homology to diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap4A) hydrolase from the yeast *Schizosaccharomyces pombe* (6). The *FHIT* gene contains the FRA3B fragile site and is the target of homozygous deletions in many human cancer cell lines (4); *FRA3B* is also the most common fragile site in humans (7). The 3p region is one of several regions of frequent loss of heterozygosity (LOH) that have been identified in head and neck squamous cell carcinomas (HNSCC) (8). The others include 9p, 11q, 13q and 17p (9).

FHIT allele alterations were detected in 55% of HNSCC cell lines using a reverse transcription-polymerase chain reaction (RT-PCR), Southern blot analysis and interphase fluorescence *in situ* hybridisation (FISH) (10). Abnormal transcription of the *FHIT* gene in HNSCC was assessed by RT-PCR amplification by Mao *et al.* (11), who suggested that loss of *FHIT* function might be important in the development of HNSCC and proposed it as a potential tumour suppressor gene for HNSCC (11).

In a more comprehensive study of nine HNSCC cell lines and 32 primary HNSCCs, Kisielewski *et al.* (12) examined the *FHIT* gene for alterations at the DNA, RNA and protein levels. The HNSCC cell lines were examined for *FHIT* transcription by Northern blot analysis and *FHIT* transcript was not detected in the seven HNSCC cell lines, suggesting little or no *FHIT* transcript. RT-PCR amplification of *FHIT* RNA from these cell lines showed complete absence of a normal-sized *FHIT* transcript in two of the cell lines; the others showed a full-size RT-PCR product,

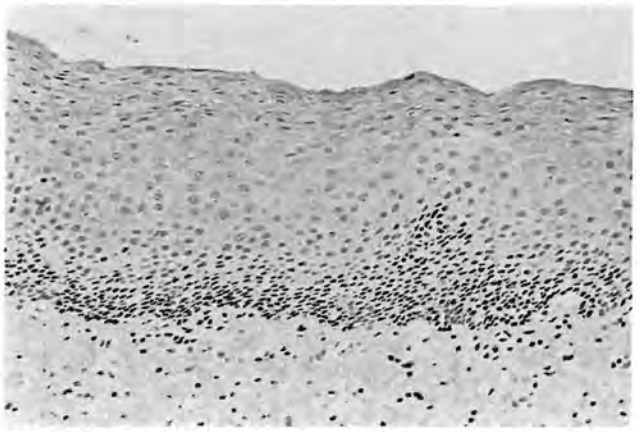

Fig. 1. Normal stratified squamous epithelium showing prominent Fhit staining in the stratum spinosum and stratum superficiale. Note the scarcity of staining in the basal and parabasal cells (×60).

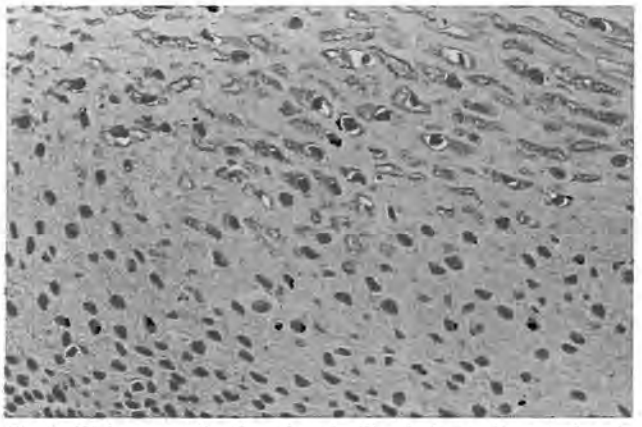

Fig. 2. Higher magnification than in Fig. 1 shows the cytoplasmic staining of the more superficial epithelial cells (×125).

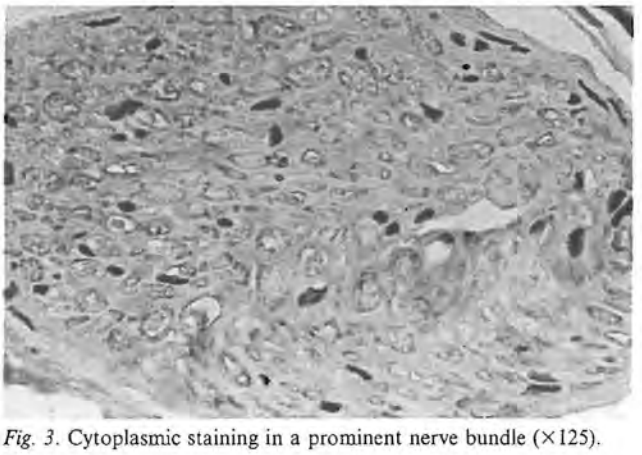

Fig. 3. Cytoplasmic staining in a prominent nerve bundle (×125).

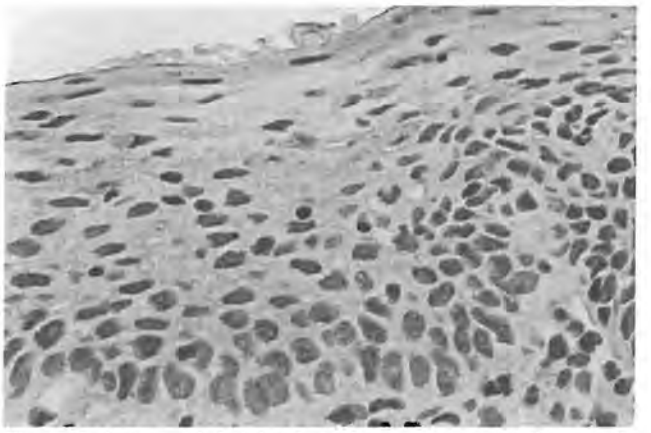

Fig. 4. Moderate epithelial dysplasia showing staining in the superficial cells but no staining in the basal and parabasal dysplastic cells (×250).

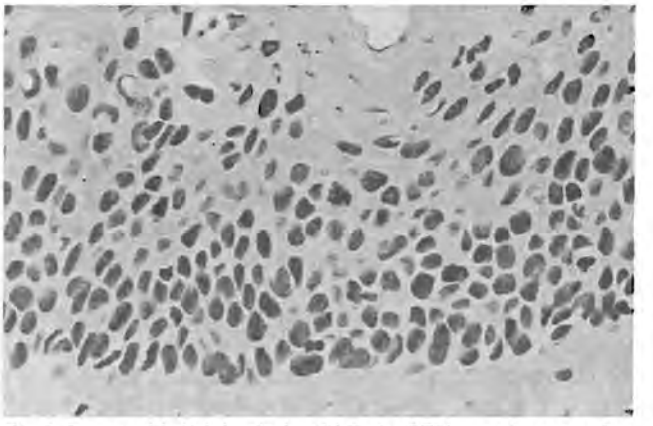

Fig. 5. Severe epithelial dysplasia with lack of Fhit protein expression in all cell layers (×250).

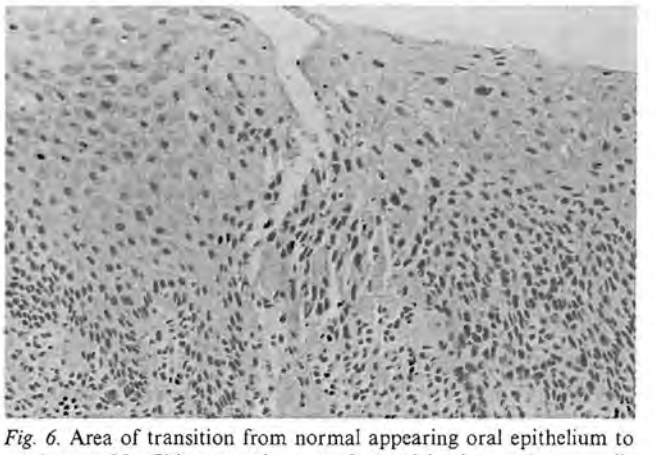

Fig. 6. Area of transition from normal appearing oral epithelium to carcinoma. No Fhit expression was detected in the carcinoma cells (×125).

demonstrating a low level of *FHIT* transcript, undetectable by Northern analysis. Six of the nine cell lines (66%) showed no Fhit protein or trace amounts. All of the primary tumours expressed very little Fhit, which could be due to contaminating non-tumour cells (12). This question would need to be pursued by immunohistochemical detection of Fhit to determine the actual level of Fhit expressed in the primary cells. It is important to resolve this question because evidence is accumulating in other tumour systems that Fhit can be inactivated in a large fraction of a number of tumour types (13,14) and at a very early stage in some, such as lung carcinomas (15).

Abnormalities of the *FHIT* gene and its expression have been reported in digestive tract (4), cervix (16), lung (15, 17), and kidney (18, 19) carcinomas.

The Fhit protein has dinucleoside 5′,5‴-P^1,P^3-triphosphate hydrolase activity *in vitro* (6) but its cellular function is thus far unknown (13).

To evaluate the possible role of Fhit

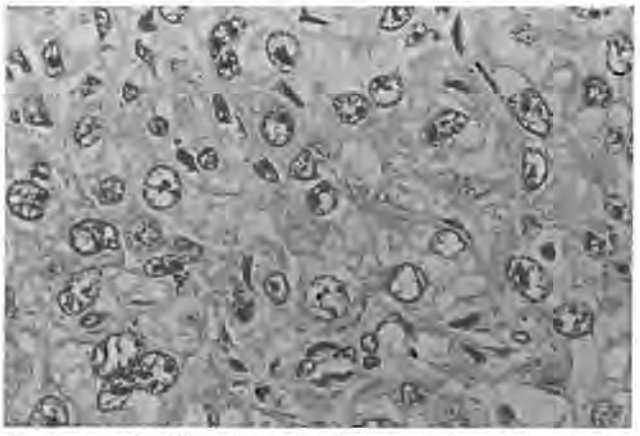

Fig. 7. A poorly differentiated OSCC without Fhit protein expression (×300).

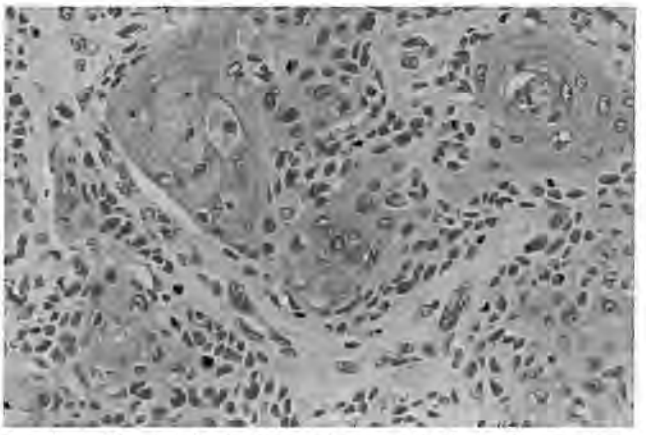

Fig. 8. Positive staining in the carcinoma cells. Note the prominent staining in the better-differentiated areas (×250).

protein in oral squamous cell carcinomas, we have studied its expression in normal and carcinoma tissues. The pattern of expression of Fhit protein in oral squamous epithelium, adjacent epithelial dysplastic lesions (when present) and squamous cell carcinoma was evaluated by immunohistochemistry using Fhit-specific antiserum (6, 20).

Material and methods

Intraoral squamous cell carcinomas were retrieved from the archives of the Departments of Oral Pathology and Anatomical Pathology, University of Pretoria. Blocks containing squamous cell carcinoma with adjacent epithelium were selected. Thirty-two cases were included in this study.

These formalin-fixed, paraffin-embedded tissue blocks were sectioned at 3 μm onto 3-aminopropyl-triethoxysilane coated slides. (SIGMA A3648), dried overnight at 37°C and then deparaffinised in two changes of fresh xylene for 5 min each. Sections were hydrated into dH2O through a series of graded alcohols. Antigen enhancement was performed by microwave heating of the sections in a saturated lead thiocyanate solution in a microwave pressure cooker. Slides were subsequently cooled for 20 min. Slides were washed three times in phosphate buffer and then treated with hydrogen peroxide for 5 min at 37°C to reduce endogenous peroxidase activity. Following additional phosphate buffer washes the slides were incubated with PBA (Coulter 0599 Universal Immunostaining kit) for 5 min at 37°C. Slides were then incubated overnight at 25°C with primary rabbit polyclonal anti-GST-FHIT antiserum at 1:600 (17, 19, 20). This antiserum is against the full length Fhit protein fused at the N-terminus to GST. Subsequent staining was performed with the Coulter 0599 Universal Immunostaining kit. Slides were then washed three times and incubated with secondary biotinylated antibody for 10 min at 37°C. After washing the slides three times in phosphate buffer they were incubated with the streptavidin-peroxidase reagent for 10 min at 37°C. Antibody localisation was effected by a 4-min incubation at 37°C with the chromogen, AEC. Finally, slides were washed in dH2O, lightly counterstained with haematoxylin, washed and then coverslipped using Faramount (Dako S3025) aqueous mounting medium. Negative controls were done by substituting the primary antibody with PBS buffer.

The intensity of Fhit protein expression was evaluated using criteria suggested by GREENSPAN *et al.* (17). The intensity of staining was recorded as: absent/weak, 1; moderate, 2; and strong, 3. The extent of immunostaining was scored based on the percentage of positive cells: <10%, 1; 10–50%, 2; and >50%, 3. The two scores were then multiplied to give a composite score (1–9) for each tumour. Composite scores of 1–3 were defined as marked reduction or absence of Fhit protein expression. The areas of the squamous cell carcinoma showing the poorest differentiation were used for evaluation of Fhit protein expression.

The sections were evaluated and scored independently by two oral pathologists. Those cases where the composite scores were not in concordance (<3 versus>3) were reviewed and scored on consensus opinion.

Results

The normal stratified squamous epithelium showed strong staining for the Fhit protein. This staining was more prominent in the stratum spinosum and in areas of keratin differentiation, with almost no staining in the basal and parabasal cells (Figs. 1 & 2). The staining was seen as a granular cytoplasmic chromogen. Staining was also seen in the excretory ducts of the underlying minor salivary glands and in nerve bundles (Fig. 3).

The intensity of staining in the normal appearing epithelium could be classified as strong in the majority of cases. Three cases showed only a moderate intensity of staining in the normal epithelium. A decrease in staining intensity was seen where the normal epithelium was associated with a dense chronic inflammatory cell infiltrate in the upper lamina propria and with exocytosis of lymphocytes into the epithelium.

Mild and moderate dysplastic changes did not appear to influence the Fhit expression of the epithelium (Fig. 4). Severe dysplastic changes, though, consistently showed absence of Fhit expression (Fig. 5). Most cases, however, showed a sudden transition from normal appearing epithelium to carcinoma, resulting in dysplastic lesions being present in only a few cases (Fig. 6).

Twenty-one (66%) of the squamous cell carcinomas had scores of three or less, indicating a distinct reduction or loss of Fhit protein expression (Fig. 7). Agreement with regard to the extent of staining for the two types of Fhit expression (≤3 and>3) was reached by the two oral pathologists in all the cases. A heterogeneous staining pattern

was observed in some of the tumours. When carcinoma cells were positive, the staining was found to be more prominent in the better-differentiated areas, especially the areas of keratinisation. (Fig. 8). Tumour grading, however, did not influence Fhit expression. Moderately to well-differentiated tumours were found to be negative, while some poorly differententiated tumours expressed Fhit protein.

Discussion

Fhit expression in tumours may be evaluated by quantification of *FHIT* mRNA expression. This may be impeded by lack of enough RNA, poor RNA quality and contaminating non-neoplastic cells in the tumour specimen (17). Immunohistochemical techniques directed against the protein product are also suitable to evaluate Fhit expression in different tumours (15, 17, 19).

The presence of Fhit protein in adjacent normal appearing epithelium served as a positive internal control for the immunohistochemical technique in this study. The extent of Fhit protein expression in various tissues has been assessed in various studies by analysis of RNA and protein levels. Epithelia of all major organs tested express Fhit protein strongly (breast, stomach, distal colon, kidney, cervix, liver) to moderately (lung, pancreas); lymphoid and brain tissue express Fhit protein less strongly and only in subsets of cells (13, 15–19, 21). The moderate staining observed in the normal epithelium in three of our OSCC cases may be either fixation or processing related.

The lack of influence of mild and moderate dysplastic changes on Fhit protein expression was to be expected. Marked Fhit protein expression in oral epithelium was consistently observed in the stratum spinosum and superficial epithelial areas. The basal and parabasal cells, which are phenotypically altered in mild and moderate epithelial dysplasia, were uniformly negative. Fhit protein expression in mild and moderate epithelial dysplasias would therefore be expected to be negative. Severe epithelial dysplasia, with atypical cells through the full thickness of the epithelium, including stratum spinosum and superficial layers, showed no Fhit protein expression. The absence of Fhit protein expression in the severe dysplastic lesions suggested that *FHIT* inactivation might occur at an early phase of oral squamous cell carcinogenesis. The number of carcinomas with adjacent dysplastic epithelium was unfortunately too small to correlate the staining pattern of the dysplasia with the carcinoma. Absence of Fhit protein expression was also found in dysplastic lesions of the lung (15).

The prominent positivity of the better differentiated squamous cell carcinoma cells may be linked to the expression pattern of the overlying epithelium, where strong positivity was absent from the basal and parabasal cells. The negative basal and parabasal cells in normal epithelium might be an indication that Fhit protein was not expressed in large enough quantities to be detected with immunohistochemistry. These negative cells had no correlation with Fhit-negative tumours, since no Fhit protein was expressed in the basal and parabasal epithelium cells of Fhit-positive tumours. This is in contrast to squamous cell carcinomas from the lung where SOZZI *et al.* (15) observed a prominent basal pattern of expression in lung squamous cell carcinomas. No staining was detected in the differentiated tumour cells in their study.

It has been suggested that *FHIT* gene involvement in a variety of malignancies is a consequence of its location in a genetically unstable region (22). All the sections in this study included normal epithelium as well as neoplastic cells. Absent or reduced Fhit protein expression was observed only in the malignant cells and in severe dysplastic lesions compared to the normal squamous epithelium. This would suggest the inactivation of both *FHIT* alleles.

The loss of Fhit protein may affect oral carcinogenesis by causing genetic instability, perhaps through loss of DNA replication control *via* its Ap4A hydrolase function. Alteration of the normal Ap4A levels in cells may compromise their ability to respond to environmental stresses caused by carcinogenic agents. A common feature of the different tumours presenting with abnormal *FHIT* is that they are carcinomas from areas directly exposed to environmental carcinogens (4).

The alterations in Fhit protein expression in many oral squamous cell carcinomas, but not in normal tissues, suggest that *FHIT* gene alterations may play a role in oral carcinogenesis. These results confirm and complement the study by KISIELEWSKI *et al.* (12), who showed that 6 of 9 (66%) HNSCC cell lines expressed no Fhit protein or only trace amounts. This fraction of Fhit-negative HNSCC cell lines is the same as our fraction of 32 Fhit- negative/reduced primary OSCCs. The former report also investigated protein expression in 32 primary HNSCCs by Western blot analysis and found that all tumours expressed low levels of Fhit. These authors discuss the fact that the low level of Fhit protein in primary tumours, and indeed the light RT-PCR bands from RNA templates of primary tumours, may indicate that *FHIT* RNA and protein expression are actually absent and that they are detecting expression from normal infiltrating stromal cells. Immunoblotting results cannot resolve this issue. Our immunohistochemistry study confirms that the majority of OSCCs have lost Fhit protein expression. Since by now hundreds of tumour cell lines and primary tumours of various types have been shown to harbour independent *FHIT* deletions in both alleles (13, 14), it is likely that the majority of OSCCs carry *FHIT* alleles that have been damaged directly by exposure to carcinogenic agents.

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FHIT RNA and Protein Expression in Oral Squamous Cell Carcinomas

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Abstract. *Background: To investigate the possible role of FHIT, a possible tumour suppressor gene, in oral carcinogenesis, we examined 17 oral squamous cell carcinomas (OSCCs) for genetic alterations. Materials and Methods: Fresh tissue was obtained during surgery, snap-frozen in liquid nitrogen and stored at -70°C. Nested PCR amplification to examine the integrity of FHIT mRNA was performed on the reverse transcribed complementary DNA obtained from the frozen normal and tumour tissue. Immunohistochemistry was done on formal in-fixed paraffin-embedded tissue protein from the same cases using a polyclonal antiserum against the full length Fhit. Results: Twelve out 17 (71%) OSCCs showed reduced or absent Fhit protein and half of the cases with reduced Fhit protein exhibited aberrant RT-PCR products. Conclusion: Immunohistochemical detection of Fhit protein expression in OSCCs is the more sensitive method to determine the status of Fhit in these tumours, in agreement with previous studies of other tumour types.*

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity and appears to be on the increase in developing countries (1). Oral cancer rank 6th behind cancer of the prostate, lung, oesophagus, bladder and colon in males in South Africa. In combination with cancers of the pharynx, these malignancies are the 4th most common in South African males (2).

Previous cytogenetic and molecular studies of oral cancer have indicated that malignant transformation in the carcinogenic pathway results from progressive accumulation of genetic changes, including inactivation of tumour-suppressor genes, amplification or over-expression of oncogenes or both, and specific chromosomal abnormalities (3). Tobacco and alcohol have been implicated as the most important aetiological agents in the majority of head and neck malignancies (4). Exposure to tobacco carcinogens may also lead to characteristic mutational events in head and neck malignancies, as have been observed with the p53 gene (5). Particular genetic alterations have also been demonstrated in other tobacco- related malignancies such as lung cancer, where alterations involving the *FHIT* gene are significantly increased in smokers compared to non-smokers (6). It has also been shown that differences in genetic changes of squamous cell carcinoma from different sites in the head and neck region do exist, suggesting intrinsic tumour properties regardless of a common aetiological agent (7).

The *FHIT* gene at 3pl14.2 covers the renal carcinoma-associated chromosome t(3;8) translocation breakpoint and the FRA3B fragile site (8). This gene encodes a protein that shows homology to diadenosine 5',5'''-P^1, P^3-triphosphate (Ap3A) hydrolase from the yeast Schizosaccharomyces pombe (9) and is itself a diadenosine polyphosphate hydrolase. Support for the tumour suppressor function of the *FHIT* gene is based on homozygous deletions within the gene in numerous tumour cell lines (8, 13), the presence of aberrant *FHIT* transcripts in different tumours (10-12), a high incidence of allelic imbalance at 3p14.2 (13, 14) and loss of Fhit protein expression in cancers.

Abnormalities of the FHIT gene and its protein expression have been reported *inter alia* in digestive tract (8), lung (15, 16), cervical (17, 18) and kidney (19, 20) carcinomas. A reduction or loss of Fhit protein expression was also found in 66% of oral squamous cell carcinomas (OSCC) (21). The aim of this study was to evaluate the pattern of Fhit protein expression in OSCC and to compare its protein expression status with detectable genetic abnormalities.

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Key Words: Oral cancer, oral squamous cell carcinoma, *FHIT* gene, Fhit expression.

Materials and Methods

Tissues. Seventeen fresh OSCC tumour samples were obtained from resection specimens, snap-frozen in liquid nitrogen and stored at -70°C to be used for isolation of RNA followed by RT-PCR analysis. The remainder of the material was fixed in 10% buffered formalin and used

for routine histological examination, followed by immunohistochemical analysis.

RT-PCR. RNA was prepared from frozen oral tissue using the RNeasy protocol for the isolation of total RNA from animal tissues (Qiagen GmbH, Germany). RT-PCR was carried out using the Access RT-PCR system (Promega Corporation, Madison WI, USA). The reaction mixture contained 5µl RNA, 200 µM of each nucleotide, 0.4 µm of each primer 5U2 (5'-CATCCTGGAAGCTTTGAAGCTC) and 3D2 (5'-CCTGTATTCTCAACCAGTGA), 1U of *Taq* DNA polymerase (Promega Corporation), 10 µl of Promega 10x bufferl and 1.5 M of $MgCl_2$ made up in a final volume of 100 µl. Amplification cycles were carried out using a Perkin-Elmer PCR 9600 machine as follows: 48°C for 45 minutes; 94°C for 2 minutes; 40 cycles denaturing at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 68°C for 1.5 minutes and one cycle extension at 68°C for 7 minutes. A nested PCR was performed using 2 µM of RT- PCR product and primers 5U1 (5'-TTCCGTAGTGCTATCTACAT) and 3D1 (5'-CAAGAGGAACTGAATCAGCATG). PCR amplification was as follows: one cycle of denaturing at 94°C for 2 minutes forty-five cycles denaturing at 94°C for 1.5 minutes annealing of primers 50°C for 1.5 minutes; and extension of the annealed primers at 72°C for 3 minutes and one cycle extension at 72°C for 7 minutes. The 700 base pair (bp) PCR products were visualised with ultraviolet light after electrophoresis through 2% agarose gels and ethidium bromide staining.

Sequencing. The PCR products of four samples yielded more than one band on agarose gel electrophoresis, namely samples 2, 8, 12 and 15. They were run on a 20% polyacrylamide gel and the bands were cut out. The DNA was extracted using the Qiaex II Gel Extraction kit (Qiagen GmbH, Germany) and the PCR was repeated using the nested primer pair. The nested PCR products were directly sequenced on the 373 DNA Sequencer (Perkin Elmer, California, USA) using the ABI Prism Dye Terminator Cycle sequencing kit (Perkin Elmer, California, USA). 5Ul and 3Dl were used as sequencing primers. The sequences were translated into amino acids using the DNAMAN computer program (Lynnon Biosoft, Canada, 1994). Alignment of the amino acid sequences was conducted using the CLUSTAL V software program.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded tissue blocks of the same OSCCs were used for immunohistochemical evaluation of Fhit protein expression. These formalin-fixed, paraffin-embedded tissue blocks were sectioned at 3 µm and mounted onto 3-aminopropyltriethoxysilane-coated slides (SIGMA A3648), de-paralfinized in xylene and rehydrated in graded alcohol. Antigen enhancement was performed by microwave heating of the sections in a microwave pressure cooker in citric acid buffer (pH 6). The slides were subsequently cooled for 20 minutes and then washed three times in phosphate buffer and treated with hydrogen peroxide for 5 minutes at 37°C to reduce endogenous peroxidase activity. Immunohistochemical detection of Fhit protein was carried out using a polyclonal antibody (ZR44) that detects the full length Fhit protein (Zymed Laboratories, South San Francisco, CA, USA). The tissue sections were treated with the non-immune blocking serum of the Zymed Histostain^{TM}-Plus detection kit (85-9043) for 10 minutes at room temperature. The sections were then incubated with the antibody for 60 minutes at room temperature. Detection of the antibody reactivity was done with the above mentioned Zymed detection kit according to the manufacturer's instructions. AEC was the chromogen of choice. Finally, the slides were washed in distilled water, lihtly counterstained with haematoxylin, washed and then coverslipped using Faramount (Dako S3025) aqueous mounting medium. Negative controls were performed by substituting the primary antibody with PBS buffer. Both the extent and intensity of Fhit protein expression was evaluated. The intensity of staining was recorded as: absent/weak, 1; moderate, 2; and strong, 3. The extent of immunostaining was scored based on the percentage of positive cells: <10%, 1; 10-50%, 2; and >50%, 3. The two scores were then multiplied to give a composite score (1-9) for each tumour. Composite scores of 1-3 were defined as marked reduction or absence of Fhit protein expression while scores >3 were considered positive for Fhit. The tumour cells at the invasive tumour front were used for evaluation of Fhit protein expression.

Table I. *RT-PCR results compared with immunohistochemistry findings. Concordant findings were observed in ten cases* and discordant findings in six cases. Simple could not be sequenced.*

Sample number	Fhit protein composite score	Interpretation of RT-PCR results
1	1 (neg)	Normal
2*	3 (neg)	Deletion within one *FHIT* allele
3	1 (neg)	Normal
4*	6 (pos)	Normal
5	2 (neg)	Normal
6	3 (neg)	Normal
7	4 (pos)	Not evaluable
8*	3 (neg)	Deletion within one *FHIT* allele
9*	6 (pos)	Normal
10	3 (neg)	Normal
11 *	1 (neg)	Deletion within one *FHIT* allele
12*	1 (neg)	Deletion within one *FHIT* allele
13*	3 (neg)	Deletion within one *FHIT* allele
14*	6 (pos)	Normal
15*	1 (neg)	Deletion within one *FHIT* allele
16*	6 (pos)	Normal
17	1 (neg)	Normal
19 (positive control)		Normal
20 (positive control)		Normal

Results

Heterogeneity or a mixed pattern of protein expression, with co-existing posotive and negative tumour cells occurred in most of the tumours. Staining was also seen in nerve bundles, skeletal muscles, inflammatory cells and promlnent in the excretory ducts of minor salivary glands when present. Fhit protein expression at the invasive front was detected in 30%(5/17) of the tumours.

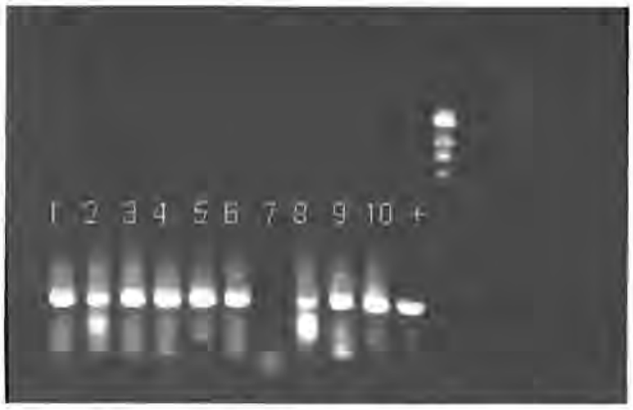

Figure 1. *Ethidium bromide-stained agarose gel of nested PCR products. All specimens 1- 'b demonstrated a normal band except 7, where no band was found. Specimens 2 and 8 also demonstrated aberrant RT-PCR products.*

Figure 2. *RT-PCR products from OSCCs. Cases 11 and 13 showed only aberranl-sized RT-PCR lel products, suggesting deletion within both FHIT alleles, cases I2 and 15 exhibited both normal IQd and aberrant-sized amplification products, suggesting a mixed population oJ cells with intact and deleted FHIT alleles. Cases 14, 16 and 17 showed only Ihe normal sized FHIT RT-PCR product.*

The agarose gels of the nested RT-PCR products are pictured in Figures 1 and 2. Aberrant transcripts were observed in 6 (35%) of the cases. All RT-PCR amplifications were repeated 3 times with similar results. All the samples had an 11 bp deletion representing an alternative splice form, when compared with the FHIT consensus sequence. Samples 2, 8, and 11 had a 296 bp deletion within the cDNA, while a 458 bp cDNA deletion was observed in samples 12 and 15. A summary of the RT-PCR amplification results and the corresponding immunohistochemistry analysis of *FHIT* protein expression are shown in Table I.

Discussion

RNA was used for genetic analysis because the *FHIT* genomic locus is very large (more than a 1000 kilobases) while the coding region (mRNA) is small (about 1 kilobase in size). Perfect correlation between the RT-PCR and immunohistochemistry results was observed in 10 cases. Immunohistochemistry for the Fhit protein was negative in all the cases where RT-PCR results were abnormal. Six cases showed normal RT-PCR results with no detection of Fhit protein expression. Several explanations were possible: it might have been that very low levels of the RNA were expressed in these cases, too low for immunohistochemical detection of the Fhit protein, thereby reflecting the difference in sensitivity of the two methods. It might also be possible that Fhit protein was absent in tumours and that the RT-PCR products detected derived from amplification of FHIT messages from normal stromal and inflammatory cells. The Fhit epitopes recognised by the antibody might also have been altered by processing or fixation (22), although this seems an unlikely explanation since all samples were treated similarly.

The *FHIT* gene is inactivated by deletion rather than mutation (8, 23, 24). The complexity of DNA lesions observed in *FHIT* gene studies may point towards protein detection as the best method to assess the level of involvement of Fhit in various human cancers. More sensitive Western blots could be used, although only immunohistochemistry can evaluate the fraction of cells expressing and their level of protein expression. Five of the cases (30%) showed Fhit protein expression by immunohistochemistry. The method of evaluating Fhit expression by immunohistochemistry is very important. The invasive tumour front was used as the preferred site to evaluate Fhit expression. This site was selected because of its appropriateness in predicting prognosis in OSCC (25). This evaluation of selected sites in a tumour overlooked the presence of heterogeneity of Fhit expression results and should be considered when comparing immunohistochemical results from different studies.

Abnormal RT-PCR products were amplified in 6/17 (35%) cases. Four of the six cases showing aberrant transcripts on RT-PCR had normal transcripts as well. This may reflect the presence of different clones in the tumour, some of which may have homozygous deletions, or contamination with normal stromal cells as the source of normal transcripts. The frequency of aberrant sized *FHIT* products in our study was higher than that reported for head and neck squamous cell carcinomas (HNSCC) in several studies. Kisielewski *et al* (26) found aberrant products in 2/9 HNSCC-derived cell lines and 4/42 primary HNSCC (three of which failed to give amplified fragments), similar to the 4/16 from Mao *et al* (12) and the 1/8 HNSCC-derived cell lines and 1/11 primary OSCC from the study by Pateromichelakis *et al* (27). Virgilio *et al* (23) observed aberrant transcripts in 15/25 (55%) in their HNSCC-derived cell lines. These differences could be attributed to different RT-PCR methods used or different cell culture conditions.

These results indicated that FHIT aberrations were frequently found in OSCC in the population sample studied. Loss of Fhit expression at the invasive tumour front was also common but may not necessarily correlate with detectable gene alterations.

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Fhit Protein Expression in Oral Epithelium: Immunohistochemical Evaluation of Three Antisera

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Abstract. *Background: A number of studies have shown that the Fhit tumour suppressor protein is abundantly expressed in normal epithelial cells of human organs and that this expression is lost or reduced in the majority of cancers arising in these epithelial tissues. A variety of antiFhit sera have been used but a systematic comparison of the different antisera has not yet been reported. Materials and Methods: We compared the Fhit expression pattern in the epithelium of fibrous epuli, oral lichen planus, oral epithelial dysplasia and oral squamous cell carcinomas (OSCC) using three different Fhit antisera. Results: The antigstFhit sera from two sources gave very similar results for all types of oral lesions except for lichen planus and showed that about 60% of OSCCs have lost Fhit expression. Conclusion: Although different staining patterns were found for the three antisera, all three could be used for evaluation of Fhit expression in OSCC.*

The *FHIT* (fragile histidine triad) gene, a candidate tumour suppressor gene, has been mapped to 3p14.2 and covers the renal carcinoma-associated chromosome t(3;8) translocation breakpoint, the FRA3B fragile site and homozygous deletions in many human cancer cell lines (1). This gene encodes a protein that shows homology to diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap3A) hydrolase from the yeast *S. pombe* (2) and the human and murine Fhit proteins are diadenosine polyphosphate hydrolases. Support for the tumour suppressor function of the *FHIT* gene is based on the presence of aberrant *FHIT* transcripts in different tumours (1, 3-5), homozygous deletions in the cDNA of many tumour cell lines (1, 6), a high incidence of allelic imbalance at 3p14.2 (1, 6, 7) and functional studies showing suppression of tumourigenicity after exogenous expression of Fhit in cancer cells (8-10).

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Key Words: Fhit protein, immunohistochemistry, oral mucosa, oral cancer.

The evaluation of Fhit protein expression using immunohistochemistry has been reported *inter alia* in lung (11-13), cervical (14-16), breast (17), kidney (18) and oral squamous carcinomas (19). Fhit protein is also expressed in the normal epithelia of all major organs tested (12, 13, 15, 19-21).

Although most immunohistochemical studies have reported a large fraction of cancers with reduced or absent *Fhit* protein relative to Fhit-positivity in corresponding normal tissues, there have been variations in the percentages of negative tumours of specific types, for example lung cancer (12, 13). Since different antibodies against Fhit protein have been used in the reported studies (16, 18, 19), differences in affinity, avidity or other variables could contribute to these discrepancies. To evaluate the staining patterns of Fhit protein in oral epithelium in different disease states and, at the same time, to compare different antisera against *Fhit*, we studied its expression using three different Fhit antisera.

Materials and Methods

Tissues. Formalin-fixed paraffin-embedded blocks of fibrous epuli, oral lichen planus, epithelial dysplasia and oral squamous cell carcinoma (OSCC) were retrieved from the files of the Department of Oral Pathology, University of Pretoria, South Africa. Fibrous epuli are reactive connective tissue lesions of the gingiva covered by normal stratified squamous epithelium. No sub-epithelium inflammatory cell infiltrate was present in the fibrous epuli included in this study. These served as the normal epithelium control. Lichen planus is an immune-mediated skin disease characterised by a dense sub-epithelial lymphocytic infiltrate and was included to evaluate the possible influence of an inflammatory cell infiltrate on the staining properties of the overlying epithelium. Ten blocks each of the fibrous epuli and lichen planus and twenty blocks each of epithelial dysplasia (varying from mild to severe) and OSCC were selected.

Immunohistochemistry. Immunohistochemical detection of Fhit protein was carried out using three different antibodies recognising human *Fhit* protein. Two Zymed polyclonal rabbit anti-*Fhit* sera (Zymed Laboratories, South San Francisco, CA, USA) were used. One of these sera (ZP54) was raised against a synthetic peptide derived from a sequence near the C-terminus of the human Fhit protein and detects

Table I. *Composite staining scores for Fhit expression in oral epithelium. The loss or reduction of Fhit expression in OSCC is expressed as a percentage.*

Antisera	Fibrous epulis (n=10)	Lichen planus (n=10)	Epithelial dysplasia (n=20)	OSCC (n=20)	%OSCC with loss/reduction
Zymed:					
GST-*Fhit*	5.4 ± 2.9	2.7 ± 1.3	6.1 ± 2.3	3.6 ± 2.2	66
Zymed:					
Fhit C-terminus	2.0 ± 1.1	0.7 ± 0.6	1.7 ± 2.2	4.1 ± 2.9	60
KCC:					
GST-*Fhit*	4.6 ± 2.4	6.2 ± 1.9	6.2 ± 2.7	4.9 ± 2.7	60

full-length Fhit protein in Western blots. The other Zymed antiserum (ZR44) also detects full-length *Fhit* protein and the full-length human Fhit fusion protein with glutathione amino S-transferase (GST) was used as immunogen. The other serum was received from the Kimmel Cancer Center (KCC) and consisted of a rabbit polyclonal anti-GST-Fhit fusion protein. Three sections (3 mm each) of each paraffin-embedded tissue block were cut and mounted onto aminopropyltriethoxysilane coated slides (SIGMA A3648), de-paraffinized in xylene and rehydrated in graded alcohol. Antigen enhancement of all sections was performed in a microwave using a pressure cooker and citric acid buffer (pH 6). Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in distilled water for 5 minutes at 37°C. The two sections of each case on which the two Zymed antisera were tested were treated with the non-immune blocking serum of the Zymed HistostainTM-Plus detection kit (85-9043) for 10 minutes at room temperature. The sections were then incubated with the two antisera (ZP54 at 1µg/ml dilution and ZR44 at 1:50) for 60 min at room temperature. Detection of the antibody reactivity was done with the above-mentioned Zymed detection kit according to the manufacturer's instructions. AEC was the chromogen of choice. The remaining section on which the antiserum from KCC was tested was treated with non-immune serum of the Vectastain Universal Elite ABC detection kit (PK-6200) for 20 minutes at room temperature. The sections were incubated overnight at 25°C with the antiserum (1:600) (19). Detection of the immunostaining was carried out by the Vectastain Universal Elite ABC detection kit according to the manufacturer's instruction. AEC was the chromogen of choice (Immunotech 0599 A Coulter Company, BP 177-13276 Marseille Cedex 9- France). All the sections were counterstained with haematoxylin, washed in tap water and mounted with Dako Faramount aqueous mounting medium. Both the extent and intensity of Fhit protein expression were evaluated. The intensity of staining was recorded as: absent/weak, 1; moderate, 2; and strong, 3. The extent of immunostaining was scored based on the percentage of positive cells: <10%, 1; 10-50%, 2 and >50%, 3. The two scores were then multiplied to give a composite score (1-9) for each lesion. Composite scores of 1-3 were defined as marked reduction or absence of Fhit protein expression. The full epithelial thickness was used for evaluation of Fhit expression in the fibrous epuli, lichen planus and dysplastic lesions, while the invasive areas of the squamous cell carcinoma showing the poorest differentiation were used. The same areas on the different sections were used when evaluating the staining of different antisera.

The one-way repeated measures analysis of variance (Anova) was used to evaluate the staining properties of the four different antibodies. Pairwise multiple comparison procedures were used to isolate the antisera that differed from the others. The Tukey Test was used for normally distributed data while the Student-Newman-Keuls method was used where normality failed.

Results

The staining properties of the three antisera in the four different lesions are reflected in Table I. The extent and intensity of staining with the Zymed C-terminus antibody were significantly lower than that of the other two antisera in the cases of fibrous epuli and oral epithelial dysplasia. The degree of epithelial dysplasia did not affect the staining scores. The staining properties of both Zymed antisera were significantly lower than the KCC antiserum in the case of lichen planus. The difference between the two Zymed antibodies for lichen planus was also statistically significant. The differences in staining scores in the OSCC group among the three antisera was not statistically significant (Figures 1, 2). A more intense staining in the better-differentiated areas of the OSCC was observed with the KCC antiGST-Fhit (Figures 3, 4).

Subtle staining differences between the antisera were noted. The Zymed antiGST-Fhit serum demonstrated strong staining of all the inflammatory cells as well as strong staining

Figure 1. *Invasive tumour front of OSCC with a combined staining score of 6 (Zymed antiGST-Fhit).*

Figure 2. *Example of a combined staining score of 9 in OSCC (KCC antiGST-Fhit).*

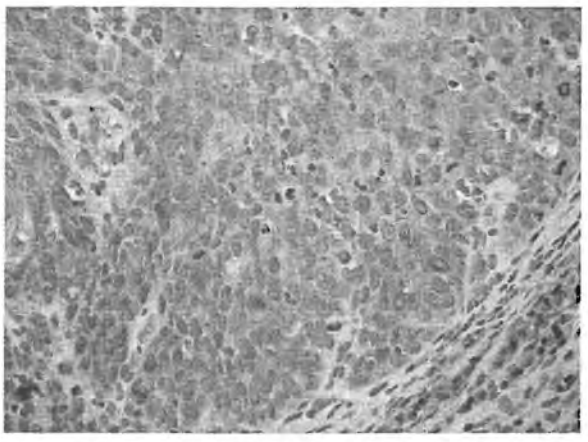
Figure 3. *Prominent staining of the better-differentiated area in OSCC with KCC antiGST-Fhit.*

Figure 4. *Same area as in Figure 3 with poor staining in the better-differentiated area with Zymed antiGST-Fhit.*

Figure 5. *Zymed antiGST-Fhit demonstrated strong staining in the inflammatory cells of lichen planus. The epithelial staining is more prominent in the basal cell region (total staining score of 6).*

Figure 6. *KCC antiGST-Fhit in the same case of lichen planus (Figure 5) showed a more prominent staining of the superficial epithelial cells and no staining in the inflammatory cells (total staining score of 9).*

Figure 7. *Strong staining in the excretory ducts of minor salivary glands with Zymed antiFhit C-terminus.*



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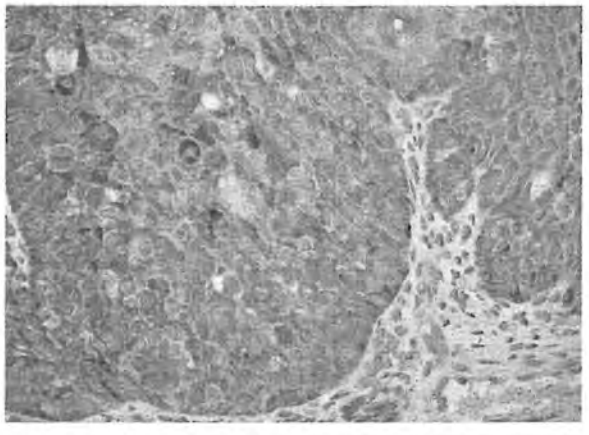

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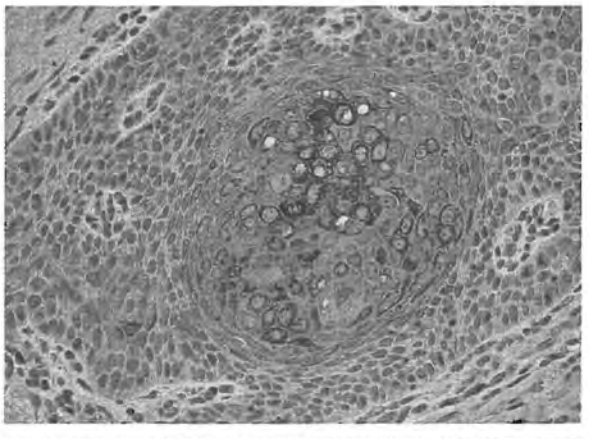

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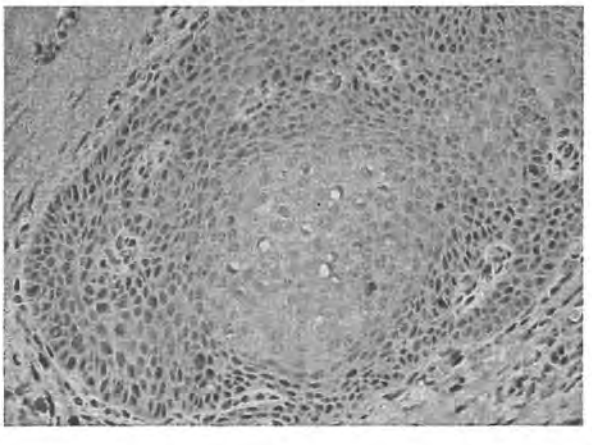

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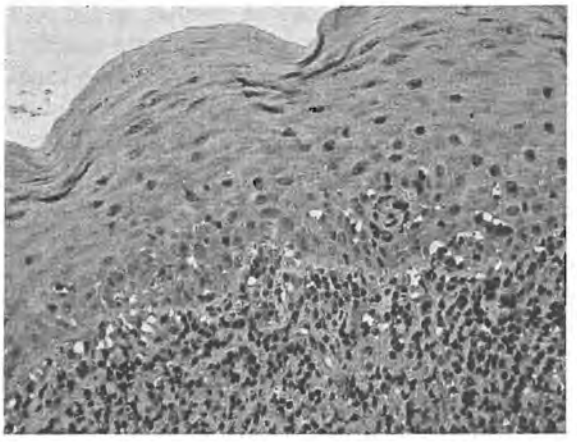

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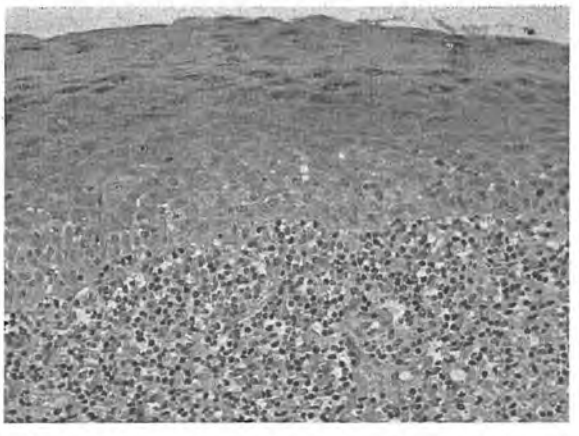

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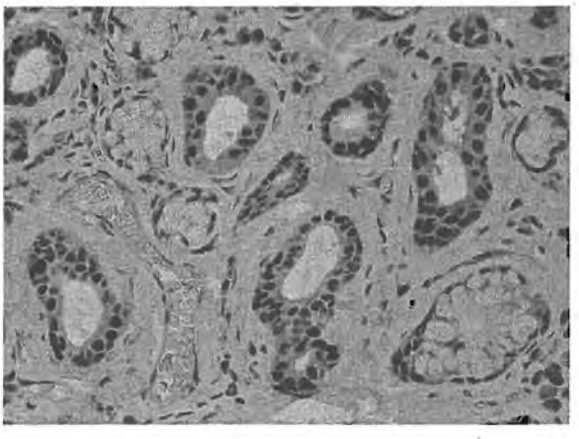

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ANTICANCER RESEARCH *21:* 2419-2424 (2001)

of the basal and parabasal cells in contrast to the KCC antiGST-Fhit that showed stronger staining in the superficial layers of the epithelium. The presence of subepithelial inflammatory cells had no influence on the staining of the epithelial cells (Figures 5, 6). Although the Zymed Fhit C-terminus antiserum showed relatively poor staining of the oral epithelium, strong staining was detected in minor salivary glands with this (Figure 7), as well as the other two antisera.

Discussion

Immunohistochemistry is the best method to evaluate protein expression in a primary tumour because the proportion of cells expressing the protein, as well as their level of expression, can be determined. This is in contrast to Western blots where some positivity from non-tumour cells is often found, even if the tumour is negative. Immunohistochemistry is also a suitable method to determine *FHIT* inactivation in tumour cells because *FHIT* is inactivated by deletion rather than mutation.

The stronger staining of the basal and parabasal epithelium using the Zymed GST-Fhit antiserum, in contrast to the KCC GST-Fhit antiserum, is important when comparing results from studies using different antisera. Although the KCC GST-Fhit antiserum appeared to be related to keratin differentiation in normal epithelium, all three antisera showed strong staining in the better-differentiated areas of the OSCC.

The reason for the relatively weak epithelial staining of lichen planus using both Zymed Fhit antisera compared to the KCC antiGST-Fhit is difficult to explain. It may be related to the closely associated lymphocyte component, although strong epithelial staining was observed with the Zymed antiGST-Fhit in dysplastic epithelium when associated with a subepithelial inflammatory cell component. The nature of the infiltrate in lichen planus, consisting predominantly of T-lymphocytes with an imbalance between T-helper and T-suppressor activity (22), compared to the mixed infiltrate associated with oral epithelial dysplasia might have influenced the staining abilities of the Zymed antiGST-Fhit. The presence of inflammatory cells in the oral dysplasia group did not influence the staining of the antisera.

The staining score for the three Fhit antisera was slightly higher in the dysplastic epithelium compared to the normal epithelium in the fibrous epuli. The composite staining score of the OSCC for the antisera except the Zymed antiFhit C-terminus, was lower than the staining score in normal oral epithelium and dysplastic oral epithelium. This difference was statistically significant for the Zymed and KCC full-length antisera. The invasive tumour front area of the OSCC was used for Fhit evaluation and generally showed less intense and less extensive expression of Fhit than the rest of the tumour. The reduction or loss of Fhit protein expression in about 60% of OSCC in our study was similar to previous reports of OSCC (19, 23) but differed from results reported by Götte *et al* (24).

Tissue manipulation, including excessive fixation and antigen retrieval, has a considerable influence on the outcome of IHC studies (25). Since all the material was retrieved from the archives of a diagnostic histopathology laboratory, most of the specimens were fixed in 10% buffered formalin for less than 12 hours and similar heat-mediated antigen retrieval performed on all cases. It is therefore unlikely that the absence of immunoreactivity when evaluating certain antisera reflects the destruction or masking of that specific target epitope. The fact that significant differences were observed among the four antisera in the non-neoplastic lesions but not in the OSCC group can therefore not be attributed to tissue manipulation.

Both the qualitative and quantitative staining patterns of the different antibodies were evaluated. This was done to include more than one parameter for the antibody comparison. It may not necessarily be important to determine both aspects when evaluating Fhit expression in all tissues. This is especially relevant to OSCC, where new tumour cell clones with different behavioural characteristics and arguably different staining patterns may arise. This necessitates the use of a well-defined area when evaluating IHC staining in solid tumours. The invasive tumour front of OSCC is an appropriate site because it has been suggested that molecular and morphological characteristics at this site reflect tumour prognosis better than other parts of the tumour (26).

In conclusion, comparison of the three Fhit antisera showed that all three could be used for evaluation of Fhit expression in OSCC. The Zymed C-terminus antibody showed weaker staining characteristics in non-neoplastic oral epithelium than the other antibodies evaluated. It would appear that loss of Fhit expression might be important in the behaviour rather than in the initiation of OSCC.

Acknowledgements

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Prevalence of EBV in Oral Squamous Cell Carcinomas in Young Patients

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Abstract. *Background: Recent studies reported a difference in the age distribution of oral squamous cell carcinoma (OSCC) between Black and White South Africans with OSCC more prevalent in Black patients under the age of 50 compared to Whites. Materials and Methods: Paraffin embedded blocks of OSCC were divided into two groups: one with a mean age of 56.2 years and the second group all younger than 40 years of age. A control group of 30 non-neoplastic intraoral lesions were selected. A standard PCR reaction was used to amplify the BAM H1 W-fragment of the EBV. Results: EBV DNA was demonstrated in 11/45 (24%) cases from the first group and in 11/45 (24%) cases from the second group. EBV DNA was present in 11/30 (37%) cases from the control group. Conclusions: This study showed that the prevalence of EBV in OSCC was not influenced by the age of the patient.*

Squamous cell carcinoma is the most common malignancy of the oral cavity. Despite the relative easy accessibility of the oral mucosa to detect premalignant lesions, recent studies suggest a global increase in the incidence of oral squamous cell carcinoma (OSCC) . This increase is especially evident in developing countries (1). The age standardised incidence rate per 100,000 for OSCC in the Black population in South Africa is 8.25 for males and 2.3 for females as obtained from the National Cancer Registry of 1988 (2). This register was the most recent and the first not to combine OSCC with squamous cell carcinoma of the naso- and oropharynx. These incidence rates are higher than findings in previous studies investigating the epidemiology of OSCC among the Black population in South Africa (3,4,5). OSCC is the 4th most common malignancy among Black males and 11th for females (2).

OSCC occurs over a wide age range with a peak in the sixth and seventh decades and found predominantly in men. Flemming *et al* (6) found a significantly different age distribution between Black and White male patients. They found that 33.4% of the Black male patients was below the age of 50 years compared to 15.6% of White males. This difference may be the result of exposure to carcinogenic agents at a young age.

'Young' in patients with OSCC is arbitrary defined in the literature as younger than 40 years of age. The reported incidences of squamous cell carcinoma in patients under 40 years of age vary between 0.4% to 3.6% of all cases (7). Several factors have been implicated as possible causes of squamous cell carcinoma in young patients. Advanced squamous cell carcinomas have been described in young patients who were regular marijuana users (8). This possible etiologic role of marijuana as a risk factor in the development of OSCC is supported by Almadori *et al* (9). Studies of OSCC in young patients report conflicting findings regarding tobacco use, a well documented carcinogenic agent. Lower tobacco use among young cancer patients was present compared to site-matched and stage-matched older patients (7,10), while Lipkin and co-workers (11) found a high exposure to tobacco and alcohol in their young head and neck cancer patients.

Epstein-Barr virus (EBV) is a double stranded DNA virus that is a member of the human herpes virus group. EBV DNA has been demonstrated in normal oral epithelium as well as in oral squamous cell carcinoma (12). Sufficient evidence exists to support a possible role for EBV in

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carcinogenesis. EBV latent membrane protein inhibits epithelial cell differentiation (13) and induces hyperplasia and aberrant expression of keratin 6 in the skin of transgenic mice (14). EBV is a common viral infection with more than 90% of the population already infected late in adolescence or early adulthood (15).

This study was undertaken to determine the presence of EBV DNA in OSCC in patients from two age categories in a Black African population sample from the North Western Transvaal and to evaluate the possible role of EBV as an etiologic agent in carcinogenesis in young patients.

Materials and Methods

Patients. Formalin fixed paraffin embedded blocks of intraoral squamous cell carcinoma were retrieved from the files of the Department of Oral Pathology at Medunsa. The sections were screened to confirm the diagnosis and divided into 2 groups.

Group 1: Forty five blocks containing OSCC without any normal appearing adjacent or overlying epithelium. The mean age of this group was 56.2 years ± 9.2. Thirty-five were male and 10 female.

Group 2: Forty five blocks containing only tumor tissue without normal epithelium. All these patients were 40 years of age or younger with a mean age of 32.4 ± 8.7. Thirty-two were male and 13 were female.

Group 3: A control group consisting of 30 non-neoplastic intraoral lesions were included. These were non-viral associated lesions, all being fibrous epuli and fibroepithelial polyps.

Two 10 µm sections were cut from each block, using a new disposable blade after each block. To evaluate the possibility of contamination at this stage, 10 µm sections of a block containing normal brain tissue were cut after every five tumor blocks and also included for evaluation. All sections were placed in separate Eppendorf tubes.

DNA extraction from the sections. Cell lysates were prepared as follows: sections were dewaxed, washed with ethanol and digested with 400 µl of lysis buffer containing proteinase K. The samples were then heated to inactivate the proteinase K, centrifuged to pellet the debris and 10 µl of the supernatants analysed by polymerase chain reaction (PCR).

PCR. Oligonucleotide primers corresponding to sequences in the internal repeat fragment (BAM H1 W-fragment) of the EBV genome were used. These primers were selected because the reiterated structure of the target sequence would theoretically enhance the sensitivity of detection. Thermal cycling was carried out using a Techne PHC-2 water cooled machine at a reaction volume of 100 µl. All specimens were subjected to two rounds of PCR amplification, first with the outer and subsequently with the inner primer sets. The sequences for the outer primer set were: 5'- CTTTAAAACTCTAAAAATCAAAACTTTAGA (+) and 5'-ACCAGAAATAGCTGCAGGACCACTTTATAC (-) and for the inner primer set: 5'-AATGGGCGCCATTTTGT (+) and 5'-TCCCTAGAACTGAC-AATT (-).

The hot start method was done before adding enzyme. Forty amplification cycles were used as follows: denaturing of DNA (94°C; 1,5 minutes), annealing of primers (60°C; 1,5 minutes) and extension of the annealed primers (72°C; 3 minutes). Reaction mixtures for the amplification contained: 10 µl of DNA; 200 µM of each nucleotide; 0,4 µM of each primer; 1U of Taq DNA polymerase (Promega); 10 µl of Promega 10 x buffer and made up to a final volume of 100 µl. Each reaction mixture was covered with two drops of mineral oil. Template DNA for the second amplification round consisted of 10 µl of reaction product from the first round. All other reagents for the second round of amplification were in the same concentrations used for the first round.

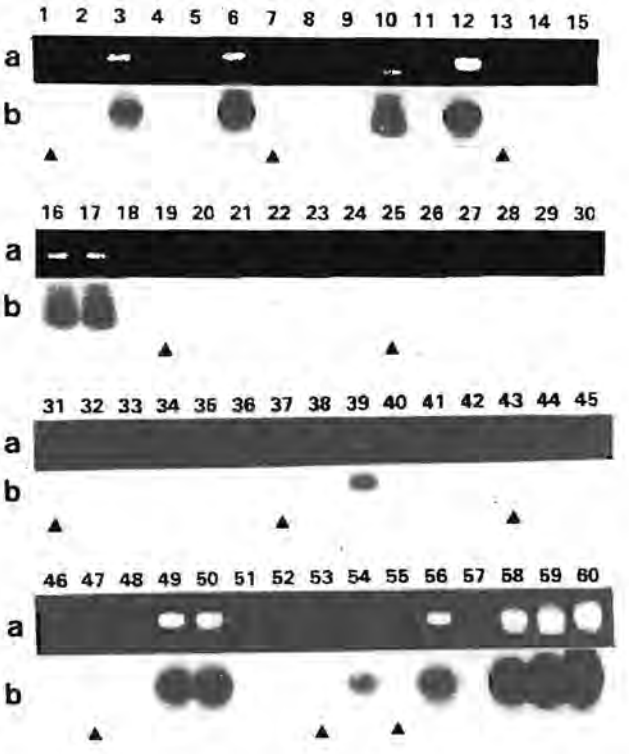

Figure 1. *Oral squamous cell carcinomas of group 1. Numbers 58, 59 and 60 represent 1, 10 and 100 genome copies respectively; number 57 designate the negative control. (a)-gel electrophoreses; (b)-hybridisation; (▲)-brain samples.*

Negative controls contained sterile water in place of template DNA. To standardise our PCR reactions the EBV Bam H1 W-fragment cloned in the pACYC 184 plasmid (courtesy of D. Neumann-Haefelin, Freiburg, Germany) was used as the positive control. To obtain a quantitative estimate of the sensitivity of our PCR method we made 10 fold serial dilutions of the plasmid in 1 µg/µl salmon sperm DNA. The dilutions containing 1, 10, 100 and 1 000 copies of plasmid/µl were included in each run. After the last round of amplification, 10 µl of the reaction product was electrophoresed on ethidium bromide stained agarose gels and evaluated under ultraviolet light. The B globin gene was used as control and only 40 amplifications were performed. This was done to determine that the DNA was amplifiable after which a more sensitive PCR was used to amplify any EBV DNA present.

Southern blot hybridisation. Southern transfer blotting was used to confirm the PCR results. The Bam H1 W fragment was used as probe and labelled using the multiprime system (Boehringer Mannheim). Hybridisation was carried out at 55°C overnight in 6 x SSC, 5 x Denhardt's solution, 0,2% SDS and 100 µg/ml salmon sperm DNA. The blots were washed at high stringency using 2 x SSC plus 0,1% SDS for 5 minutes at 65°C, then 0,2 x SSC and SDS at 65°C for 5 minutes, followed by 15 minutes at 65°C.

Results

All the brain samples placed randomly between the study samples were negative on PCR. The sensitivity was such that

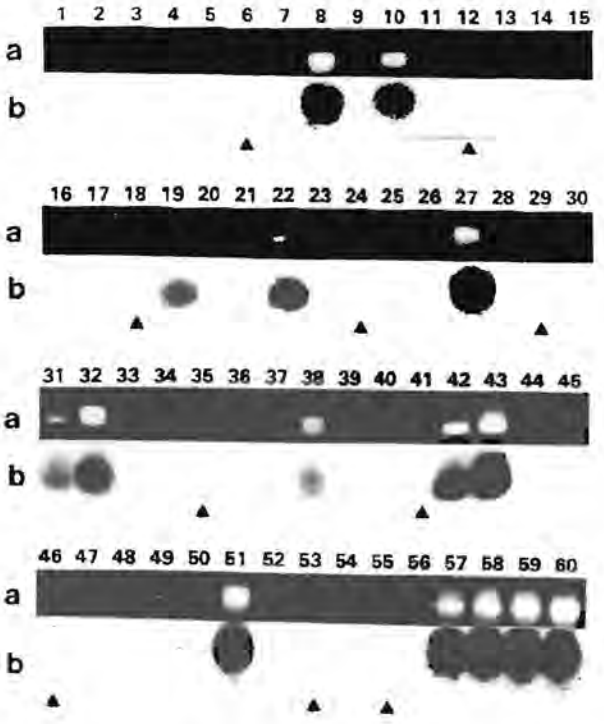

Figure 2. *Squamous cell carcinomas of patients 40 years and younger. Numbers 57, 58, 59 and 60 represent 1, 10, 100 and 1000 genome copies respectively, while number 56 designate the negative control. (a)-gel electrophoreses; (b)-hybridisation; (▲)-brain samples.*

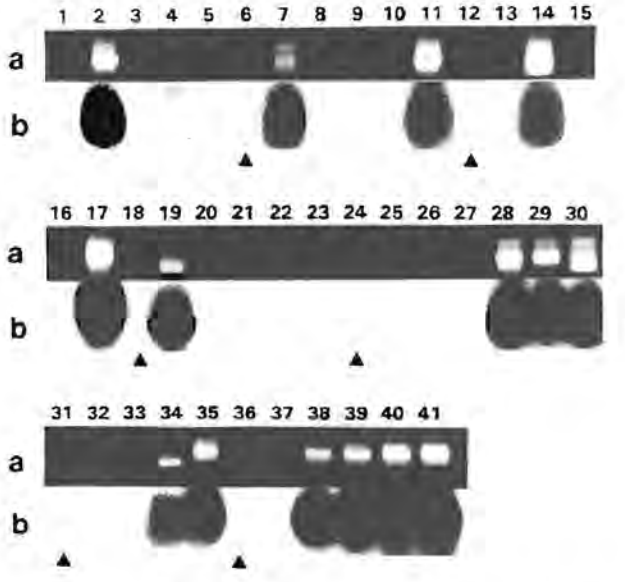

Figure 3. *Contol group. Numbers 38, 39, 40 and 41 represent 1, 10, 100, and 1000 genome copies respectively and number 37 the negative control. (a)-gel electrophoreses; (b)-hybridisation; (▲)-brain samples.*

1 copy of plasmid DNA could be detected in each reaction. EBV DNA was demonstrated in 11/45 blocks - 24% of the first group (Figure 1), in 11/45 blocks - 24% of the carcinomas in the patients younger than 40 years (Figure 2)and in 11/30 blocks - 37% in the control group (Figure 3).

Discussion

PCR was chosen for evaluating our samples, because it is the most sensitive diagnostic tool available. Further, the Bam W region was chosen as the specific genome region for amplification as this DNA segment is reiterated more than 10 times and this should provide a more readily detectable sequence than a single copy viral gene (16). Amplification of a second region within the EBV genome was not performed to confirm the presence of viral DNA. We plan however, as an extension of this study, to do EBV subtyping on all the positive cases. The sensitivity was optimised by adjusting the reaction to detect 1 gene copy per reaction mix.

EBV was found to be the etiologic agent of infectious mononucleosis (17) as well as being closely associated with Burkitt's lymphoma (18), undifferentiated nasopharyngeal carcinoma (19) and oral hairy leukoplakia in immunodeficient patients (20). An association of some other epithelial tumors with EBV has been suggested recently on the basis of molecular biological techniques. The presence of EBV DNA in tonsilar carcinomas (21), epithelial thymic carcinomas (22), and undifferentiated salivary gland carcinomas (23) has been reported. EBV DNA has been demonstrated in 43% squamous cell carcinomas of the uterine cervix and 8% of CIN II and CIN III lesions while no EBV DNA could be demonstrated in normal cervical epithelium (24).

EBV DNA was demonstrated in carcinomas in both age groups to the same extent (24%). This implies that EBV did not play a more important role, if any, in the carcinogenesis process of the young patients compared to the older age group. The 37% positivity of the control group consisting of normal oral epithelium was higher than previously found in normal oral mucosa with PCR (12). The possibility of contamination of saliva containing EBV or latently infected B lymphocytes in tissue blocks may result in a higher positive rate. Contamination by saliva is a dilemma when smears are used to evaluate epithelial cells for the presence of EBV DNA, while the imbedding process and sectioning of tissue blocks exclude this possibility when archival material is being investigated. B lymphocytes are present to various degrees in almost all squamous cell carcinomas adjacent to the infiltrating tumor islands. The magnitude of lymphocytic infiltration in the two carcinoma groups was the same and was found to a lesser extent in the fibroepithelial polyps and fibrous epuli, the lesions used in the control group.

Lymphocytic infitration could therefore not be responsible for the differential sensitivity between the carcinoma groups and the control group. Niedobitek and Young (25) argue that B lymphocytes alone are responsible for lifelong persistence of the virus in seropositive persons. This point of view is challenged by Nicholson and Crawford (26) who reason that the persistent low-grade replication and shedding of EBV in the epithelial cells of the oropharynx (27), uterine cervix (28) and male genital tract (29) cannot be explained by EBV persistence in B lymphocytes only. This argument will bring EBV in line with other herpes viruses such as cytomegalovirus that is also secreted into body fluids by replicating in the lining epithelial cells (26).

The positivity rate of EBV DNA in normal oral epithelium is consistent with the concept that oral epithelium may act as a reservoir of viruses in clinically healthy patients. The anatomical site as well as the use of sensitive techniques seems to influence the detection of EBV DNA in normal epithelium. EBV DNA was demonstrated in 70% of nasopharyngeal tissue obtained from asymptomatic seropositive individuals (30). It is also possible that the geographical area may influence the presence of EBV DNA in normal oral epithelium. All the patients in our study are of a rural Black origin, which may be a contributing factor.

Heterogeneity in the PCR product size was observed. The weaker PCR reactions appeared to be slightly smaller in size than the strong positive reactions. The smaller size fragments were formed after the second round of amplification and the larger after the first round of amplification. The small size fragments were weak implying that only a small amount of EBV DNA was present in the original tissue. This indicated that the PCR method used can achieved different levels of sensitivity. This exquisitely sensitive PCR technique may detect viral genomes from small numbers of latently infected lymphocytes infiltrating the neoplasms.

One has to be circumspect in drawing conclusions from PCR analysis, especially when studying ubiquitos viruses such as EBV. Corrobotive evidence from other molecular techniques such as in situ hybridization is necessary before substantiating an aetiologic role for EBV (31). A possible role of EBV in the multistep process of squamous cell carcinogenesis can not be excluded although it would appear not to influence the age distribution of this disease. It is possible that the EBV DNA detected in the tumor cells was merely passengers stemming from neoplastic change of latently infected oral epithelial cells.

Acknowledgements

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Prevalence of Epstein-Barr virus in nasopharyngeal carcinoma in a SouthAfrican population sample

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This study was undertaken to determine the presence and localisation of Epstein-Barr virus (EBV) in squamous cell nasopharyngeal carcinoma (SCNPC) and non-keratinising nasopharyngeal carcinoma (NKNPC) of a South African population sample and to evaluate the possible role of EBV as an aetiological agent in the carcinogenesis of these tumours. Forty-five cases of NPC were selected from the archives of the Departments of Oral Pathology at the Medical University of South Africa and the University of Pretoria as well as the Department of Anatomical Pathology at the University of Pretoria. Viral expression of the smaller nuclear RNAs (EBER-1 and EBER-2) was detected by in situ *hybridisation (ISH) with fluorescein-conjugated oligonucleotide probes. All the specimens of NKNPC produced positive ISH signals with the EBER-1/ EBER-2 antisense probe mixture, whereas the SCNPC specimens produced negative ISH signals. In 14 of the 45 cases epithelial dysplasia was detected in adjacent epithelium, none of which produced positive ISH signals. EBV is present in all the undifferentiated NPCs evaluated, which indicates an important role in its pathogenesis in this country. EBV probably enters the pathogenetic process at a later stage as no positive ISH signals were produced by* in situ *lesions. Larger studies including a higher number of SCNPC cases should be conducted to further investigate the possible role of EBV and other aetiological factors in the pathogenesis of this subgroup of tumours.*

Introduction

The World Health Organization (WHO) (1991) separated nasopharyngeal carcinoma (NPC) into two groups: squamous cell nasopharyngeal carcinomas (SCNPC) and non-keratinising nasopharyngeal carcinomas (NKNPC) with the latter subdivided into differentiated and undifferentiated carcinoma, and lymphoepitheliomas included under NKNPC.[1,2]

NPC is an epithelial tumour that occurs worldwide, the incidence of which differs markedly in geographical areas and population groups.[3] NPC is rare among Europeans and North American Caucasians, with an age-adjusted incidence of less than 1/100 000. It has a high incidence in southern China and South East Asia, where it represents 25% of all cancers.[4] In southern China the age-adjusted incidence is approximately 25/100 000 for males. This tumour is also common in other Chinese populations and in Alaskan Eskimos and occurs with intermediate incidence in Mediterranean Africans.[5] NPC is found at a decreasing rate in succeeding generations of Chinese in Hawaii and in California.[6]

The frequency of NPC cases reported in a study conducted in the Sudan at two different treatment centres was 5.8% and 7.2%, the highest frequency reported outside the Chinese population up to 1983.[7] In this study 12.1% to 14% of NPC occurs in children 14 years and younger, which makes it the most common childhood malignancy in the Sudan. North African NPC is closely associated with Epstein-Barr virus (EBV) and most patients have high antibody titers to EBV antigens.[8]

EBV is an ubiquitous virus infecting more than 90% of the world adult population and it is associated with an increasing number of neoplastic conditions including Burkitt's lymphoma, other B- and T-cell non-Hodgkin's lymphomas, Hodgkin's disease as well as epithelial neoplasms occuring in the nasopharynx and elsewhere.[9]

Studies on EBV gene expression in latently infected B-lymphocytes *in vitro* have identified six nuclear proteins (called EBV nuclear antigens) and three membrane proteins (called latent membrane proteins) which are likely to mediate EBV-induced effects on cell proliferation.[10,11] The most abundantly expressed viral transcripts, though, are the non-polyadenylated polymerase III transcripts, called EBV-encoded small nuclear RNA (EBER) 1 and 2.[12] Despite their abundance the EBERs do not code for protein and the function of these RNAs is unknown. It has been proposed that they may be active during lytic replication. EBERs are expressed early after infection and can be detected predominantly in the nucleus.[13,14]

The precise mechanism of EBV in carcinogenesis is still unclear. EBV produces the latent membrane protein-1 (LMP-1), which may induce dedifferentiation in non-malignant keratinocyte cell lines.[15] The EBV-encoded protein is also involved in the induction of bcl-2, thereby preventing EBV-infected cells from undergoing programmed cell death.[16] Interleukin-10 (IL-10) expression by EBV-infected NPC cells, with its local immunosuppressive action, may also play a role in tumour development.[17]

The question as to the association of SCNPCs with EBV has been subject to controversy. SCNPCs from different geographical regions vary in their EBV association. SCNPCs from Hong Kong, a country with a high NPC incidence, were invariably EBV-positive, but only a few cases from China, which has an intermediate incidence of NPC and the United Kingdom, which is a low-risk area for NPC, were EBV-

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associated.[18]

Currently there are little data available on the association between EBV and NPC in South Africa. The aim of this study was to determine the prevalence of EBV in NPC in a South African population sample using *in situ* hybridisation (ISH), and to evaluate the possible role of EBV as an aetiological agent in carcinogenesis in this sample group.

Materials and methods

Patients

Formalin-fixed paraffin-embedded tissue blocks and histological sections of all cases of nasopharyngeal carcinoma were retrieved from the archives of the Departments of Oral Pathology at the Medical University of South Africa (MEDUNSA) and the University of Pretoria, as well as the Department of Anatomical Pathology at the University of Pretoria. The blocks included biopsy as well as excision specimens. The material represented NPCs diagnosed over a period of 11 years from 1985 to 1996 inclusive. A 4 µm section from each block was stained with haematoxylin and eosin and screened to confirm the diagnosis of NPC with use of the WHO (1991) criteria.[1] The diagnosis was made independently as well as on a consensus basis by the authors.

In situ hybridisation (ISH)

To detect expression of the smaller nuclear RNAs (EBER-1 and EBER-2), ISH with fluorescein-conjugated oligonucleotide probes was used. The probes were obtained commercially and consisted of a mixture of EBER-1 and EBER-2 (Hybaid, Dako Corp). Probes were labelled with fluorescein isothiocyanate (FITC). Hybridised probes were detected with rabbit F(ab') anti-FITC conjugated to alkaline phosphatase. 5-Bromo-4-chloro-3-indolyl and nitroblue tetrazolium chloride were applied as chromogen. The EBV-infected cell line P3HR-1 served as a positive control, while a block of human brain served as a negative control. ISH was performed using the OmniSlide System (Hybaid, Teddington, Middlesex, United Kingdom). All glassware was treated with di-ethyl pyrocarbonate to prevent RNA-ase activity. The slides were examined under a Nikon Alphaphot YS light microscope. Intensity and localisation of staining were noted. Positive signals were regarded as dark brown to black staining in the nuclear area of the tumour cells with nucleolar sparing.

Results

EBER-ISH

Forty-five cases of NPC were retrieved from the archival material and included 24 differentiated NPCs, 17 undifferentiated NPCs and four SCNPCs. Hybridised signals were observed in the tumour cells of all differentiated and undifferentiated NPCs while the SCNPCs yielded no signals. The signals were restricted to nuclei of malignant epithelial cells with relative nucleolar sparing. No tumour-infiltrating lymphocytes that hybridised with the EBER probes could be detected (Figure 1). Signal intensity varied between specimens and ranged from dark brown to black stains. Discrete tumour cell islands could be seen in most cases,

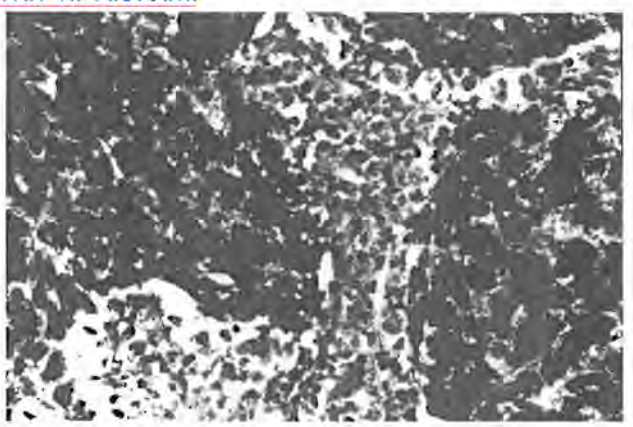

Figure 1: **EBER *in situ* hybridisation of undifferentiated NPC. Hybridisation signals are restricted to tumour cells with no staining of surrounding lymphoid stroma. (Original magnification x75)**

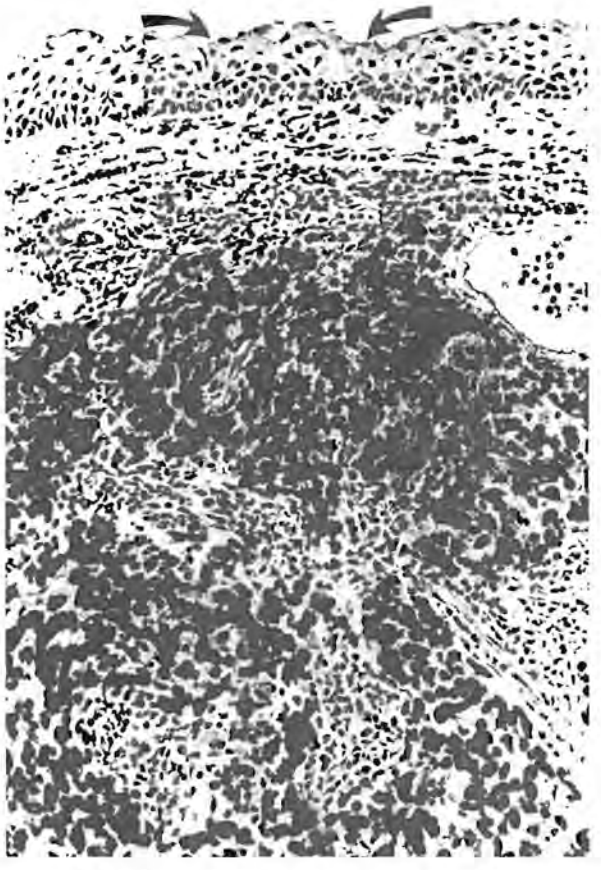

Figure 2: **EBER *in situ* hybridisation of undifferentiated NPC. Sheet of positive staining malignant cells with normal overlaying epithelium (arrows). (Original magnification x50)**

while some specimens demonstrated sheets of positive staining malignant cells (Figure 2). Of the 45 cases, 14 showed features of dysplasia in adjacent epithelium, none of which produced positive hybridisation signalling. No background staining could be seen on any of the slides. The P3HR-1 cell line stained intensely positive while the brain block demonstrated no hybridisation signal.

Discussion

The main advantage of using ISH above the polymerase chain reaction (PCR) is the fact that tissue morphology is conserved, allowing localisation of the hybridised probe to certain cells or even subcellular regions. This phenomenon was used before to localise EBER-1 and EBER-2 in human B lymphocytes, in an attempt to elucidate the function of EBERs in EBV infection.[14] Because EBERs are expressed in high copy numbers, they are easily detected by ISH using probes and therefore serve as a sensitive marker for latent EBV infection. PCR can be applied to viral detection in formalin-fixed specimens, but the high sensitivity of PCR and the inability to identify the particular cells infected, render the technique somewhat problematic in its application to tumours arising from mucosal surfaces from which EBV is regularly shed, even in persons without malignancy. ISH has the virtue of identifying infected cells rather than free virus.

In all forms of NPC, the same set of specific EBV genes are expressed with an apparent decrease in EBER abundance in areas of differentiation. The demonstration of clonal EBV genes supports earlier reports suggesting that regardless of the state of differentiation, NPC is a clonal expansion of EBV-infected cells.[5,19] The monoclonality of resident EBV genome has been interpreted as evidence that the viral infection occurred before the expansion of the malignant clone, therefore highlighting an essential role of EBV in the oncogenic process. In this study of NKNPCs the prevalence of EBV particles in the form of EBER-1 and EBER-2 correlated with the findings reported in other studies.

A characteristic morphological feature of undifferentiated NKNPCs is the presence of an intense lymphoid stroma.[1] It has been suggested that the presence of a lymphoid stroma might be a requirement for undifferentiated NPC growth at least in certain stages of tumour development.[20] It was also reported that NPC cells do not express the EBV receptor, but that a secretory component (SC) protein is being expressed on all NPC cells but not in untransformed metaplastic epithelial cells. From this was concluded that EBV could not infect untransformed nasopharyngeal squamous metaplastic epithelia, but could enter NPC cells through IgA-mediated endocytosis.[21] In another study the expression of EBER-1 in NPC tissue of 140 primary and 11 metastatic tumours to lymph nodes was investigated. The authors reported that the EBER-1 signal could be detected in 135 out of 140 primary NPCs of all histological types and 10 out of 11 metastatic tumours. The authors suggested that EBER-1-ISH be performed on routinely processed specimens whenever NPC is suspected.[22]

No data are available on the prevalence and incidence of NPC in South Africa. Data extracted from the National Cancer Registry database suggest that the coloured male population may be at moderate risk of developing NPC. From the results of this study it is clear that EBV is present in a large number of cases, if not all, of NKNPCs in the South African population and it can therefore be concluded that EBV plays an important role in the carcinogenesis of the tumour in this country. NKNPC is therefore potentially a preventable disease if vaccination programmes are implemented in population groups at risk.

This study included only four cases of SCNPCs, none of which yielded a positive signal with ISH. The study has to be extended to include a larger number of cases before any meaningful conclusions could be derived from this fact. It does however suggest that the pathogenesis of SCNPC is more heterogeneous than that of NKNPC. Factors that can substitute for EBV infection in the pathogenesis of EBV-negative SCNPC need to be identified. Such factors have recently been suggested. HPV-11- and HPV-16-DNA were detected in some EBV-negative SCNPCs.[23] A strong association was found between SCNPC and a history of smoking and alcohol consumption.[24]

Of the 45 cases 14 showed features of epithelial dysplasia in epithelium adjacent to the primary tumour. None of these lesions produced ISH signals, which suggests that EBV probably enters the pathogenetic process at a relatively late stage.

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ANTICANCER RESEARCH *20*: 1953-1958 (2000)

Epstein-Barr Virus Strain Characterisation in South African Patients with Nasopharyngeal Carcinomas

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Abstract. *Epstein-Barr virus (EBV) has been implicated in various diseases, among others, nasopharyngeal carcinoma (NPC). In this study we investigated the frequency and subtype distribution of EBV in 39 NPCs. The presence of EBV was detected by using a nested PCR to amplify the Bam Hl W-fragment of the genome. Two regions were targeted for subtype analysis, namely the EBNA-2A and EBER regions. PCR was used to amplify these regions, and the EBER region was sequenced to detect subtype specificity. The results showed that EBV could be detected in 82% (31/38) of the tumours. In 15 of these, EBNA subtypes could be identified of which 14 were subtype A and one tumour had both subtypes A and B present. The EBER region was amplified in 21 samples. The majority of cases(18/21) demonstrated a mutation profile which consisted of 5 type B and one type A mutations. The consensus type is therefore type B. In conclusion: a strong association was found between EBV and NPCs in our group of patients and their "consensus" genotype was A/B based on the two genome areas investigated.*

Epstein-Barr virus (EBV) belongs to the human gamma-herpesvirus subfamily, is the etiologic agent of infectious mononucleosis (1), has a clearly established association with endemic Burkitt's lymphoma (2), nasopharyngeal carcinoma (NPC)(3) and secondary B-cell proliferation in immuno-suppressed individuals (4). Based on the organisation of the Bam Hl WYH gene region that encodes for the EBV nuclear antigen 2 (EBNA-2), two distinct types of EBV (type A and B) have been identified (5-7). These type-specific differences were also demonstrated by RFLP analysis and DNA sequencing of the two small RNA-encoding regions (EBERs) of EBV (8-9).

In addition to the genetic variation, biological differences between type A and B strains have been noted. Cell lines containing a type B virus display a lower growth rate than those containing a type A virus, which has been attributed to a reduced transformation capacity of type B viruses (10). Markedly different frequencies of the two types are also found in different geographical locations (11). Type A predominates in Western and Chinese communities, whilst type B has been found mainly in Central Africa and New Guinea (12-13). Co-infection with the two types is often found in immunosuppressed individuals (14).

The purpose of this study was to detect the frequency and subtype distribution of EBV DNA in South African naso-pharyngeal carcinomas (NPC), by specifically investigating the EBNA-2A and the 190 bp EBER fragments.

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Key Words: Epstein-Barr virus, subtypes, nasopharyngeal carcinoma.

Materials and Methods

Patients. Formalin-fixed paraffin-embedded blocks of NPCs were retrieved from the archives of the Departments of Oral Pathology and Anatomical Pathology (University of Pretoria) as well as the Department of Oral Pathology (MEDUNSA). Thirty eight blocks were selected which represented biopsies from 24 Black males and 14 Black females. Haematoxilin and eosin stained sections from all the blocks were used to classify the NPCs according to the World Health Organisation classification.

Preparation of cell lysates. Two 10 μm sections were cut from each block, using a new disposable blade each time. To evaluate the possibility of contamination at this stage, 10 μm sections of a block containing normal brain tissue were cut after every nine tumour blocks and also included for evaluation. All sections were placed in separate microfuge tubes. Cell lysates were prepared as follows: sections were dewaxed, washed with ethanol and digested with 400 μl of lysis buffer containing proteinase K (15). The samples were then heated to inactivate the proteinase K, centrifuged to pellet the debris and stored at -20°C until use. Ten μl of the supernatants were analysed by polymerase chain reaction (PCR).

PCR. EBV was detected in the samples by using a nested PCR reaction targeting the internal repeat fragment (Bam Hl W-fragment) of the genome. This fragment is reiterated ten times per genome, which would theoretically enhance the sensitivity of the detection (16). Thermal cycling was carried out using a Perkin Elmer GeneAmp 9600 PCR system. All specimens were subjected to two rounds of PCR amplification, first with the outer (EBV-1 and -2) and subsequently with the inner primer (EBV-3 and 4) sets (Table I). Preceding the amplification, the reaction mixture was heated to 94°C for two minutes.

Fourty amplification cycles were done as follows: denaturing of DNA (94°C; 1.5 minutes), annealing of primers (55°C; 1.5 minutes) and extension of the annealed primers (72°C; 3 minutes). The final elongation step was extended for 10 minutes. Reaction mixtures for the amplification contained: 10 µl of cell lysate; 200 µM of each nucleotide; 0.4 µM of each primer; 1.5 mM $MgCl_2$; 1 U of Taq DNA polymerase (Promega Corporation, Madison WI, USA); 10 µl of Promega 10 x buffer and made up to a final volume of 100 µl. Template DNA for the second amplification round consisted of 10 µl of reaction product from the first round. All other reagents for the second round of amplification were used in the same concentrations as the first round.

Reagent controls contained sterile water instead of template DNA. To standardise our PCR reactions, the EBV *Bam* HI W-fragment cloned in the pACYC 184 plasmid (courtesy of D. Neumann-Haefelin, Freiburg, Germany) was used as the positive control. To obtain a quantitative estimate of the sensitivity of our PCR method, we made 10-fold serial dilutions of the plasmid in 0.2 µg/µl salmon sperm DNA. The dilutions containing 1, 10, 100 and a 1 000 copies of plasmid/10 µl were included in each run.

EBV subtyping was carried out by determining divergence of the EBNA-2 region using the PCR method by Borisch *et al.* (17). The primers (Gen1, Gen2) were used as outer primers, while EBNA2 A-1, A-2 and EBNA2 B-1, B-2 were used as nested primers to determine subtype A and B specificity. The PCR was done as described above using the generic outer primers. After the first PCR reaction, 10 µl of PCR products were mixed with EBNA2-A or EBNA2-B specific primers. Annealing for the nested reactions were done at 55°C and 48°C for the A and B subtypes respectively, and all reaction times for the cycles were shortened to one minute each.

EBER amplification was carried out by using the EBER primers P1 arid P3 as a prenested reaction, followed by P1 and P2 in a heminested reaction as described (9). The mixtures were subjected to 40 cycles of amplification (one minute at 94°C, one minute at 40°C (P1 & P3) or 55°C (P1 & P2), and 1.5 minutes at 72°C.

Spot blot hybridisation. The final amplification products were spot blotted onto Hybond-N paper (Amersham Pharmacia Biotech, Buckinghamshire, England) and the sheets were hybridised with the corresponding 31-end labeled oligo probes. The hybridisation signals were detected with the DIG DNA detection kit (Boehringer Mannheim, Mannheim, Germany).

Sequencing. All samples that gave a positive result with the heminested EBER primers (P1/P2), were sequenced to identify type specific mutations (8), using cycle sequencing and the dye terminator method on an automated ABI DNA Sequencer model 373 (Perkin Elmer).

Table I. *List of primers and probes used in this study.*

Primer/Probe name	Sequence
EBV- 1	CTTTAMACTCTAAAAATCAAAACTTTAGA
EBV-2	ACCAGAAATAGCTGCAGGACCACTTTATAC
EBV-3	MTGGGCGCCATTTTGT
EBV-4	TCCCTAGMCTGACMTT
PROBE-5	TATCTTTAGAGGGGAAAAGAGGMTMG
EBNA2 Gen1	AGGGATGCCTGGACACMG
EBNA2 Gen2	GTGCTGGTGCTGCTGGTGG
EBNA2 A-1	TCTTGATAGGGATCCGCTAGGATA
EBNA2 A-2	ACCGTGGTTCTGGACTATCTGGATC
PROBE A	CTCTGTCACMCCGAGGCTTACC
EBNA2 B-1	CATGGTAGCCTTAGGACATA
EBNA2 B-2	AGACTTAGTTGATGCCCTAG
PROBE B	AGGCCTACTCTTCCTCMCCCAG
EBER P1	GTGGTCCGCATGTTTTGATC
EBER P2	GCMCGGCTGTCCTGTTTGA
EBER P3	GTGTCTACCTGMCTMGAC
EBER P4	CCTAGTGGTTTCGGACACAC
PROBE P5	MCGGGGCTTMCGTTGCAT

Results

The nested *Bam* HI W-fragment of EBV could be detected in 31/38 (82%) of tumour samples. Fourteen (45%) of the tumours carried an EBNA type A virus while both EBNA A and B types could be identified in one tumour (patient 29) (Table II). Sixteen (52%) of the tumours could not be typed with the EBNA primers.

The EBER primers were able to amplify DNA in a larger percentage (21/31 :68%) of tumour samples (Figure 1). These fragments were further analysed by sequencing for either type 1 or 2 specific, mutations at nucleotide positions 6806, 6884, 6886, 6911, 6927 and 6944 (9) (Table II). According to Sample *et al* (18), type 1 and 2 donate type A and B respectively. The majority of cases (18/21: 86%) demonstrated a similar mutation profile which consisted of 5 type 2(B) mutations (positions 6808, 6884, 6886, 6911 and 6944) and only one type 1 (A) mutation (position 6927). EBV strains from two tumours showed an additional type 1 mutation at position 6944 (patients 29 and 35), while a third strain, (patient 20) had three type 1 and three type 2 mutations. Therefore the "consensus" EBER type in the majority of cases is that of type 2(B), while one tumour can be classified as a 1/2(A/B) hybrid strain. Patient 6 demonstrated two additional mutations at positions 6806 (T to G transversion) and 6939 (G to A transversion).

In 15 patients both the EBNA-2 and EBER regions could be amplified, but in none of these did the types correspond, as most of the EBNA's were type A, while the consensus type for the majority of the EBER's were type B.

Discussion

Our study shows a strong association (82%) between EBV positivity and NPCs. As in other studies, this association is found in non-keratinising and undifferentiated NPCs (19,20).

Table II. *EBV subtype and sequence analysis of the study population.*

Patient nr	Age/Sex	Histology	Bam W	EBNA-A	EBNA-B	EBER	Substitution at positions*									
							6806	6808	6866	6884	6886	6908	6911	6927	6939	6944
1	25/M	undif	pos	neg	neg	neg										
2	21/F	nonker	pos	pos	neg	pos		B		B	B		B	A		B
3	31/F	nonker	pos	neg	neg	neg										
4	29/F	undif	pos	neg	neg	neg										
5	17/M	nonker	pos	neg	neg	neg										
6	67/M	nonker	pos	neg	neg	pos	T-G	B		B	B		B	A	G-A	B
7	25/M	nonker	pos	pos	neg	pos		B		B	B		B	A		B
8	43/F	nonker	pos	neg	neg	pos		B		B	B		B	A		B
9	28/F	undif	neg	neg	neg	neg										
10	23/M	nonker	pos	neg	neg	neg										
11	44/M	nonker	pos	neg	neg	neg										
12	66/F	nonker	pos	neg	neg	pos		B		B	B		B	A		B
13	?/M	undif	pos	pos	neg	pos		B		B	B		B	A		B
14	?/M	undif	pos	pos	neg	pos		B		B	B		B	A		B
15	19/M	undif	pos	pos	neg	pos		B		B	B		B	A		B
16	17/M	nonker	pos	pos	neg	pos		B		B	B		B	A		B
17	38/M	undif	pos	pos	neg	pos		B		B	B		B	A		B
18	70/M	nonker	pos	neg	neg	neg										
19	21/F	undif	pos	neg	neg	neg										
20	46/F	undif	pos	pos	neg	pos		B		A	A		B	A		B
21	60/M	nonker	ne,q	neg	neg	neg										
22	17/M	undif	pos	neg	neg	pos		B		B	B		B	A		B
23	23/M	nonker	pos	pos	neg	pos		B		B	B		B	A		B
24	52/M	undif	neg	neg	neg	neg										
25	72/M	nonker	pos	neg	neg	neg										
26	17/M	undif	pos	pos	neg	pos		B		B	B		B	A		B
27	60/F	undif	pos	pos	neg	pos		B		B	B		B	A		B
28	54/F	undif	pos	pos	neg	pos		B		B	B		B	A		B
29	61/F	nonker	pos	pos	pos	pos		B		B	B		B	A		A
30	17/M	undif	pos	pos	neg	pos		B		B	B		B	A		B
31	39/M	undif	pos	ne,q	neg	pos		B		B	B		B	A		B
32	23/F	undif	pos	pos	neg	pos		B		B	B		B	A		B
33	25/M	undif	pos	neg	neg	neg										
34	66/M	nonker	neg	neg	neg	neg										
35	33/M	nonker	pos	neg	neg	pos		B		B	B		B	A		A
36	53/F	sqcell	neg	neg	neg	neg										
37	41/F	undif	neg	neg	neg	neg										
38	44/M	sqcell	neg	neg	neg	neg										

Undif- undifferentiated; Nonker - nonkeratinising; Sqcell - squamous cell carcinoma
*The letters A or B specify the base found in either B95-8 (A) or AG876 (B) prototype. According to Sample *et al.* (1990), type 1 and 2 donate type A and B respectively.

Except for 2 cases, all the carcinomas were histologically characterised as either non-keratinising or undifferentiated. It is possible that a higher positivety could be found if fresh tissue were investigated, because parafffin embedded tissue usually yield less sensitive PCR results.

According to our knowledge, this is the first study to type EBV strains from Southern Africa. As in other studies, no type-specific correlation was found between the two genome regions targeted for analysis (21,22). Type A virus strains predominated with the EBNA-2 analysis. Fifty two percent of our strains could not be typed using these primers. The inability to amplify the EBNA-2 region was also found in a significant number of cases in another study (23). One explanation for this is that clinical isolates are more heterogenous than the laboratory prototype strains used for designing the prime pairs. Type A is commonly found in Western Countries as well as in sero-positive individuals from Japan (24). Type A is also the predominant subtype found in Asian NPC (25-27). The finding of type A variants in NPC is in keeping with their better transforming ability in cell culture (10).

Analysis of the EBER region showed a distinct combination of type 1 and 2 mutations with the majority of the mutations belonging to type 2 (B). Type B is the traditional type found in African Burkitt's lymphoma and cases from New Guinea (8). More recent studies have found dual carriage especially in HIV-1 carriers (28-31) Sculley *et al.* (32) also showed that type B infection in HIV-positive subjects was six- fold higher than in the general population.

These results indicate that the EBV strains analysed here has arisen by recombination between viral types A and B. Due to the type A conformation of their EBNA gene, these subtypes may show the biologic behaviour of the type A EBV prototype, but is genetically distinct. They contain "hybrid" sequences of A and B types in their EBER gene, and thus the "consensus" genotype is A/B. A breakdown in the patient's immunological barriers has been proposed for the presence of several EBV variants in the same patients due to superinfection. This in turn may lead to the generation of new variants by recombination between endogenous and superinfecting viral genomes (29, 33).

The EBV strains in the carcinomas that were investigated all seemed to harbour an identical strain (with minor point mutations) in their EBER region and can be interpreted as follows: It could be: (1) a more "oncogenic" strain which lead to carcinogenesis in a subgroup of the general population; (2) a strain that commonly circulate in Southern-Africa (geographic location) or (3) the predominant strain circulating in Black South Africans (ethnically linked). To find answers to these questions, EBV strains from patients without NPCs as well as from other ethnic groups in South Africa should be analysed. It will also be interesting to evaluate the impact of the HIV epidemic in the country, which is considered to be the fastest growing epidemic worldwide (34), on EBV strain evolution and recombination.

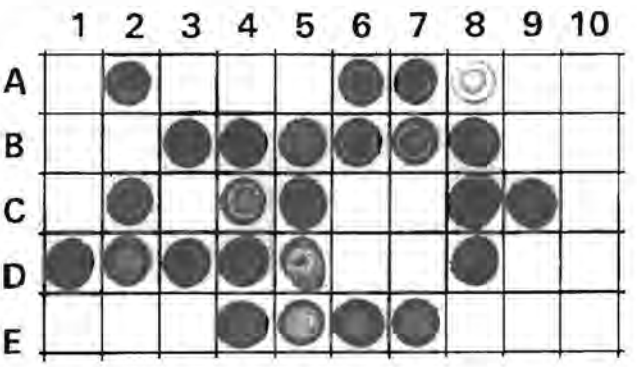

Figure 1. *Hybridisation results on EBER PCR results. Row A1-9 correspond to patient samples 1-9; B1-9 to samples 10-18; C1-9 to samples 19-27; D1-9 to samples 28-36; E1-2 to samples 37 an 38; E3 is a negative reagent control; E4, 5, 6 and 7 correspond to 1, 10, 100 and a 1000 plasmid copies/μl and A10, B10, C10 and D10 were negative controls from normal brain tissue.*

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DNA content and histologic grading of oral squamous cell carcinoma

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Abstract *Background.* Grading of oral squamous cell carcinomas was initiated by Broders. His system is based on the proportion of highly differentiated cells within the entire tumor. This grading has not been generally accepted for clinical use due to poor prognostic value and reproducibility. This study was undertaken to determine the inter-observer reproducibility of the invasive cell grading method on oral squamous cell carcinomas and to correlate this with the DNA ploidy status.

Methods. Fifty formalin-fixed paraffin-embedded blocks that contained both tumor and adjacent normal epithelium were randomly selected. This grading was performed independently by two Oral Pathologists and the inter-observer agreement calculated. The DNA ploidy analysis was performed on a flow cytometer.

Results. Thirty-two tumors were graded as poorly differentiated and 18/50 as moderately. Thirteen of 25 poorly differentiated tumors had an aneuploid DNA content compared with 9/18 of the moderately differentiated group. The inter-observer correlation of the total malignancy score and individual morphologic features was highly significant.

Conclusions. This study showed that the invasive cell grading method is reproducible but no correlation was found between the grading results and ploidy status. *(Eur J Lab Med 1995;1:117-120).*

Introduction

Various methods for the histopathologic grading of squamous cell carcinoma of the oral cavity exist. Broders, in 1920, proposed a method based on the percentage of highly differentiated cells throughout the tumor[1]. Jacobsson et al.[2] introduced a multifactorial grading system evaluating the most invasive parts of the biopsies. This grading method was modified by Anneroth et al.[3] to evaluate three morphologic features in the less differentiated parts of the tumor as well as three histologic features evaluating the relationship between the tumor cells and underlying connective tissue. These six features are: degree of keratinization, nuclear polymorphism, number of mitoses, pattern of invasion, stage of invasion and leukocyte infiltration. A modification of this method was described by Bryne et al.[4] where only the most anaplastic areas in the most invasive sites are evaluated. This modified system of Bryne and co-workers proved to be of high prognostic value[4,5].

DNA content analysis of solid tumors has been studied by flow cytometry, but this technology has only been in widespread use since the recent development of reliable preparation techniques and data analysis programmes. DNA analysis of oral squamous cell carcinoma has been investigated as a possible indicator of prognosis or to predict tumor behavior[6,7]. The frequency of aneuploid tumors increases with a decreasing degree of histologic differentiation[6], while patients with aneuploid primary tumors are also found to have a significantly higher incidence of lymph node metastases when compared to patients with diploid tumors[8].

This study was undertaken to determine the inter-observer reproducibility of the invasive cell grading method modified by Bryne et al.[4] on oral squamous cell carcinomas and to correlate this with the DNA ploidy status.

Materials and Methods

Formalin fixed paraffin-embedded biopsy specimens of tumors diagnosed as intraoral squamous

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cell carcinoma were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa. These cases were randomly screened to confirm the diagnosis and for the presence of normal oral epithelium overlying or adjacent to the carcinoma. The first 50 cases in the screening process containing tumor tissue with normal overlying or adjacent epithelium were included in this study.

These tumors were graded in the most anaplastic areas of the deep invasive margins according to five morphologic features described by Bryne et al.[4]: degree of keratinization, nuclear polymorphism, number of mitoses, mode of invasion and plasma-lymphocytic infiltration. Each of these features was scored from 1 to 4 according to the criteria proposed by Anneroth et al.[3]. The scores were added to obtain a total malignancy score for each tumor. The gradings were performed independently by two authors (W.F.P. v.H. and E.J.R). The values for each of the parameters, as well as the total malignancy score of each tumor were subjected to correlation evaluation using Pearson's product moment correlation coefficient. The average total score of each tumor from the two investigators was used to separate the cases into 3 groups: a well differentiated group (score 5-8); a moderately differentiated group (score 9-12) and a poorly differentiated group (score 13-20). These groups were so selected because patients with these scores had shown a significantly different prognosis in a previous study[5]. The average score evaluating the plasma-lymphocytic cell infiltrate was also determined to separate the tumors into two groups: one with an average score of 3 and more and another with an average score of less than 3.

Two 100 μm sections from each paraffin embedded block including both the normal epithelium and tumor tissue were cut and prepared for flow cytometry analysis according to the Heiden method[6]. The nuclei were stained with propidium iodide using a Coulter DNA prep system instead of DAPI-phosphate. The cells were then analyzed on an Epics Elite flow cytometer (Coulter Electronics, Hialeah, Fl) which had been calibrated with chicken red blood cells and DNA check beads. The Elite was operated at 15 mW and emitted an Argon ion laser at 488 nm. The data rate varied between 20-200 events/second and 10000-20000 events were collected on a single parameter histogram. Cell data were collected in list-mode fashion and the DNA histograms were analyzed using a multicycle DNA analysis software programme (Phoenix Flow Systems, San Diego, CA). By convention, when using paraffin embedded tissue, the first peak was considered to be the normal DNA diploid peak representing the G0/G1 phase of the cell cycle. DNA aneuploidy was reported when at least 2 separate G0/G1 peaks were demonstrated. The coefficient of variation (CV) was calculated using the width of the peak (number of channels) at 61% of the maximum peak height divided by the peak height channel number, multiplied by a factor of 2.

Table I. Inter-observer correlation of the total malignancy score and individual morphologic features.

	Inter-observer correlation coefficient	Significance
Total Score	0.55	<0.001
Degree of keratinization	0.52	<0.001
Nuclear polymorphism	0.31	=0.018
Number of mitosis	0.38	=0.0037
Pattern of invasion	0.63	<0.001
Lymphoplasmacytic infiltration	0.61	<0.001

Results

The interobserver correlation and the statistical analysis of the total grading score and the individual morphologic features are given in Table I. All the tumors were grouped into either poorly differentiated or moderately differentiated categories. No tumor had a total score lower than 9. The distribution of aneuploidy within these two categories of tumors was almost equal (Table II): DNA ploidy analysis was only possible in 25 of the 32 tumors in the poorly differentiated group (Figures 1 and 2). The mean CV obtained was 6.6% ± 4.1.

Discussion

Most malignant neoplasms are polyclonal, implying the presence of heterogeneous cell populations with different biologic characteristics. Morphologic identification of specific tumor cells with a metastatic potential is not possible although poorly differentiated cell populations are often associated with an increased metastatic potential[3]. The most anaplastic areas in the most invasive sites were evaluated in this study because it has been shown that poorly differentiated areas in the superficial aspect of a tumor do not necessarily reflect an aggres-

Table II. Differentiation and ploidy distribution.

	Total malignancy score			
	5 - 8	9 - 12	13 - 20	Total
Number of cases	0	18 (36%)	32 (64%)	50
Diploid DNA content	–	9 (50%)	12/25 (48%)*	21
Aneuploid DNA content	–	9 (50%)	13/25 (52%)*	22

* Ploidy analysis was only possible on 25 of the 32 poorly differentiated tumors.

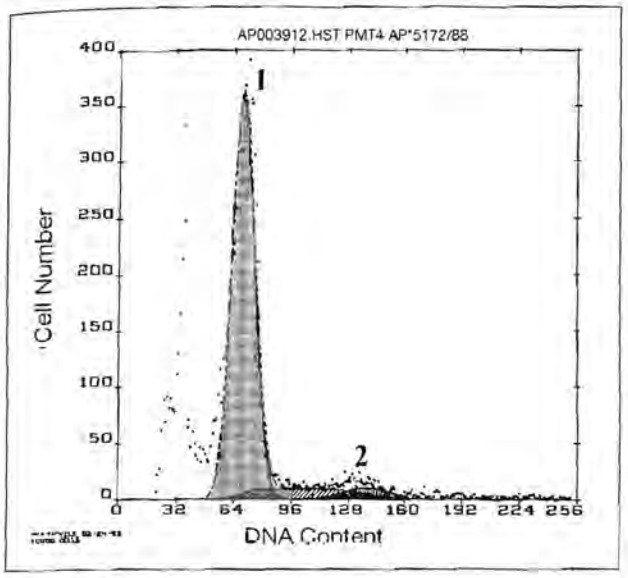

Figure 1. DNA histogram of a diploid oral squamous cell carcinoma. Peak 1 represents the diploid G_0/G_1 cells while peak 2 represents the tetraploid G_2 and mitosis cells.

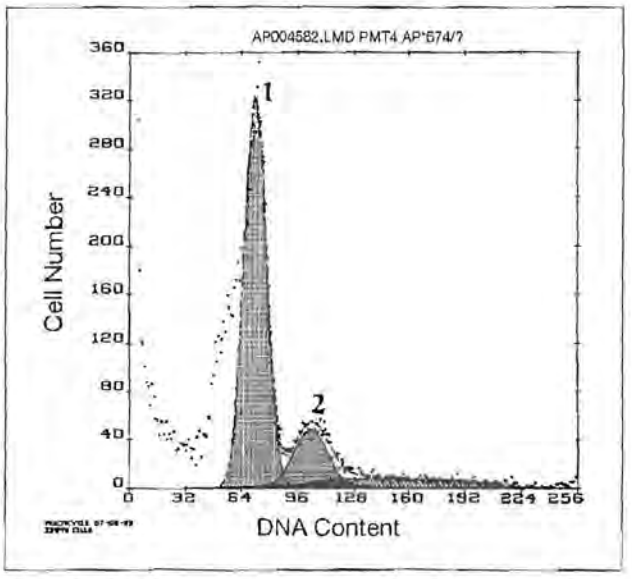

Figure 2. DNA histogram of an aneuploid oral squamous cell carcinoma. The two G_0/G_1 peaks (1 and 2) are clearly visible.

sive behavior and grading thereof may not give an accurate assessment of the prognosis. Increased labelling of bromodeoxyuridine[10] as well as Ki-67[11] and H-ras mRNA[12] expression in the cells of the invasive margin suggest that these cells may predict tumor behavior more accurately than evaluating of the total tumor cell population. The deep invasive cells also generally appeared histologically less differentiated than the cells in the superficial areas. Biopsy specimens were used in this study as this is usually the only non-clinical parameter available to predict tumor behavior. The question remains whether a biopsy could be regarded as representative of the different tumor cell populations. Grading of excision specimens results in a higher malignancy score than biopsies, although the biopsies could still be used to predict the behavior and prognosis significantly[4]. The most invasive margins in a biopsy can only be verified if a fragment of the overlying oral epithelium is included. Biopsies consisting only of tumor tissue cannot be evaluated by this grading method.

The inter observer correlation coefficient regarding the total malignancy score was highly significant. The worst correlation, although still significant, was found when evaluating the number of mitotic figures and the nuclear polymorphism. The evaluation of the mitotic count was omitted in another study which used the same grading method[5]. This omission resulted in an improved inter-observer reproducibility without influencing the prognostic value of the total malignancy score[5]. Less subjective methods of determining the proliferative activity of tumors exist, e.g. in vitro bromodeoxyuridine[13] and PCNA[11] labelling. These techniques should be incorporated if the proliferative activity of tumors are to form part of any grading method. A better correlation in the evaluation of nuclear polymorphism should be possible with proper training and standardization of observers.

It was unfortunately not possible to separate the patients in the present study into groups according to their prognosis and tumor behavior because a high percentage of patients were lost to follow-up. The total malignancy score was, however, divided into 3 groups based on other studies which used this grading method[5]. These groups had significant differences in behavior and prognosis. Almost two thirds of the patients in the present study were categorized into the poorly differentiated group. The vast majority of the patients at our hospital present with advanced tumors of more than 4 cm in largest diameter. This may be the reason why no tumor was grouped into the well differentiated category as aneuploidy has been shown to be an ultimate event of oral squamous cell carcinoma progression[8]. Aneuploidy has also been linked to a decrease in histologic differentiation of oral squamous cell carcinoma[6].

The distribution of aneuploidy between the two groups of tumors in the present study was almost equal. Most studies evaluating these two parameters found an increased number of aneuploid tumors in poorly differentiated carcinomas[6,7,14], however the grading method in these studies evaluated the whole tumor section and not the most anaplastic area in the most invasive site. Comparison with other studies is therefore only possible where similar grading methods had been used. Since only the most anaplastic areas in the most invasive sites

were evaluated, it was possible that a small number of cells which might influence the grading score were not detected with flow cytometry using paraffin blocks. Cusick et al.[14] demonstrated that the identification of closely associated aneuploid stemlines is directly related to the CV as well as the percentage of aneuploid nuclei present. It is possible that with a mean CV of 6.6% as found in the present study, tumors classified as diploid may contain undetectable peri-diploid and small aneuploid cell populations. DNA differences less than 15% cannot be detected with flow cytometry where the CV, an indication of the sensitivity, is more than 7%[15]. The CVs of paraffin-embedded tissues are often higher than those obtained using fresh material from the same tissue[16]. Although acceptable CVs are possible with paraffin-embedded tissues[9], it is recommended that fresh tissue be used as far as possible to improve the sensitivity of the measurements.

This study found that the invasive cell grading method is reproducible but no correlation was found between the grading results and the ploidy status.

Acknowledgments

We would like to thank Mrs. C.S. Begemann for secretarial functions. This study was supported by the Medical Research Council and National Cancer Association of South Africa.

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Lack of correlation between DNA ploidy, Langerhans cell population and grading in oral squamous cell carcinoma

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van Heerden WFP, Raubenheimer EJ, van Rensburg EJ, le Roux R: Lack of correlation between DNA ploidy, Langerhans cell population and grading in oral squamous cell carcinoma. J Oral Pathol Med 1995; 24: 61–5.

This study was undertaken to determine the inter-observer reproducibility of the invasive cell grading method on oral squamous cell carcinomas and to correlate this with the DNA ploidy status and Langerhans cell (LC) population. Fifty formalin-fixed paraffin-embedded blocks that contained both tumor and adjacent normal epithelium were randomly selected. DNA ploidy analysis was performed on a flow cytometer and the LC population was determined using an immunohistochemical technique with anti-S100 and anti-HLADR primary antibodies. The inter-observer correlation of the total malignancy score and individual morphologic features was highly significant. Thirty-two of the 50 tumors were graded as poorly differentiated and 18/50 as moderately differentiated. Thirteen of 25 poorly differentiated tumors had an aneuploid DNA content compared with 9/18 of the moderately differentiated group. No statistical differences in the LC counts between the poorly and moderately differentiated and aneuploid and diploid carcinomas were found. This study showed that the invasive cell grading method is reproducible but no correlation was found between the grading results and the ploidy status or LC count.

Key words: DNA ploidy; grading oral cancer; Langerhans cell

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The prognosis of patients with oral squamous cell carcinoma is determined mainly by morphologic characteristics and extent of the tumor, as well as by the host response. DNA content analysis of solid tumors has been studied by flow cytometry, but this technology has only been in widespread use since the recent development of reliable preparation techniques and data analysis programmes. DNA analysis of oral squamous cell carcinoma has been investigated as a possible indicator of prognosis or to predict tumor behaviour (1, 2). Patients with aneuploid primary tumors have a significantly higher incidence of lymph node metastases when compared with patients with diploid tumors (3). The frequency of aneuploid tumors also increased with decreasing degree of histologic differentiation (1).

Various grading systems for oral squamous cell carcinoma exist. BRODERS' method is based on the percentage of highly differentiated cells present throughout the tumor (4). This system has limited clinical use due to a poor prognostic value (5). ANNEROTH *et al.* (6) recommended a method evaluating six morphologic features in the most poorly differentiated parts of the tumor. A modification of this method was described by BRYNE *et al.*, where only the most anaplastic areas in the most invasive sites are evaluated; this modified system proved to be of high prognostic value (5, 7).

Langerhans cells (LCs) are bone marrow-derived dendritic cells (8) which form an integral part of all stratified squamous epithelia. LCs express the major histo-compatibility complex (MHC) class II antigens and are therefore capable of antigen presentation (9). It has been postulated that a depletion in LC numbers, resulting in an impairment of the local immune response, may predispose involved tissue to neoplastic change (10). The presence of a high number of LC was found to be a favourable prognostic factor in a variety of malignancies (11–14). Conflicting results were obtained when the number of LCs was correlated with the histological grading of tumors (11, 15).

This study was undertaken to determine the inter-observer reproducibility of the invasive cell grading method on oral squamous cell carcinomas and to correlate this with the DNA ploidy status and LC population.

Material and methods

Formalin-fixed-paraffin-embedded biopsy specimens of tumors diagnosed as intraoral squamous cell carcinoma were retrieved from the files of the De-

partment of Oral Pathology, Medical University of Southern Africa. These cases were randomly screened for the presence of normal oral epithelium overlying or adjacent to the carcinoma. The first 50 cases in the screening process were included in this study.

These tumors were graded in the most anaplastic areas of the deep invasive margins according to five morphologic features described by BRYNE *et al.* (5): degree of keratinization, nuclear polymorphism, number of mitoses, mode of invasion and plasma-lymphocytic infiltration. Each of these features was scored from 1 to 4 according to the criteria proposed by ANNEROTH *et al.* (6). The scores were added into a total malignancy score for each tumor. The gradings were performed independently by two authors (W.F.P. v H and E.J. R). The values for each of the parameters, as well as the total malignancy score of each tumor, were subjected to correlation evaluation using Pearson's product moment correlation coefficient. The average total score of each tumor from the two investigators was used to separate the cases into 3 groups: a well differentiated group (score 5–8); a moderately differentiated group (score 9–12) and a poorly differentiated group (score 13–20). These groups were so selected because they had shown a significantly different prognosis in a previous study (7). The average score evaluating the lymphoplasmacytic cell infiltrate was also determined to separate the tumors into two groups: one with an average score of 3 and more and another with a score of less than 3.

Two 100 μm sections from each paraffin-embedded block were cut and prepared for flow cytometry analysis according to the HEIDEN method (16). The nuclei were stained with propidium iodide using a Coulter DNA prep system instead of DAPI-phosphate. The cells were then analysed on an Epics Elite flow cytometer (Coulter Electronics, Hialeah, Fl) which had been calibrated with chicken red blood cells and DNA check beads. The Elite was operated at 15 mW and emitted an Argon ion lazer at 488 nm. The data rate varied between 20–200 events/second and 10,000–20,000 events were collected on a single parameter histogram. Cell data was collected in list-mode fashion and the DNA histograms were analysed using the Multicycle DNA analysis software programme (Phoenix Flow Systems, San Diego, CA). By convention, when using paraffin-embedded tissue, the first peak was considered to be the normal DNA diploid peak representing the G0/G1 phase of the cell cycle. DNA aneuploidy was reported when at least two separate G0/G1 peaks were demonstrated. The coefficient of variation (CV) was calculated using the width of the peak (number of channels) at 61% of the maximum peak height divided by the peak height channel number, multiplied by a factor of 2.

The LCs were demonstrated using an ABC immunoperoxidase technique with monoclonal antibodies diluted at 1:50 against HLA-DR (Dakopatts, Denmark) and polyclonal antibodies against S-100 proteins (Immunon, Lipshaw) on 5 μm sections. S-100-positive cells were considered to be LCs and not melanocytes when: 1) they were located in the suprabasal layers of the epithelium; 2) the whole cell body was visible; and 3) at least one dendritic process was present (17). Only HLA-DR positive cells with at least one associated dendritic process were counted as LCs. The LCs were counted along the entire length of the normal epithelium using high power (×400) magnification. The LCs were expressed per mm basement membrane length (BML) and epithelial surface length (ESL) as measured on a VIDS II Image Analysis System (Analytical Measuring Systems Ltd, Essex). The LCs in the carcinoma tissue were expressed as LCs per mm^2 tumor tissue. The Mann-Whitney U Test was used to compare LC density in the various tumor groups because the data was not normally distributed, while the correlation analysis was evaluated using the Spearman correlation coefficient method.

Results

The inter-observer correlation and the statistical analysis of the total grading score and the individual morphologic features are given in Table 1. All the tumors were grouped in either poorly differentiated or moderately differentiated categories. No tumor had a total score lower than 9. The distribution of aneuploidy between these two categories of tumors was almost equal (Table 2). DNA ploidy analysis was only possible in 25 of the 32 tumors in the poorly differentiated group (Figs. 1 & 2). The mean CV obtained was 6.6%±4.1.

The LC population counts in the tumors (Fig. 3) and adjacent normal epithelium are given in Table 3. The correlation between the LC counts in

Table 1. Inter-observer correlation of the total malignancy score and individual morphologic features

	Inter-observer correlation coefficient	Significance
Total score	0.55	<0.001
Degree of keratinization	0.52	<0.001
Nuclear polymorphism	0.31	=0.018
Number of mitoses	0.38	=0.0037
Pattern of invasion	0.63	<0.001
Lymphoplasmacytic infiltration	0.61	<0.001

Table 2. Differentiation and ploidy distribution

	Total malignancy score			
	5–8	9–12	13–20	Total
Number of cases	0	18 (36%)	32 (64%)	50
Diploid DNA content	–	9 (50%)	12/25 (48%)	21
Aneuploid DNA content	–	9 (50%)	13/25 (52%)	22

*Ploidy analysis was only possible on 25 of the 32 poorly differentiated tumors.

Table 3. Mean LCs count in the carcinomas and adjacent epithelium

	S-100	HLADR
Tumor	12.3±23.7/mm^2	1.67±6.0/mm^2
Epithelium BML	1.8±2.6/mm	0.2±0.4/mm
ESL	3.9±5.1/mm	0.5±1.0/mm

BML: basement membrane length.
ESL: epithelial surface length.

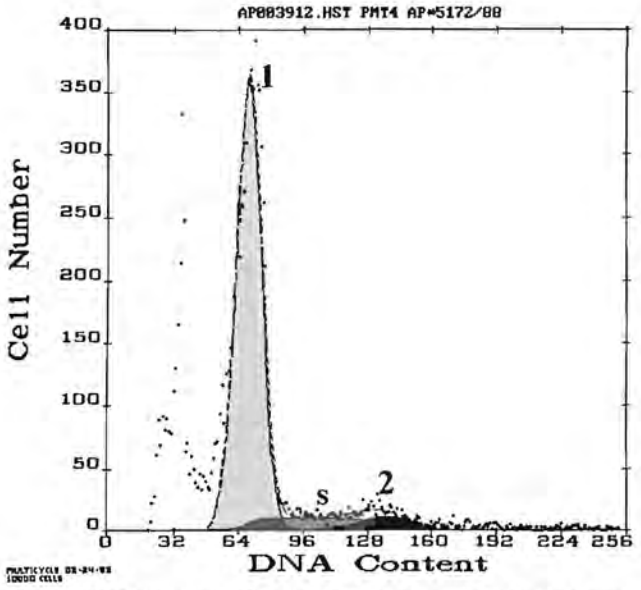

Fig. 1. DNA histogram of a diploid oral squamous cell carcinoma. Peak 1 represents the diploid G_0/G_1 cells, while peak 2 represents the tetraploid G_2 and mitosis cells. Cells in S-phase with intermediate DNA content are displayed between the two peaks.

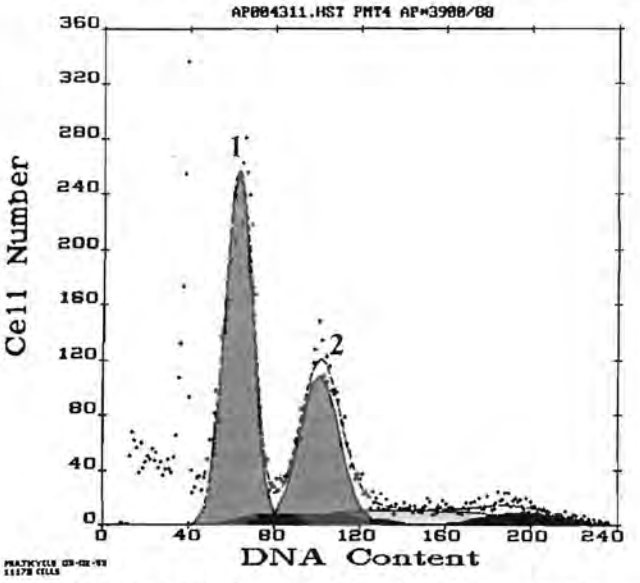

Fig. 2. DNA histogram of an aneuploid oral squamous cell carcinoma. The two G_0/G_1 peaks (1 and 2) are clearly visible.

the epithelium and tumor tissue as demonstrated by S-100 was statistically significant but not those demonstrated by antibodies against HLA-DR. The mean LC count in the aneuploid and diploid, and the poorly differentiated and moderately differentiated, carcinomas are shown in Table 4. The differences in LCs between the aneuploid and diploid group, as well as between the poorly differentiated and moderately differentiated tumors, when evaluating both methods of detection, were statistically not significant. The mean LC counts obtained from both detection methods were higher in the tumors associated with a more prominent lymphoplasmacytic cell infiltrate adjacent to the infiltrating tumor margins than in those carcinomas with an average lymphoplasmacytic score of 3 and 4 (Table 5). These differences however, were statistically not significant.

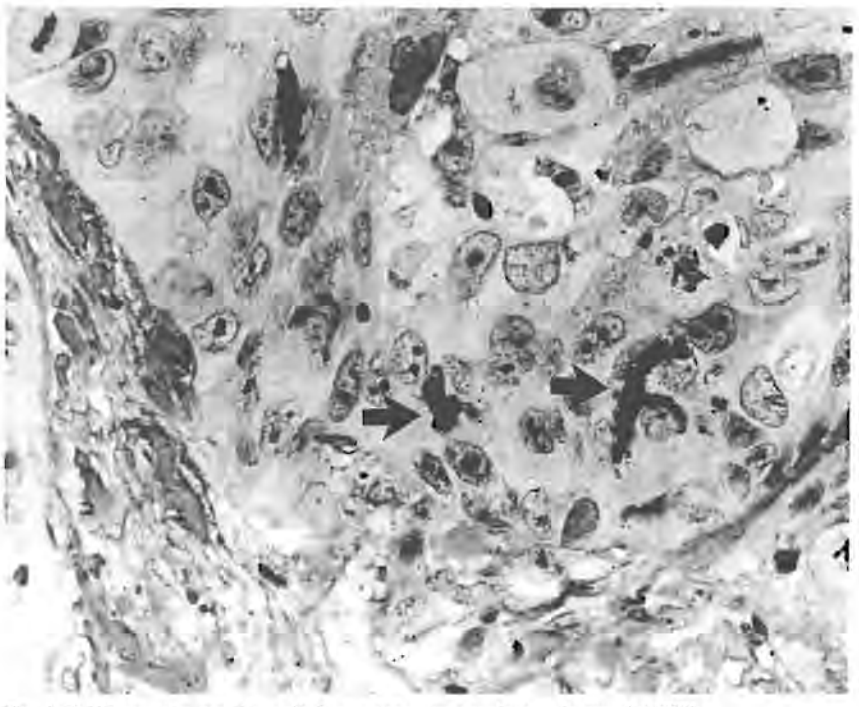

Fig. 3. S-100 protein-positive cells in a squamous carcinoma (arrows) ×200).

Discussion

Most malignant neoplasms are polyclonal, implying the presence of heterogeneous cell populations with different biologic characteristics. Morphologic identification of specific tumor cells with a metastatic potential is not possible, although poorly differentiated cell populations are often associated with an increased metastatic potential (6). The most anaplastic areas in the most invasive sites were evaluated in this study because it has been shown that poorly differentiated areas in the superficial aspect of a tumor do not necessarily reflect an aggressive behaviour and grading thereof may not give an accurate assessment of the prognosis. Increased labelling of bromodeoxyuridine (18) as well as Ki-67 (19) and H-ras mRNA (20) expression in the cells of the invasive margin suggest that these cells may predict tumor behaviour more accurately than evaluating the total tumor cell population. The deep invasive cells also generally appeared histologically less differentiated than the cells in the superficial areas. The question remains whether a biopsy could be regarded as representative of the different tumor cell populations.

Table 4. Langerhans cell distribution between aneuploid, diploid, poorly differentiated and moderately differentiated tumors

	S-100 (/mm² tumor)	HLA DR (/mm² tumor)
Aneuploid carcinomas	16.9±30.1	0.8±2.6
Diploid carcinomas	5.7±8.6	2.1±8.0
Poorly differentiated	11.8±25.9	0.7±2.2
Moderately differentiated	13.8±22.5	3.0±8.7

Table 5. Mean LC counts in tumors according to the lymphoplasmacytic cell infiltration

	S100	HLA DR
Lymphoplasmacytic score 3+4	9.2±19.8	0.52±1.5
Lymphoplasmacytic score 1+2	15.3±26.9	2.67±8.0

Grading of excision specimens results in a higher malignancy score than biopsies, although the biopsies could still be used to predict the behaviour and prognosis significantly (15). The most invasive margins in a biopsy can only be verified if a fragment of the overlying oral epithelium is included. Biopsies consisting only of tumor tissue cannot be evaluated by this grading method.

The inter-observer correlation coefficient regarding the total malignancy score was highly significant. The worst correlation, although still significant, was found when evaluating the number of mitotic figures and the nuclear polymorphism. The evaluation of the mitotic count was omitted in another study which used the same grading method (7). This omission resulted in an improved inter-observer reproducibility without influencing the prognostic value of the total malignancy score (7). Less subjective methods of determining the proliferative activity of tumors exist, e.g. *in vitro* bromodeoxyuridine (21) and PCNA (19) labelling. These techniques should be incorporated if the proliferative activity of tumors is to form part of any grading method. A better correlation in the evaluation of nuclear polymorphism should be possible with proper training and standardization of observers.

It was unfortunately not possible to separate the patients in the present study into groups according to their prognosis and tumor behaviour because a high percentage of patients were lost to follow-up. The total malignancy score was, however, divided into 3 groups based on other studies which used this grading method (7). These groups had significant differences in behaviour and prognosis. Almost two-thirds of the patients in the present study were categorized in the poorly differentiated group. The vast majority of the patients at our hospital present with advanced tumors of more than 4 cm in largest diameter. This may be the reason why no tumor was grouped in the well-differentiated category, as aneuploidy has been shown to be an ultimate event of oral squamous cell carcinoma progression (3). Aneuploidy has also been linked to a decrease in histologic differentiation of oral squamous cell carcinoma (1).

The distribution of aneuploidy between the two groups of tumors in the present study was almost equal. Most studies evaluating these two parameters found an increased number of aneuploid tumors in poorly differentiated carcinomas (1, 2, 22). Since only the most anaplastic areas in the most invasive sites were evaluated, it is possible that a small number of cells which might influence the grading score were not detected with flow cytometry using paraffin blocks. CUSICH *et al.* demonstrated that the identification of closely associated aneuploid stemlines is directly related to the CV as well as the percentage of aneuploid nuclei present (23). It is possible that with a mean CV of 6.6% as found in the present study, tumors classified as diploid may contain undetectable peri-diploid and small aneuploid cell populations. DNA differences less than 15% cannot be detected with flow cytometry where the CV, an indication of the sensitivity, is more than 7% (24). The CVs of paraffin-embedded tissues are often higher than those obtained using fresh material from the same tissue (25). Although acceptable CVs are possible with paraffin-embedded tissues (16), it is recommended that fresh tissue be used as far as possible to improve the sensitivity of the measurements.

Differences between the mean LC counts in both the epithelium and carcinoma tissue existed between the two antibodies used for immunohistochemistry. More LCs were detected with antibodies against S-100 protein than with those directed against HLA-DR. It is possible that melanocytes were not distinguished in the normal epithelium even though they are seldom found suprabasally (17). Not all LCs express HLA-DR antigens. WALSH and coworkers postulated that LCs acquire T6, then HLA-DR, and subsequently HLA-DQ, in their differentiation process (26).

The LC count in normal epithelium in the present study differed from other published reports (27, 28). This could be because of the processed nature of the tissue used in our study. Higher counts are found when evaluating HLA-DR on frozen sections than paraffin-embedded blocks (29).

More LCs were generally counted in the moderately differentiated tumors than in the poorly differentiated group, using both detection methods, but this difference was not statistically significant due to high standard deviations. A statistically significant difference was not present in the mean LC counts between aneuploid and diploid tumors. The immune functions of LCs due to their expression of MHC class II antigens are closely related to the presence of T-lymphocytes (30). More LCs/mm² were found in the tumor tissue in those carcinomas associated with a dense lymphoplasmacytic cell infiltrate (scores 1 and 2) than in those with a scanty cell infiltrate (scores 3 and 4). This correlation, although positive, was not significant. The correlation between the number of LCs in the tumor tissue and in the adjacent/overlying epithelium was statistically significant when evaluating both detection methods. It would appear that factors influencing the LC numbers were affecting both the normal epithelium and tumor tissue.

This study found that the invasive cell grading method is reproducible but no correlation was found between the grading results and the ploidy status or LC count.

Acknowledgements – We thank Mrs C. S. BEGEMANN for secretarial assistance. This study was supported by the Medical Research Council and National Cancer Association of South Africa.

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Flow cytometric cellular DNA content and lymph node metastasis in squamous cell carcinoma of the oral cavity

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Abstract. This prospective DNA flow cytometric study on 386 primary squamous cell carcinomas of the oral cavity showed that only 18% of the patients with diploid primary tumors had lymph node metastasis on admission compared to 52% of those with aneuploid carcinomas. The aneuploid group without evidence of lymph node involvement at the time of primary tumor treatment carried a 3-fold increased risk for developing late metastasis (23%) compared with the diploid group (8%). The clinical manifestation of occult metastasis in patients with diploid carcinomas was delayed by about two years compared to the aneuploid group. These ploidy-specific differences of the metastatic behaviour held true even if stratified with respect to tumor stage, histological grade and tumor localization. These results provide substantial evidence that cells with gross DNA content aberrations have a significantly higher probability of successfully producing a metastatic colony than flow cytometrically diploid tumor cells. An excellent 5-year survival rate of 90% in the diploid N0 group in contrast to 52% in aneuploid N0 cases and an even worse survival rate of 21% in patients with lymph node involvement at presentation underline the clinical importance of these findings.

Introduction

DNA flow cytometry is a time-saving method to directly and reproducibly identify gross karyotypic rearrangements provided that it results in a measurable deviation from the DNA content of normal diploid values. We have suggested that aneuploid tumor cell lines develop from flow cytometrically diploid progenitor cells in squamous cell carcinoma of the oral cavity (1,2). Although structural chromosome rearrangements have frequently also been detected in diploid tumor cells (3), aneuploid transformation does actually reflect a dramatic reorganization of the tumor cell genome in which gains and losses of chromosomes substantially contribute to the expression of an altered DNA content (4).

Whatever aneuploidy means in cytogenetic terms, a close association with an increased biological malignancy seems to be evident. Patients with diploid oral carcinomas have an excellent 5-year survival rate of about 90% independent of whether initially treated by surgery alone or with preoperative induction chemotherapy while only about 40% of the patient with aneuploid primary tumors, treated similarly, are long-term survivors (2,5,6). These results suggest a pronounced capability of aneuploid tumor cells to evade local control. This hypothesis is supported by studies reporting the incidence of lymph node metastasis to be between 56% and 82% in patients with aneuploid primary oral carcinomas and only 19% to 24% in the diploid group (7-10).

Since conflicting results have also been reported (11-13), the present prospective study of 386 cases was conducted to determine if aneuploid transformation in the primary tumor is associated with a higher frequency of lymphatic metastasis in patients with oral cancer. Special attention has been directed, firstly, to unequivocally assessing lymph node invasion by thorough histopathological examination, secondly, to predicting occult lymph node involvement by monitoring of delayed metastasis and, thirdly, to evaluating the influence of the sensitivity of the flow cytometrical measurements on the interpretation of the results.

Materials and methods

Patients. A total number of 386 consecutive patients with primary and previously untreated squamous cell carcinomas of the oral cavity were the cohort in this prospective study. The carcinomas were localized as follows: upper and lower lip (19), tongue (76), floor of the mouth (142), hard and soft palate (33), buccal mucosa (20), spread over two or more anatomical regions (83) and other sites (13). The majority (279 cases) were treated by surgery alone. Fourty patients underwent intra-arterial regional induction chemotherapy

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Key words: head and neck carcinomas, DNA flow cytometry, metastasis, prognosis

Table I. Flow cytometrical DNA ploidy status of the primary tumor, cervical lymph node involvement and type of initial lymph node treatment in 386 patients with oral squamous cell carcinoma.

	diploid		aneuploid		
	N0	N+	N0	N+	total
ipsilateral radical neck dissection	9	7	55	95	166
bilateral radical neck dissection	-	1	-	4	5
ipsilat. radical neck diss. + contralat. submandibular triangle dissection	6	2	11	29	48
ipsilat. submand. triangle diss.	12	2	25	4	43
bilateral submand. triangle diss.	5	-	11	2	18
clinically positive nodes, no surgery[a]	-	1	-	28	29
clinically negative nodes, no surgery	28	-	49	-	77
total	60	13	151	162	386

[a]palliative treatment or treatment refused

with 5-fluorouracil and cisplatin while 23 received epirubicin and cisplatin preoperatively. Fourty-one were submitted for radiation therapy. Three patients refused treatment. The therapeutic modalities employed in cases with positive cervical lymph nodes are listed in Table I. The follow-up ranged between 1 and 103 months (mean: 20.4 months; SD: 19.7).

Sample preparation. Tumor samples were taken by incisional biopsy or were removed postoperatively from the resected tumor. After addition of 1 ml 0.9% sodium chloride (NaCl), the tissue pieces were immediately homogenized by thoroughly mincing with surgical scalpels. The nuclei were extracted by incubation in acid pepsin solution (0.5 g pepsin dissolved in 100 ml of 0.05 N HCl) at room temperature with careful stirring for 5 minutes. Remaining tissue fragments were removed by a 50-micron nylon mesh. The nuclei suspension was centrifuged (500 x g, 10 minutes), fixed with 70% ethanol, and stored at -20°C.

DNA specific staining. The ethanol was removed by centrifugation (500 x g, 10 minutes). After 3 minutes additional pepsination, the cells were resuspended in a solution containing 10 mg ethidium bromide (Serva; Heidelberg, Germany), 4 mg mithramycin (Serva), 500 mg magnesium chloride ($MgCl_2$) dissolved in 1,000 ml Tris buffer (pH 7.5). The minimum incubation time was 30 minutes.

DNA flow cytometry. Flow cytometry was carried out using a PAS II flow cytometer equipped with a high-pressure 100 W mercury lamp (Partec; Münster, Germany). The filter combination used was a BG 12 excitation filter, a dichroic mirror TK 500 and a RG 590 barrier filter (Partec). A flow rate of about 100 counts per second was maintained by vacuum adjustment. The presence of cells with diploid DNA content could be verified in all tumor samples by addition of human lymphocytes or normal tissue cells in a control measurement.

Histogram analysis. DNA histograms of at least 10,000 counts were plotted. The diploid cell population was used as an internal reference standard for the calcuation of the DNA index of the aneuploid clone. The DNA index was expressed by the quotient of the respective modal peak values and reflected the discrepancy between the aneuploid DNA content and the normal diploid DNA value (DNA index = 1.0). The sensitivity of the flow cytometric measurements, which was reflected by the coefficient of variation of the mean, ranged between 0.9% and 5.3% with a mean value of 2.3% (SD: 0.9).

Statistical methods. The chi-square probability test was used to determine distribution differences in stratified subgroups. Survival curves were calculated by the product limit method of Kaplan and Meier and a log rank test was used for comparison between the curves. No corrections were made for deaths unrelated to cancer. In order to avoid influences of different treatment strategies, only patients who were treated by surgery alone were taken into account for the survival analysis. The Kaplan-Meier approach was also applied to calculate the time-adjusted risk of late metastasis development using the intervals defined by the actual time between the primary intervention and the first diagnosis of delayed lymph node involvement by histology. Patients who developed local recurrences before or simultaneously with delayed appearence of lymph node metastasis were excluded as well as cases where a histological examination of the lymph node status were not available.

Results

Histopathological examination provided confirmation of lymph node metastasis in 146 patients with oral squamous

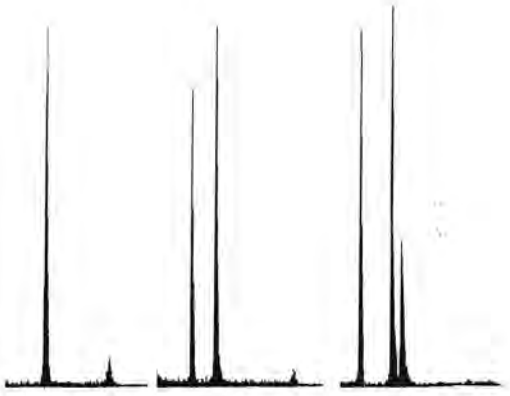

Figure 1. Representative DNA histograms of oral carcinomas. Diploid tumors (left) are characterized by a prominent peak representing the cells in the G1/G0-phase of the cell cycle. The much smaller peak with exactly double the DNA content of the first one contains cells in G2-phase and mitosis. The counts plotted between both peaks reflect cells in S-phase characterized by intermediate DNA values. Aneuploid tumors show additional cell lines with abnormal DNA contents. The cell cycle phase distributions of the diploid and aneuploid cell populations are more or less overlapping depending on the respective DNA indices of the aberrant clones. A DNA histogram of a tumor with an aneuploid cell line with a DNA index of 1.46 (middle) and a biclonal DNA histogram with two aneuploid cell lines expressing DNA indices of 1.71 and 1.93 (right) are shown.

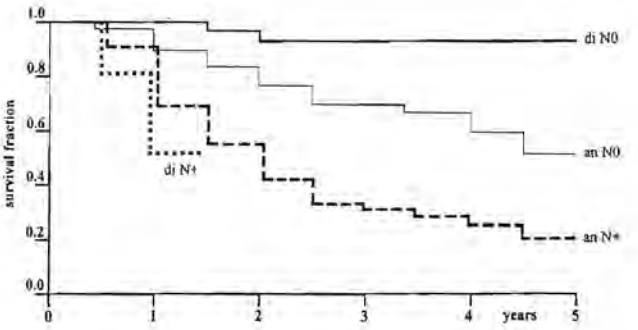

Figure 2. Overall survival of 279 oral carcinoma patients who underwent initial treatment by radical primary tumor surgery accompanied with a neck dissection if indicated with respect to lymph node involvement at presentation and DNA ploidy status of the primary tumor.

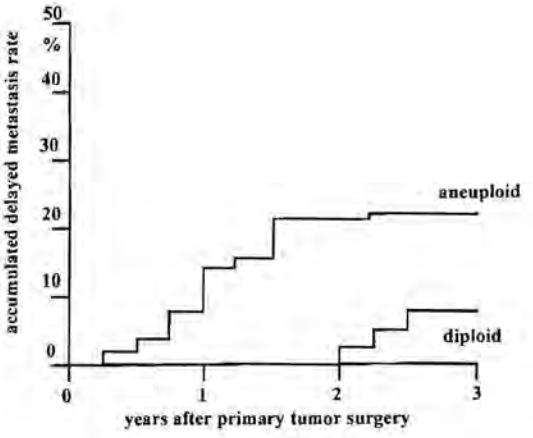

Figure 3. Time-adjusted delayed metastasis rate in patients without clinical or histological evidence of lymph node involvement at primary tumor surgery with respect to the DNA ploidy status of the primary tumor. Patients who showed lymph node involvement after or simultaneously with the development of a second primary tumor have not been taken into account.

cell carcinoma (Table I). The lymph nodes of 134 patients who underwent a radical or modified neck dissection contained no metastatic deposits. Histopathological findings were not available from 29 patients with clinical evidence of metastatic disease who refused any treatment or were referred directly for palliative radiation therapy as well as from 77 patients who remained untreated due to lack of clinical evidence of lymph node involvement.

Seventy-three of the 386 primary tumors (19%) consisted exclusively of flow cytometrically diploid cells. A single cell population characterized by an abnormal DNA content was detected in 273 cases (71%) and 40 tumors (10%) expressed two or more aneuploid cell lines (Fig. 1). Clinical or histopathological evidence of lymph node involvement was found in only 18% of the patients with diploid primary tumors, but in 52% of the aneuploid group (P<0.001). Even if only the cases in which histopathological findings were available (n=280) were considered, lymph node metastasis were detected in 27% of the diploid and 57% of the aneuploid group (P<0.001).

The negative influence of cervical lymph node involvement at admission on the outcome of the disease in patients who were initially treated by surgery alone was substantiated by the 5-year overall survival rate of only 21% in the aneuploid N^+ group and a comparably poor prognosis also of the few diploid N^+ cases (Fig. 2). The prognosis of the aneuploid N0 group, with a 5-year survival rate of 52%, turned out to be significantly more favourable (P<0.001). Ploidy-specific differences in the follow-up may be suggested by the excellent 5-year survival rate of 90% of the diploid N0 group (P<0.001).

Late metastasis were observed in 9 of the 77 N0 patients (12%) who had undergone neck dissection at primary tumor surgery (compare Table I). Also in 18 of the 134 patients (13%) who were treated by radical or modified neck dissection, but turned out to be free of lymph node involvement by histology in the respective resection field, lymph node metastasis appeared later in initially untreated neck areas. Although late metastasis has been proven in only 5% of the diploid N0 group and 16% of the aneuploid N0 group (P<0.05), the three-year accumulated risk of delayed metastasis was estimated to be 8% in patients with diploid carcinomas and 23% in the aneuploid group (P<0.01) if corrected for losses during follow-up as well as for the influence of death before clinical manifestation of lymph node disease (Fig. 3). Moreover, occult metastasis in patients who suffered from diploid primary tumors reached a clinically detectable size about two years later than those with aneuploid tumors.

The close association between the DNA ploidy status of the primary tumor and metastatic behaviour remained significant even if stratified with respect to tumor size or with degree of histological differentiation (Table II). The higher incidence of metastasis in diploid carcinomas of the

Table II. Correlation between lymph node status, DNA ploidy of the primary tumor with tumor stage and histological grade. The number of N+ cases at primary tumor surgery as well as the actually observed cases with delayed metastasis were considered.

	N0		N+			
	d	a	d	a	sum	p-value
T1	28	32	2	11	73	0.05
T2	24	81	11	104	220	0.01
T3	5	14	3	71	93	0.01
G1	19	15	2	10	46	0.025
G2	34	87	13	115	249	0.001
G3	4	25	1	61	91	0.025
sum	57	127	16	186	386	0.01

Table III. Correlation between lymph node involvement at presentation and the DNA ploidy status of the primary tumor at different anatomical sites of the oral cavity.

	diploid		aneuploid	
	n	%N+	n	%N+
lips	12	8	7	14
tongue	19	11	57	49
floor of the mouth	23	23	120	48
palate	8	13	25	56
buccal mucosa	6	33	14	36

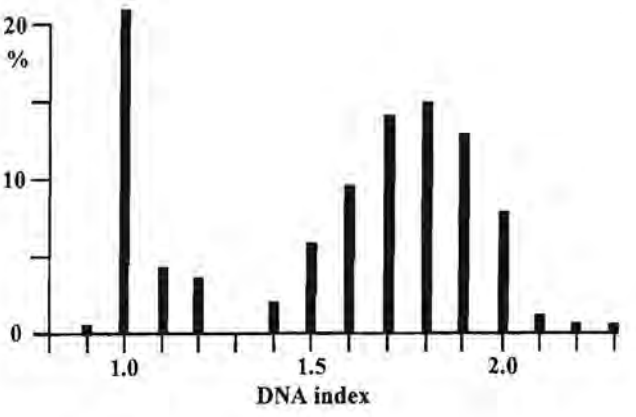

Figure 4. DNA index frequency distribution pattern of all monoclonal oral carcinoma cases.

buccal mucosa was statistically not significant while the low metastatic potential of lip carcinomas was confirmed (Table III). Although the DNA indices of the monoclonal aneuploid cases varied in a wide range from 0.9 to 2.2 (Fig. 4), and inspite of the presence of two or more aneuploid cell lines in some tumors, the proportion of cases with lymph node metastasis was nearly identical in all subgroups (Table IV). Only in tetraploid cases, lymph node involvement appeared to occur significantly more frequently than in all other aneuploid tumors ($P<0.05$). Cases classified as diploid, but measured with a relatively low sensitivity (CV>4%), developed lymph node metastasis at nearly the same frequency as the aneuploid group.

Discussion

An incidence of lymph node metastasis at the time of primary tumor treatment of only 18% as well as a 3-year

Table IV. Correlation between lymph node metastasis and DNA ploidy profile of the primary tumor.

	N0	N+ at presentation	N+ delayed	N+ (%)
diploid	57	13	3	21.9
CV<4%	52	6	2	13.3
CV>4%	5	7	1	61.5
aneuploid	127	162	24	59.4
multiclonal aneuploid	21	17	2	47.5
monoclonal aneuploid	106	145	22	59.4
DI<1.25	11	16	1	60.7
DI<1.45	2	5	0	71.4
DI<1.55	8	12	0	60.0
DI<1.65	13	18	3	61.8
DI<1.75	19	28	3	62.0
DI<1.85	23	23	6	55.8
DI<1.95	21	20	5	54.3
DI<2.05	6	19	4	79.3
DI>2.05	3	4	0	57.1

delayed metastasis rate of only 8% in patients with primary carcinomas consisting exclusively of flow cytometrically diploid cells was found compared to 52% and 23% respectively in those with aneuploid tumors. This provide substantial evidence for a close association between aneuploidy formation and the expression of the metastatic behaviour in squamous cell carcinoma of the oral cavity. The existence of cell lines with almost identical DNA contents in both primary tumors and their synchronous metastasis in spite of a wide range of individual DNA quantities, may largely exclude the possibility of dissemination of diploid tumor cells with a subsequent evolution of cell strains with identical aneuploidy profiles in the corresponding lesions (8).

Metastatic spread from true-diploid carcinomas must be considered to be less frequent than could be determined. This is suggested by a significantly lower proportion of metastasis in diploid oral carinomas measured with a CV<4% (13%) compared to the diploid group where a sensitivity threshold of 4% was exceeded (62%). A certain number of the latter cases has obviously been falsely classified as diploid, most likely due to the inability of discriminating tumor cell populations with only small deviations of their DNA contents from normal diploid cells. However, occasional dissemination from flow cytometrically diploid oral carcinomas cannot completely be excluded and should be accepted.

An excellent survival rate of 90% in the diploid N0 group provides striking evidence that malignancy progression can completely be interrupted by local surgery alone in the vast majority of cases if treated before aneuploid cell lines have emerged. A 5-year overall survival rate of only 52% in the aneuploid N0 group confirmed the deteriorating influence of late metastasis as well as of local recurrence development, a complication which has also been monitored predominantly in aneuploid oral carcinomas (14). Cervical lymph node involvement at presentation resulted in a poor 5-year survival rate of only 18%.

It has been suggested that aneuploid tumor cell lines originate from diploid progenitor cells as a result of abnormal mitosis (2,15). Convincingly, the contribution of numerical chromosome aberrations is required to substantiate the development of extensive DNA content abnormalities, an assumption that has been confirmed by comparisons with karyotype analyses (3,4,16-18). Although an increasing emergence of gene-dose effects should be expected, this type of gene expression alone does not explain the induction of the metastatic phenotype because even great karyotypic differences, as reflected by the wide range of DNA content aberrations, did not result in a significant change of the metastatic behaviour, perhaps with some reservation of tetraploid DNA amounts.

The existence of additional cytogenetic changes were suggested from absence of nodal involvement in approximately every third aneuploid oral carcinoma. This implies a certain delay between aneuploidy formation and the definite expression of the metastatic phenotype, a period which can be used for successful local intervention. The development of metastasis from diploid tumors provides further evidence that metastasis-associated genes are occasionally activated even in absence of gross karyotypic changes. The extensive reorganization of the genome by aneuploid transformation whether due to simultaneous accumulation of mutations or as a result of gene-dose effects contribute to an acceleration of the expression of metastatic behaviour. This is underlined by the high incidence of metastasis in patients with aneuploid tumors and can also be concluded from the observation that late clinical appearence of metastasis from diploid primary tumors is delayed by two years in comparison to the aneuploid group.

In conclusion, the present study demonstrate the advantages of DNA flow cytometry to easily and reliably screen biopsies from the primary oral carcinoma to determine the relative risk of metastasis spread. Advances in unveiling the concrete cytogenetic nature of altered DNA contents and the role the underlying karyotypic changes may play in the induction of metastatic growth, may translate into further improvements in patient care.

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Flow cytometric DNA ploidy and recurrence development in squamous cell carcinoma of the oral cavity

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Abstract. This prospective study on 348 patients with oral squamous cell carcinoma who underwent radical surgery established a close association between the DNA ploidy status of the primary tumor and the risk of local recurrence development. Nine percent of patients with flow cytometrically diploid tumors developed a recurrence compared to 46% of those with aneuploid tumors. This correlation held true even if evaluated with respect to tumor stage or histological grade. Thirteen percent of the diploid and 59% of the aneuploid group showed lymph node metastasis. These results provide substantial evidence that cytogenetic events that underlie aneuploidy formation from initially diploid progenitor cells are functionally linked to the development of tumor cell populations that have the capability to establish independently growing colonies in foreign tissues.

Introduction

DNA flow cytometric studies on squamous cell carcinoma of the oral cavity have shown that early tumor stages consist predominantly of diploid cells while cell lines with aberrant DNA content are frequently expressed in advanced lesions (1-5). This implies that aneuploid cell strains develop from diploid progenitor cells, usually after the tumors have advanced towards a clinical detectable size. A close correlation between the degree of DNA content aberration and the respective chromosome number suggests an abnormal mitosis as the underlying cytogenetic event responsible for the development of tumor cell clones with altered DNA content (6,7). Although flow cytometrically diploid tumor cells frequently possess structural chromosome aberrations (8), it appears that a change of the nuclear DNA content reflects a fundamental reorganization of the tumor cell genome.

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Key words: head and neck carcinomas, DNA flow cytometry, recurrence, metastasis

There is evidence indicating that the cytogenetic changes that are associated with aneuploid transformation contribute to the behavior of oral cancer. Patients with aneuploid tumors, when compared to patients with diploid carcinomas of the same stage and histological grade, have approximately a 3-fold increased risk to develop cervical lymph node metastasis (3,9,10). These results, which are in agreement with data presented by others (4,11,12), indicate that the development of cell populations with aberrant DNA content is associated with an increased potential of successfully producing distant colonies.

Since 1986, DNA flow cytometry has been a regular part of our diagnostic procedures in cases of oral cancer. This study was done to evaluate the implications of the DNA ploidy status of primary oral squamous cell carcinomas on the development of local recurrences.

Materials and methods

Three hundred and forty-eight consecutive patients, all of whom had undergone radical primary tumor surgery accompanied by radical neck dissection if indicated, were included in this study. Patients who were selected for adjuvant treatment protocols were excluded as well as those who were treated palliatively. Tumor staging and histological grading were done according to conventional criteria. The surgical margins were examined by frozen sections and the resection was adjusted if indicated.

The methods used for sample preparation and DNA flow cytometry have recently been described in detail (2,9,10). In short, tumor samples were immediately homogenized by thoroughly mincing with a surgical scalpel. The nuclei were extracted by incubation in acid pepsin solution (0.5 g pepsin dissolved in 100 ml of 0.05 N HCl) at room temperature with careful stirring for 5 min. Remaining tissue fragments were removed by a 50-micron nylon mesh. The nuclei in suspension were fixed with 70% ethanol and stored at -20°C. For DNA specific staining, the cells were pepsinated for 3 min and resuspended in a solution containing 10 mg ethidium bromide (Serva, Heidelberg, Germany), 4 mg mithramycin (Serva), 500 mg magnesium chloride ($MgCl_2$) dissolved in (1 l) Tris buffer (pH 7.5). The minimum incubation time was 30 min. Flow cytometry was carried out using a PAS II or a PAS III flow cytometer equipped with a high-pressure 100 W mercury lamp (Partec, Münster, Germany).

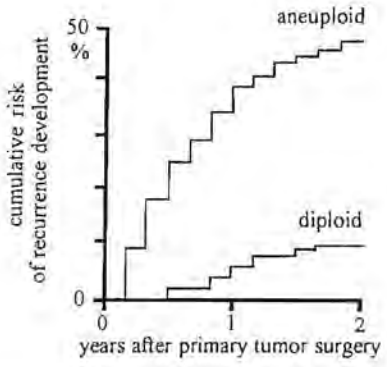

Figure 1. Recurrence development with respect to the DNA ploidy status of the primary tumor.

In order to exclude influences due to loss during follow-up or other causes of death, the cumulative risk of recurrence development was expressed in a percentage at a particular time period according to the Kaplan-Meier method. A log rank test was used to compare the differences between the life-table probabilities of the respective cohorts.

Results

Seventy-two oral carcinomas consisted exclusively of flow cytometrically diploid cells (21%). An additional cell population with aneuploid DNA content was expressed in 276 cases (79%). The risk to develop juxtaprimary recurrences during the first two-year interval after primary tumor surgery was only 9% in patients with diploid primary tumors while 46% of the aneuploid group relapsed during the same period ($P<0.001$; Fig. 1). This correlation held true ($P<0.01$) even if evaluated with respect to tumor stage or degree of histological differentiation (Fig. 2). The recurrence rates of the diploid cases ranged between 0% and 11% if stratified with respect to stage or histological grade without showing statistically significant differences between these subgroups. Aneuploid T1 tumors (37%) and low grade (G1) lesions (28%) relapsed less frequent than advanced aneuploid carcinomas ($P<0.01$). The recurrence rates were nearly identical in aneuploid T2 (52%) and T3 cases (45%) as well as in intermediate grade (G2) (50%) and high grade (G3) cases (48%).

Although the local recurrence rates were significantly lower ($P<0.001$) in patients with carcinomas of the T1 category (26%) or with well differentiated tumors (14%) compared to patients with advanced tumors of the T2 (43%) and T3 category (40%) or of the G2 (42%) and G3 group (48%) (Fig. 3), these differences were obviously influenced predominantly by the composition of diploid and aneuploid cases in the respective subgroups. The proportion of diploid cases actually decreased with staging from 41% in the T1 group to 16% in T2 carcinomas and 12% in the T3 category. The percentage of diploid cases also decrease in the various tumor grades from 50% in well differentiated lesions to 21% in moderately differentiated tumors and 6% in poorly differentiated carcinomas (Table I).

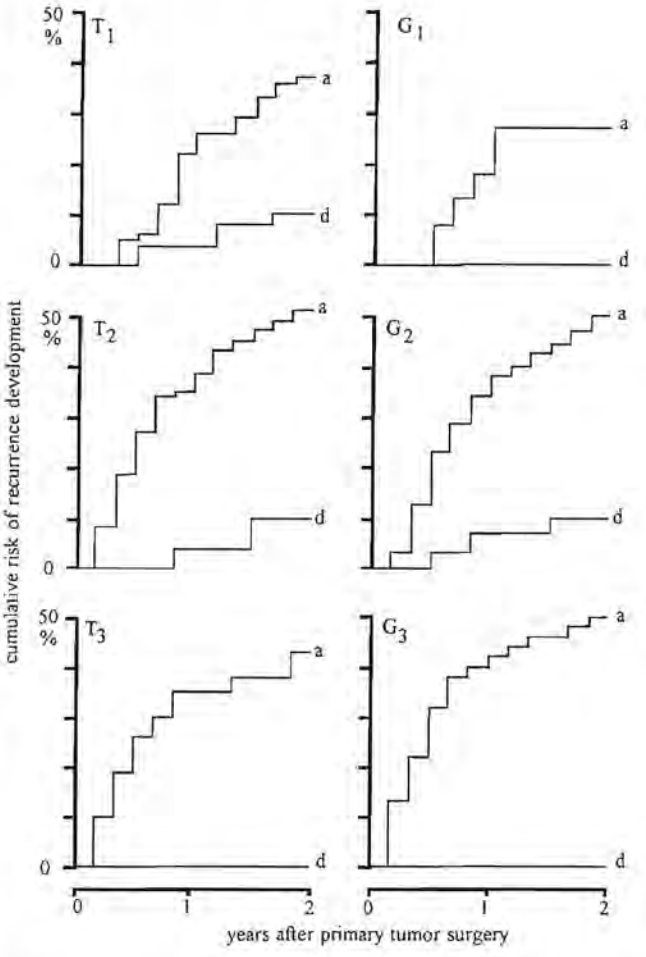

Figure 2. Recurrence development with respect to the DNA ploidy status of the primary tumor as well as with respect to tumor stage and histological grade.

Table I. Clinical and histopathological features related to the DNA ploidy status of the primary tumor.

	Diploid	Aneuploid	Total
Stage			
T1	31	45	76
T2	33	172	205
T3	8	59	67
Histological differentiation			
well	19	19	38
moderate	48	184	232
poor	5	73	78
Lymph node metastasis			
absent	63	113	176
present	9	163	172
total	72	276	348

Only 13% of the patients with diploid primary tumors had histologically verified lymph node metastasis at the time of primary tumor surgery in contrast to 59% of the aneuploid group ($P<0.001$, chi square probability test). The two-year cumulated risk of recurrence development was 36% in the

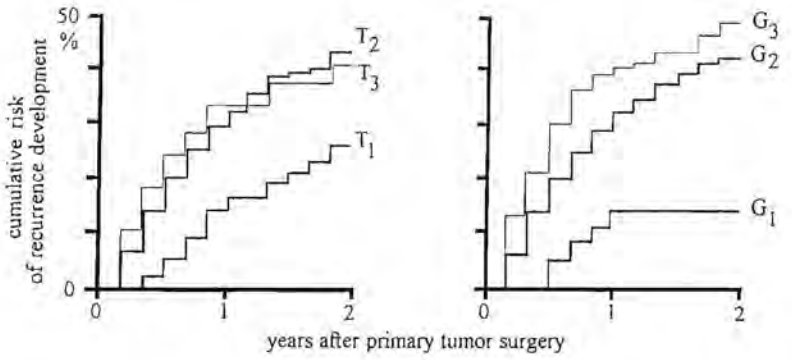

Figure 3. Recurrence development with respect to tumor stage and histological grade.

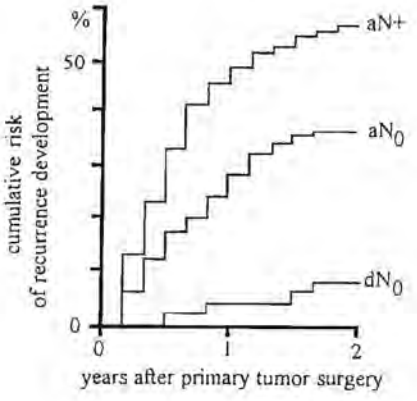

Figure 4. Recurrence development with respect to the DNA ploidy status of the primary tumor as well as with respect to lymph node involvement at primary tumor surgery.

aneuploid N0 group and 61% in the aneuploid N^+ group ($P<0.001$) while only 8% of the diploid N0 cases relapsed ($P<0.001$; Fig. 4). The low number of diploid cases with lymph node metastasis did not warrant an evaluation of the time-adjusted risk of recurrence development in this group. A remarkable difference between the diploid N^+ and the diploid N0 group was obvious because only one out of 9 patients with lymph node involvement was actually afflicted with recurrent disease.

Discussion

This study confirmed previous results of a striking ploidy-specific difference in the metastatic behavior in oral squamous carcinoma (3,9,10). As we have recently reported, lymph node involvement, which is almost exclusively found in the aneuploid group, is an important contributing factor to the poor 36% 5-year survival rate in these patients (2). In contrast, 88% of the diploid group were long-term survivors. In our previous report on the same patient sample (10), we found a dramatical difference in the outcome between patients with diploid tumors who were not afflicted with lymph node metastasis on admission (90%) and the aneuploid N0 group (52%). Although a delayed metastasis rate of only 9% in diploid N0 cases has been found and which was consistent with their excellent prognosis, the fatal outcome in half of the aneuploid N0 group could not solely be explained by their delayed metastasis rate of 23% (10).

This study provided striking evidence that the progression of oral squamous cell carcinoma could be interrupted by local surgery alone if executed before the establishment of aneuploid cell lines and confirmed a close association between DNA ploidy status and the risk of local recurrence development. Although only 9% of the patients with diploid carcinomas relapsed, 46% of those with aneuploid tumors developed recurrent tumor over the same period. An even more pronounced difference, namely 4% in case of diploidy and 71% in case of aneuploidy, has been reported in a study on 76 patients who had undergone resection with curative intent (4). In line with these results, we previously found a complete lack of recurrence in patients with diploid tumors and a 38% relapse rate in the aneuploid group in 53 oral carcinoma patients who were treated by intra-arterial induction chemotherapy prior to radical surgery (13). The 5-year survival rates of these patients were 90% in the diploid and 18% in the aneuploid group (13,14), very similar to those in this study and who underwent surgery alone.

As the number of diploid cases markedly decreased with progression of the disease, one could propose that the close correlation between the DNA ploidy status of the primary tumor and the risk of recurrence development simply reflects stage-specific differences in therapeutic success. This is obviously not the case in our study. The recurrence rate remained negligibly low in advanced diploid tumors while even small and well-differentiated aneuploid lesions, which had been managed with a comparable radicality to diploid tumors of the same stage, showed a dramatically higher risk of recurrent disease. These observations suggested that malignant regrowth emanating from the primary tumor continuum represented a less important source for the evasion of local control during recurrence development compared to the intrinsic property of aneuploid tumor cells.

Tumor invasion is certainly a prerequisite for tumor regrowth in cases of 'complete' surgical removal. No attempts have been made, however, to histologically examine the tumor-host interface. One reason is the well-known problem to definitely diagnose malignant invasion beyond the resection margins (15,16). There is also mounting evidence from histological studies that recurrent disease does not always occur if invaded host tissue has been left behind

(17). It can therefore not be excluded that flow cytometrically diploid tumor cells are capable of invasion. The conclusion that diploid cells possess a largely reduced colony forming potential is rather convincing as not only the risk of recurrence development but also the metastasis rate was very low in patients with these tumors.

To explain the lack of cervical lymph node involvement in a third of patients with aneuploid carcinomas, we have already postulated a certain delay between aneuploidy formation and the definite expression of the metastatic phenotype (10). The capacity to generate juxtaprimary tumor regrowth is not necessarily simultaneously induced with aneuploid transformation as may be concluded from a relapse-free follow-up period experienced in roughly half of the aneuploid group that had undergone surgery. Such a delay that was probably due to the requirement of further cytogenetic changes, would potentially allow successful local intervention. It was however, difficult to discriminate between patients that have been treated curatively because of the inability of the tumors to relapse and those who could be cured in spite of the potential of tumor regrowth. Since as many as 61% of the aneuploid cases with lymph node metastasis upon admission developed local recurrences, it appears to be conclusive that when clones that share the capability to survive and grow in foreign tissues have emerged, the tumor can indeed only occasionally be brought under local control. Although there was a trend towards improved surgical success during the complete removal of small and well differentiated aneuploid lesions, the recurrence rates in more advanced cases were completely identical irrespective of the tumor stage or grade. This partly meets the expectation that treatment failure is closely linked with the accessibility and extension of the lesion. The actual surgical success is however mainly due to the fact that a certain number of aneuploid tumors at the time of intervention, have not yet generated cellular phenotypes with the capacity to establish independently growing colonies.

These considerations are certainly of profound clinical importance. We can firmly propose an excellent success rate for standard local surgical intervention in patients with flow cytometrically diploid oral carcinomas. Surgery alone is not recommended for the aneuploid N^+ group. Whether more radical surgical procedures may improve the cure rate in the aneuploid N0 cases is speculative as the need for wide uninvolved margins always competes with the prerequisite to preserve the vital anatomical structures of the head and neck region. It therefore seems desirable to distinguish patients of the aneuploid group who can successfully be treated by standard surgery alone from those in whom adjuvant therapeutic strategies should be taken into consideration.

The observation that two of three patients with metastatic oral carcinoma suffer from local recurrences supports the idea that cells that share the capability to establish secondary malignant colonies have in the majority of cases acquired this characteristic at the primary tumor site and not after having detached from it. In order to select patients at high-risk for metastasis and recurrence development, the only information required is cytometric data on that which is easily accessible: the primary tumor. This should stimulate our ambition to explore the cytogenetic and phenotypic changes involved in dissemination of neoplastic cells.

Acknowledgments

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The Value of DNA Flow Cytometry in Predicting the Development of Lymph Node Metastasis and Survival in Patients with Locally Recurrent Oral Squamous Cell Carcinoma

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BACKGROUND. DNA flow cytometry studies of squamous cell carcinoma of the head and neck have shown that patients with diploid tumors have favorable prognoses, whereas the outcomes of those with aneuploid tumors are poor. This study was conducted to examine the importance of DNA ploidy in patients with locally recurrent oral carcinoma.

METHODS. DNA flow cytometry was performed on 93 primary oral carcinomas and their subsequent recurrences.

RESULTS. Eight patients with diploidy of both the primary tumor and the recurrence never developed lymph node metastasis. The 5-year overall survival rate of this group was 87%. For 80 aneuploid primary carcinomas, recurrences developed that were also aneuploid. Only 31% of these patients were 5-year survivors ($P < 0.001$). Lymph node metastasis at presentation was found in 55% of this group, whereas 13% of initially lymph node negative patients presented with regional disease at second surgery. Five of 13 diploid primary tumors recurred with aneuploid cell lines. Three of these five patients died, two with regional metastasis. The 5-year survival rate of patients with aneuploid recurrences who were never afflicted with lymph node involvement (41%) was better ($P < 0.05$) than the 5-year survival rate of those with metastasis at presentation or at second surgery (26%).

CONCLUSIONS. The excellent prognosis of patients with diploid primary tumors can be reestablished by treating local recurrences with radical surgery, if the surgery is performed before aneuploid cell lines have emerged. It appears that aneuploid tumor cell lines acquire unique properties that make them capable of invasion and metastasis. *Cancer* **1997;79:2309–13.** © *1997 American Cancer Society.*

KEYWORDS: head and neck carcinoma, oral carcinoma, DNA flow cytometry, aneuploidy, recurrence, metastasis, prognosis.

DNA flow cytometry studies provide substantial evidence that the development of aneuploid tumor cell populations from diploid progenitor cells contribute significantly to the behavior of oral carcinoma.[1,2] With radical surgery, a 5-year survival rate of nearly 90% was achieved for patients with diploid primary squamous cell carcinoma, in contrast to a rate of approximately 30% for patients with aneuploid primary tumors. This suggests that prognosis can be improved by local intervention alone before the development of aneuploid tumor cell lines.[1–3] In fact, the risk of recurrence at the primary site has been reported to range between 46% and 71% for surgically resected aneuploid oral carcinomas, compared with between 4% and 9% for diploid tumors.[4,5] This study involved a homogenous series of patients

TABLE 1
Clinical and Histopathologic Characteristics of 13 Diploid and 80 Aneuploid Primary Oral Carcinomas

Characteristic	Aneuploid	Diploid
Localization		
Mobile tongue	15	3
Base of the tongue	34	4
Floor of the mouth	16	3
Palate	11	2
Buccal mucosa	4	1
T-classification		
T1	10	5
T2	49	6
T3	16	1
T4	5	1
Histologic differentiation		
High	5	0
Moderate	48	11
Poor	27	2

who were treated with surgery of both the primary and secondary tumors with curative intent.The DNA ploidy status of subsequent lesions was compared with that of primary lesions and used to analyze the predictive importance of DNA flow cytometry in patients with locally recurrent oral carcinoma.

MATERIALS AND METHODS

Ninety-three consecutive patients with squamous cell carcinoma of the oral cavity, all of whom underwent surgery of locally recurrent tumor subsequent to radical primary tumor dissection between March 1986 and December 1995, composed the cohort of this prospective study. Only cases in which the second surgery had been undertaken with curative intent were considered. Surgical revisions made earlier than 2 months after primary treatment were excluded. Initial and second intervention were accompanied by a radical or modified neck dissection in cases of suspect lymph nodes, as evidenced by palpation, ultrasound, or computed tomography.

Tumor staging and histopathologic grading was done according to conventional criteria. Only cases in which microscopic findings were available were defined as lymph node positive (N+) without further stratification with respect to site or size of the involved lymph nodes. Patients who did not undergo neck treatment were defined as N0, as were those without microscopic evidence of lymph node involvement. The clinical and histopathologic characteristics of the patients at initial treatment are summarized in Table 1.

DNA flow cytometry was performed in both the primary tumor and the corresponding secondary tumor. Tumor samples were collected by incisional biopsy or were removed postoperatively from the resected tumor. The tissue pieces were immediately homogenized by thorough mincing with surgical scalpels. The nuclei were extracted by incubation in acid pepsin solution containing 0.5 g pepsin dissolved in 100 mL of 0.05 N hydrochloric acid (HCl) at room temperature, with careful stirring for 5 minutes. Remaining tissue fragments were removed with a 50-μm nylon mesh. The nuclei in suspension were spun down (500 $\times$ g, 10 minutes), fixed in 70% ethanol, and stored at -20 °C.

For DNA flow cytometric analysis, the nuclei were pelleted (500 $\times$ g, 10 minutes) and resuspended in 0.2 mL acid pepsin solution at room temperature to dissociate aggregates. After 3 minutes incubation, 4.8 mL of DNA specific staining solution was added, which contained 10 mg ethidium bromide (Serva, Heidelberg, Germany), 4 mg mithramycin (Serva), and 500 mg magnesium chloride ($MgCl_2$) dissolved in 1 liter Tris buffer (pH 7.5). For samples analyzed after 1991, a DNA staining solution containing 0.2 mg 4',6-diamidino-2-phenylindole (DAPI; Serva) and 11.8 g citric acid trisodium salt dihydrate (Serva) dissolved in 100 mL distilled water (pH 8.0) was used. The minimum incubation time at room temperature was 30 minutes for ethidium bromide/mithramycin and 10 minutes for DAPI, respectively.

Flow cytometry was carried out using a PAS II or a PAS III flow cytometer equipped with a high-pressure 100 W mercury lamp (Partec, Münster, Germany). The filter combination used for ethidium bromide/mithramycin was BG-12 for excitation, a dichroic mirror TK-500, and a RG-590 barrier filter. For DAPI, a UG-1 excitation filter, a dichroic mirror TK-420, and a GG-435 barrier filter were used (all filters were purchased from Partec). DNA histograms of at least 10,000 cells were plotted. The diploid cell population was used as an internal reference standard for the identification of aneuploid clones. In cases in which the diploid cell population could not unambiguously be identified, human lymphocytes were added in a control measurement. The DNA index was expressed by the quotient of the respective modal peak values and reflected the discrepancy between the aneuploid DNA content and the normal diploid DNA value (DNA index = 1.0). The coefficient of variation (CV) of the ethidium bromide/mithramycin measurements ranged between 1.1% and 5.4%, with a mean value of 2.4% (standard deviation [SD] = 1.1). For DAPI, the CV ranged between 0.8% and 2.7%, with a mean value of 1.6% (SD = 0.8).

Survival curves were calculated by the Kaplan-Meier product limit method, and a log rank test was

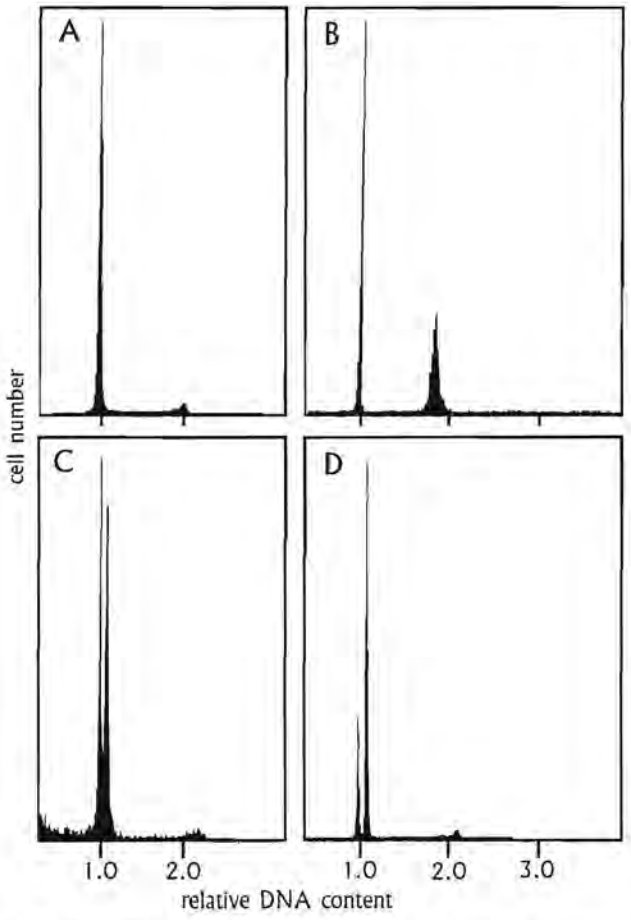

FIGURE 1. DNA histograms of primary oral carcinomas and their subsequent recurrences are shown. A diploid primary tumor (A) is shown, which developed a local recurrence characterized by an aneuploid tumor cell line with a DNA index of 1.85 (B). The lower panels demonstrate a primary tumor (C) and its subsequent local recurrence (D), both of which expressed an aneuploid cell line (DNA index = 1.08) with identical DNA contents.

used to compare the differences between the life-table probabilities of the respective cohorts. The chi-square probability test was used to determine distribution differences between stratified subgroups.

RESULTS

Thirteen primary tumors consisted exclusively of flow cytometrically diploid cells. Aneuploid cells were expressed in 80 carcinomas. There were no significant distribution differences with respect to localization and histologic grade between diploid and aneuploid cases (Table 1). The relative number of diploid lesions decreased with T classification, but this difference was not statistically significant ($P > 0.1$). Five of 13 diploid primary carcinomas recurred with aneuploid cell lines (Fig. 1), whereas 8 secondary tumors consisted of diploid cells. All the recurrences of the 80 aneuploid tumors had aneuploid DNA content. Fifty-nine (74%) of these expressed an identical DNA content aberration (Fig. 1), whereas a change in the DNA profile was observed in 21 cases.

TABLE 2
Lymph Node Involvement and Flow Cytometric DNA Ploidy in Primary Oral Carcinomas and Their Corresponding Recurrences

DNA ploidy		Lymph node status[a]			
		Initial treatment		Second surgery	
Primary tumor	Recurrence	N0	N+	N0	N+
Diploid	Diploid	8	0	8	0
Diploid	Aneuploid	4	1	2	3
Aneuploid	Aneuploid	36	44	26	54

[a] Only cases in which microscopic findings were available were defined as N+. Patients who did not undergo neck treatment and those without microscopic evidence of lymph node involvement were defined as N0.

Six of 8 diploid (75%) and 58 of 85 aneuploid recurrences (68%) had a maximum diameter of 2 cm. Six aneuploid second tumors (7%) were highly differentiated, 45 (53%) were moderately differentiated, and 34 (40%) were poorly differentiated. In the diploid group, 5 (63%) were moderately differentiated and three (37%) were poorly differentiated. These differences were not statistically significant.

Only 1 of 13 patients (8%) with diploid primary tumors had lymph node metastasis on admission (Table 2). No lymph node involvement was found in patients with diploid primary and secondary tumors. In contrast, 44 of 80 patients (55%) with aneuploid primary tumors presented with lymph node metastasis at initial surgery, whereas 10 initially lymph node negative patients (13%) developed regional disease during recurrence growth. Three of the five patients who developed aneuploid recurrences from diploid primary tumors had lymph node metastasis at second surgery. Thus, no patient who presented with diploid recurrent tumor developed regional disease, whereas only 26 of 80 aneuploid cases (33%) never developed lymph node metastasis.

The 5-year overall survival rate of patients who underwent radical surgery of diploid recurrent tumor was 87% (Fig. 2). Only 31% of those with aneuploid recurrence were long term survivors ($P < 0.001$). Three of the 5 patients who developed aneuploid recurrences from diploid primary tumors died of cancer after 7, 15, and 22 months, respectively.

The 5-year survival rate for patients with aneuploid recurrences who were never afflicted with lymph node metastasis (41%) appeared to be more favorable

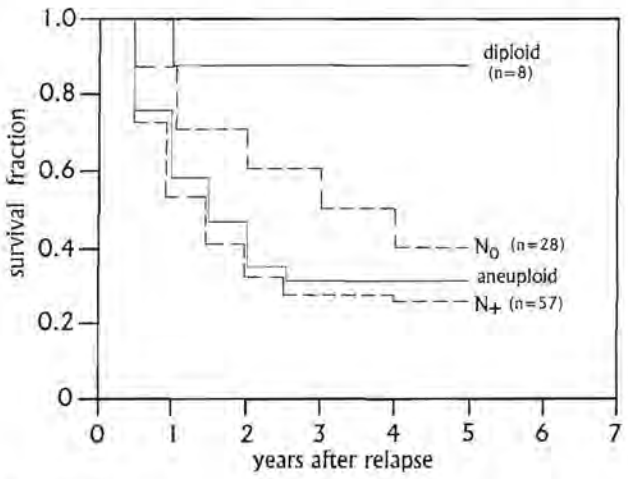

FIGURE 2. Overall survival rates of patients with diploid and aneuploid locally recurrent oral carcinomas are shown. All patients of the diploid group were lymph node negative. The aneuploid lymph node negative group (N0) had no evidence of lymph node involvement at initial surgery or at second surgery. The aneuploid lymph node positive group (N+) had lymph node metastasis either at presentation or at second surgery.

($P < 0.05$) than the rate for patients who had a history with lymph node involvement (26%). In the latter group, the issue of whether regional metastasis had developed at presentation or at second surgery was not statistically significant (data not shown).

DISCUSSION

In a prospective study of 348 patients who had undergone surgical resection of their primary oral squamous cell carcinomas, we found constantly low recurrence rates of less than 10%, independent of tumor stage or histologic grade, in those with flow cytometrically diploid tumors, compared with an increase in the local relapse rate of approximately fivefold among those with aneuploid tumors belonging to respective subgroups.[4] Thus, different stage specific recurrence rates [6,7] appear to reflect decreasing proportions of aneuploid cases with tumor progression[1,8] rather than difficulties in achieving a complete resection of advanced lesions. These findings indicate a pronounced capability of aneuploid tumor cells to evade local control.

The current study provides further evidence of this hypothesis. Although the number of patients with diploid recurrence was low, a favorable 87% 5-year survival rate indicated that the already excellent outcome of radical primary tumor surgery[1-3] could largely be reestablished by a second local intervention, provided that aneuploid tumor cell populations had not emerged during recurrence development. Thus, there was no evidence of a significantly different pattern of host tissue invasion in diploid tumors that recurred compared with those that did not. A restrained propensity of flow cytometrically diploid tumor cells to establish independently growing colonies was also emphasized by a regional metastasis rate of only 9% among patients with diploid primary oral carcinoma, an observation that is in agreement with results from larger series.[2,5,9,10] Beyond that, patients with diploid recurrent tumors never developed metastatic disease.

Aneuploid tumor cells have a pronounced potential of invasive growth and metastatic spread. In support of previous results,[2,11] about half of the aneuploid carcinomas in this study were associated with lymph node metastasis at presentation. Another one-fourth of the aneuploid lymph node negative group presented with involved lymph nodes at second surgery. In addition, two of the five patients with aneuploid recurrences derived from diploid primary tumors developed regional disease. A fivefold increase in the local recurrence rate was observed among the group with aneuploid primary carcinomas as compared with the group with diploid tumors;[4] in accordance with this, aneuploid second tumors were found at a 10-fold frequency than diploid local recurrences. The difference was due to initially diploid primary tumors, which recurred with aneuploid tumor cell lines.

A high degree of concordance between the DNA profiles of primary tumors and those of their corresponding recurrences indicated a definite cytogenetic stability of initially established aneuploid clones, in spite of a great variability of individual DNA contents.[1] This observation has also been made with respect to synchronous lymph node metastases.[11] This was in spite of changes in the DNA contents of approximately one of three cases, and may reflect a selection of aneuploid subclones with secondary cytogenetic alterations that have made advantageous phenotypic adaptations to the therapeutically modified microenvironment. However, the development of second primary tumors, although their incidence is assumed to be less frequent than the proportion of second local tumors with changed DNA contents,[12] cannot be ruled out either. The 5-year survival rate of 31% observed among patients with aneuploid recurrent oral carcinomas implied that only a minority of patients can be cured by local intervention if aneuploid tumor cells have developed. However, there was evidence of improved therapeutic success in patients who were never afflicted with lymph node metastasis. Although the outcome of surgery is excellent in diploid primary as well as in diploid recurrent oral carcinoma, the poor prognosis of those patients whose initially diploid tumors recur

with aneuploid tumor cell populations emphasizes the importance of a complete resection during initial treatment.

In conclusion, DNA flow cytometry is a useful tool for selecting oral carcinoma patients who are at high risk of lymph node involvement and therefore should be considered for neck dissection, and it is also useful for identifying those who have a promising outcome even if treated by local intervention alone. As the prognosis of patients with recurrent head and neck tumors is poor and meaningful prognostic factors are largely lacking,[13] the application of DNA flow cytometry may contribute to an improved outcome in locally recurrent disease.

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Injury to the permanent tooth germ following trauma to the deciduous predecessor

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Report of a Case

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Key words: Injury, tooth germ, trauma

SUMMARY

Trauma to the underlying permanent tooth germ following mechanical trauma to the deciduous predecessor may cause several enamel and dentine abnormalities which may present as local or diffuse defects on the tooth-crown after eruption of the tooth. A case of localized enamel and dentine hypoplasia is presented together with a very unusual pulpo-gingival soft tissue tag on the labial surface of the permanent tooth.

OPSOMMING

Die tandkiem van 'n sekondêre tand mag sodanig deur meganiese trouma aan die oorliggende primêre voorganger beskadig word dat verskeie afwykings van glasuur en dentien later in die sekondêre tand voorkom. Hierdie afwykings mag gelokaliseerd of verspreid op die tandkroon sigbaar wees na erupsie. 'n Geval van gelokaliseerde glasuur- en dentien-hipoplasie word beskryf, tesame met 'n baie ongewone pulpo-gingivale sagte weefsel aanhangsel wat op die labiale oppervlakte van die kroon van die sekondêre tand voorgekom het.

INTRODUCTION

Trauma to the deciduous dentition occurs predominantly in children from 1½ to 2½ years of age. Without differentiating between upper and lower anterior teeth, van Gool (1973) reported that the deciduous central incisors were involved four times more frequently than the lateral incisors.

The germs of the permanent incisors are initially situated lingual to the apices of the deciduous teeth. During their further development the tooth germs are gradually positioned more closely to the resorbing roots of the deciduous teeth. A predominantly axially directed force may be transmitted via the apex of the displaced deciduous tooth to the uncalcified permanent tooth germ. If the ameloblasts are injured, enamel hypoplasia may arise and in the case of concurrent injury to the odontoblasts, would extend into the dentine. The developmental defects in the crown will be dependant on the severity and direction of the traumatic insult and the stage to which the process of amelogenesis has progresses. These developmental injuries may be simple or complex, and extensive or local (Torneck, 1982).

The most common clinical manifestation of injury to the crown of a developing permanent tooth is an area of whitish discoloration caused by an insufficient degree of calcification (Andreasen & Ravn, 1973). It may appear as a small dot or a large area in the enamel that does not change after scaling or prophylaxis. Other possible defects included partial crown or root duplication (Williamson, 1961), interruption or cessation of root completion (Pindborg, 1970), and occurence of an odontoma-like structure (Rodda, 1960). All the above authors mentioned dilaceration and disturbance of enamel formation as possible defects. No reference was made to the disturbance of dentine formation or soft tissue reaction.

The present report is concerned with mechanical trauma as the cause of the observed enamel and dentine defects.

CASE REPORT

A white boy, aged 9, presented with an unusual pulpo-gingival soft tissue tag on the labial aspect of the left maxillary central incisor. A history of trauma to the primary maxillary incisor, occuring at 2 years of age, was given by the mother. The patient's main complaint was sensitivity and discomfort on brushing the tooth.

Clinically, a tag of soft tissue, arising from the labial gingiva extended as far as the middle third of the labial aspect of the tooth where it entered the tooth substance (Fig. 1). The colour of the soft tissue tag was slightly

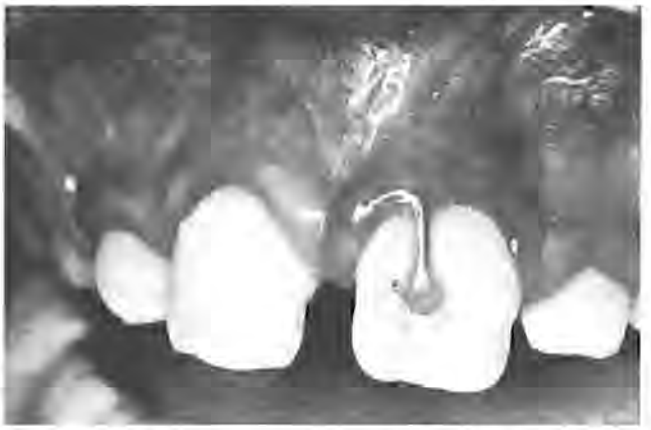

Fig. 1: **Soft tissue tag extending from the gingiva to the middle third of the crown of the left maxillary incisor, entering the pulp chamber in that area.**

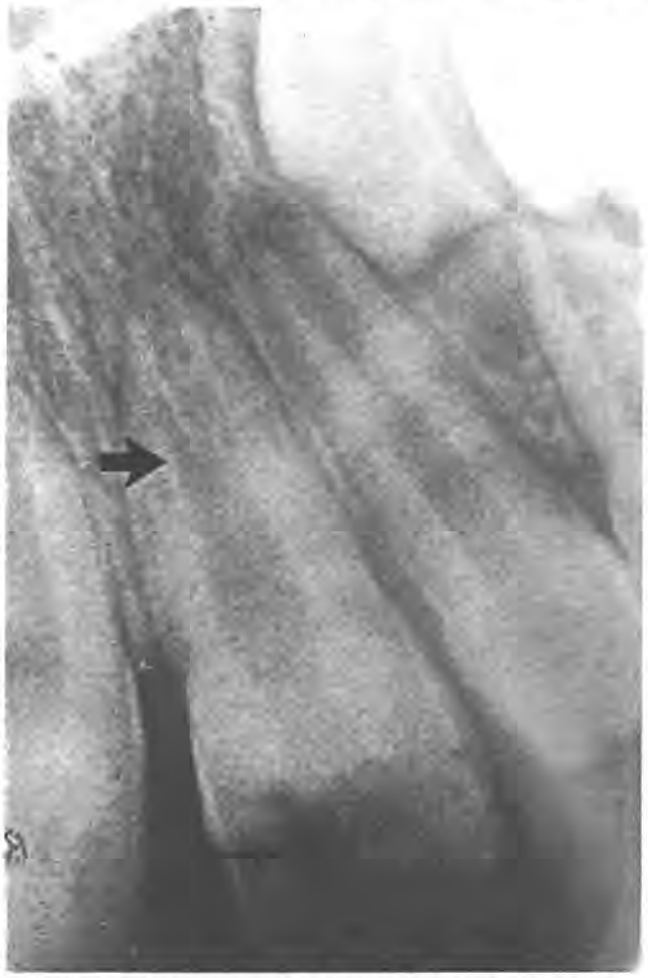

Fig. 2: **Radiolucency, representing the area of penetration of the soft tissue tag (arrow). An accessory canal is also visible in the apical third of the root (broad arrow).**

more red than the gingival soft tissue. Hypoplasia was evident in the enamel surrounding the entrance of the tag into the tooth substance. The tooth-mobility was normal and the tooth reacted positively to electrical sensitivity tests.

Radiographically the point of penetration was visible as a radiolucent area mesial to the pulpal chamber of the tooth. Root formation appeared to be complete. An accessory canal was also evident in the apical third of the root (Fig. 2).

Prior to root canal treatment an access cavity was prepared palatally in the tooth when it was established that the soft tissue tag was continuous with the pulp.

The soft tissue tag was then surgically removed from its pulpal connection and from the gingiva. On histological examination it was established that the soft tissue tag was completely covered by nonkeratinized epithelium and that the central core was made up of connective tissue which contained prominent blood vessels (Fig. 3). On completion of the root treatment, light cured restorative material was used to restore the hypoplastic defect on the labial surface of the tooth.

DISCUSSION

The reported case is unique in that both enamel and dentine defects appeared together with the very unusual soft tissue connection between the pulp and gingiva.

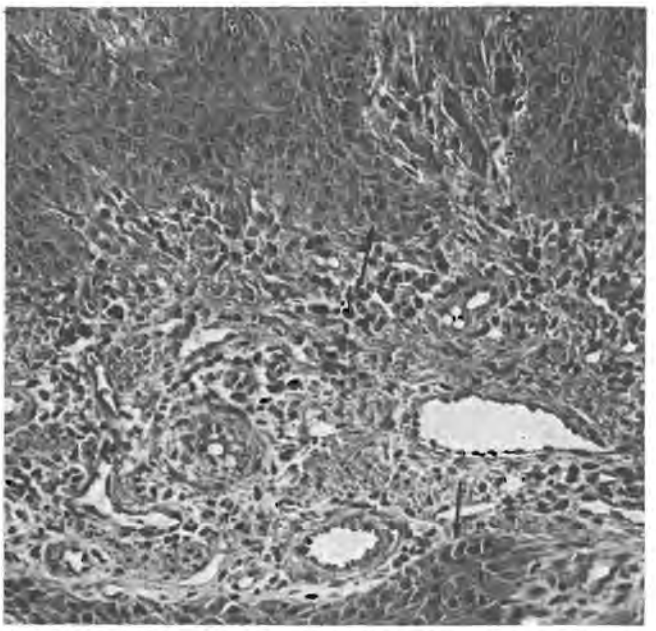

Fig. 3: **Histological appearance of the soft tissue tag. The covering epithelium (arrows) and prominent blood vessels within the central connective tissue of the tag are visible (H&E, x250).**

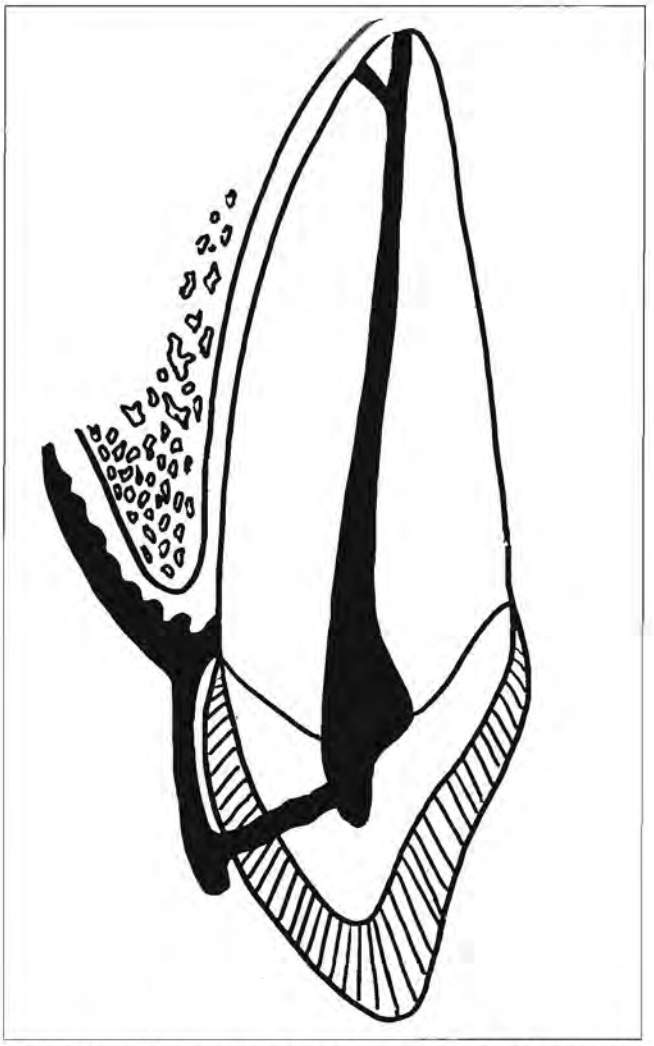

Fig. 4: **Schematic illustration of the soft tissue tag extending between the pulp and gingiva.**

Enamel matrix is laid down in a modified lamellar pattern and calcification normally takes place along the long axis of the tooth, proceeding from the incisal edge cervically. The position of the lesion on the tooth corresponded with the area of the tooth that had been forming and calcifying when the child was 2 years old (Wheeler, 1968). Severe insults either greatly disturb enamel production or produce death of the ameloblasts (Ten Cate, 1985). Odontoblasts are just as sensitive to insult (Linde, 1984). As a result of trauma to both the ameloblasts and odontoblasts that were actively forming enamel-and-dentine matrix at that time, subsequent necrosis and loss of the cells in that particular area of the forming tooth must have taken place. During the healing process, a fibrovascular connection was established between the dental papilla and the surrounding tooth follicle in the same location. In the final stage of the emergence of the tooth into the oral cavity the epithelium of the oral mucous membrance could have proliferated in an apical direction and merged with the epithelial cells of the reduced enamel epithelium in the affected area (Ten Cate, 1985) thus forming the attachment of the tag to the gingiva (Fig. 4).

CONCLUSION

This case clearly illustrates that trauma to the deciduous teeth may cause both enamel and dentine defects of the permanent successors, while abnormalities in the soft tissue configuration may also occur.

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Amelogenesis imperfecta: multiple impactions associated with odontogenic fibromas (WHO) type

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Keywords: Amelogenesis imperfecta; fibroma; odontogenic fibroma

SUMMARY

Three types of amelogenesis imperfecta (AI) are recognised, namely hypoplastic, hypomature and hypocalcified varieties. We report on two cases of hypoplastic AI, the type which occurs most frequently. Both patients presented with multiple impacted permanent teeth. Odontogenic fibromas of the WHO type were found to be associated with the crowns of all the impacted teeth and are considered to have prevented normal eruption. Dentinal dysplasia found only in the furcation area of the multirooted impacted teeth was evident. The macroscopic, microscopic and radiological appearance of the affected teeth, pericoronal lesions and interradicular dentinal dysplasia are described, and the most likely origins of the odontogenic fibromas and calcifications observed, are discussed.

OPSOMMING

Drie tipes amelogenese imperfekta (AI) word aangetref namlik hipoplasties, hipovolwasse en hipogekalsifiseerde tipes. Hierdie artikel beskryf twee gevalle van hipoplastiese AI, die mees algemene tipe. Altwee pasiënte het veelvuldige geïmpakteerde tande gehad. Odontogene fibrome, WGO tipe, is aangrensend tot die geïmpakteerde tandkrone gevind en het moontlik erupsie vertraag. Dentinale displasie is slegs in die furkasiegebied van die geïmpakteerde molaartande gevind. Die makroskopiese, mikroskopiese en radiologiese beelde van die betrokke tande, perikoronale letsels en dentinale displasie word beskryf en die moontlike oorsprong van die odontogene fibrome en kalsifikasies wat waargeneem is, word bespreek.

INTRODUCTION

Amelogenesis imperfecta (AI) is an inherited, congenital defect that primarily affects enamel formation and which is not accompanied by morphologic or metabolic defects in other body systems except abnormal tooth form or eruption (Witkop, 1989). The recent classification of Witkop (1989) describes different types of AI according to the predominant clinical and histological characteristics as well as the mode of Mendelian inheritance. The enamel abnormality can be either hypoplastic, hypomature, hypocalcified or a combination of these with autosomal dominant, autosomal recessive, sex-linked dominant or sex-linked recessive modes of inheritance (Table I). The hypoplastic type is characterised by thin, hard enamel of normal radiographic translucency. This type is the result of insufficient matrix formation with normal mineralisation. Hypomature enamel is a result of a defect in the formation of crystalline apatite in various parts of the enamel rods and sheaths. The enamel is of normal thickness with a mottled appearance, is slightly softer than normal and chips off the dentine. Radiographically it has approximately the same density as dentine. Hypocalcified enamel develops to a normal thickness but is lost soon after eruption. It is the result of defective mineralisation of the formed matrix and radiographically the enamel is less radiodense than dentine (Witkop and Sauk, 1976).

The combined prevalence of all types of AI has been reported to be 1:14 000 in the United States (Witkop and Sauk, 1976); 1:8 000 in Israel (Chosack *et al*, 1979) and 1:4 000 in Sweden (Sundell and Valentin, 1986). The most common type of A1 is the hypoplastic variety with a reported prevalence that varies from 1:8 800 (Chosack *et al*, 1979) to 1:6 700 (Sundell and Valentin, 1986). Impacted teeth are often associated with the smooth hypoplastic type and, less frequently, with the rough hypoplastic type (Witkop and Sauk, 1976).

The purpose of this paper is to report two cases of rough hypoplastic amelogenesis imperfecta associated with impacted teeth and pericoronal odontogenic fibromas of the WHO type.

CASE 1

A 14-year-old girl presented for treatment with the main complaint of delayed eruption of her teeth. The child had no systemic abnormalities. Intraoral examination revealed thin, hard enamel on all the erupted teeth. The enamel surface varied from smooth to rough and had a yellow-white colour. The teeth failed to meet at the interproximal contact points. The patient had 5 sisters of whom 3 had AI with the same enamel appearance. The mother had normal teeth but the father was edentulous. His teeth had been extracted at a young age. This mode of inheritance was suggestive of an autosomal dominant inheritance pattern.

Radiographic examination revealed the normal number of teeth, of which 13 were unerupted, including the 4 developing third molars. Dilated follicles or cyst-like lesions were apparent as well demarcated radiolucencies with sclerotic margins associated with the crowns of the unerupted teeth (Fig 1). No well developed enamel could be seen. The roots of the molar teeth showed gross disfigurement with structures suggestive of pulpal calcifications.

Table 1: **Classification of amelogenesis imperfecta according to Witkop (1989)**

Type I	– Hypoplastic
IA	– hypoplastic, pitted autosomal dominant
IB	– hypoplastic, local autosomal dominant
IC	– hypoplastic, local autosomal recessive
ID	– hypoplastic, smooth autosomal dominant
IE	– hypoplastic, smooth X-linked dominant
IF	– hypoplastic, rough autosomal dominant
IG	– enamel agenesis, autosomal recessive
Type II	– Hypomaturation
IIA	– hypomaturation, pigmented autosomal recessive
IIB	– hypomaturation, X-linked recessive
IID	– snow capped teeth, autosomal dominant
Type III	– Hypocalcified
IIIA	– autosomal dominant
IIIB	– autosomal recessive
Type IV	– Hypomaturation-hypoplastic with taurodontism
IVA	– Hypomaturation-hypoplastic with taurodontism, autosomal dominant
IVB	– Hypoplastic-hypomaturation with taurodontism, autosomal dominant

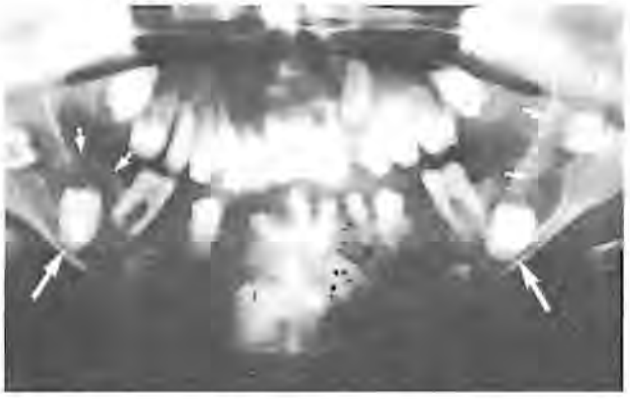

Fig. 1: **Case 1. Pantomograph showing unerupted teeth with pericoronal radiolucencies (small arrows). No evidence of enamel is present and the unerupted molar teeth show gross root disfigurement (large arrows).**

Fig. 2: **Ground section of an unerupted molar tooth with irregular enamel and globular calcifications (bold arrows). Note the straight dentinoenamel junction (fine arrows) × 100.**

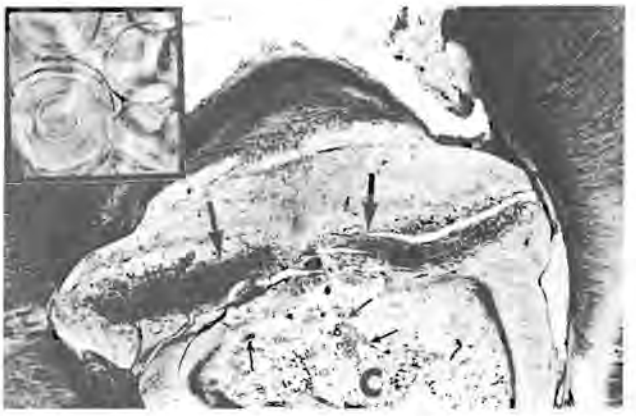

Fig. 3: **Interradicular dentine dysplasia (bold arrows) associated with hypercementosis (c) and globular calcifications (fine arrows). Ground section, unstained × 10. Inset: Calcified globules with an onion-like appearance. Ground section, unstained × 200.**

All the unerupted teeth with the associated pericoronal tissue were removed surgically under general anaesthesia. The bone was found to be normal in texture and no excessive haemorrhage was encountered. Post-operative healing was uneventful.

Light microscopy of ground sections of the molar teeth showed irregular hypoplastic enamel with globular calcifications. The dentinoenamel junction lacked the normal scalloping (Fig 2). The dentine of the crowns and roots showed no abnormalities. An irregular mass of dentine was present in the interradicular area at the level of root bifurcation in all the molar teeth. Hypercementosis, consisting of cellular cementum extending into the interradicular space of the roots, was present.

Calcified globules with an onion-like appearance were present in the cementum in close association with the irregular dentine (Fig 3). False pulpal stones, not associated with the dentinal wall, were observed.

Scanning electron microscopy (SEM) of the outer enamel surface showed irregular globular and linear masses in association with depressions (Fig 4). SEM of the fractured surface confirmed the straight dentinoenamel junction and showed normal dentine. The enamel had voids and loss of structure with a resulting honeycomb appearance throughout its full thickness (Fig 5).

The pericoronal tissue consisted of fibrous tissue that varied in cellularity. No evidence of a cystic lining was found. Odontogenic epithelial cell rests were scattered in the connective tissue. These epithelial cells appeared to be inactive with no peripheral pallisading of ameloblast like cells. Some of the epithelial cells had a vacuolated appearance. Two types of calcifications were present in the fibrous tissue. The most common type consisted of psammomatous lamellar bodies with an eosinophilic centre and a more basophilic peripheral zone. The second type consisted of eosinophilic material with a fibrillar matrix and peripheral tufts resembling Sharpey's fibres. Both types were closely associated with the odontogenic epithelial cell rests (Fig 6). The lesions were considered to be odontogenic fibromas, WHO type.

CASE 2

A 26-year-old black female reported to the hospital, requesting that she be fitted with full upper and lower dentures. The patient was clinically edentulous and had marked vertical enlargement of the entire alveolar ridge in all four quadrants. No abnormalities were found on systemic examination.

Radiological examination confirmed the enlargement of all four quadrants with both maxillary tuberosities markedly overdeveloped. There was evidence of recent tooth extractions in the mandible in the form of healing sockets and 13

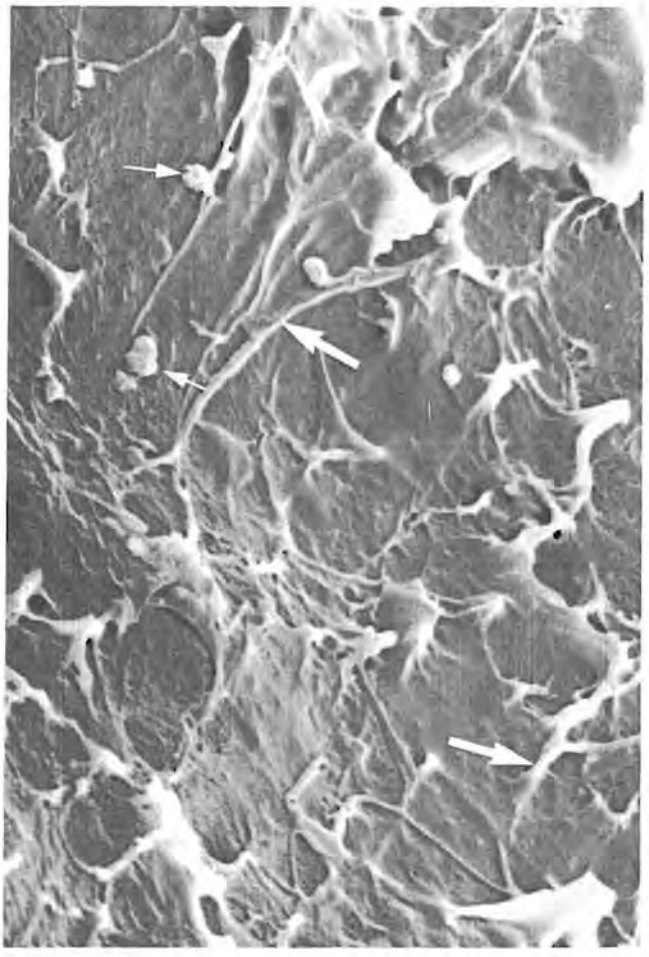

Fig. 4: **The outer enamel surface showing globular (fine arrows) and linear (bold arrows) masses associated with depressions ×2 000.**

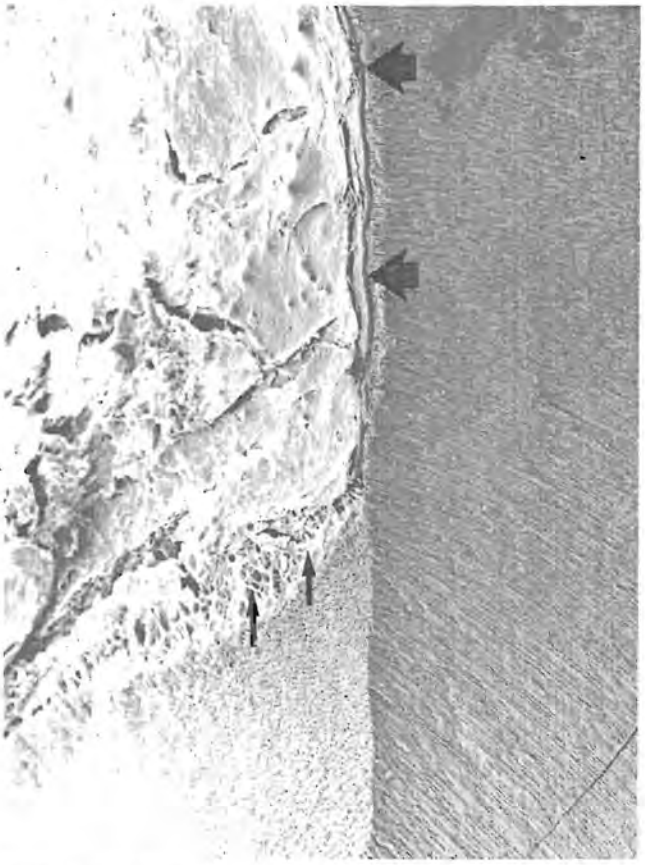

Fig. 5: **SEM of the fractured surface confirmed the straight dentinoenamel junction (bold arrows) and a honeycomb appearance in the enamel (fine arrows) × 72.**

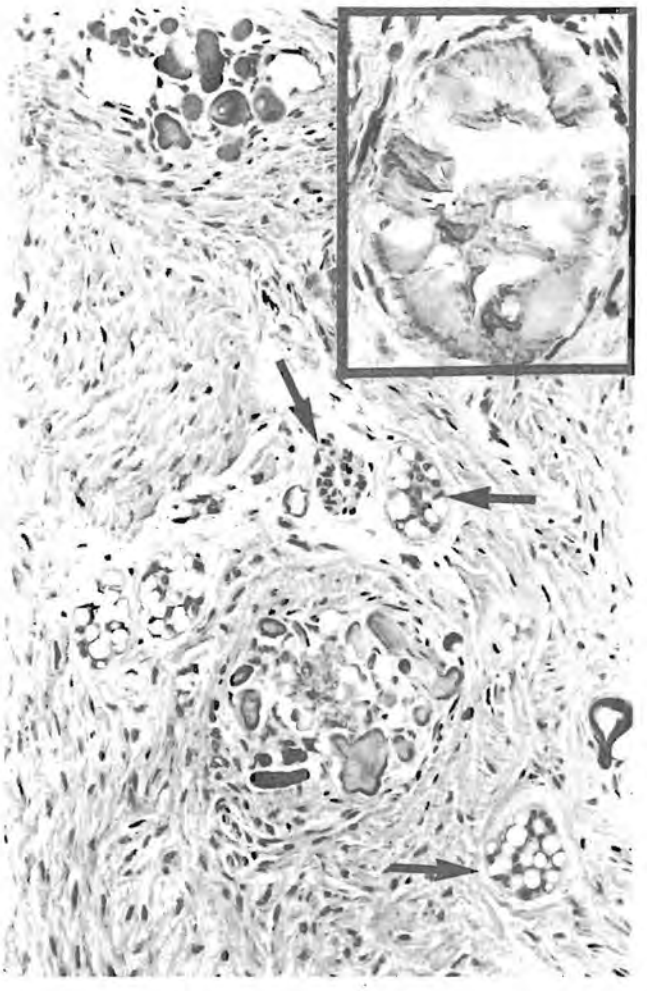

Fig. 6: **Odontogenic epithelium (arrows) associated with psammomatous calcifications in a fibrous stroma. H and E × 100. Inset: Fibrillar calcification with peripheral tufts. H and E ×200.**

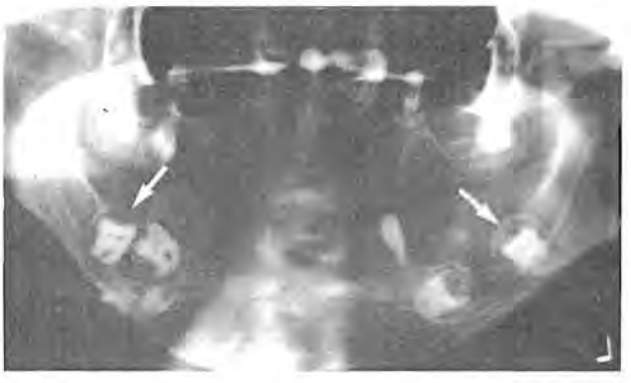

Fig. 7: **Case 2. Pantomograph showing impacted teeth with pericoronal radiolucencies (arrows).**

impacted teeth could be observed within the four quadrants. The enamel of the crowns of all teeth appeared markedly hypoplastic, with abnormally shaped pulp chambers which were smaller than normal. The roots of the teeth were malformed, shorter than normal, with occasional dilaceration. The crowns of the impacted teeth were surrounded by what looked like hyperplastic follicles. The follicular spaces were less radiolucent than normal (Fig 7). Radiological examination of the skeleton showed no abnormalities.

Macroscopic examination of an impacted molar tooth revealed thin, hard enamel with a granular appearance. The enamel could easily be chipped off. Microscopic examina-

tion of a 50 μm ground section showed normal dentine with an almost flat dentinoenamel junction. The enamel was thinner than normal and short curling enamel rods were seen. These were covered by irregular globular calcified masses (Fig 8). These features were consistent with rough hypoplastic amelogenesis imperfecta. The mode of inheritance could not be established. The pericoronal lesions had the same microscopic appearance as in case 1 (Fig 9).

DISCUSSION

Calcifications associated with odontogenic epithelial remnants have been reported in odontodysplasia, impacted dens in dente, congenitally absent teeth in which there is an attempt at tooth formation and several types of AI (Witkop and Sauk, 1976). Odontogenic epithelium was present in 60 cases and calcifications in 54 cases of the 130 cases of opercula of impacted third molars (Cutright, 1976). Gardner and Sapp (1973) described two types of calcifications designated types A and B associated with the soft tissue and a periapical area of an involved tooth of a patient with regional odontodysplasia. The type A and B calcifications are similar in appearance to the two types that were found in our cases. Calcifications are also frequently found in the excised gingivae covering unerupted teeth in patients with AI (Nakata, Kimura and Bixler, 1985; Bab *et al* 1985, Ooya, Nalbandian and Noikura, 1988).

Our radiological differential diagnosis of pericoronal radiolucent lesions was dilated dental follicles, hyperplastic dental follicles, follicular cysts or odontogenic fibromas. Normally some teeth have dilated follicles in the preeruptive phase but according to Shear (1983) it does not signify a cyst unless the pericoronal width is at least 3-4 mm as measured on a radiograph. The hyperplastic follicle presents macroscopically as a solid rather than cystic lesion and no signs of a cyst can be seen microscopically. The histological appearance of hyperplastic follicles and odontogenic fibromas are similar. According to Gardner (1980) the distinction is based on the size and location of the lesion. The follicles are invariably associated with the crowns of unerupted teeth whereas it is not necessarily true for odontogenic fibromas. Sandler *et al* (1988) reported a case of a 16-year-old boy with 13 unerupted teeth, each one associated with hyperplastic pericoronal tissue that had histological features suggestive of the WHO type of odontogenic fibroma. The erupted as well as removed impacted teeth in their case were macroscopically normal. Gardner (1980) considers the WHO type of odontogenic fibroma to be a fibroblastic neoplasm. The pericoronal location of the tumours in our two patients suggested a follicular origin. The association of this fibroma-like tissue with impacted and unerupted teeth in AI suggested a hamartomatous lesion rather than a neoplasm. It is possible that the WHO type of odontogenic fibromas associated with impacted teeth, as in our cases, have a different histogenesis than the tumours described by Doyle, Lamster and Baden (1985), as none of their 6 cases was in a pericoronal location. Dunlap and Barker (1984) consider the central odontogenic fibroma of the WHO type to be the morphologic and histogenetic counterpart of the peripheral odontogenic fibroma. The authors postulated an ectomesenchymal-epithelial interaction in the histogenesis of this tumour. The close association of calcifications with odontogenic epithelium in both our cases supported their theory.

Fig. 8: **Thin abnormal enamel covered by irregular globular calcifications (bold arrows). Unstained × 100.**

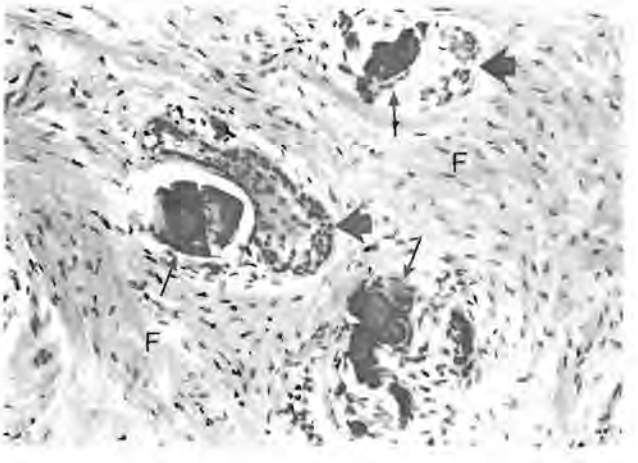

Fig. 9: **Odontogenic epithelium (bold arrows) closely associated with psammomatous calcifications (fine arrows) in a cellular fibrous tissue (F). H and E × 150.**

AI associated with interradicular dentinal dysplasia has been reported by Nakata *et al* (1985). They suggested 3 possible mechanisms for the presence of dysplastic dentine: resorption of the interradicular area followed by secondary calcification; gene influence on matrix formation in this area; and secondary calcification for some unknown reason. No sign of resorption of roots or crowns of the impacted teeth in our cases was found. No abnormalities in the roots of single rooted teeth were seen on radiological and microscopic examination. This is an indication that the underlying cause is likely to be associated with the process of root branching. A genetic influence responsible for the abnormal interradicular dentine is unlikely since the abnormal

dentine present in our first patient did not occur in her 3 sisters who had AI. They had no other dental abnormalities or impacted teeth. The erupted molar teeth of the first patient showed no radiological evidence of root abnormalities. The association between the interradicular abnormalities and impactions was unclear. No abnormalities apart from AI could be seen on the impacted single rooted teeth. It is unlikely that a disturbance affecting the eruption occurred first and then caused a secondary abnormality of the interradicular area of the impacted teeth as suggested by Nakata *et al* (1985). It has been shown that eruption proceeds normally in the absence of root formation (Cahill and Marks, 1980). Both erupted and impacted molars in the AI patient reported by Nakata *et al* had interradicular dentinal dysplasia. The odontogenic fibromas WHO type associated with the pericoronal areas were probably the main reason for the impaction of teeth in both our cases. The suggested follicular origin of the odontogenic fibromas as a hamartomatous growth under the influence of the follicular epithelium supported this statement as Cahill and Marks (1980) have shown that a dental follicle is required for the eruption of a tooth.

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REGIONAL ODONTODYSPLASIA ASSOCIATED WITH SOFT TISSUE SWELLING

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Odontodysplasia is an uncommon developmental anomally with an unknown cause affecting both dentin and enamel of a group of adjacent teeth. The maxilla is involved twice as often as the mandible[1]. The condition is more common in the anterior than in the posterior regions and affects women more frequently than men (ratio 1.4:1)[1].

This condition has been reported under a variety of names, such as odontogenic dysplasia[2], localised arrested tooth development[3] and ghost teeth[4]. Regional odontodysplasia however, has become the accepted terminology, because the condition tends to affect several adjacent teeth within a particular segment or region of the jaw. Radiographically, the affected teeth demonstrate a ghost-like appearance with little demarcation between enamel and dentin, wide pulpal chambers and open apices. Clinically, the teeth appear discoloured, hypocalcified and hypoplastic. Delayed eruption of affected teeth is common.

The purpose of this article is to report a case of regional odontodysplasia associated with a soft tissue tumour.

CASE REPORT

A healthy 8-year-old female presented at the clinic complaining of a painless tumour in the anterior mandible. She first noticed the lesion 4 months previously and it progressively increased in size over this period.

Examination revealed a round, bony hard tumour of 2 cm in size on the alveolus between the 41 and first 84 (Fig. 1). The tip of a tooth had erupted through the posterior aspect of the tumour. The mucosa was slightly erythematous in one area and pigmented in another, without any ulceration being present.

Radiographs revealed a mixed dentition, with the developing canines and premolar teeth, and 31 and 32 appearing normal. The 84, 85 and 46 were in their normal positions. The tooth of which the tip alone was visible was found to be the 83. The 41 and 42 were totally embedded in the tumour and of decreased radiodensity while their roots were hypoplastic (Fig. 2). The tumour itself was well circumscribed and had a ground-glass appearance.

Under general anaesthesia the mucosa was relfected from the lingual and buccal aspects of the tumour. It shelled out easily from a bony cavity in the alveolus, with the malformed 41 and 42 completely enclosed. The wound was sutured primarily and healing was un eventful.

The surgical specimen consisted of 2 teeth embedded in a firm fibrous tumour. The teeth showed variable degrees of surface hypoplasia and were decalcified for histological examination.

Microscopic examination of the teeth and associated soft tissue showed hypoplastic dentin with a prominent predentine layer and numerous interglobular masses. The pulpal horns were high and pulp stones were present. The reduced enamel epithelium around the unerupted teeth showed numerous calcifications (Fig. 3). The adjacent tumour consisted of cellular fibrous tissue with numerous islands of small round amorphous calcifications distributed throughout the fibrous tissue. Odontogenic epithelium islands were noted in areas, the majority however, were associated with the calcifications. this fibrous tissue mass was well demarcated from the cortical and medullary bone. The diagnosis of regional odontodysplasia was confirmed.

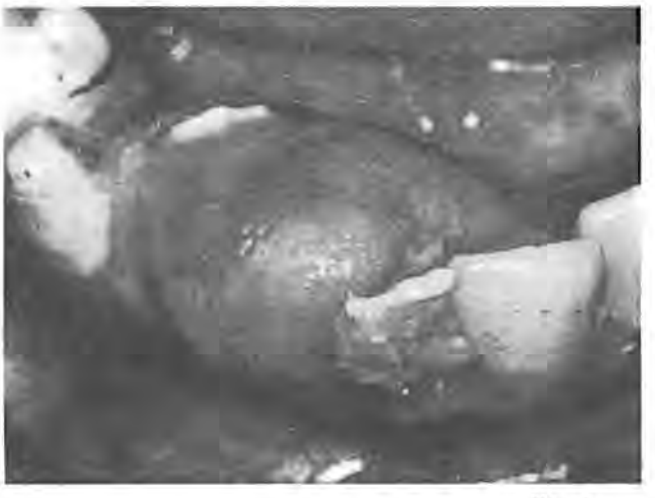

Fig. 1

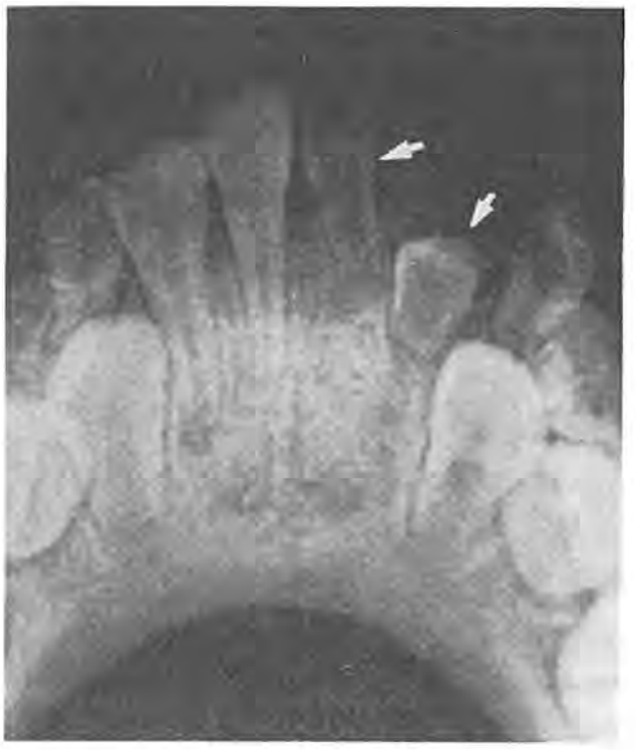

Fig. 2

DISCUSSION

This case is unusual in that the patient complained about a soft tissue tumour and was unaware about the associated "dental problem". Regional odontodysplasia has been reported in association with ipsilateral hypoplasia of the face[5], epidermal nevus syndrome[6] and hydrocephalus[7]. In a recent review of the world literature, 109 cases of regional odontodysplasia were described and it was found that none were associated with a soft tissue tumour[1]. Neupert & Wright however, later described a case of regional odontodysplasia in the maxilla associated with a soft tissue swelling with similar histologic features as the present case[8].

The majority of cases reported in the literature were treated by extraction of the affected teeth. These teeth are frequently painful and associated with abcess formation due to the inability of the aberrant enamel and dentine to resist bacterial invasion. Crawford and Aldred are of the opinion that noninfected affected teeth be saved wherever possible[1]. However, facial cellulitis appear to be a complication if these teeth are retained[8].

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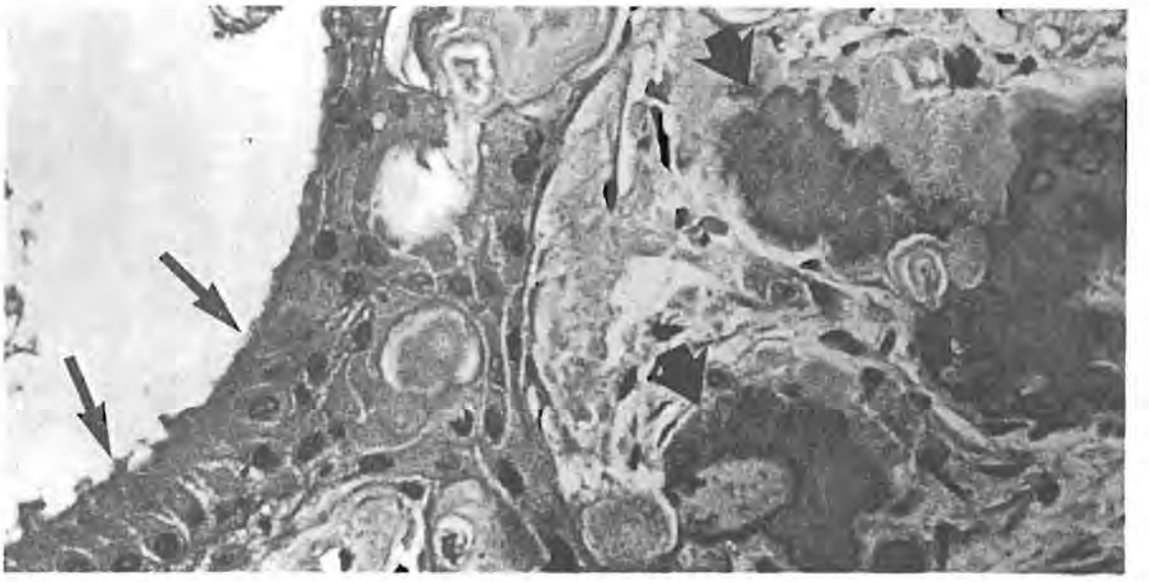

Fig. 3

The use of tricure glass ionomer cement as an apical sealant after apicoectomy

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Keywords: apicoectomy; glass ionomer cement; sealing ability.

SUMMARY

The adaptation and sealing ability of a tricure glass ionomer material (Vitremer), used in a retrograde cavity was assessed and compared with amalgam. Fifty single-rooted, extracted teeth were prepared and filled endodontically. All teeth underwent root resection and retrograde cavities were prepared. The teeth were divided into two groups. One group of 10 teeth received a layer of varnish and an amalgam filling in a retrograde cavity, while the other group of 40 teeth received a layer of primer and a glass ionomer filling. All the teeth were placed in an aqueous solution of Procion Brilliant Blue for 7 days whereafter ground sections were prepared. Micro leakage was determined according to the extent of dye penetration using an image analysis system. The results showed that significantly less dye penetration was observed in teeth filled with the glass ionomer cement than in those with the amalgam.

OPSOMMING

Die adaptasie en seëlvermoë van 'n drieledige-kuuraksie glasionomeer materiaal (Vitremer) wat in 'n retrograde kawiteit gebruik is, is ondersoek en met amalgaam vergelyk. Vyftig enkelwortel verwyderde tande is endodonties gevul en in twee groepe verdeel. Een groep, bestaande uit 10 tande, het 'n laag vernis en amalgaamvulsel in 'n retrograde kawiteit ontvang, terwyl die groep van 40 tande een laag "primer" en glasionomeer vulsel ontvang het. Al die tande is in 'n oplossing van Procion Brilliant Blue in water vir 7 dae geplaas waarna slypsels voorberei is. Mikrolekkasie is bepaal na gelang van die mate van kleurstof-penetrasie soos vasgestel deur 'n beeld analise sisteem. Die resultate het getoon dat kleurstof-penetrasie betekenisvol minder was met die glasionomer sement in vergelyking met amalgaam.

INTRODUCTION

An ideal material for retrograde apical seal has yet to be found. Studies have shown that amalgam does not provide an effective apical seal (Abdal, Retief and Jamison, 1982; Stabholtz *et al.*, 1985; Szeremeta-Browar, van Cura and Zaki, 1985). Further investigation into seeking an alternative retrograde root filling material would improve endodontic treatment. Vitremer light-curing glass ionomer cement may prove to be an effective sealant that can be used in the moist environment found in root resection. This material exhibits almost no water sensitivity after 20 seconds of irradiation and is the only restorative material that forms a strong bond to dentine in a moist environment (Katsuyama, Tatsuya & Benji, 1993). The material has been shown to be biocompatible by both cell toxicity and biological testing (Zetterquist, Anneroth and Nordenram, 1987)

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Previous studies (Chong, Pitt Ford and Watson, 1991; 1993) using a light-cured glass ionomer cement as a retrograde filling material showed that good adaptation was achieved to one cavity wall, but gaps were observed on the opposing wall.

We postulate that it is important to prepare a retrograde cavity when using glass ionomer material because at present it is still questionable whether the marginal seal will be maintained satisfactorily over a long period of time. This uncertainty is due to dimensional changes, such as from shrinkage at the time of setting, dissolution and exposure to various harmful factors such as external forces (Katsuyama *et al.*, 1993).

The purpose of this study was to assess the sealing ability of a tricure glass ionomer material (Vitremer) when used as a retrograde root cavity filling material.

MATERIALS AND METHODS

Fifty extracted single rooted human teeth consisting of incisors, canines and premolars were collected from the Department of Maxillofacial and

Oral Surgery at Medunsa and stored in de-ionized water at 37°C. An access cavity was prepared through the crown into the pulp space of each tooth. The root canals of all the teeth were prepared and filled with laterally condensed Gutta-Percha and Roth Root Canal Sealer (Roth International, Chicago, IL). The access cavity was sealed with IRM temporary filling material (L.D. Chaulk Co, Milford, DE). The external surfaces were coated with a layer of nail varnish. The teeth were then stored in de-ionized water at 37°C for 7 days. All teeth were subsequently resected with an ISO size 009 plain cut tungsten carbide bur (H23L) (Komet, Brassler, Lengo, Germany) at high speed (300 000rpm) with water coolant. Approximately 3mm of the root apex was removed and the root surface was bevelled labially at approximately 45° to the long axis of the root. All the resected root surfaces were of approximately similar dimension. The teeth were then divided into two groups:

Group 1: 10 teeth for conventional amalgam fillings

Group 2: 40 teeth for tricure glass ionomer fillings

In both groups a single surface retrograde cavity was prepared in each tooth with an ISO size 016 round diamond bur (801)(Komet) at high speed with water coolant to a depth of between 2 and 3mm, measured from the labial margin of the cavity. The cavity was circular in cross-section, with a diameter of approximately 1,6mm. All the teeth were rinsed with water and dried with air from a three-in-one syringe before placement of the test materials.

Group 1:

The retrograde preparations received one application of cavity varnish (Copalite; H.J. Bosworth Co, Skokie, IL USA) applied with a paper point and blown dry to evaporate solvent, followed by a retro filling of high copper dispersed phase amalgam alloy (Dispersalloy; Johnson & Johnson, East Windsor, N.J. USA) mixed according to the manufacturer's instructions. The amalgam was carried to the retrograde cavities with an amalgam carrier and the condensation was done manually.

Group 2:

The primer was applied and air dried immediately. The Vitremer tricure glass ionomer material (3M Dental Products, St Paul, Minnesota, USA, Batch no: 19930116) was mixed and placed in bulk, according to the manufacturer's instructions. The glass ionomer cement was cured for 10 seconds using a light-curing unit (Chaulk Max; Caulk, Dentsply, Milford, DE, USA).

After material placement, all teeth from both groups were stored in an aqueous solution of Procion Brilliant Crestal Blue (Merck; Fedlife Park, Midrand, RSA) at 37°C for 7 days. The pH of the solution was 7,59. Vitremer tricure glass ionomer material sets by means of exposure to visible light. It also has two self-curing mechanisms to provide a relatively rapid set where light does not penetrate and thus allows for bulk placement. It also shows much less contraction shrinkage than the previous light-cured glass ionomers (3M Dental Products, St. Paul, Minnesota, USA; Vitremer Technical Product Profile, 1992, 0.8)

After the storage period of 7 days the teeth from both groups were embedded in resin and sectioned longitudinally in a buccal-lingual plane with a low speed diamond saw (Low Speed Isomet Saw; Beuhler, Lake Bluff, IL, USA). A one millimetre section was cut off each tooth and then ground on carborundum paper to approximately 30µm thickness. The sections were subsequently mounted onto microscope glass slides with mounting media. The amount of leakage was scored according to the extent of dye penetration along the cavity walls. The extent of dye penetration was measured using an image analysis system (FIPS; Wirsam, Auckland Park, RSA) connected to a Nikon Opthiphot microscope (IMP; Pretoria, RSA). For every tooth, two measurements were obtained from both sides of the retrograde cavity walls and expressed in micrometer penetration. Dye penetration beyond the prepared cavity was not measured. An average measurement was then calculated for each tooth.

The results from the dye leakage study were analysed using the Student's t-test for uncorrelated data.

RESULTS

Three of the 40 teeth filled with Vitremer and one tooth filled with amalgam could not be used for dye penetration analysis, as they fractured during the cutting and grinding process. The dye penetration was present as a well defined blue line on the resected root surface and along the cavity walls. The mean distance of dye penetration in the group with amalgam fillings was 1774µm ± 947µm. These measurements ranged from 816µm to 3137µm (Fig. 1). The mean distance of dye penetration in the group with tricure glass ionomer fillings was 83,8µm ±185µm. The measurements ranged from 14,9µm to 823,5µm (Figs. 2 and 3). The mean difference of dye penetration between these two groups was statistically highly significant ($p<0,001$).

DISCUSSION

Bacterial invasion of the pulp space through dentinal tubules have been described in previous studies (Hoshino *et al.*, 1992; Kiryu, Hoshino and Iwaku, 1994). The development and maintenance of a hermetic seal is considered to be a major prerequisite for success in root canal treatment. The

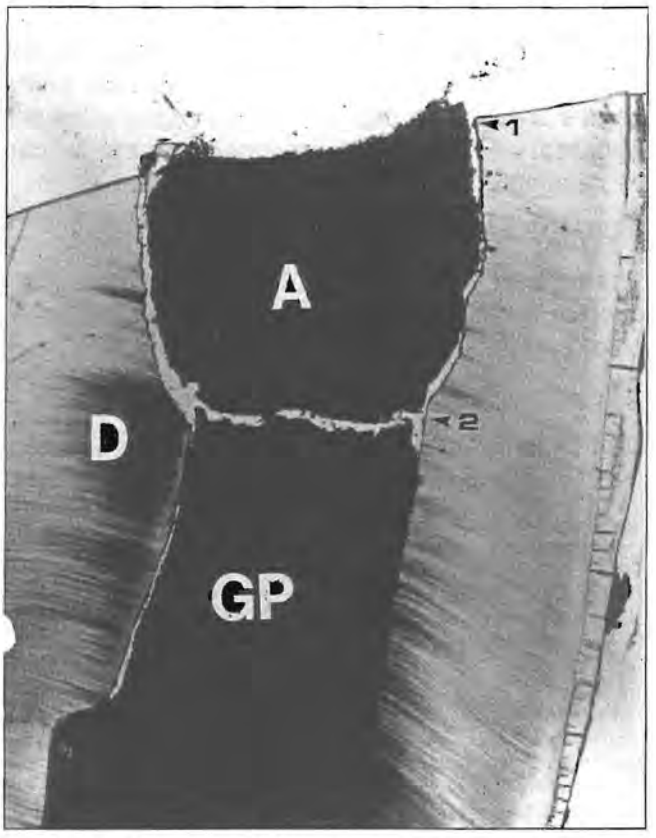

Fig 1: **Photomicrograph of a ground section showing the amalgam retrograde filling (A) and Gutta Percha (GP). Dye leakage was observed extending the full length of the amalgam filling (1 to 2). Dentine = D. (Original magnification: x5).**

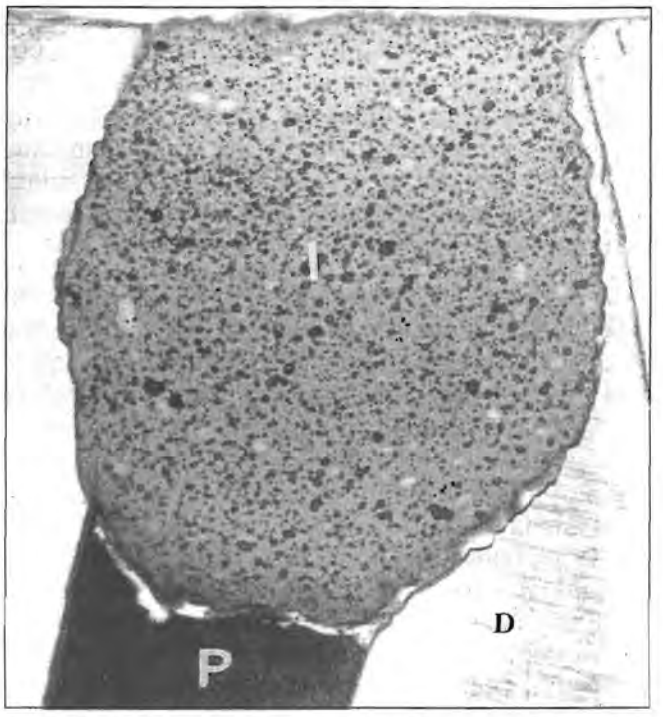

Fig 2: **Photomicrograph of a ground section showing the glass ionomer retrogade filling (I) and Gutta Percha (P). Dentine = D. (Original magnification x10).**

evaluation of the quality of the root canal filling using leakage tests is therefore still relevant (Wu and Wesselink, 1993). Glass ionomer materials, including cements, are technique sensitive (Bowen and Marjenhoff, 1992). The necessity to have a clean, dry dentinal surface on which to place these materials, remains the major problem to the clinician. Special instruments are available to facilitate debridement and obturation of the root canal space from a retrograde direction (Flath and Hicks, 1987), whilst root end isolation techniques for retrograde fillings in order to obtain and maintain a sterile, dry environment, have been described (Guerra, 1992).

Contrary to the findings of Chong *et al.*, (1993) using a light-cured glass ionomer material, the tricure glass ionomer material in this study was observed to be well adapted to both the cavity walls in all specimens. This finding is in line with the statement of the manufacturers that this material undergoes less polymerization contraction than previous glass ionomers and can be placed in bulk. The suggestion by Watson (1990) that a light-cured glass ionomer material should only be used in thin layers, is therefore not applicable to this specific material according to our results.

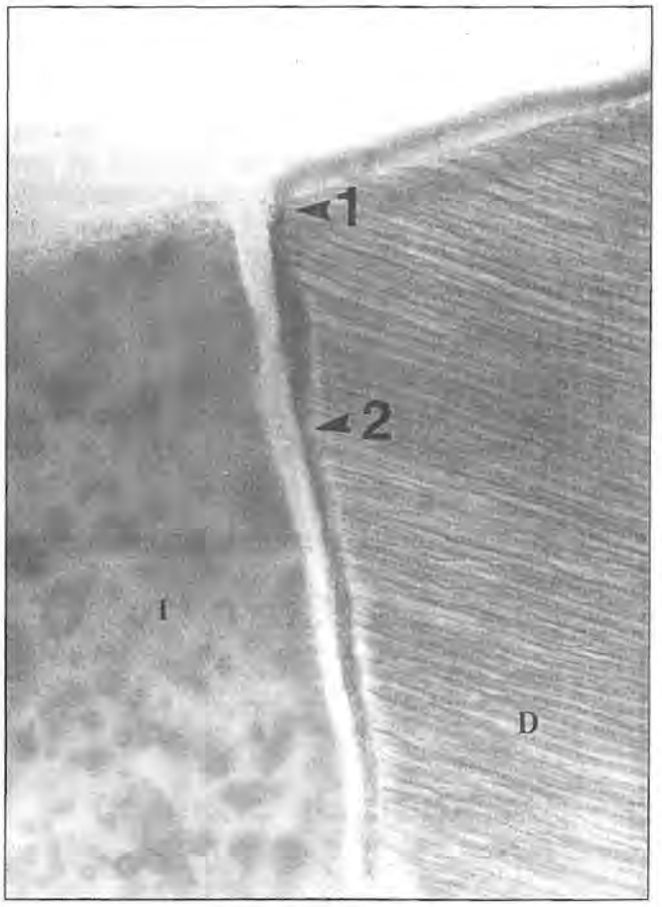

Fig 3: **Photomicrograph of a ground section showing the extent of dye penetration (from 1 to 2) with a glass ionomer filling (I). Dentine = D. (Original magnification x100).**

In this study the glass ionomer material was placed in bulk and cured for only 10 seconds (i.e., 50 per cent of the time recommended by the manufacturers) before being immersed in the dye. This was done in an attempt to make the study clinically more relevant since it is often difficult to isolate the apex of a tooth and keep it dry for any length of time.

Measurement of dye penetration by using an image analysing system linked to a light microscope is the method of choice. It measures the actual dye penetration in scientific units and is therefore reproducible and allows for meaningful statistical analysis. The dye penetration results in this study indicated that a 3mm retrograde amalgam filling with one layer of varnish did not give a predictable seal, implying that communication between the periapical area and the pulp chamber was therefore still possible. This is demonstrated by the mean depth of dye penetration around amalgam (1774µm) which means that in most cases the dye leakage reached the base of the cavity (2000-3000µm deep). The results also demonstrated that it was possible to seal the canal orifice of a retrograde preparation to prevent the dye from reaching the pulp chamber by the bulk placement of a tricure glass ionomer material *in vitro*. This is demonstrated by the mean depth of dye penetration around Vitremer (185µm) which indicates that dye penetration never extended to the cavity floor. *In vivo* studies are necessary to determine the clinical relevance of these observations. The changing of formulae, as well as the addition of new chemicals to dental products, can serve as potential irritants and sensitizing agents. It is therefore important to do biological testing before clinical application of any new material.

CONCLUSION

A 3mm retrograde amalgam filling with one layer of varnish did not give predictable seal *in vitro*. Communication between the pulp chamber and the periapical tissue may therefore still be possible immediately post operatively. Bulk placement of a 3mm tricure glass ionomer material filling gave a predictable seal *in vitro*. *In vivo* studies are necessary before this glass ionomer material can be recommended for routine clinical use.

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SHORT COMMUNICATION

AMINO ACID COMPOSITION OF DENTINE IN PERMANENT HUMAN TEETH

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(Accepted 20 August 1991)

Summary—Dentine of permanent mandibular incisors from nine individuals was hydrolysed and the amino acid composition determined by ion-exchange chromatography against a standard calibrant of 41 amino acids. Nineteen amino acids were detected, including small quantities of 1-methylhistidine and asparagine, two amino acids whose existence had apparently not been recorded before in human dentine. The total content of hydroxylysine plus lysine varied between 2.6 and 3.3 residues per 100 (SD, 0.74) in different teeth, which therefore did not support previous studies that had proposed a constant total value. This and other quantifiable differences between present and previous findings may be the result of the different methods and the influence of dietary and other regional factors on dentinogenesis.

Key words: human dentine, amino acids.

Dentine is the major component of teeth, responsible for most of the weight, volume and overall shape (Butler, Munksgaard and Richardson, 1979). Ninety per cent of the organic matrix of human dentine consists of collagenous proteins (Jones and Leaver, 1974) and the remaining 10% is made up of non-collagenous proteins, proteoglycans, glycoproteins and lipids (Avery, 1987). Various analytical methods, some of which are historic, have been used for determination of amino acids in dentine, namely: microbiological assay (Hess, Lee and Neidig, 1952), quantitative paper chromatography (Battistone and Burnett, 1956) and ion-exchange chromatography (Eastoe, 1963).

Our objective now was to determine the amino acid composition of human dentinal matrix with a modern technique and to compare it with previously published data.

Permanent mandibular incisors were extracted from nine bodies of known age and sex in the Forensic Medicine mortuary of Ga-Rankuwa Hospital, situated 32 km north of Pretoria. Before processing, the crowns and cementum were removed with a dental bur, and the pulp with an endodontic file. Pieces of radicular dentine of approx. 0.20 g were washed, dried and hydrolysed in 6 M hydrochloric acid (HCl) for 24 h at 110°C. The hydrolysates were neutralized with neutralizer (Spitz, 1973) and citrate buffers in the ratio 1:2:2, filtered (Millex-GS 0.22 μm) and then diluted further 1:1 with the citrate buffer. Calibrants containing 41 amino acids were prepared and diluted as above. The amino acids of dentine and the calibrants were separated in duplicate by ion exchange on a Beckman 6300 amino acid analyser which incorporates a 25-cm lithium column and a four-buffer system. Chromatograms thus obtained were integrated and quantitated with a Hewlett-Packard 3390A integrator and the results expressed as a per cent residues detected. The results were tabled as the average of the total number of residues per 100 and the SD for each amino acid was calculated.

Nineteen amino acids were detected (Table 1). Asparagine and 1-methylhistidine, which have not previously been identified in human dentine, were present in small quantities. Asparagine was present in all our hydrolysates. In a serial study of hydrolysis, asparagine was detected only after 16 h and remained present in all acid hydrolysates for 24 h. In hydrolysates stored at 4°C, asparagine could be detected over as long as 12 months. No explanation for this phenomenon could be found. Acid hydrolysis of a pure mixture of aspartic acid, asparagine, glutamic acid and glutamine showed complete hydrolysis of asparagine and glutamine within 30 min. The concentration of aspartic acid and glutamic acid increased and high levels were detected under these conditions. It is suggested that asparagine in dentine may be 'protected' against complete hydrolysis. Furthermore, our monitoring system may have been more sensitive and with improved resolution as larger quantities of aspartic acid, glutamic acid, arginine, leucine, iso-leucine and valine were found than previously reported (Table 1).

Linde (1984) reported that the total content of hydroxylysine plus lysine in dentine is constant at 3.5 residues per 100. In our study, we found a variation between 2.6 and 3.3 residues per 100 (SD, 0.74), supporting Eastoe's (1963) finding of a variation in the total content of hydroxylysine and lysine over a

Table 1. Comparison of our findings (average of nine cases, with SD) with those of previous investigations (expressed as residues per 100)

Human dentine amino acids	Hess *et al.* (1952)	Battistone and Burnett (1956)	Eastoe (1963)	Linde (1984)	Present study (1992)	
					$\bar{X}$	SD
Aspartic acid	4.4	5.4	5.5	4.5	5.9	0.06
Hydroxyproline	10.3	11.6	10.1	9.6	10.4	0.24
Threonine	2.7	2.0	1.9	1.8	2.1	0.11
Serine	3.4	3.0	3.8	4.1	4.0	0.28
Asparagine	—	—	—	—	0.3	0.08
Glutamic acid	7.4	7.6	7.3	7.2	8.8	0.15
Proline	14.5	9.7	11.5	11.9	11.8	0.28
Glycine	30.9	31.3	31.9	33.4	30.1	0.30
Alanine	9.8	11.2	11.2	10.2	8.6	0.31
Valine	2.6	2.5	2.5	2.3	3.0	0.15
Methionine	0.35	0.46	0.52	0.7	0.5	0.05
Iso-leucine	1.0	*	1.0	1.0	1.3	0.00
Leucine	2.8	*	2.6	2.4	3.0	0.04
Phenylalanine	1.2	*	1.4	1.4	1.5	0.05
Hydroxylysine	0.64	0.71	0.84	1.5	1.1	0.11
Lysine	2.4	2.2	2.3	2.0	2.1	0.17
1-Methylhistidine	—	—	—	—	0.3	0.07
Histidine	0.54	0.43	0.53	0.4	0.3	0.19
Arginine	4.4	5.0	4.7	5.2	5.6	0.17

*Total of leucine, iso-leucine and phenylalanine is 6.4.

comparatively narrow range (2.91–3.35 residues per 100). The presence of two amino acids that have not hitherto been reported in dentine as well as other differences between our study and earlier ones may, amongst other factors, be the result of different methods. The more efficient buffer system and the modern lithium columns that we used facilitate the separation of isomers and increase the resolution of the various amino acids. The reported differences in the amino acid content of human dentine may also reflect dietary and other regional factors that may influence the formation of dental hard tissues.

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9 The Effect of Modern Dentine Bonding Systems on Human Dentine.
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Most modern dentine bonding systems contain primers or cleansers which are used to remove or alter the smear layer or dentine before resin application. The purpose of this study was to assess the effect of four modern dentine bonding systems (DBS), three with primers/cleansers and one without, on the appearance of human dentine.

Denthesive Bond (D, Kulzer), Pertac Universal Bond (P, E.S.P.E.), Prisma Universal Bond 3 (PUB3, Dentsply) and Syntac (S, Vivadent) were included in the study. The crowns of 80 sound, human, molar teeth were imbedded in rings, leaving the occlusal surfaces projecting. These surfaces where ground wet on 220 grit SiC paper to expose superficial dentine. Fifteen dentine surfaces were then treated with each of the four DBS. Cylinders of matching composites were thereafter bonded to these surfaces, using a rubber split mould. After 24 hours storage in water the specimens were stressed to failure using a shear load in an Instron. Fracture sites were evaluated in a SEM. Five abraded dentine surfaces were treated with the respective primer/cleanser of each of 3 DBS. Dentine treated with the D and S cleanser/primer, and debonded specimens of D and S, displayed open dentinal tubules. Subsequent application of an adhesive closed almost all the tubules. Pieces of fractured dentine were attached to some composite stubs, showing resin tags clearly penetrating deep into, and even through the dentine fragments. It can be concluded that only 2 of the 4 DBS tested opened the dentinal tubuli.

10 Evaluation of the Effectiveness of an Oral Health Preventative Programme. S.Dhansay*, R. Lalloo, A. Bawa, M.H. Moola. University of the Western Cape

This study was designed to measure the effectiveness of an oral health preventative programme based in a school population in the Cape Peninsula. The objective of the study was to measure the difference between schoolchildren who were exposed to the programme and children who were not.

Three experimental (programme) and two control schools were selected for the study. A total of 110 children in the experimental group and 102 in the control group were examined (Total = 212) in the age group 11-12 years. The examiners were calibrated for reproducibility using WHO (1986) criteria for dental caries. The results of the study showed a mean DMF(S) of 7.8 (±3.1) for the experimental schools and 24.5 (±8) for the control schools. 84% of the experimental group and 60% of control group were caries free. 98% of the experimental group and 65% of experimental group had an M component of 0.42% of the control group; 8% of experimental group had at least one 1st molar tooth decayed. These results clearly showed that the programme is successful in a community based school dental service.
This project is supported by an MRC grant.

11 Film Thickness Evaluation - Implementing the BENCOR MULTI-T System. C.H. DRIESSEN*, F.A. DE WET and W.J.C. COETZEE. Faculty of Dentistry, University of Pretoria, South Africa.

Various techniques have been described for the determination of film thickness of dental cements. The purpose of this study was to assess the effectivity of the BENCOR MULTI-T system, and to explore the effect of load variation on the film thickness data. Four luting cements were included in this study i.e. X-R IONOMER (X), a glass ionomer, Mirage-FLC (M), a dual cure resin; UNITY (U), a self curing resin; and POLY-F Plus (P), a polycarboxylate. The BENCOR MULTI-T device (Driesson, 1990) was used for testing. The system is based upon direct force application derived from any calibratable source through it's active rod onto the specimen material placed between two 22mm0 glass discs. The latter is covered with teflon discs for the purpose of: (1) simulating oral temperature, (2) transfering visible light (if needed) for the curing of a VLC material and (3) protecting the 160µm glass discs from direct metal impact. Measuring of the specimens was done by electronic digital calipers with control by reflex microscope data and SEM image observation. Ten samples of each product were tested using 10Kg and 15Kg forces in order to assess the effect of different loads on the film thickness. All data were statistically analysed. It was found that the tecnique enabled operators to measure film thickness of dental cements accurately, easily, fast and with only small variations. Statistical analyses showed a significant difference ($p < 0.05$) between application of 10Kg and 15Kg load of (X)[25,76:19,60µm] and (U)[28,60:14,20µm] but not for (M)[31,14:30,80µm] and (P)[36,00:34,00µm]. It can be concluded that the BENCOR MULTI-T system is able to easily assess the film thickness of dental cements, and that a 15Kg load reduces film thickness compared to the 10Kg load.

12 Cross-infection Risks Associated with High-speed Dental Handpieces. C.H.J. HAUMAN.* Department of Oral Pathology, Faculty of Dentistry, University of Stellenbosch, Tygerberg, South Africa

Dental handpieces are particularly prone to contamination with patient material, which can then be transmitted to the next patient. The common approach of disinfecting handpieces by external chemical wiping in combination with flushing may pose unacceptably high risks to those individuals treated soon after infected patients.
The aim of this study was to evaluate the efficacy of chemical disinfection of high-speed handpieces. Autoclaved high-speed handpieces were contaminated with an overnight culture of *Staphylococcus aureus* and dried in a hot air oven for 90 minutes. The outer surfaces of equal numbers of these handpieces were wiped with 70% alcohol, alcohol-in-hibitane and Asepsys (iodophor). The front, back and sides of the heads of these handpieces were pressed onto the surface of blood agar plates. In addition, contaminated handpieces were attached to the dental unit and water was flushed through the handpiece onto the surface of blood agar plates for 2 seconds. Handpieces used in the Tygerberg Dental Hospital were tested in a similar way after routine lubrication and alcohol swabbing. Residual contamination of handpieces after flushing for specific periods of time were also tested. After overnight incubation, growth was recorded. Although the numbers were reduced, *S. aureus* could still be cultured from the outer surfaces of artificially contaminated handpieces after wiping with all three disinfectants. Handpieces used in the clinic yielded virtually no growth from the external surfaces after routine cleansing. Confluent or 2+ growth was obtained with samples from the interior surfaces of both artificially contaminated handpieces and handpieces from the clinic. *S. aureus* was still present on the internal surfaces of artificially contaminated handpieces after flushing for 5 minutes.
Sterilisation of both the internal and external surfaces of handpieces is necessary to exclude the risk of cross-infection in the dental surgery.

13 Effect of Respiratory Acidosis on Faecal Fluoride Excretion in Rats. S.D. JANSE VAN RENSBURG* and C.A. VAN DER MERWE. University of Pretoria, and Medical Research Council, Pretoria, South Africa.

Respiratory acidosis is characterised by a primarily increased PCO_2 with a compensated increased $[HCO_3^-]$ which may effect the pH of blood. As the permeation of fluoride (F) through epithelia is dependent on the pH, as well as F concentration, respiratory acidosis could have an effect on faecal F excretion, and therefore on the F balance of the body. Sixteen young adult female Sprague-Dawley rats were used in this study. They were divided into a control group (Group A; receiving a normal atmospheric gas mixture) and an experimental group (Group B; subjected to an atmospheric CO_2 content of 7%). All the rats were fed a low F diet (< 1ppm) and received water with a fluoride content (Fl) of 20ppm *ad lib* for 7 weeks. Water and food consumption were monitored daily. F intake/rat/day via water and food was calculated from the data. After sedation, blood was collected anaerobically in heparinised syringes from the descending aorta. Faeces were collected from the large intestine. The [F] of the faeces was determined potentiometrically after HMDS diffusion. Blood gas analysis were done using the ABL blood gas analyser. The data were subjected to the Mann-Whitney procedure to detect differences between the groups, and to multiple regression analysis to explain the variation in the [F] of the faeces. Although the F intake via the water differed significantly ($p < 0,05$) between the groups, there was no significant difference between the F intake via food ($p = 0,197$) or the total F intake ($p = 0,071$). There was a significant difference ($p < 0,05$) in the [F] of the faeces (A: $\bar{x}$ = 148,33 ppm; B: $\bar{x}$ = 213,20 ppm) between the groups. Taking the [F] of faeces as the dependent variable, 68,89% of the variation in the [F] of faeces in the experimental group could be explained by the combination of the independent variables H_2CO_3 or PCO_2 and total F intake. A 46,92% association between the variables was found in the control group. Respiratory acidosis enhanced faecal excretion of fluoride in rats. The higher [F] of the faeces in the experimental group could possibly be dye to net secretion of fluoride into the gut lumen. This project was partially supported by a grant from Afrox Pty Ltd.

14 Inorganic Contents of Opaque and Translucent Radicular Dentine. F.S. NKHUMELENI*, E.J. RAUBENHEIMER, W.F.P. VAN HEERDEN, M.L. TURNER and M.J. DREYER. Depts. Oral Pathology and Chemical Pathology, MEDUNSA, P O Medunsa.

This study was undertaken to compare the calcium, magnesium, phosphorus, zinc and fluoride contents of opaque and translucent radicular dentine. Twelve mandibular incisors were utilized. The crowns and cementum were removed using a dental bur. The specimens were then hydrolysed individually in 1M perchloric acid. Calcium, zinc and magnesium were determined utilising the ammonium phosphomolybdate calorimetric method and fluoride by ion selective electrode method. The mean values (mg/g) of opaque and translucent dentine respectively were :

Calcium (Ca)	244.65	(SD ± 3.60)	and	244.17	(SD ± 4.10);
Magnesium (Mg)	8.25	(SD ± 0.42)	and	6.97	(SD ± 0.69);
Fluoride (F)	0.22	(SD ± 0.01)	and	0.27	(SD ± 0.02);
Phosphorus (P)	127.70	(SD ± 2.43)	and	125.00	(SD ± 2.33);
Zinc (Zn)	0.14	(SD ± 0.01)	and	0.20	(SD ± 0.03).

The Mann Whitney test showed that there was a significant difference between the Mg, F and Zn contents of opaque and translucent dentine ($p<0.05$). Our findings do not support those of Moore and Leaver (1974) who found that only Calcium values were significantly lower in translucent dentine.

15 Enamel Surface Roughness after CO_2 Laser Radiation, in vitro Assessment. S.H. PAN*, C. BAKER, J. DE VRIES, P.J. BECKER and S.S. MASHELE. Dept. of Operative Dentistry, Faculty of Dentistry, MEDUNSA, S.A.

Recently, a CO_2 laser has been used successfully to enhance dental bonding. Knowledge of surface roughness is essential to reduce bond failures. The objectives were to: 1) determine the surface roughness after conventional drilling with/without laser radiation and with/without acid etching using the Bendix Profilometer. 2) examine under Scanning Electron Microscope (SEM) the ultrastructure of the enamel surface after CO_2 laser radiation. Thirty-two human maxillary central incisor teeth were selected and stored in 10% buffered formalin. Conventional drilling and combined treatment with laser radiation were randomised on the labial enamel surface in the vertical dimension of 3 x 5mm. Laser radiation was set at a repeat pulse energy intensity of below 3W for a period of 10 seconds. Acid etching was performed on half of the specimens and the surface roughness was measured with the Bendix Profilometer. The SEM assessment was also noted. The experiment was designed as a randomised block but test results showed that roughness caused by laser produced the maximum measurable roughness of 10 micron for each sample point. The analysis then focused on conventional high/low speed drilling and no significant difference was found between acid etching techniques, ($p = 0,4270$) or between drilling speeds ($p = 0,7355$). Compared to laser the roughness after conventional drilling was clinically less.
An improved understanding of roughness caused by CO_2 laser radiation on enamel surfaces may lead to possible clinical application in aesthetic restorative dentistry.

16 Relation Between Blood, Molar, Cortical Bone and Trabecular Bone Lead Levels of Rats. R.J. ROSSOUW* and S.R. GROBLER, Faculty of Dentistry, University of Stellenbosch, Tygerberg, South Africa

Bone is the major reservoir of body lead stores. Loosely and deeply bound lead compartments in bone provide short- and long-lived sources of this toxic element to blood and soft tissues.

Several groups of inbred BD-IX rats were continuously exposed to nebulized aerosols of lead for different time periods. Furthermore, the effect of different post-exposed periods on the lead concentrations were also investigated. The lead concentrations were determined in blood, molars, tail vertebra and iliaca crest of the rat. The graphite furnace atomic absorption spectrophotometer was used.

The blood, molar, tail and iliaca crest lead levels differed significantly ($p < 0,05$) among the exposed groups. However, only the blood and iliaca crest lead concentrations differed significantly ($p < 0.05$) in the post-exposure periods.

It is concluded that lead is absorbed in the apatite crystal of different kinds of bone. Furthermore, cortical bone is identified as long term storage reservoirs of lead in the body.

This study was supported by the MRC.

Giant ossifying fibroma: a clinicopathologic study of 8 tumors

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van Heerden WFP, Raubenheimer EJ, Weir RG, Kreidler J: Giant ossifying fibroma: a clinicopathologic study of 8 tumors. J. Oral Pathol Med 1989; 18: 506–509.

Clinical, radiographic and microscopic features of 8 ossifying fibromas diagnosed in 7 patients and measuring more than 8 cm in greatest diameter, were reviewed. The tumors occured in both juvenile and middle aged patients and all lesions in women involved the maxilla. The abundance of fibrous connective tissue and resorption of mineralized deposits are indicative of altered cellular differentiation and proliferative activities in large ossifying fibromas. Focal areas of aneurysmal bone cyst formation were identified in the majority of lesions.

Key words: fibro-osseous lesion; giant ossifying fibroma; juvenile aggressive ossifying fibroma.

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Ossifying fibromas are generally regarded as slow growing and well circumscribed jaw tumors which contain foci of trabecular and spherical calcifications resembling bone and cementum respectively (1). They are reported to be more common in blacks, occur frequently in women and the majority of lesions involve the mandible (1, 2).

Although no convincing definition of giant ossifying fibromas are to be found in the literature, these neoplasms were reported by various authors as large tumorous fibro-cemento-osseous proliferations (3–7). Unfortunately, many of the reported cases are not documented satisfactorily. HAMNER *et al.* (1968) however, arbitrarly defined giant lesions as those exceeding 2 × 2 cm in size or involving the space occupied by two or more teeth.

There is no published series of giant ossifying fibromas in the literature. Therefore, this study was undertaken to determine the clinical and radiographic appearances and the microscopic features of the large ossifying fibromas diagnosed by the Department of Oral Pathology at the Medical University of Southern Africa (Medunsa) over a 6-yr period.

Material and methods

All the cases diagnosed as ossifying fibroma over the last 6 yr were retrieved from the files of the Department of Oral Pathology, Medunsa, Most patients seen at the hospitals served by the department are black and of rural origin. The pathology reports were reviewed and all lesions with a longitudinal diameter of 8 cm or more as measured on the excision specimen, were included in this study. Radiographs were available in all the selected cases. Histologic evaluation was done by means of light microscopy. Three specimens (Cases 1, 3, 4) were bivalved in the longitudinal diameter, and a 5 mm slice of the entire cut surface was obtained by means of a band-saw. The slice was then radiographed and blocked into multiple squares, each of which was numbered on a scheme corresponding to the radiograph and processed for routine light microscopy. Representative histologic sections were avialable in the remaining cases.

Results

Seven patients from a total of 30 cases of ossifying fibroma were found to have tumors larger than 8 cm in greatest diameter (Fig. 1). The age, sex, site and size of the tumors are indicated in Table 1. Case 5 presented with a mandibular and a maxillary tumor, both exceeding 8 cm. Signs and symptoms varied, the most common of which was swelling. At the age of 2 yr, Case 1 presented with a mandibular tumor 4 cm in diameter. Biopsy showed a benign fibro-osseous proliferation and due to parenteral refusal the lesion was followed over a period of 7 yr during which it became less radiopaque and tripled in diameter. The duration of the other lesions could not be determined reliably. Although the post-operative follow-up is in some cases as short as one year, none of the lesions have recurred.

All tumors involved the tooth-bearing areas of the jaws and were well demarcated with scattered foci of radiopacities (Fig. 2). Root resorption were present in three cases.

Case 1 was treated with hemimandibulectomy. Enucleation of the tumor was done in the other cases. No cortical perforation was present, only expansion in all directions. On cut surface, the tumors had a gray-white color with a gritty consistency. Cystic spaces representing aneurysmal bone cyst changes and measuring up to 1 cm in diameter could be seen focally in six tumors.

The histologic features correlated with the radiographic appearance of the corresponding area. The inconspicuous radiodense areas consisted of woven trabecular bone, although a few lamellar bony trabeculae and psammomatous calcifications were also found. Active resorption of the trabeculae with accumulation of osteoclast type giant cells were evident in all tumors (Fig. 3). Vascularity was more prominent in the areas of resorption and the fibrous component adjacent to these areas were cellular.

The radiolucent zones consisted

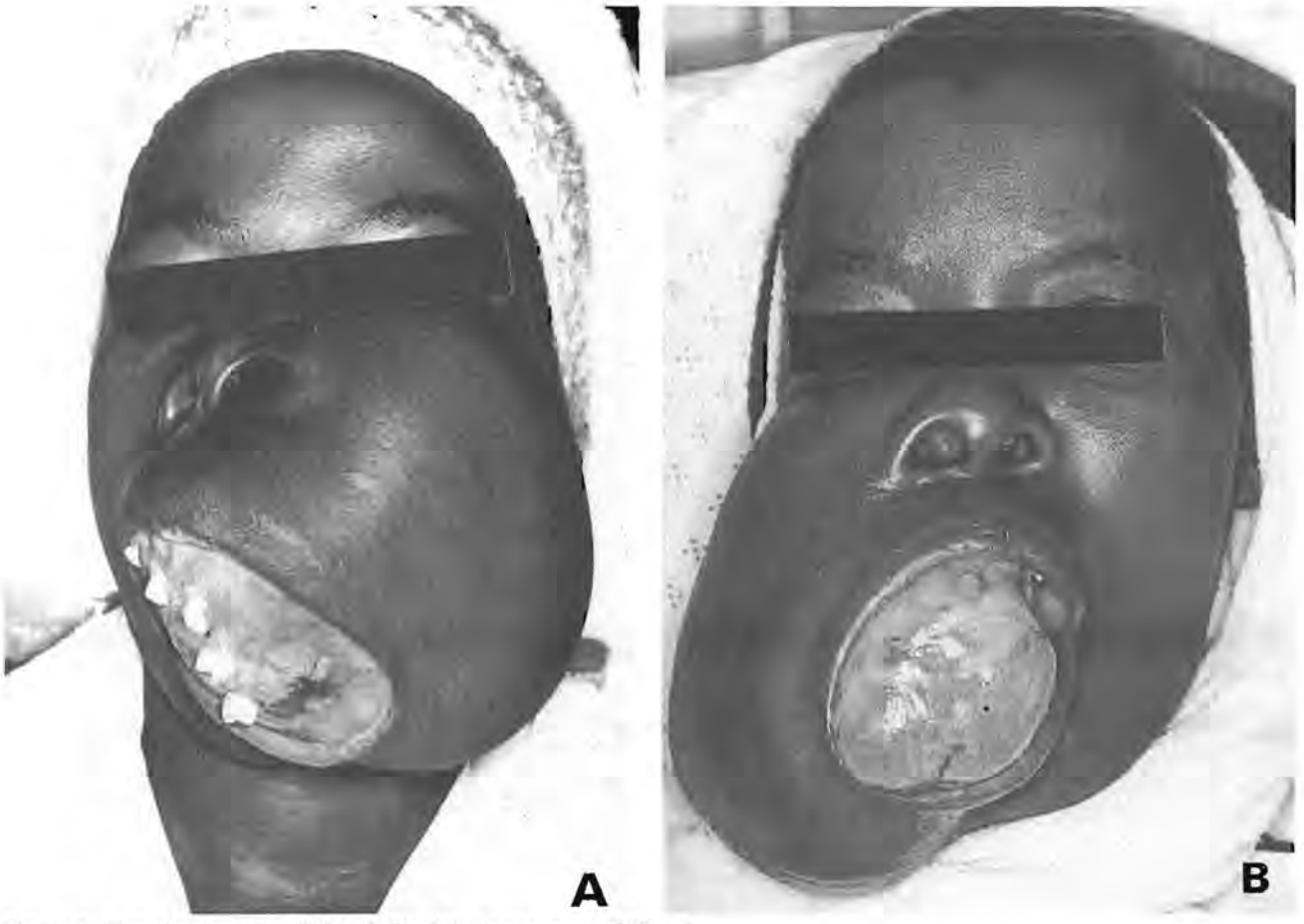

Fig. 1. A, clinical appearance of Case 3. B, clinical appearance of Case 4.

mainly of fibrous tissue. The stroma varied from mature collagen to tissue with a cellular storiform pattern (Fig. 4). Small amounts of mineralized tissue, mainly of a psammomatous cementum-like nature were present in the fibrous tissue. The aneurysmal bone cyst changes were found in areas where the fibrous tissue had a loose, edematous structure.

Discussion

The sizes of the eight tumors described surpass that of all giant ossifying fibromas reported in the literature. HAMNER *et al.* (3), defining 'giant' lesions as those exceeding 2 × 2 cm in diameter, found 17% of their cases of cemento-ossifying fibromas to have reached these dimensions. Seven (or 23%) of our collection of ossifying fibromas had a diameter of more than 8 cm. If the criteria of *Hamner et al.* (3) had to be applied to our collection of ossifying fibromas, almost all of the 30 cases diagnosed in our department over the last 6 yr will be regarded as 'giant'. The large dimensions of our tumors is related to the rural character of the populations served where proper diagnosis and treatment is often delayed through tribal customs.

Table 1. Clinical data of the seven patients.

Case No	Age	Gender	Site	Size
1	9	M	Mandible	12 cm
2	7	W	Maxilla	8 cm
3	13	W	Maxilla	13 cm
4	46	W	Maxilla	15 cm
5	40	M	Mandible	8 cm
			Maxilla	10 cm
6	49	M	Mandible	10 cm
7	57	W	Maxilla	10 cm

The age range of our patients was 7–57 yr with an age peak in the first and fifth decades, a distribution corresponding to that generally reported for ossifying fibromas (1). The occurrence of large ossifying fibromas in young children is of particular interest as it is believed that these tumors require many years of growth to attain large dimensions (2). One of our cases, diagnosed at 2 yr of age, showed an increase of 8 cm in diameter over a follow-up period of 7 yr. As far as we can ascertain, this represents the youngest age at which an ossifying fibroma had been diagnosed. Five of the eight tumors and all lesions in women involved the maxilla. This is in contrast to the generally held view that ossifying fibromas occur more frequently in the mandible (2).

Radiographically, the large ossifying fibromas in our study contain relatively less mineralized tissue than smaller lesions. This finding is substantiated by the microscopic appearance of the giant lesions where the balance of cellular activity favor fibrous tissue formation and bone resorption at the expense of new bone formation. Although the majority of our lesions showed foci of aneurysmal bone cyst formation, STRUTHERS & SHEAR (8) found this change to occur in only 4% of their ossifying fibromas and *Eversole et al.* (9) noted aneurysmal bone cyst features in three of their 64 cases. The high prevalence of aneurysmal bone cyst formation in our lesions is probably due to the prominent fibrous com-

ponent which contains more loose edematous areas than is found in smaller ossifying fibromas. This feature is not responsible for the giant dimensions, as the foci of aneurysmal bone cyst change are limited and the cystic spaces are of relative small size.

HAMNER *et al.* (3) stated that ossifying fibromas containing cementum are larger and more aggressive than pure ossifying or cementifying lesions. HALL *et al.* (10) consider mixed cementifying and ossifying fibromas as a potentially aggresive variant of ossifying fibroma. WALDRON & GIANSANTI (11) however stated that a separation into cementifying and ossifying types is artificial as they could find no difference in the behaviour of tumors with these histologic designations. Cementum-like as well as osseous deposits were present in all tumors in our series and we believe that if representative tissues of ossifying fibromas are taken for microscopic examination, most tumors will be found to be of a mixed nature.

Our study furthermore suggests that ossifying fibromas with a gigantiform growth potential are characterized by the appearance of large fibrous areas which are represented radiographically by less radiodense areas. This is in contrast to the normal progression of these lesions where the islands of mineralizations are reported to increase in size and coalesce resulting in a more radiopaque lesion (2).

The microscopic appearance of the giant lesions does not resemble that of juvenile aggressive ossifying fibromas. The criteria defined by WALDRON (12) for the diagnosis of juvenile aggressive ossifying fibroma include a cellular vascular stroma with varying amounts of giant cells and little collagen production. Osteoid lined by osteoblasts are usually present. These lesions furthermore appear most often in young patients – predominantly younger than 20 yr and almost always below 40 yr of age (13). None of the lesions in the three young patients in our series can be classified as the aggressive variant because of the prominent fibrous tissue and collagen component and the scarcity of osteoid formation. This however does not exclude the possibility that at some earlier stage our lesions may have had the microscopic features of juvenile aggressive ossifying fibromas.

This study suggests that the shift in cellular activity from osteoblastic in small ossifying fibromas to fibroblastic in the giant lesions represents a phenomenon associated with gigantiform tumor enlargement.

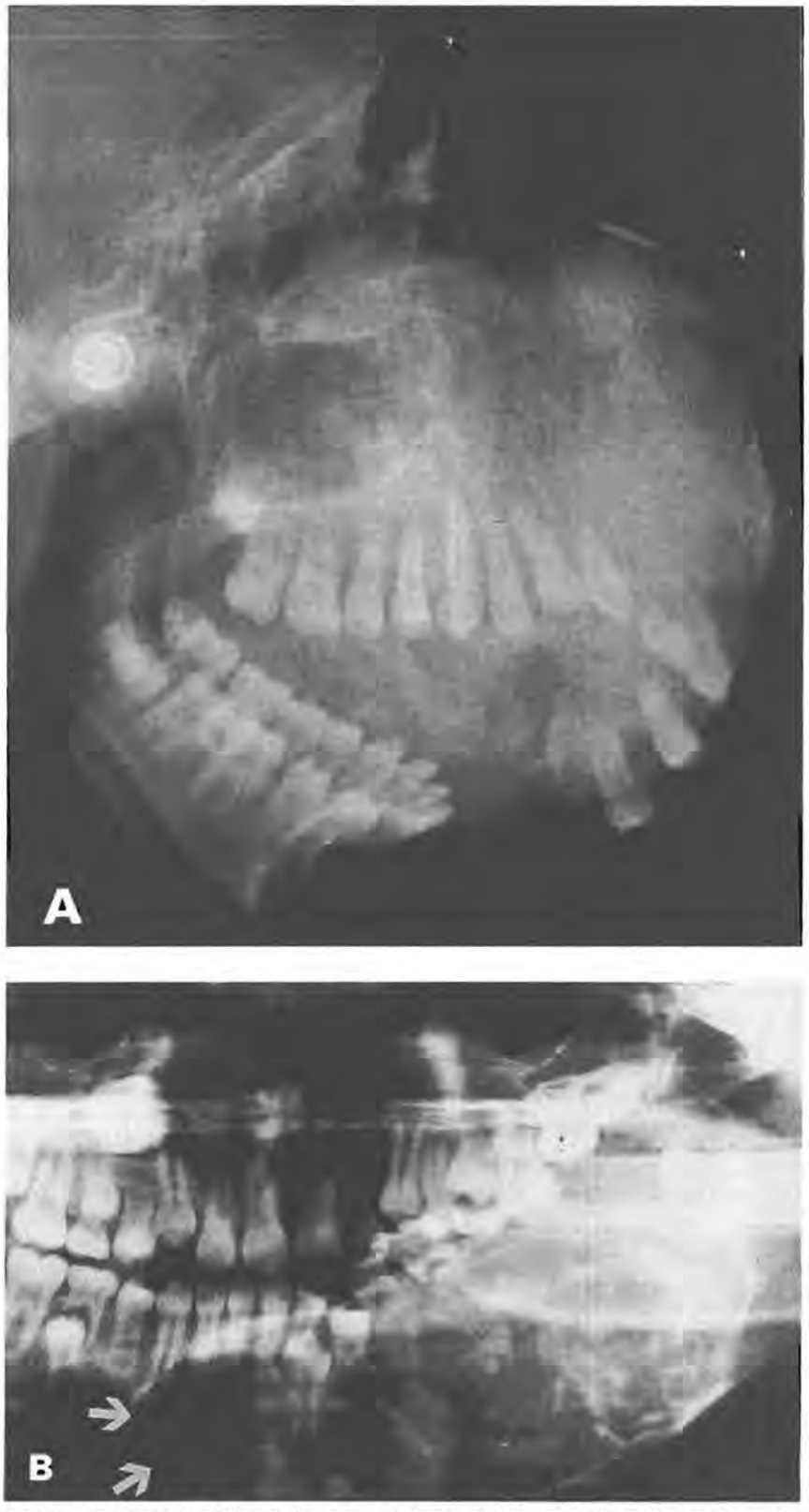

Fig. 2. A, radiograph of Case 3 shows a well demarcated lesion with smokescreen appearance and irregular radiolucent areas. B, Prominent lytic areas are present in Case 1 (arrows).

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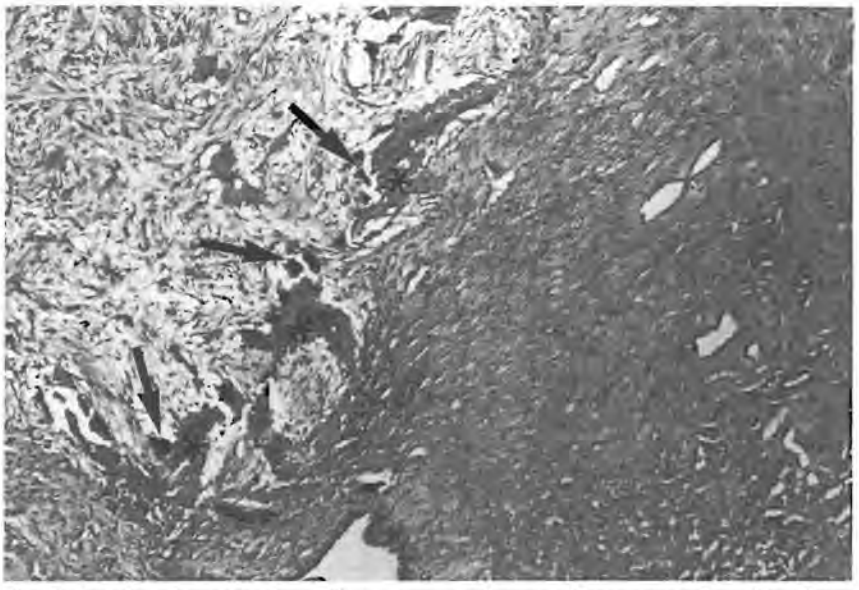

Fig. 3. Interface between dense fibrous zone and area of bone resorption. Note bony trabeculae (asterisks) and osteoclasts (arrows) in loose fibrous tissue. H&E, × 40.

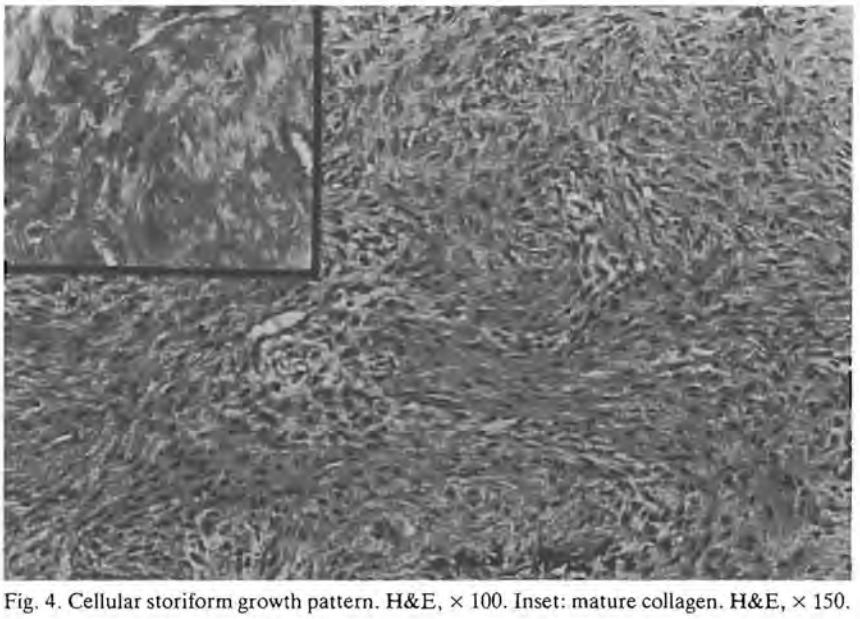

Fig. 4. Cellular storiform growth pattern. H&E, × 100. Inset: mature collagen. H&E, × 150.

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Adenomatoid odontogenic tumour: a report of two large lesions

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Adenomatoid odontogenic tumours with a diameter of more than 4 cm are uncommon. Two cases, both measuring in excess of 7 cm, are described and the differential diagnosis discussed. The progressive growth and cortical perforation in these two cases support the view that it is a benign neoplasm rather than a hamartoma.

Keywords: Odontogenic tumours; maxilla

The adenomatoid odontogenic tumour (AOT) is a rare, benign odontogenic tumour of which approximately 170 verifiable cases have been reported in the English literature. It occurs most often in young females and commonly involves the anterior maxilla. Although many AOTs are detected during routine radiographic examination, patients may present with a gradually enlarging, painless swelling which can lead to facial asymmetry. Radiographs generally show a clearly demarcated, radiolucent lesion surrounding an unerupted tooth, usually a maxillary canine[1,2]. Radiopaque foci frequently occur in the tumour. The size of an AOT varies between 1.5 and 3.0 cm but some as large as 9.0 cm have been reported[3]. It is usually diagnosed radiographically as a follicular, lateral periodontal[1], residual[4] or 'globulo-maxillary' cyst[2,5]. If calcification is present, the differential diagnosis should also include calcifying odontogenic cyst, central ossifying fibroma, calcifying epithelial odontogenic tumour and ameloblastic fibro-odontome. Microscopically, AOTs are characterized by a well-defined fibrous capsule surrounding sheets, strands and nodular masses of epithelial cells which form tube-like structures and rosettes[1]. The purpose of this paper is to report two large AOTs with diameters of more than 7 cm.

Case reports

Case 1

A 12-year-old black female presented complaining of a maxillary swelling obstructing her nose; the duration of the swelling was unknown. On examination, a maxillary tumour, extending from the lower border of the right eye and crossing the midline of the face, was present. The size of the lesion interfered with lip closure. The nose was deviated, the nasal passage obstructed and on palpation the bony cortex was perforated, resulting in fluctuation. The skin overlying the lesion had three parallel scars (each 4 cm long). Intra-oral examination showed mobile and displaced maxillary permanent incisors and a primary canine, and bulging of the right palatal shelf and buccal plate (Figure 1). Radiographs revealed a well-circumscribed unilocular radiolucency containing the crown of the unerupted permanent canine (Figures 2, 3). A clinical diagnosis of follicular cyst was made and the lesion was enucleated through an intra-oral approach. The specimen submitted for pathological examination consisted of a cystic lesion measuring $12 \times 10 \times 10$ cm which contained a normal maxillary canine. The lining of the cyst contained multiple nodules measuring up to 5 mm in diameter. Microscopic examination showed an epithelial lining containing nodular masses of odontogenic epithelial cells forming rosettes and pseudoglandular spaces. A diagnoses of AOT was made.

Case 2

A 9-year-old black female presented with a complaint of swelling in the right maxilla obstructing her nose. The lesion was painless and had been present for 3 years. On examination, there was a 9×8 cm swelling in the right maxilla, which had elevated the right ala (Figure 4). The maxillary permanent central incisors,

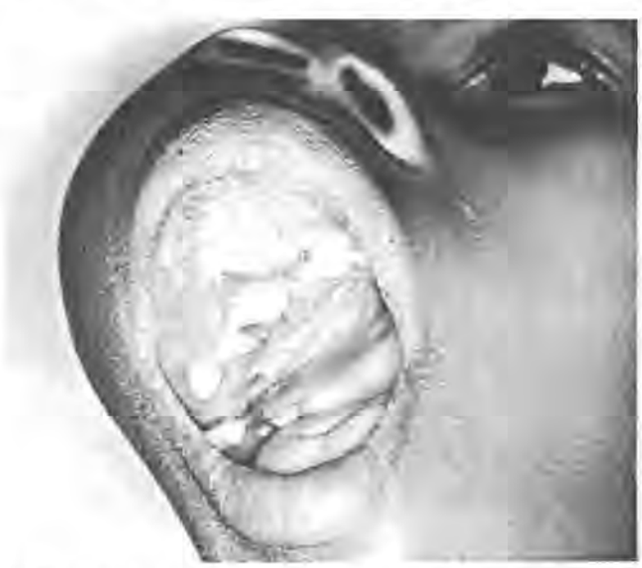

Figure 1 Case 1. Intraoral view of the tumour

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Figure 2 Case 1. Lateral cephalometric radiograph. Note the displacement of the permanent maxillary canine (arrow)

primary canines and molars were erupted and the palatal shelf and buccal plate were expanded by a firm swelling. Radiographically, there was a well-circumscribed radiolucent lesion, surrounding the crown and neck of an unerupted maxillary canine; the developing maxillary premolars were dilacerated and the second incisor displaced and impacted (Figure 5). In the absence of calcification in the wall of the lesion, a clinical diagnosis of a follicular cyst was made. The lesion was enucleated and an opened cyst, 8 cm in diameter, surrounding the crown and neck of an unerupted maxillary canine and containing mural granules, was submitted for pathological examination. Microscopically, the cyst wall consisted of abundant connective tissue lined by thin and inactive odontogenic epithelium which surrounded nodular masses of epithelial cells exhibiting rosettes and pseudoglandular structures. A diagnosis of AOT was made.

Discussion

As far as can be ascertained, Case 1 represents the largest AOT reported in the English literature.

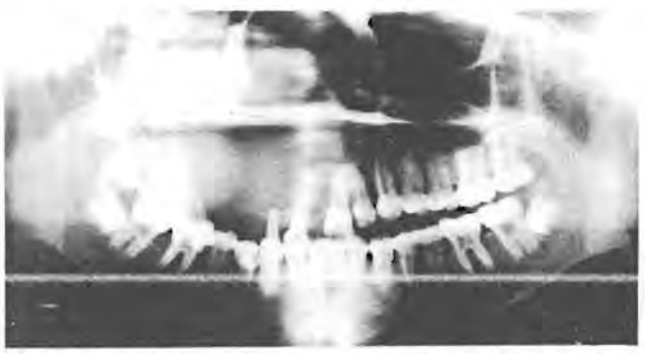

Figure 3 Case 1. Panoramic radiograph. Note the well circumscribed, expansile lesion in the right maxilla with displacement of the roots of involved teeth as well as the canine

Figure 4 Case 2. Frontal photograph

Another large tumour had a diameter of 9 cm and formed part of a series of 13 cases occurring in Nigerian patients[3]. The large size of our two cases could be related to their more rapid growth in younger patients, certainly, the average age is higher in previous reports. However, the size may also result from a delay in seeking proper dental treatment. This view is supported by the presence of linear scars on the skin overlying the tumour in Case 1, an indication of regular visits to tribal medicine men before seeking hospital treatment.

The histogenesis of the AOT is unknown but the possibilities range from the dental lamina to reduced enamel epithelium[6–8]. One investigator[9] suggested that the epithelial rests of Malassez at the apex of deciduous teeth is the progenitor tissue. His argument is based, in

Figure 5 Case 2. Panoramic radiograph. Note the dilacerated first and second permanent maxillary premolars (arrows)

part, on the fact that AOTs have never occurred in association with impacted deciduous teeth nor in areas not preceded by deciduous teeth. The existence of those lesions not associated with an unerupted tooth and therefore not arising from the reduced enamel epithelium, may be explained on this basis.

Courtney and Kerr[10] from a study of 20 AOTs, as well as others[11–13], believe the lesion to be an hamartoma rather than a benign neoplasm. However, hamartomas have a limited growth potential and progressively differentiate into more mature tissue with ageing[14]. Our cases do not support a limited growth potential as postulated by Saito *et al.*[4] nor did they exhibit maturation into more differentiated dental tissues. We therefore believe the lesion to be a benign neoplasm. The growth potential of AOTs is supported by Ajagbe *et al.* and others[15, 16] and earlier detection is likely to be the reason for the small size of most cases reported in the literature.

Radiographically both of our cases resembled a follicular cyst, the most common lesion to consider in the differential diagnosis of AOT. The well demarcated radiolucency associated with an AOT is reported to extend more apically on the root of the associated unerupted tooth than in the case of a follicular cyst[17]. Another feature that could be helpful in distinguishing between these two lesions is the virtual absence of root resorption in AOTs[18]. The dilaceration of the permanent premolars in Case 2 is most likely the result of pressure exerted by the enlarging tumour on the roots of the developing teeth.

Nasal obstruction is a common complaint in patients with maxillary AOTs measuring 5 cm or more in diameter[3, 19]. Furthermore, erosion of bone has been reported in a large AOT and actual perforation has led to it being described as a 'fluctuant mass'[19]. Our Case 1 also exhibited this feature but we do not agree with Poulson and Greer[20] that its presence warrants the exclusion of an AOT and the consideration of a more aggressive tumour in the differential diagnosis.

Acknowledgement

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Diffuse peripheral odontogenic fibroma: report of 3 cases

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Since peripheral odontogenic fibroma (POF) is characteristically described as a solitary lesion and no diffuse POF had been reported in the literature, our cases should be considered as extremely unusual. Three diffuse cases of POF are described of which one case was seen in association with ocular and skin lesions. The question arises whether POF should be considered as a true odontogenic tumor rather than a diffuse hamartomatous lesion caused by uncontrolled induction of the gingiva. It is also possible that such lesions could be part of a yet undescribed syndrome.

Key words: hamartoma; mouth, neoplasms; odontogenic fibroma; peripheral odontogenic lesions.

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The odontogenic fibroma is defined by the World Health Organization as a benign odontogenic neoplasm of fibroblastic origin characterized by relatively mature collagenous fibrous tissue and varying amounts of odontogenic epithelium, with the potential to occur in either a central or extraosseous location. The extraosseous counterpart is designated peripheral odontogenic fibroma (POF) (1).

All the POF's described in the literature presented as single, exophytic tumors which frequently prompted a clinical diagnosis of localized gingival hyperplasia. Diffuse involvement of the gingiva has not yet been reported. Three cases with the histologic appearance of POF with diffuse involvement of the gingiva, of which one case was associated with dermatological and ocular abnormalities, are presented.

Material and methods

Case 1

An 8-yr-old Black girl presented with diffusely enlarged gingiva in both jaws, causing delayed eruption of the perma-

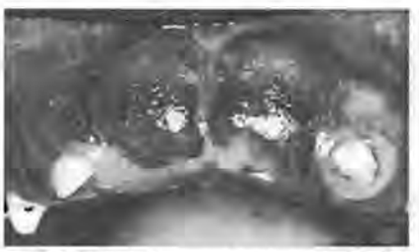

Fig. 1. Clinical photograph of Case 1 showing diffusely enlarged gingivae in both jaws.

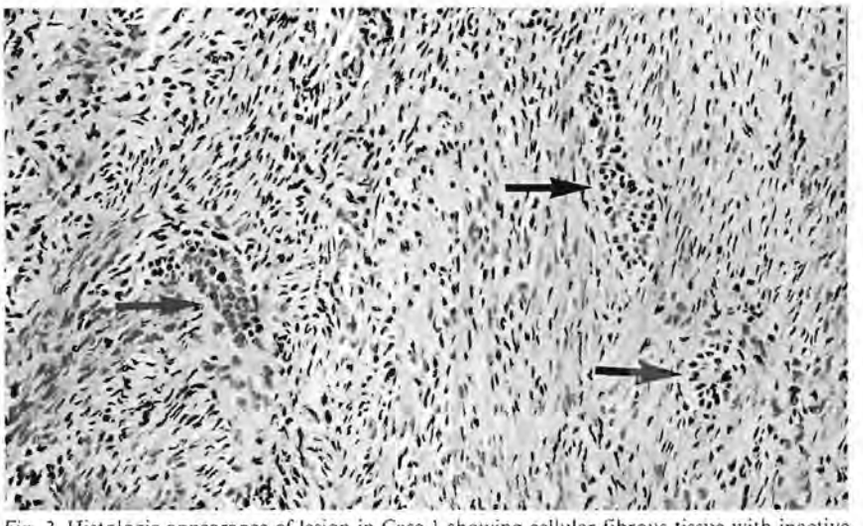

Fig. 2. Histologic appearance of lesion in Case 1 showing cellular fibrous tissue with inactive odontogenic epithelium arranged in nests and strands (arrows).100.

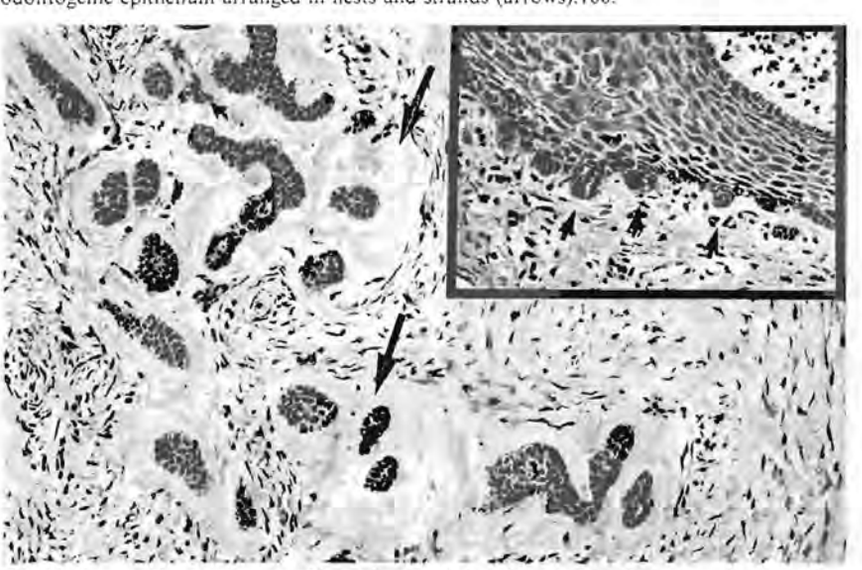

Fig. 3. Microscopic examination of lesion in Case 2 showing strands of odontogenic epithelium with prominent hyalinization (arrows).100. Inset: budding of overlying epithelium (arrows).100.

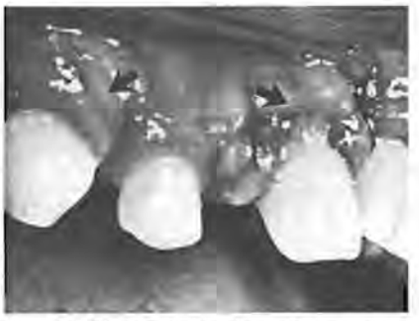

Fig. 4. Diffuse nodular gingival hyperplasia (arrows) in Case 3.

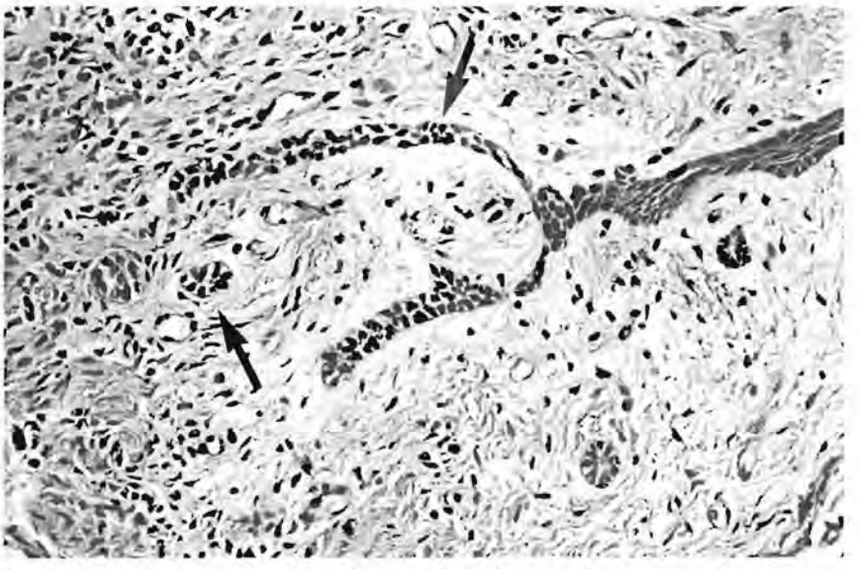

Fig. 6. Histologic appearance of tumor in Case 3 showing islands and strands of odontogenic epithelium (arrows) scattered in connective tissue.100.

nent incisors. Nodules were present in the gingival masses which were firm in consistency (Fig. 1). The duration of the lesions could not be determined and no evidence of a family history was found. Radiographic examination showed no bone involvement or disturbance in tooth development. Gingivectomy of the hyperplastic tissue was done and the tissue sent for histologic examination.

Microscopically, the lesion consisted of cellular fibrous tissue with myxomatous areas. The odontogenic epithelium appeared inactive and was arranged in cell nests and strands (Fig. 2). No hard tissue formation was seen. The overlying epithelium was hyperplastic without evidence of downward proliferation of the rete ridges.

Case 2

A 56-yr-old black woman presented with diffuse gingival hyperplasia of both jaws resulting in enlarged alveolar ridges. The duration of the lesions was not known. The mandibular canines were displaced. All teeth were severely afflicted by plaque and calculus deposits. A biopsy of the lesion was performed and oral hygiene procedures implemented.

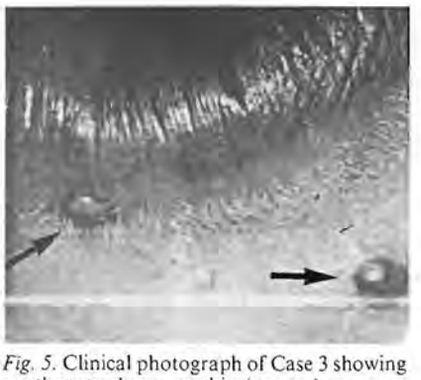

Fig. 5. Clinical photograph of Case 3 showing xanthogranuloma on skin (arrows).

Microscopically the gingival enlargement resulted from a proliferation of loose cellular connective tissue with scattered islands of inactive odontogenic epithelium. Hyalinization and calcifications were present in relation to the odontogenic epithelial rests. Budding of the overlying oral epithelium (Fig. 3) and focal areas of chronic inflammation were seen.

Case 3

A 3-yr-old white boy presented with diffuse nodular maxillary and mandibular gingival hyperplasia which became evident soon after birth (Fig. 4). The normal eruption pattern was disturbed but no other abnormalities were found radiographically. The patient also had small nodular skin lesions diagnosed as xanthogranulomas (Fig. 5), as well as corneal opacities. Corneal transplants were done in the eye lesions which could not be diagnosed as any specific pathological entity as yet. The oral lesions were clinically diagnosed as gingival hyperplasia and a biopsy performed.

Microscopically the lesion was composed of cellular fibrous tissue with islands and strands of odontogenic epithelium scattered in the connective

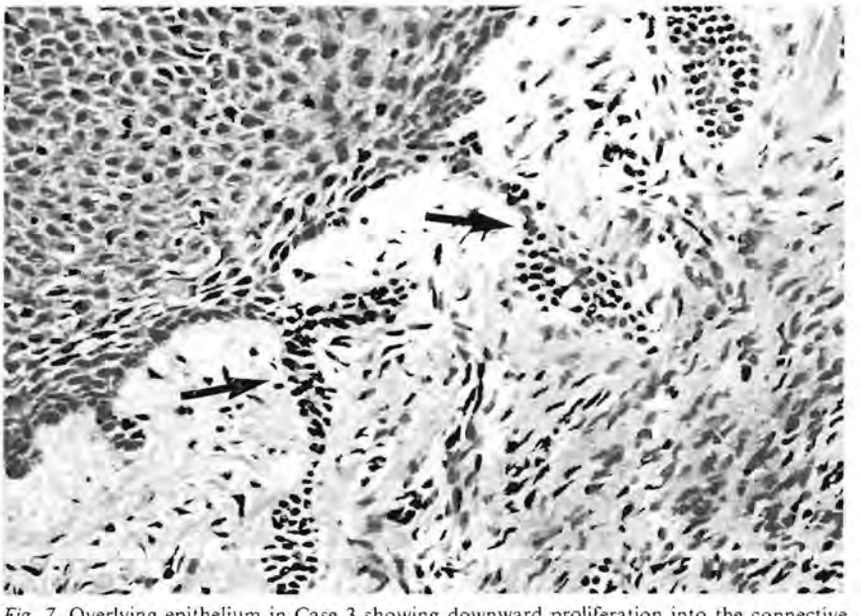

Fig. 7. Overlying epithelium in Case 3 showing downward proliferation into the connective tissue (arrows).200.

tissue (Fig. 6). No mineralized matrix formation was evident and the surface epithelium exhibited mild hyperplasia with downward proliferation (Fig. 7). A diagnosis of POF was suggested and gingivectomy of the hyperplastic tissue was performed. All the tissue submitted exhibited similar microscopic features.

Discussion

Not one of the accepted cases of POF in the literature were described as a diffuse gingival lesion. Furthermore, POF was never before described in relation to any other lesions as was seen in Case 3.

The question arises as to whether POF is a true neoplasm or whether it should be regarded as an hamartomatous developmental anomaly. The diffuse involvement of the gingiva in our three cases supports the possibility that POF does have an hamartomatous origin rather than being a true benign neoplastic lesion. We agree however, that the distinction between an hamartoma and a benign neoplasm is at best difficult and is differently interpreted.

The authors are of the opinion that POF should be considered as solitary or diffuse hamartomatous lesions which are caused by uncontrolled induction in the gingiva in a local or diffuse manner. Furthermore, the possibility that POF is an hamartomatous growth, which could be part of a yet undescribed syndrome, cannot be excluded, and should be investigated.

Acknowledgments – The authors thank C. S. BEGEMANN for secretarial services.

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Peripheral dentinogenic ghost cell tumor

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Raubenheimer EJ, van Heerden WFP, Sitzman F, Heymer B: Peripheral dentinogenic ghost cell tumor. J Oral Pathol Med 1992; 21: 93–5.

A case of dentinogenic ghost cell tumor, that has originated peripherally in the jaw, is presented and the literature reviewed with particular reference to the origin of the tumor. The total number of central and peripheral cases reported in the English literature is 10 and although mucosal infiltration is common, peripheral origin of the neoplasm could be verified in only 3 cases.

Key words: dentinogenic ghost cell tumors; jaws, neoplasms; odontogenic tumors

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The calcifying odontogenic cyst is a unique jaw lesion described as a distinct entity in 1962 by GORLIN *et al.* (1). In a study of 16 cases by PRAETORIUS *et al.* (2), it became evident that this group of lesions contains two entities, a cyst (Type I) and a neoplasm (Type II), and for the latter the term 'dentinogenic ghost cell tumour' was proposed. The neoplasm occurs predominantly in later life and consists microscopically of ameloblastoma-like odontogenic epithelial proliferations infiltrating the bone and connective tissue. Ghost-cells are present as well as varying amounts of dentinoid the latter being closely associated with odontogenic epithelium.

The purpose of this paper is to present an unique case of a dentinogenic ghost cell tumour originating in an extraosseous location.

Case report

An 82-yr-old man presented in the Department of Dental Surgery and Radiology, University of Ulm, complaining of a slow growing nodule on the mandibular right alveolar ridge. The lesion started 6 yr ago after extraction of the mandibular right canine. On examination the patient was found to be edentulous. A 6 mm broad based polypoid lesion was located on the mandibular right alveolar ridge. Radiographic examination revealed no underlying bone involvement (Fig. 1) and a clinical diagnosis of a peripheral giant cell granuloma was made. During surgical removal, the lesion was found to be located within the alveolar mucosa and the alveolar bone was not involved.

The surgical specimen measured 6 × 6 × 4 mm and had a firm consistency with foci of calcifications. Microscopic examination revealed hyperplasia of overlying epithelium and a solid tumor, composed of odontogenic epithelium associated with calcifications in the subepithelial connective tissue (Fig. 2). The neoplastic epithelium showed a well defined cuboidal to cylindrical basal cell layer closely associated eosinophilic cells with abrupt keratinization, resembling ghost cells (Fig. 3). Although the epithelial cells displayed nuclear pleomorphism, no mitotic figures were present. In focal areas, stellate reticulum-like differentiation as well as the formation of dental lamina-like structures were observed. Masses of acellular calcified material, resembling dentinoid, were evident in close association with the epithelium (Fig. 3). The surrounding connective tissue contained strands of inactive epithelium associated with small globular dentinoid deposits. Slight inflammation with vasodilatation and edema was present and a diagnosis of peripheral dentinogenic ghost cell tumor was made. Six year follow-up after removal failed to reveal a recurrence.

Discussion

Peripheral occurence of the cystic types of calcifying odontogenic cysts (Type I) is well documented in the English literature (1, 3). This may result from cortical bone perforation by a central lesion or more rarely, true peripheral origin from gingival epithelial remnants (4).

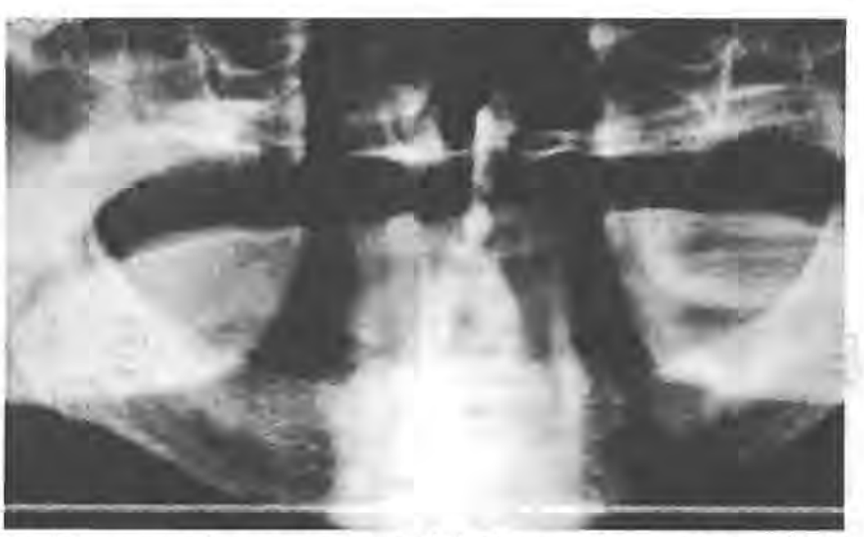

Fig. 1. Panoramic radiographic view showing lack of bony involvement of the mandibular right alveolar ridge.

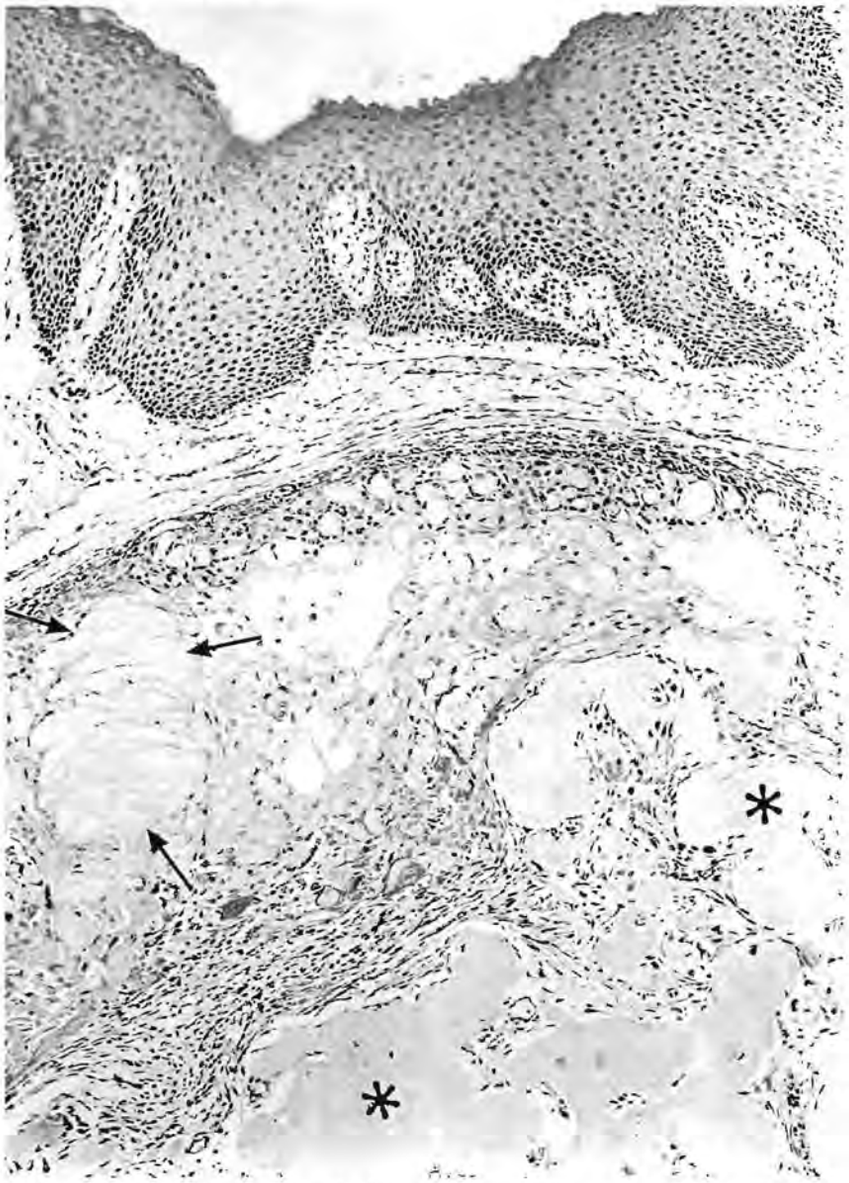

Fig. 2. Well encapsulated tumor with ghost cell formation (arrows) and dentinoid deposits (asterisks). H&E stain, ×30.

Conflicting data on the origin of the solid type calcifying odontogenic cyst (Type II) or dentinogenic ghost cell tumor are present in the literature. A recent review article summarizes the clinico-pathological features of 10 cases published in the English literature. In this article gingival swelling is considered to be the most frequent clinical feature. Radiographically all cases presented as lucencies with poorly defined margins (5). An earlier paper, reviewing 5 cases, proposed that dentinogenic ghost cell tumors usually occur peripherally and on the gingiva (6). Analysis of the original publications, mostly case reports, proves the discrepancy to lie in the interpretation of the clinical descriptions. Both cases reported by PRAETORIUS (2) are described as being 'extraosseous', despite radiographic signs of bony and dental involvement. Although peripheral involvement is probably implied by the authors, true peripheral origin of these lesions need to be questioned. The first of two cases reported by FEJERSKOV & KROGH (7) is described as an exophytic palatal mass. Unfortunately roentgenograms were not available and central origin of this case can therefore not be excluded. Central dentinogenic ghost cell tumors were also reported by GÜNHAN & SENGÜN (8) – 1 case, – TAJIMA (9) – 1 case – and COLMENERO *et al.* (5) – 1 case –, bringing the total number of central dentinogenic ghost cell tumors in the literature to 7. The 'peripheral odontogenic tumor with ghost-cell keratinization' reported by VULETIN *et al.* (10) contained no dentinoid deposits and exhibited odontogenic epithelium surrounded by cellular fibroblastic tissue, resembling and primitive dental pulp. On microscopical grounds, this lesion can not be classified as a dentinogenic ghost cell tumor since ghost cells are found in many other odontogenic neoplasms (2).

Peripheral presentation of dentinogenic ghost cell tumors is related to their infiltrative behaviour and although this feature appears to be common, true peripheral origin is not as frequently reported. ABRAHAMS & HOWELL (11) describe a dentinogenic ghost cell tumor located entirely extraosseous and palatal to a maxillary cuspid. Peripheral dentinogenic ghost cell tumors involving only the gingiva or alveolar mucosa, with radiographic support, were also reported in the lingual mandibular left premolar region (6) and the anterior part of the maxilla (12). This brings the number of true peripheral tumors to four, including our case.

The average age reported in the literature is 50, the oldest being 72 and the youngest 17 (5). Our patient, with an age of 82 yr, represents the most advanced age at which a dentinogenic ghost cell tumour has been diagnosed. Although the central tumors have a high rate of recurrence after removal (5), long term follow-up of our case and lack of proof of recurrence of any of the other peripheral dentinogenic ghost cell tumors suggests a favourable course for the peripheral type.

Acknowledgments – We are indebted to Ms C. S. BEGEMANN for secretarial assistance.

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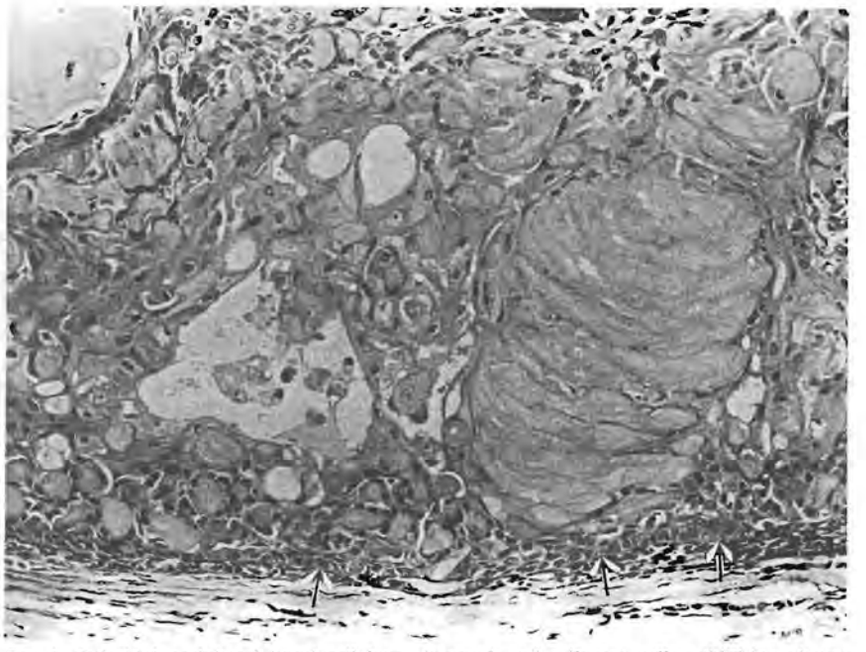

Fig. 3. Cuboidal peripheral basal cell layer (arrows) and adjacent cells exhibiting abrupt keratinization with ghost-cell formation. H&E stain, ×150.

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Infrequent clinicopathological findings in 108 ameloblastomas

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One hundred and eight ameloblastomas diagnosed in a rural black Africa population were analysed for clinicopathologic findings other than those classically described. One patient had a polycystic ameloblastoma adjacent to an ameloblastic fibroma. Two other polycystic ameloblastomas showed aneurysmal bone cyst formation and one mandibular tumour was diagnosed as a keratoameloblastoma. Microscopic changes resembling an adenomatoid odontogenic tumour were present in association with two unicystic ameloblastomas and a HPV18-positive verrucous lesion occurred in the lining of a cystic space of a polycystic ameloblastoma. Two ameloblastomas contained eosinophilic granules in all tumor cells and melanocytes were diffusely present in another. One case exhibited a focus of mucous cell metaplasia. Two polycystic ameloblastomas showed diffuse interstitial ossification. One mandibular tumor was diagnosed as a desmoplastic ameloblastoma and another as an odontoameloblastoma. This study demonstrated that although ameloblastomas are regarded as a fairly homogeneous group of neoplasms, detailed investigations prove clinicopathologic diversity in a significant number of lesions.

Key words: ameloblastoma; infrequent findings; jaw tumors; odontogenic tumors.

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Ameloblastoma is the most common neoplasm affecting the jaws. It is derived from odontogenic epithelium and although located primarily intraosseously, peripherally occuring ameloblastomas involving soft tissue only have occasionally been reported. Two clinicopathologic variants of intraosseous ameloblastomas are commonly recognised. Polycystic ameloblastomas occur mainly in the body and ascending ramus of the mandible and most patients with this type present in the 4th decade of life (1). Unicystic ameloblastomas present on average a decade earlier (2) and are generally associated with a lower post-operative recurrence rate then the polycystic types. They can be divided microscopically into Groups 1, 2 or 3 depending on either the presence of a non-proliferating lining, intraluminal proliferations or mural invasion respectively (2). Ameloblastomas may occur more frequently in black Africans than in other racial groups (3).

Several recently published large series on ameloblastomas make no mention of coincidental and infrequent clinicopathologic findings (2–5) and most information about these is obtained through individual case reports. The secretion of interleukin-1 and a parathyroid hormone-like substance by an ameloblastoma was alleged to be the cause of hypercalcemia in one patient (6). A multilocular lytic mandibular lesion in a patient with hyperparathyroidism was proven microscopically to represent an ameloblastoma associated with a brown tumor of hyperparathyroidism (7). The occurrence of an ameloblastoma in a patient with the basal cell nevus syndrome (8) appears to be a sporadic rather than a regular feature. Other tumors that have been reported to be associated with ameloblastomas include the calcifying odontogenic cyst (9), acinic cell carcinoma and adenolymphoma of salivary gland origin (10), osteogenic sarcoma (11), traumatic neuroma (12) and aneurysmal bone cyst (13). HPV capsid antigen was proven positive with the immunoperoxidase staining technique in 3 out of 10 ameloblastomas in children (14) and mucormycosis infection was reported to have been superimposed on an ameloblastoma in an elderly diabetic woman (15). Stromal desmoplasia in a significant number of ameloblastomas has led to the use of the term 'desmoplastic ameloblastoma' (16) and extensive interstitial bone formation in ameloblastomas has recently been reported in two Japanese patients (17, 18). A case of papilliferous keratoameloblastoma was reported by ALTINI *et al.* (19) and other microscopic rarities include melanocytes between (20), and granular cell change in all tumor cells (21).

The purpose of this study was to determine the spectrum of uncommon clinical and pathological findings in a large sample of ameloblastomas diagnosed in a rural black African population.

Material and methods

Clinical records, radiographs and hematoxylin and eosin-stained microscopic slides of biopsies and surgical resections of 108 primary intraosseous ameloblastomas were scrutinized for extraordinary and coincidental pathologic features. At least four wax blocks were available in most cases. The following special staining techniques were employed on selected cases: Mucicarmine for epithelial mucins, Masson-Fontana for melanin, Perl's Prussian blue for hemosiderin pigment and the *in situ* hybridization technique for the presence of HPV antigen. All cases were diagnosed and treated in the Medunsa Dental Hospital which serves a black and mainly rural African community.

Results

The sample consisted of 108 ameloblastomas of which 75 were polycystic and 33 unicystic. All cases originated intraosseously. The sex and age distributions are shown in Table 1. All polycystic and 29 of the unicystic ameloblastomas occurred in the mandible and four unicystic ameloblastomas presented as maxillary swellings. The left mandible was involved in 65 cases, right mandible in 26 and symphysis area in 13 cases. Twelve polycystic and 4 unicystic ameloblastomas perforated the bony cortex and caused soft tissue ulceration. A mandibular ameloblastic fibroma in a 19-year-old woman was adjacent to and continuous with a polycystic ameloblastoma (Fig. 1). Aneurysmal bone cyst changes were identified in the latter patient as well as in another polycystic ameloblastoma in a 20-year-old woman. A polycystic ameloblastoma which involved the anterior mandible of a 13-year-old girl was associated with a compound odontome (Fig. 2).

Microscopically, 47 polycystic ameloblastomas were follicular, 23 plexiform and 5 were of mixed follicular and plexiform patterns. The stellate reticulum showed no differentiation in 35 cases, 22 cases presented with acanthomatous differentiation, 9 cases with granular cell differentiation, 6 cases with both granular cell and acanthomatous differentiation, and basal cell differentiation was seen in one case. A follicular ameloblastoma showed acanthomatous differentiation and foci of mucous cell metaplasia (Fig. 3). One tumor, which occurred in the mandible of a 57-year-old woman, showed extensive keratinisation and was diagnosed as a keratoameloblastoma. The unicystic ameloblastomas showed mural invasion in 15 cases and intraluminal proliferation in one case. The remaining 17 unicystic ameloblastomas were lined by non-infiltrative epithelium. In 9 of the latter group, less than 3 blocks were available for microscopic examination. Microscopic changes resembling an adenomatoid odontogenic tumor were presention the walls of two unicystic ameloblastomas (Fig. 4). HPV type 18 was identified in a verrucous lesion which occurred in a polycystic ameloblastoma and in one case melanocytes were uniformly present between the neoplastic epithelial cells. In two patients aged 15 and 26 years respectively, ameloblastic epithelium contained eosinophilic granules in all tumor cells (Fig. 5). Hemosiderin pigment was identified in the cytoplasm of neoplastic odontogenic epithelial cells next to an area of hemorrhage. A desmoplastic re-

Table 1. Sex and age distribution

	N	Sex M	Sex F	Average age (range in years)
Total sample	108	50	58	29.3 (8–84)
Polycystic	75	33	42	35.4 (13–84)
Unicystic	33	17	16	16.5 (8–37)

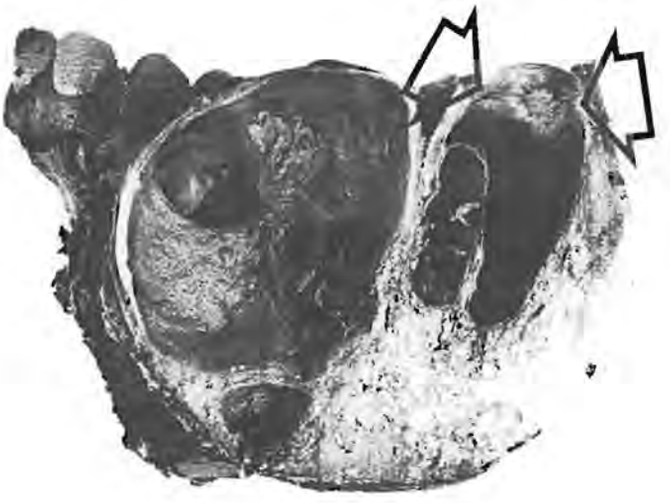

Fig. 1. A resected specimen showing a polycystic ameloblastoma associated with an ameloblastic fibroma (arrows).

Fig. 2. Panoramic radiograph of the odontoameloblastoma.

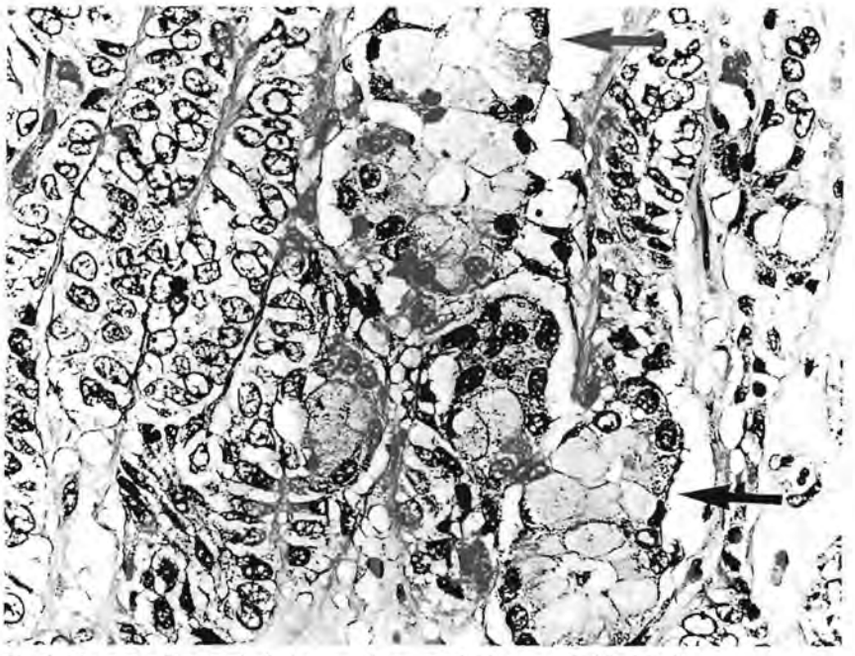

Fig. 3. Mucous-cell metaplasia (arrows) in an ameloblastoma (H&E, X240).

action was a common feature in the extraosseous component of ameloblastomas which perforated the bony cortex. Desmoplasia of the intrabony part of ameloblastomas was variable both between tumors and within the same tumor and depended upon the degree of inflammation. Only one case was diagnosed as a desmoplastic ameloblastoma on the basis of a uniform and mature connective tissue proliferation in the absence of inflammation and which impinged upon the neoplastic epithelial component (Fig. 6). Two polycystic ameloblastomas were associated with diffuse interstitial bony deposits which led to radiographic diagnoses of fibro-osseous lesions.

Discussion

Large series published on ameloblastomas often make no mention of features other than those classically described and most infrequent findings are re-

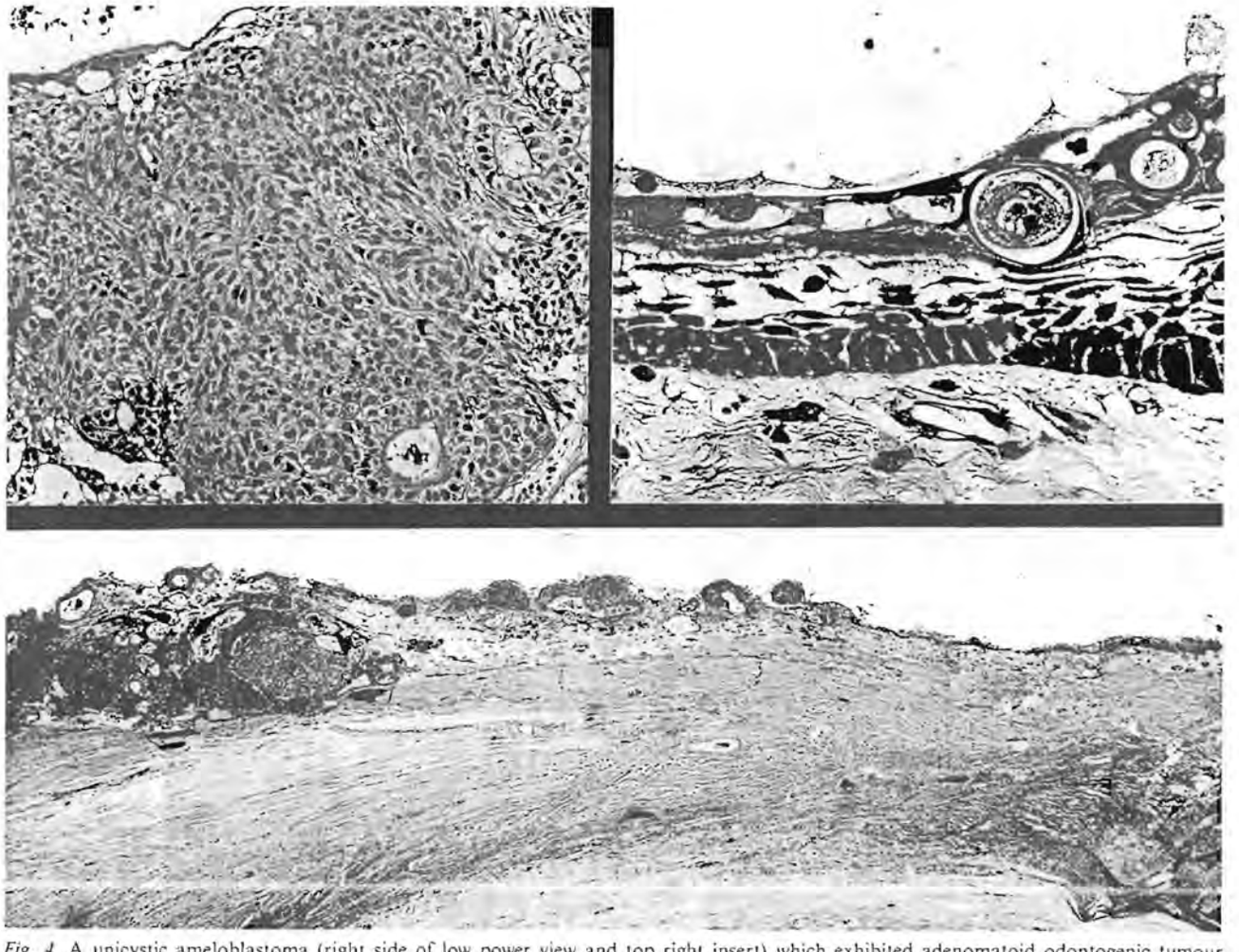

Fig. 4. A unicystic ameloblastoma (right side of low power view and top right insert) which exhibited adenomatoid odontogenic tumour differentiation (left side low power view and top left insert). (H&E, ×40, X120, X200).

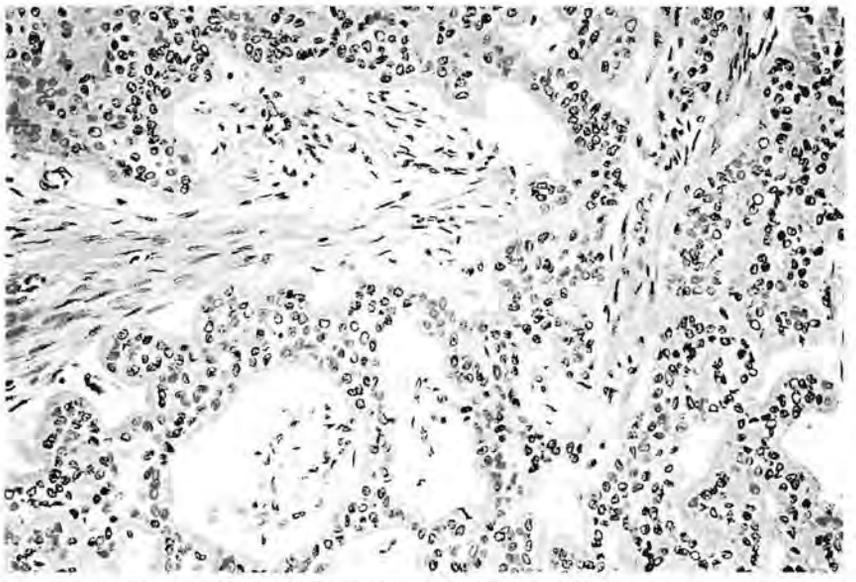

Fig. 5. A plexiform proliferation of ameloblastic epithelium exhibiting granules in all tumour cells. Typical features of ameloblastoma were not present (H&E, ×180).

Fig. 6. Desmoplastic ameloblastoma characterized by compressed epithelial strands in a mature connective tissue stroma. Typical features of ameloblastoma were not present (H&E, ×180).

ported as case studies. This has led to a generally accepted view that ameloblastomas are fairly homogeneous in their clinical and pathologic presentation. This review was undertaken to establish the spectrum of extraordinary and coincidental clinical and pathologic findings in a large collection of ameloblastomas diagnosed in a rural African population.

In the total sample, the left mandible was affected 2.5 times more commonly than the right. Although there appears to be no apparent explanation for left mandibular predominance in our study, this finding is supported by a large Japanese series in which only 40% occurred on the right hand side (4).

The subclassification of unicystic ameloblastomas according to the microscopic appearance of the lining (2) was found to be impractical. Although areas of intraluminal proliferation or mural invasion positively confirmed unicystic ameloblastomas in Groups 2 and 3 respectively, the subdivision into Group 1 lesions was found to be of limited value unless the whole tumor was processed for microscopic examination. In the case of large unicystic ameloblastomas, this was either impractical or even impossible, making the microscopic identification of foci of mural invasion in larger lesions unlikely. This dilemma is clearly illustrated in our study where fewer than three wax blocks were available for microscopic examination in 9 cases ultimately subclassified as Group 1 unicystic ameloblastomas.

Tumors which occur within bone generally predispose to pathologic fracture and aneurysmal bone cyst formation (22). Both these findings are, however, infrequently reported in association with ameloblastomas. The presenting symptom in only three of our patients was directly associated with pathologic fracture of the mandible. Aneurysmal bone cysts are reported to be rare in the jaws, occur mainly in young patients, and approximately one-third are found in association with other pathologies (23). A microscopic study of 42 ameloblastomas found hallmarks of aneurysmal bone cysts in 7 (13). Although the frequencey of aneurysmal bone cyst change in our study was not as high, both our cases occurred in young patients. The coexistence of aneurysmal bone cyst with ameloblastoma is significant because of the excessive bleeding which may be encoutered during surgery.

The association of an ameloblastic fibroma with ameloblastoma has not previously been reported. The example described here could be regarded as coincidental, as the patient was at an age when ameloblastic fibroma occurs most frequently. Simultaneous occurrence of ameloblastoma and odontoma is rare (24). These tumors, which have been designated as odontoameloblastomas, consists of epithelial proliferations typical of ameloblastomas associated with highly differentiated dental tissues either scattered throughout the tumor or, as in our case, as a single radiopaque mass (1). Squamous metaplasia is a well described and variable feature in ameloblastoma. Extensive squamous change, where follicles consist entirely of squamous epithelium with only traits of the original ameloblastomatous structure, is less frequent (23). One tumor in our series, which occurred in an elderly woman patient, exhibited this change and was diagnosed as a kera-

toameloblastoma. Although adenomatoid odontogenic tumor-like differentiation has been reported in a dentigerous cyst (25), this change in a unicystic ameloblastoma has not yet been described. Both our examples occurred in the anterior mandible and this, together with the hitherto undescribed phenomenon of mucous cell metaplasia, illustrates the differentiation protential of ameloblastic epithelium.

The presence of an HPV-induced verucca in the epithelial lining of a polycystic ameloblastoma adds an interesting parameter to the study of papillomavirus-induced epithelial proliferations. These lesions occur commonly on the skin and lining mucosa, to our knowledge this case, which was published recently (26), represents the first description of a verucca in a cystic epithelial tumor, the lining of which was within bone and not in contact with the oral mucosa. The origin of the melanocytes in odontogenic tumors and cysts is speculative. The cells of the neural crest interact in the development of teeth and it is not surprising that melanocytes can occur in odontogenic tissues. Although it is believed that melanotic odontogenic tumors occur more frequently in black patients (25), the number of cases reported is too low to draw conclusions. Our one melanotic ameloblastoma in the 108 cases studied places the frequency of this phenomenon below 1% in a black African sample and thus can hardly be regarded as common. The presence of hemosiderin in the neoplastic epithelium adjacent to an area of hemorrhage emphasizes the phagocytic capacity of neoplastic odontogenic epithelium. Varying amounts of granular cells are seen in the stellate reticulum of granular cell ameloblastomas. However, the presence of granules in all cells, including the peripheral layer, is rare. We believe this feature, which was present in two of our cases, represents full expression of granular cell differentiation. The plexiform granular cell odontogenic tumor reported by ALTINI *et al.* (1986) (27) is probably an example of this variant.

Quantification of stromal desmoplasia was found to be difficult as it varied between tumors and within the same tumor. Generally, ameloblastomas which infiltrate soft tissue and those which are inflamed exhibit more desmoplasia than others. Terminology such as "desmoplastic ameloblastoma' (16) should be used with great circumspection as the intensity of the desmoplasia is variable and forms part of a continuous spectrum of stromal fibroplastic reaction in ameloblastomas. We suggest that certain criteria must be applied before the diagnosis of a desmoplastic ameloblastoma is made. These should include the absence of inflammatory changes and cortical bone perforation as well as the uniform presence of a mature, diffuse collagenous stromal tissue compressing the neoplastic epithelial component into strands. The two tumors which contained extensive interstitial bony deposits resemble the Japanese cases published recently (17, 18) and their clinical and radiographic differentiation from benign fibro-osseous proliferations is a pitfall to be avoided. The prominent bone formation appeared to be reactive in nature, probably linked to a process of interstitial connective tissue metaplasia rather than a result of induction, as the bony deposits were generally separated from the neoplastic epithelium by a broad band of inactive connective tissue. Unlike a recently published paper (28), we believe the desmoplastic and osteoplastic ameloblastomas are not distinct clinicopathologic entities but rather variants of the microscopic spectrum of stromal reactions in ameloblastomas.

This study demonstrates that although ameloblastomas are generally regarded as a homogeneous group of neoplasms, detailed investigations prove clinicopathologic diversity in a significant number of tumors. Many of these changes emphasize the differentiation potential of neoplastic odontogenic epithelium and add interesting parameters to the study of tissue reactions associated with this common odontogenic tumor.

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Detection of human papillomavirus DNA in an ameloblastoma using the in situ hybridization technique

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Van Heerden WFP, van Rensburg EJ, Raubenheimer EJ, Venter EH: Detection of human papillomavirus DNA in an ameloblastoma using the in situ hybridization technique.

HPV type 18 DNA was identified in an intrabony ameloblastoma using radiolabelled in situ hybridization. The viral DNA was found in a verrucous lesion in a cystic area of the tumor. The absence of HPV DNA in other epithelial areas of the ameloblastoma is suggestive of a secondary infection. HPV is not considered to be an etiological factor in the pathogenesis of this ameloblastoma.

Key words: ameloblastoma; DNA; human papillomavirus type 18; in situ hybridization

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Human papillomaviruses (HPVs) are DNA viruses that infect only squamous epithelium at selected locations in the skin and mucosa. The virus usually infects the basal cell layers and viral DNA are observed in low copy numbers in these cells. An increase in copy numbers of replicating viral DNA are found in the more differentiated epithelial cells while production of viral particles is restricted to the fully differentiated superficial epithelial cells (1). Since this state of differentiation has not yet been achieved in culture, it has not been possible to reproduce HPV in the laboratory to study their natural life cycle (2). These viruses induce papillomatous, hyperplastic or verrucous lesions depending on the site of infection and the HPV type implicated. HPV involvement in upper respiratory and digestive tract lesions like focal epithelial hyperplasia, squamous cell papillomas, laryngeal papillomas, leukoplakia and squamous cell carcinoma has been demonstrated by means of immunohistochemical, DNA hybridization and polymerase chain reaction studies (2–6). More than 60 types of HPV have been isolated to date, of which types 1, 2, 4, 6, 7, 11, 13, 16, 18, 32 and 57 were found in the different oral lesions (7).

The association between HPV and odontogenic tumours and cysts has not been studied to a great extent. HPV 16 DNA has been demonstrated in an odontogenic keratocyst using Southern blot hybridization (8), while KHAN found HPV capsid antigen in 3 out of 10 ameloblastomas in young persons (9). The purpose of this study was to investigate an ameloblastoma with typical HPV histologic changes for HPV DNA using the in situ hybridization technique. This is a sensitive technique and has the advantage of localizing viral DNA in tissue sections to the extent of detecting them at the resolution of single cells.

Material and methods

A 25-yr-old man reported to the Maxillofacial and Oral Surgery clinic complaining of a painless, bony hard swelling in the anterior mandible. Examination showed a tumor extending from the right mandibular angle to the contralateral first molar region. Expansion of the lingual and buccal cortices was evident with thinning and erosion of the buccal cortex in the right premolar area. Radiographs showed a well-circumscribed, multilocular lesion with root resorption of the associated teeth. No signs of mucosal ulceration were present.

After an incisional biopsy, a diagnosis of a follicular aneloblastoma was made and the tumor was resected through an intraoral approach. The specimen was fixed in 10% buffered formalin for pathologic examination.

Macroscopic examination showed a gray-white solid tumor with cystic areas of varying size. A papillomatous lesion presenting as a luminal growth was present in one cyst. Microscopy revealed a follicular ameloblastoma with acanthomatous as well as granular cell differentiation (Fig. 1). The papillomatous lesion showed verrucous hyperplasia with hyperparakeratosis, elongation of the rete-ridges and groups of koilocytes lying in the upper part of the epithelium. These features resembled those of a verruca vulgaris (Fig. 2).

The biopsy material containing the papillomatous lesion, as well as blocks exhibiting the characteristic ameloblastomatous epithelium and including areas of granular cell and acanthomatous differentiation, were examined for the presence of HPV antigen using an ABC immunoperoxidase kit for the HPV group specific antigen (Lipshaw Corporation, Detroit) as well as in situ hybridization.

For HPV typing, the specific DNA probes of HPV 2, 6, 7, 11, 13, 16, 18 and 30 cloned in either pBR322 or pUC19 were used (kindly provided by Dr E-M de Villiers, Human Papillomav-

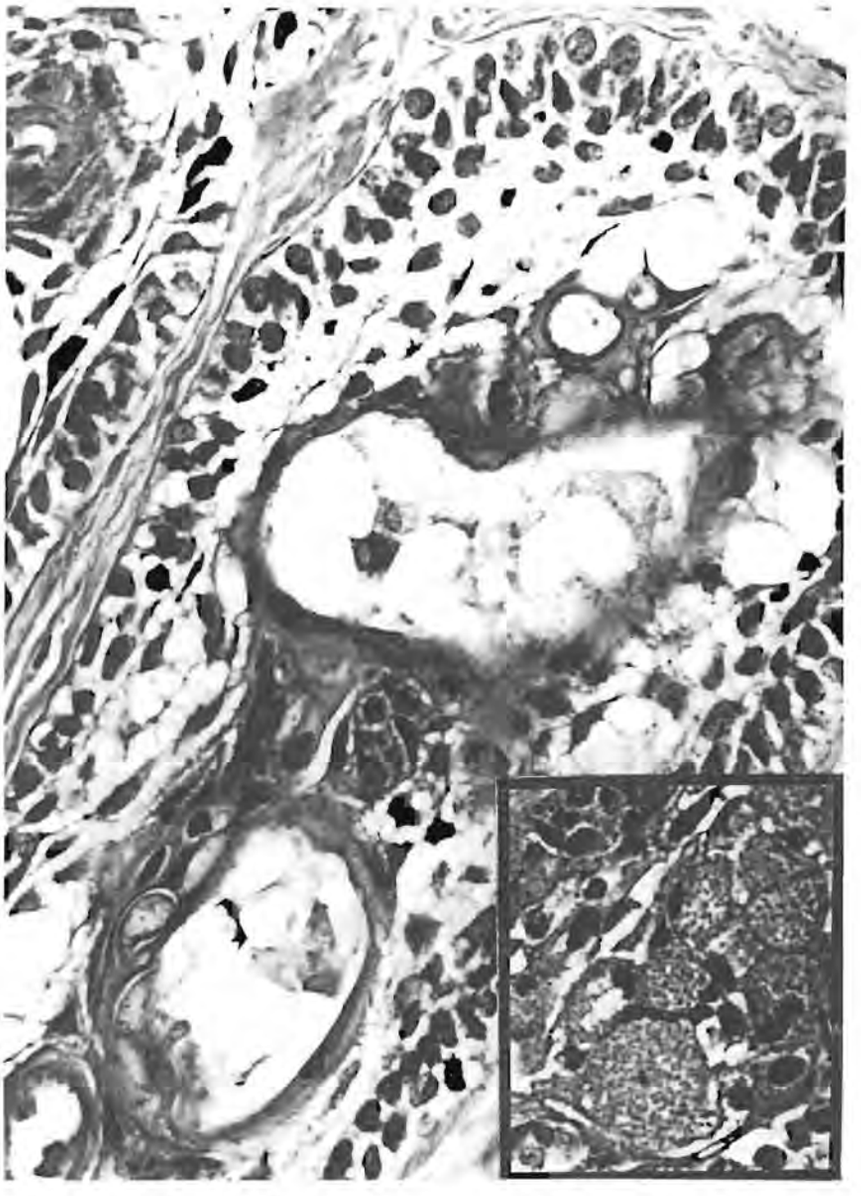

Fig. 1. Follicular ameloblastoma with central acanthomatous differentiation. HE × 200. *Inset.* Areas of granular cell differentiation. HE × 200.

irus Reference Centre, DKFZ, Heidelberg, Germany). The probes were labelled with ^{32}p dCTP using the multiprime DNA labelling system (Amersham, U.K.). The labelled probes had a specific activity of $2–5 \times 10^8$ counts/min/μg of DNA. For in situ hybridization 5–10 ng of each probe was used on individual sections.

pBR322 and pUC19 vectors served as negative control probes. Two paraffin embedded tissue sections (a cervical intraepithelial neoplasia and vulvar carcinoma positive for HPV 6 and 16 respectively) were used as positive control slides.

The tissue sections were incubated at 60°C overnight using 3-aminopropyltriethoxysilane coated slides (10). Slides were deparaffinised and rehydrated by sequential immersion into xylene (3 × 10 min) and ethanol. They were then incubated in 0.2 N HCl for 20 min at room temperature and transferred to 2 × SSC at 70°C for 10 min. The tissue sections were digested with a 2 × SSC, 0.1% SDS buffer solution containing Proteinase K (Boehringer, Mannheim, Germany) at a concentration of 0.01 μg/ml at 37°C for 30 min. Sections were post-fixed for 5 min in 4% paraformaldehyde, 2 × SSC and 5 mM $MgCl_2$; 5 min in 50% formamide, 2 × SSPE and acetylated (2 × 5 min). The slides were prehydridised (50% formamide, 10% dextran sulfate, 2 × SSPE, 100 mM glycin, 0.1% SDS, 2% 50 × Denhardts, 10 mM Tris pH 7.4 and 200 μg/ml salmon sperm DNA) for 30 min at 52°C prior to the application of the probe solution. Heat-denatured probe solution (either HPV 2, 6, 7, 11, 13, 16, 18 or 30) was added to each section and slides were incubated for 16 hours at 52°C in a humidified chamber.

Following hybridisation, the slides were washed twice in a 2 × SSPE/50% formamide solution and once in 50% fomamide, 0.1% SDS, 2 × SSC, each wash for one hour at 37°C. Slides were dehydrated through graded ethanols containing 0.3 M NH_4 acetate. Slides were dipped in LM-1 emulsion (Amersham, UK), following instructions of the manufacturer. After exposure, slides were developed, fixed and counterstained with hematoxylin-eosin. The presence of HPV DNA sequences in the lesions was indicated by the condensations of black silver grains superimposed on the nuclei of cells.

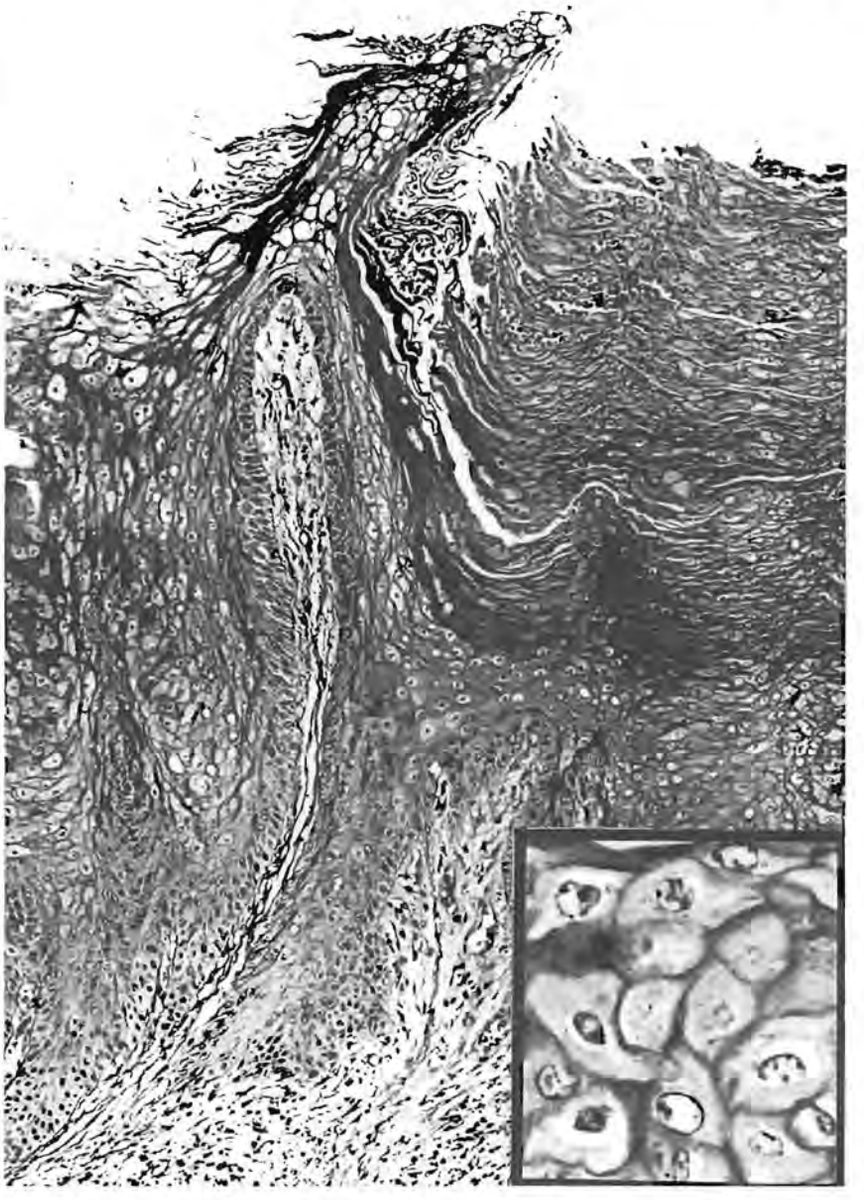

Fig. 2. Micrograph of the papillomatous lesion showing parakeratosis, epithelial papillary processes and elongation of rete-ridges. HE × 100. *Inset.* High power detail of koilocytes showing enlarged cells with slightly irregular nuclei surrounded by a halo. HE × 250.

Results

Immunohistochemical examination of both the papillomatous lesion and typical ameloblastoma areas was negative. The in situ hybridization technique revealed HPV DNA type 18 in the papillomatous lesion (Fig. 3). The blocks containing the typical ameloblastoma features, including foci of granular cell and acanthomatous differentiation, were negative for the HPV DNA types examined.

Discussion

Radiolabelled HPV DNA in situ hybridization was used instead of the more commonly used biotinylated DNA in situ hybridization because it is a more sensitive method to detect HPV DNA (11). The sensitivity of the radiolabelled HPV DNA probe is 20–100 genome copies per cell compared to the 100–800 genome copies per cell of the biotin-labelled HPV DNA probe (1). The negative immunohistochemical staining in our study may be due to the fact that this technique identifies only the productive phase of the viral infection. Furthermore, as this method is based on an antigen-antibody reaction, the target antigenic determinants may be distorted by heating in paraffin, fixation in formalin or digestion by trypsin (12).

The presence of HPV type 18 DNA in a primary intrabony tumor of odontogenic epithelial origin is difficult to explain. Contact between the tumor epithelium and the oral mucosa may have facilitated cross-infection between oral epithelium and the ameloblastoma. Although no ulceration of the oral mucosa or skin was noted in this patient, expansion of the buccal and lingual cortices with erosion of the buccal cortex in the right premolar area were present. This area corresponded with the location of the papillomatous lesion in the ameloblastoma. The specimen was thoroughly examined for the presence of similar lesions without success, supporting the link between the HPV-associated lesion and the eroded bone cortex. Direct contact between tumor epithelium and surface epithelium could not be demonstrated.

Cox *et al.* demonstrated HPV 16 DNA in an odontogenic keratocyst

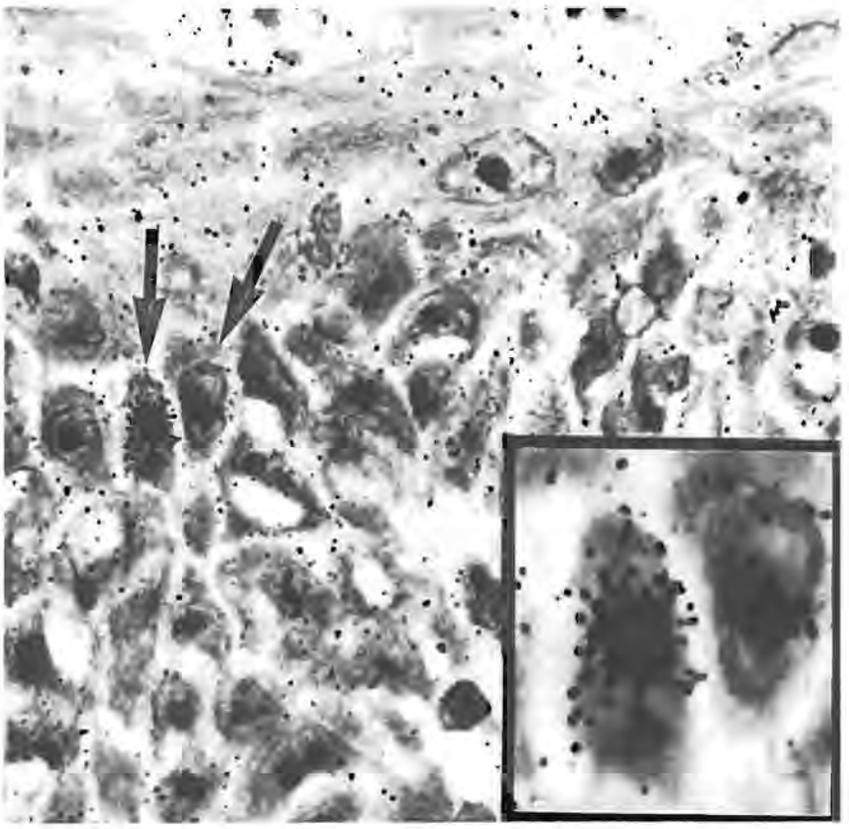

Fig. 3. Micrograph of the verrucous lesion subjected to in situ hybridization shows the presence of HPV 18 DNA as condensations of black silver grains superimposed on the nuclei (arrows). ×200. *Inset.* High power detail of positive cells. ×400.

lacking the typical histologic features of an HPV infection (8). This HPV was implicated in the pathogenesis of the odontogenic keratocyst because the keratin-producing lining of the cyst provided squamous epithelium for viral persistence as well as completion of the virus life cycle. HPV 18 has an even higher oncogenic potential than HPV 16 (13), and has also been detected in oral epithelial dysplasias and oral squamous cell carcinomas (14). In our case HPV DNA was detected only in the solitary papillomatous lesion and not in the other epithelial regions permissive for viral infections, i.e. the acanthomatous and granular cell areas. We feel that the restriction of HPV DNA positivity to the verrucous lesion represents a secondary infection and is therefore not an etiological factor in the pathogenesis of this ameloblastoma.

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Journal of the Dental Association of South Africa

Clinico-pathological study of 30 unicystic ameloblastomas

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Keywords: ameloblastoma; odontogenic tumour.

SUMMARY

The clinico-pathological records of 30 unicystic ameloblastomas collected over a period of 10 years were studied. The mean age at diagnosis was 18,0 years (SD ±8,1), most lesions were located in the mandible and were frequently associated with impacted teeth, root resorption and tooth displacement. The unicystic ameloblastomas in 11 patients (4 females and 7 males) exhibited invasion of the fibrous wall, 4 cases (1 female and 3 males) showed intra-luminal proliferation and the remaining 15 specimens (9 females and 6 males) were lined by non-proliferating ameloblastic epithelium. Two cases recurred 3 and 7 years after initial surgical removal. This study reveals the potential aggressive behaviour of unicystic ameloblastomas and underlines the importance of a thorough microscopic examination for sub-classification.

OPSOMMING

Die klinies-patologiese rekords van 30 unisistiese ameloblastome, wat oor 'n tydperk van 10 jaar versamel is, is bestudeer. Die gemiddelde ouderdom by diagnose was 18,0 jaar (SD ±8,1). Die meeste letsels was in die mandibula en was met geïmpakteerde tande, wortelresorbsie en tandverplasing geassosieer. In 11 pasiënte (4 vroulik, 7 manlik) het die unisistiese ameloblastome die fibreuse wand binnegedring, in 4 gevalle (1 vroulik, 3 manlik) was intraluminale proliferasie teenwoordig en in die oorblywende 15 gevalle (9 vroulik, 6 manlik) was die sist deur 'n nie-prolifererende ameloblastiese epiteel. In twee gevalle het herhaling onderskeidelik 3 en 7 jaar na die aanvanklike chirurgiese verwydering voorgekom. Hierdie studie bevestig die potensiële aggressiewe gedrag van unisistiese ameloblastome en beklemtoon die noodsaaklikheid van deeglike mikroskopiese ondersoek vir subklassifikasie.

INTRODUCTION

The unicystic ameloblastoma is a unilocular, cystic epithelial odontogenic tumour initially described by Robinson and Martinez in 1977. Males and females are affected approximately equally. The lesions usually occur in the mandible and especially in the molar-ramus area, while the maxilla is only occasionally affected (Ackerman, Altini and Shear, 1988). They usually occur in the second to fourth decades and the mean age at the time of diagnosis is reported to be 23,8 years. The lesions appear radiologically as a well defined unilocular radiolucency of varying size (Ackermann *et al.*, 1988). When associated with an unerupted tooth, the appearance closely resembles a dentigerous cyst. Involvement of the roots of teeth may give it a radicular cyst-like appearance (Lucas, 1984) and in many cases can only be distinguished from odontogenic keratocysts by microscopic examination. Unicystic ameloblastomas are divided into three groups. Group 1 include the simple cystic lesions lined by an epithelium that does not infiltrate into the fibrous cyst wall. Lesions in Group 2 show intra-luminal epithelial proliferation and the epithelium in Group 3 lesions invade the fibrous cyst wall. Group 1 and 2 lesions may be treated by enucleation, whereas Group 3 lesions should be treated more radically to prevent recurrences (Ackermann *et al.*, 1988).

As a rule unicystic ameloblastomas behave more aggressively than other odontogenic cysts. It is important therefore to recognise the clinical features which may facilitate an accurate diagnosis of the condition. This study was undertaken to determine the clinico-pathological features of unicystic ameloblastomas in a rural black population.

MATERIALS AND METHODS

Microscopic sections of all unicystic lesions that were biopsied between 1982 and 1992 at Medunsa were retrieved and re-evaluated. The unicystic ameloblastomas were subdivided into three groups using the criteria of Ackermann *et al.*, 1988. Clinical data and radiographs were obtained from patient files. The site of occurrence was designated as molar-ramus, premolar or incisor according to the centre of the radiolucent lesion on a panoramic radiograph.

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RESULTS

Thirty cases were diagnosed as unicystic ameloblastoma, 16 were males and 14 females. The mean age of the patients was 18,0 years (SD =±8,1) (range 8-43 years) and 63,3 per cent occurred in the second decade (Fig. 1). Twenty seven of the lesions were present in the mandible and only 3 in the maxilla (Fig. 2). The lesions varied in size from 2,5-12 cm mesio-distally and 22 of the lesions were more than 5 cm in diameter on the panoramic radiographs. One mandibular tumour was associated with a pathological fracture.

Radiologically, 11 of the lesions were associated with impacted teeth (Fig. 3), 13 with root resorption, 15 with tooth displacement and 8 with tooth displacement and root resorption. The impacted teeth associated with the lesions were mainly the mandibular third molars (n=7), followed by mandibular second molars (n=3) and mandibular canines (n=2). Two of the 3 maxillary lesions presented in the "globulo-maxillary" area. Of the 14 lesions in females, 9 were classified as Group I (Fig. 4), 1 as Group II (Fig. 5) and 4 as Group III. There were 6 Group I lesions and 7 Group III lesions amongst the 16 males, the remaining 3 were Group II lesions (Fig. 6).

In 7 unicystic ameloblastomas, 50 per cent or more of the lining was a nondescript type of epithelium (Fig. 7). Three out of 4 Group III lesions had a plexiform intra-luminal proliferation, the other had multiple mural nodules projecting into the lumen (Fig. 8). Inflammation in 3 lesions was associated with extensive epithelial arcading and 4 showed sub-epithelial hyalinization. Two cases recurred as polycystic ameloblastomas 3 and 7 years after surgical treatment respectively. The first was originally classified as Group III (Fig. 9), whereas the other case was a Group I unicystic ameloblastoma.

DISCUSSION

Since the original description of unicystic ameloblastoma (Robinson and Matinez, 1977) various reports on the aggressive behaviour of this cystic lesion have appeared (Ackermann *et al.*, 1988; Gardner, Morton and Worsham, 1987; Kahn, 1989; Keszler and Dominques, 1986; Punnia-Moorthy, 1989). Two patients in our study, which extends over a period of 10 years, presented 3 and 7 years after initial surgery with recurrences. Both recurrences exhibited the growth pattern of a polycystic ameloblastoma. Although Ackermann *et al.*, (1988) propose a more radical form of treatment for Group III lesions, a Group I lesion recurred in our study and this emphasizes the potentially aggressive behaviour of all unicystic ameloblastomas and

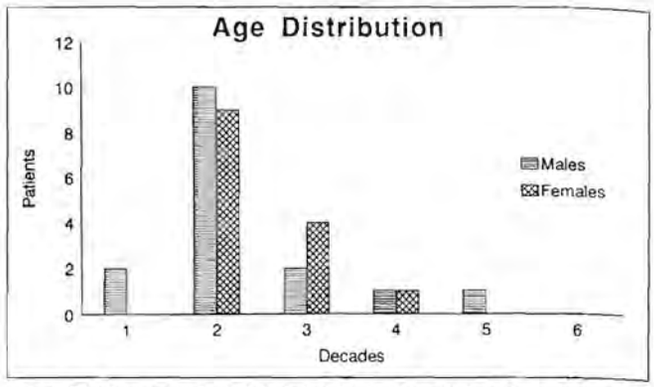

Fig. 1: The histogram of the age distribution of males and females.

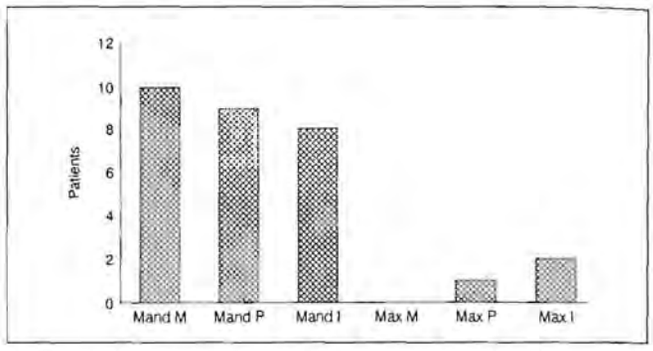

Fig. 2: The site distribution of 30 unicystic ameloblastomas. Mand=mandibular, Max=maxilla, M=molar ramus area, P=premolar area, I=incisor area.

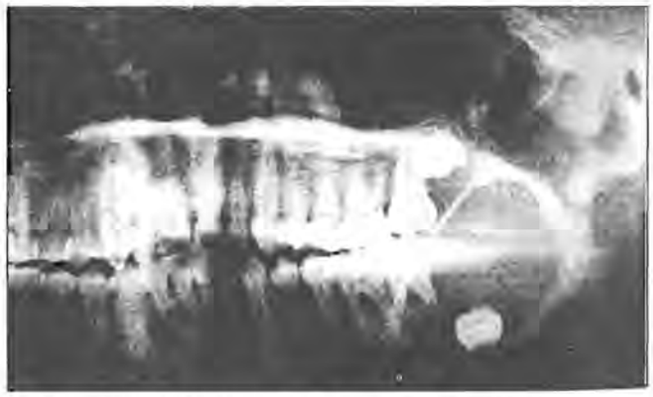

Fig. 3: Panoramic radiograph of an unicystic ameloblastoma associated with an impacted mandibular third molar.

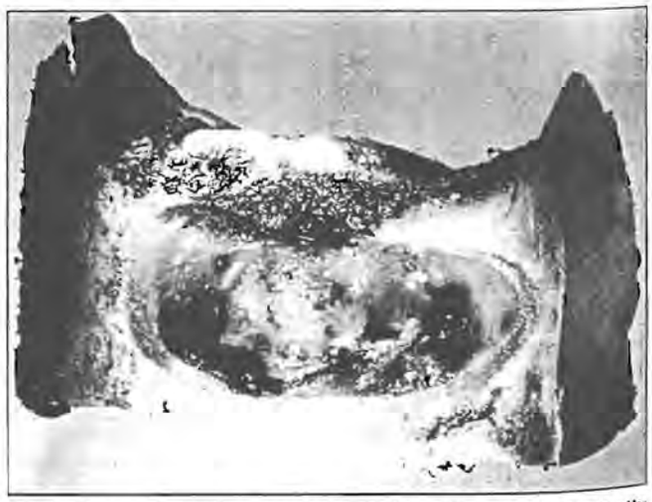

Fig. 4: Section through a resected mandible with a microscopically confirmed Group I lesion. Note the simple unicystic cavity.

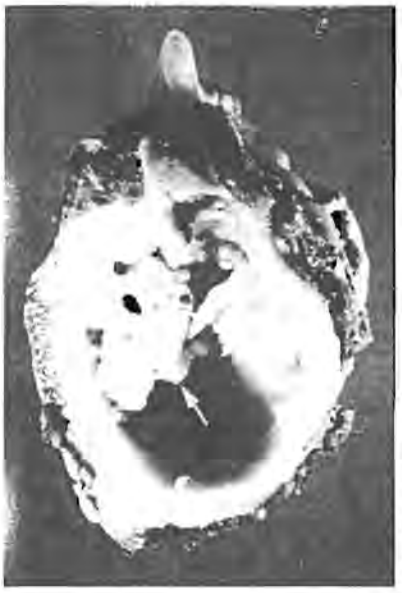

Fig. 5: Cross section through a microscopically confirmed mandibular Group II lesion. Note the intra-luminal proliferation (arrow).

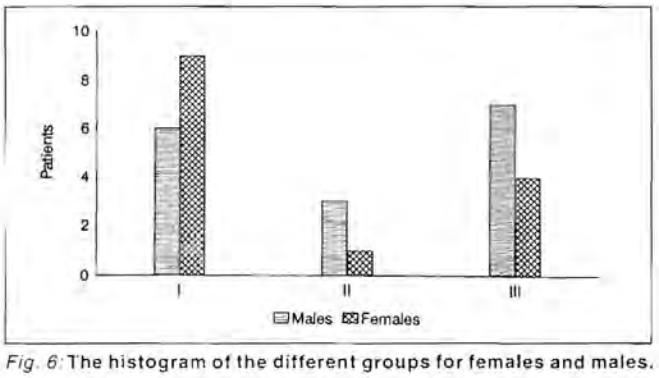

Fig. 6: The histogram of the different groups for females and males.

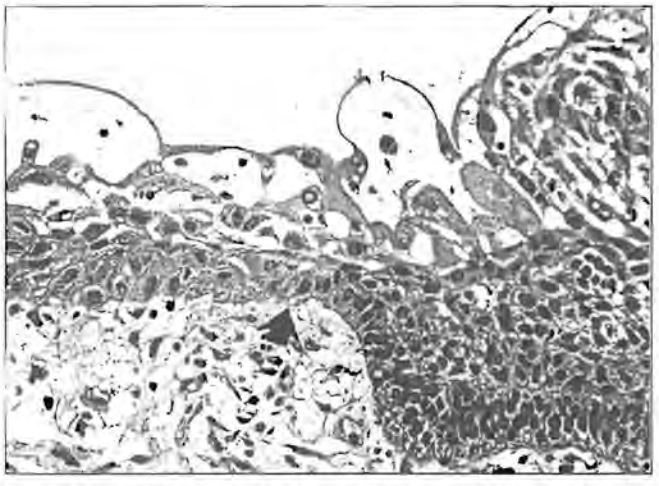

Fig. 7: Photomicrograph of a Group I lesion. Note the nondescript epithelium (left) and the sharp transition (arrow) to typical ameloblastic epithelium. (HE, X300).

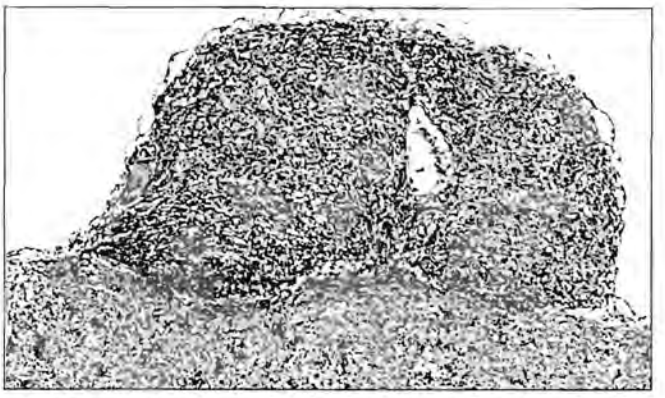

Fig. 8: The lining of a Group II lesion showing an intra-luminal nodular proliferation (HE, X100).

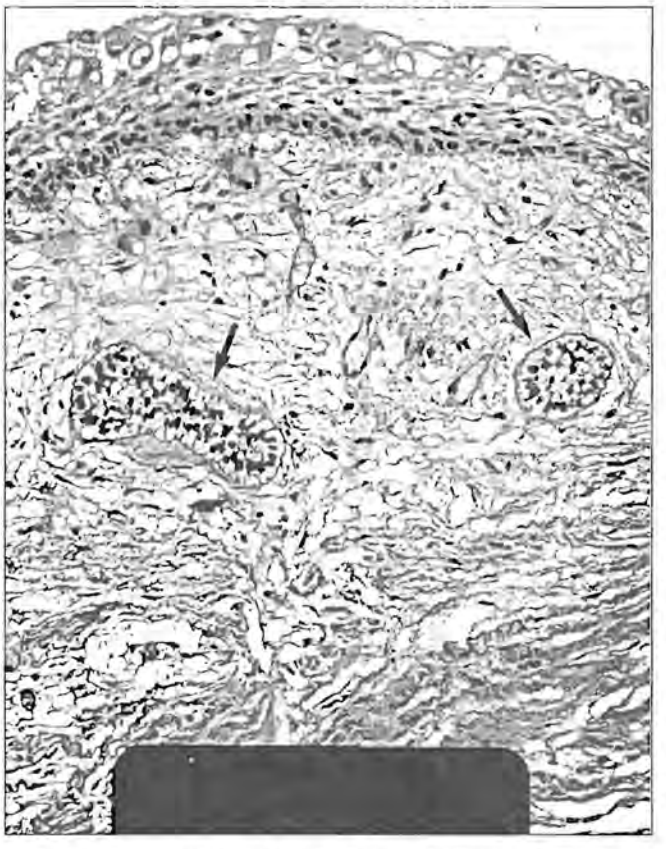

Fig. 9: The lining of the Group III lesion that recurred. Note the islands of ameloblastic epithelium in the connective tissue wall (arrows). (HE, X180).

highlights the importance of complete surgical removal. This recurrence may, on the other hand, reflect an inherent weakness in the proposed sub-grouping of unicystic ameloblastomas. If the whole cyst wall is not examined microscopically, an exercise which is highly impractical if not impossible in larger examples, mural invasion cannot be excluded categorically. The diagnosis of an unicystic ameloblastoma on a small biopsy specimen is not recommended. Moreover, the frequent occurrence of nondescript epithelium and inflammation may mask the typical characteristics of the ameloblastic epithelial lining. This microscopic sub-classification of unicystic ameloblastomas should therefore not be attempted on anything less than a thorough microscopic examination of the whole cyst wall. After such an examination the number of lesions placed in Group III would probably increase.

Our study supports the finding that there is an equal sex distribution for the unicystic ameloblastoma as well as a tendency for it to occur in young patients. Our cases however, presented on average 6 years earlier than those of Ackermann *et al.*,

(1989), probably because patients were seeking treatment sooner and had easier access to the hospital in the years 1981-1991. Most tumours occurred in the mandible and maxillary involvement was less common. A large number of mandibular lesions could be easily mistaken for dentigerous cysts, because of their association with impacted molars and canines. This is related further to the frequent occurrence of root resorption, a feature often found in dentigerous cysts (Shear, 1992). In order to establish a correct diagnosis, microscopic examination of all cystic jaw lesions is mandatory.

Group I lesions predominated in our study and then followed Group III and lastly Group II unicystic ameloblastomas. This is in contrast to Ackermann's 1988 study in which Group III lesions were most frequent. The ratio between female and male in Group I lesions was 1,5:1 and in Group III lesions 1:1,75. The significance of this finding is not known.

It is important to note that all unicystic ameloblastomas, irrespective of grouping, are neoplastic in nature and will recur if incompletely removed.

Although limited evidence is available on recurrences of unicystic ameloblastomas, it appears as if the latter may present either as a regrowth of the original unicystic lesion or as a multicystic ameloblastoma.

ACKNOWLEDGEMENT

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Serving Dentistry — ISO TC106

The 30th meeting of the International Standards Organisation's Technical Committee took place in Ottawa, Canada from 10-15 October 1994.

The countries represented were Australia, Brazil, Canada, China, France, Germany, Hong Kong, Italy, Japan, Netherlands, Norway, South Africa, Switzerland, Sweden, Thailand, United Kingdom and USA. The new South African flag was given a place of honour at the centre of the display seen in our photograph, which was taken at the conclusion of the opening ceremony. **Dr John Stanford,** Chairman of the Committee since 1991 is seen in the front of the picture with his predecessor, **Prof Pierre Laplaud** (1982-1990). the South African Bureau of Standards is a Participating Member of the Committee and Dr Heydt was their representative at the meeting. A detailed report on the work of the Committee will appear in a forthcoming issue of the ***JOURNAL***.

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JOURNAL OF CRANIO-MAXILLO-FACIAL SURGERY

A retrospective analysis of 367 cystic lesions of the jaw—the Ulm experience

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SUMMARY. **Out of 846 cyst-like lesions of the jaws, 367 cases were retrieved from the files of the Department of Oral and Maxillofacial Surgery at the University of Ulm and classified according to the new World Health Organization's classification for odontogenic tumours and cysts. Radicular and residual cysts comprised 56.9%, dentigerous cysts 21.3%, odontogenic keratocysts 10.6%, unicystic ameloblastomas 4.1%, nasopalatine duct cysts 2.7%, glandular odontogenic cysts 1.6% and paradental cysts, traumatic bone cyst, calcifying odontogenic cyst and lateral periodontal cyst each less than 1% of the sample. Nearly one third of the specimens were obtained from military patients; despite an expected bias towards young males, unicystic ameloblastomas presented one and a half decades later than is generally reported.**

INTRODUCTION

Before the recent adoption of the revised World Health Organization's classification of odonotogenic tumours and cysts (*Kramer* et al., 1992), epidemiological studies on cystic jaw lesions were difficult to interpret due to the omission of recently described entities, which had not been taken up in any classification system. Examples of these are the paradental cyst which arises from odontogenic epithelial residues stimulated into activity by inflammation (*Craig*, 1976) and the aggressive glandular odontogenic cyst, the exact origin of which is less clear (*Shear*, 1992). The calcifying odontogenic cyst, which is now classified as an odontogenic tumour, occurs both in neoplastic and cystic subtypes (*Hong* et al., 1991). Unicystic ameloblastomas are divided into three subtypes, a division which is based on the histological nature of its epithelial lining. Type I unicystic ameloblastomas exhibit a simple ameloblastic epithelial lining whereas Type II shows intraluminal proliferation and Type III mural invasion. The latter type is reported to be associated with a higher recurrence rate (*Ackermann* et al., 1988).

The purpose of this study was to revise and reclassify cystic lesions of the jaws diagnosed and treated in the Department of Oral and Maxillofacial Surgery, University of Ulm, over the last 5 years.

MATERIAL AND METHODS

The clinical examination forms and radiographs of all cystic lesions affecting the jaws were retrieved from the files of the Department of Oral and Maxillofacial Surgery at the University of Ulm, Germany. 367 out of 846 microscopic sections were supplied for re-examination by the Department of Pathology at the Bundeswehrkrankenhaus Ulm as well as from the University Department. Only cases on which clinical information, a radiograph and representative microscopic sections where available, were included in the study. Each case was re-evaluated and classified independently according to the criteria set in the second edition of the World Health Organisations classification of jaw cysts and tumours (*Kramer* et al., 1992) by two oral pathologists.

RESULTS

367 cases were included in the study and 22 excluded due to a lack of radiographs and/or unrepresentative microscopic sections. Nearly one third of the cases

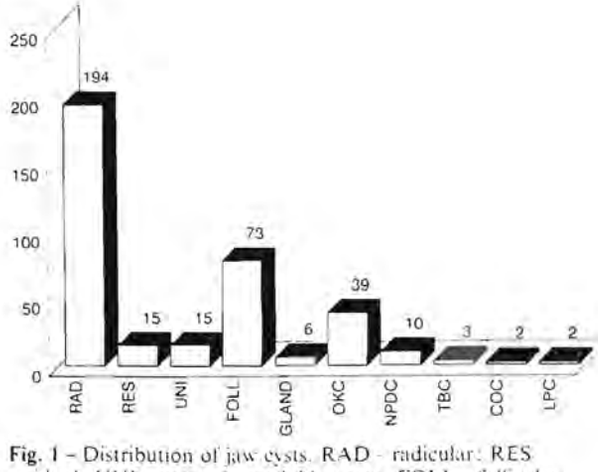

Fig. 1 – Distribution of jaw cysts. RAD – radicular; RES – residual; UNI – unicystic ameloblastomas; FOLL – follicular; GLAND – glandular odontogenic; OKC – odontogenic keratocyst; NPDC – nasopalatine duct cyst; TBC – traumatic bone cyst; COC – calcifying odontogenic cyst; LPC – lateral periodontal cyst.

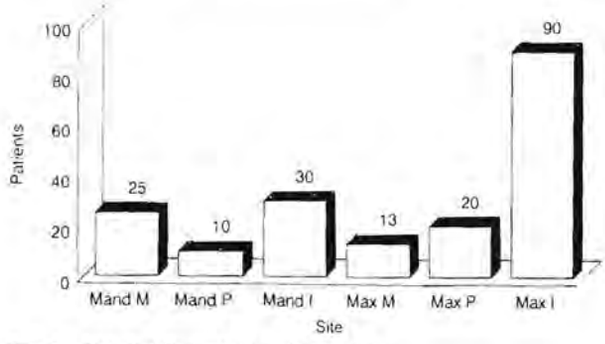

Fig. 2 – Site distribution of radicular cysts.

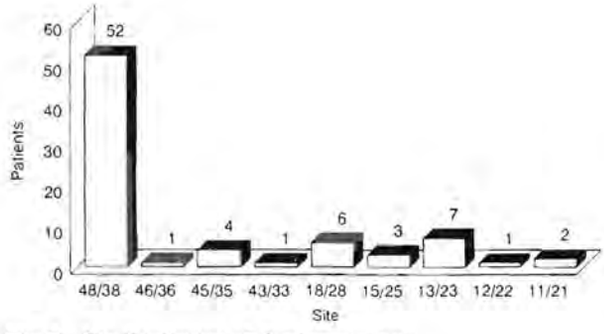

Fig. 3 – Site distribution of dentigerous cysts.

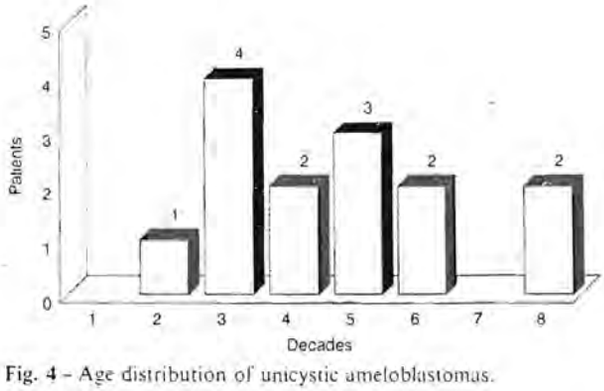

Fig. 4 – Age distribution of unicystic ameloblastomas.

recorded were military patients treated in the Bundeswehrkrankenhaus. The distribution of cystic jaw lesions in this study is reflected in Figure 1.

Radicular (n = 194) and residual cysts (n = 15) comprised 56.9% of lesions diagnosed. The mean age at presentation of radicular and residual cysts was 34.4 years (SD = 14.2) and 52.7 years (SD = 13.2) respectively. Radicular cysts occurred most commonly in the maxillary incisor region (Fig. 2).

Dentigerous cysts (n = 78) comprised 21.3% of the sample and presented at a mean age of 37.1 years (SD = 15.3 years). The mandibular third molars were most frequently involved (Fig. 3).

Three patients out of a total of 39 with odontogenic keratocysts, the latter comprising 10.6% of the sample, suffered from the basal cell naevus syndrome. The mean age at presentation of odontogenic keratocysts was 40.3 years (SD = 19.5 years) and the majority of cases (n = 21) involved the mandibular molar area. In 10 of those cysts, X-ray examination showed teeth or rudiments inside the cavity which led to a primary misdiagnosis of dentigerous cyst.

Unicystic ameloblastomas (n = 15) comprised 4.1% of the sample and presented at a mean age of 40.7 years (SD = 18.8 years). The youngest patient was in the second decade of life whereas 2 cases presented in the eighth decade (Fig. 4). 8 unicystic ameloblastomas occurred in the mandibular molar area, 6 of which appeared radiographically as dentigerous-like cysts, and 4 lesions affected the maxilla. 13 unicystic ameloblastomas were lined by non-invasive odontogenic epithelium (Type I) and 2 cases exhibited foci of mural invasion (Type III).

The mean ages at presentation of nasopalatine duct cysts (n = 10, 2.7% of the sample) and glandular odontogenic cysts (n = 6, 1.6% of the sample) were 44.9 years (SD = 13.5 years) and 46 years (SD = 14.3 years) respectively. The paradental cyst (n = 3), traumatic bone cyst (n = 3), calcifying odontogenic cyst (n = 2) and lateral periodontal cyst (n = 2) each contributed to less than 1% of the sample. No examples of gingival cysts of infants and adults, eruption cysts and nasolabial cysts were found in this study.

DISCUSSION

Accurate diagnosis of cystic lesions of the jaw is crucial as various types are aggressive and may lead to local recurrence if incorrectly diagnosed and inappropriately treated. Many cystic lesions of the jaw share clinical and radiographic features and microscopic examination forms an important part of the diagnostic process. For this purpose, an in-depth knowledge of an internationally accepted classification system, such as that proposed by the World Health Organization (*Kramer* et al., 1992) is essential.

The description of new cyst entities in combination with the new WHO-classification on the one side and improbable lack of diagnosed ameloblastomas on the other prompted this retrospective investigation. It shows the incapability of a general pathologist to make a correct and specific diagnose of jaw cysts and necessitates cooperation with an experienced oral pathologist.

Due to their association with the ghost cell odontogenic tumour, the calcifying odontogenic cyst is no longer grouped amongst cysts in this classification but is classified as a benign tumour originating from the odontogenic apparatus. This cystic tumour, as well as the odontogenic keratocyst (*Brown*, 1970; *Niemeyer* et al., 1985), unicystic ameloblastoma (*Ackermann* et al., 1988) and glandular odontogenic cyst (*Patron* et al., 1991), are notorious for their aggressive behaviour and high recurrence rates (*Machtens* et al., 1972). This implies that in the present study, 17% of the total sample of cystic jaw lesions, required more than simple enucleation as a curative surgical procedure. Type III unicystic ameloblastomas, of which 2 cases were diagnosed in this study, exhibit infiltrative features and should be treated

similarly to the polycystic types, with wide excision or even resection of the involved jaw segment (*Ackermann* et al., 1988). These results have induced a recall of those patients with diagnosed aggressive cysts or ameloblastomas in order to prove the necessity for further treatment.

A large percentage of patients in this study were military personnel and our data is probably biased towards young males. The high mean age of 40.7 years for unicystic ameloblastomas was therefore surprising as these cystic tumours are reported to occur most frequently in the first half of the third decade (*Robinson* and *Martinez*, 1977; *Gardner*, 1981; *Ackermann* et al., 1988). As no literature is available on unicystic ameloblastomas in the German population, this finding may point towards an older age incidence for unicystic ameloblastomas in Germany. Unicystic ameloblastomas frequently involved the mandibular molar area where impaction of a mandibular third molar in the cyst wall was common. Unless these lesions are examined microscopically, they will be misdiagnosed as dentigerous cysts.

The frequency of the different cyst types encountered in this study, as well as the sites of involvement of radicular, residual, dentigerous and odontogenic keratocysts and unicystic ameloblastomas, are in agreement with the recent literature (*Shear*, 1992). The lack of examples of gingival cysts of infants and adults and eruption cysts, as well as the infrequent occurrence of paradental cysts is the result of exclusion of all cases without a microscopic diagnosis. Most of these lesions either go unnoticed or are not submitted for microscopic examination after removal and are probably more common than is reflected in a study of this nature.

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SPECIAL ARTICLE

Classification of Odontogenic Cysts of the Jaws

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Recent developments in the classification and diagnosis of cysts of the jaws necessitate a revision of the topic. This paper discusses the revised World Health Organisation classification of odontogenic cysts and illustrates short descriptions of cyst types with appropriate examples.

INTRODUCTION

Cysts are pathological, fluid filled cavities lined by epithelium. They are more common in the jaws than in any other bone because of the epithelial rests remaining in the tissue after dental development. Cysts of odontogenic origin are the most common cause of chronic swelling of the jaws and have been recognised as diagnostic problems for a long time. During the past few years, numerous articles have appeared regarding the pathogenesis, behaviour, diagnosis and treatment of the different types of jaw cysts and various new concepts have since emerged. In order to standardize the diagnoses of jaw cysts, utilization of uniform diagnostic criteria is essential. The purpose of this article is to present the revised World Health Organisation's classification of odontogenic cysts of the jaws and to illustrate the typical features with appropriate examples obtained from the files of the Department of Oral Pathology, Medical University of Southern Africa.

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CLASSIFICATION

The classification of the odontogenic cysts of the jaws is based on that recommended in the World Health Organization's (WHO) publication *Histological Typing of Odontogenic Tumours*[1] and a recently published textbook on oral cysts[2] (Table 1). The histogenetic division into 'Developmental' and 'Inflammatory' groups remain unchanged.

Table 1.

1. Developmental
- 1.1 Gingival cyst of infants
- 1.2 Odontogenic keratocysts
- 1.3 Dentigerous (follicular) cyst
- 1.4 Eruption cyst
- 1.5 Lateral periodontal cyst
- 1.6 Gingival cyst of adults
- 1.7 Botryoid odontogenic cyst
- 1.8 Glandular odontogenic cyst

2. Inflammatory
- 2.1 Radicular cyst (apical and lateral)
- 2.2 Residual cyst
- 2.3 Paradental cyst
- 2.4 Inflammatory collateral cyst

This classification excludes the calcifying odontogenic cyst (which is now categorized as an odontogenic tumour) as well as other cystic tumours like the unicystic ameloblastoma. It is furthermore noteworthy that the concept of cysts developing in the closure lines of embryologic processes (such as median palatine cyst, median mandibular cyst and globulo-maxillary cyst) which were previously classified as of non odontogenic origin, has been rejected after detailed clinical[3,4] and embryological[5,6] studies.

DEVELOPMENTAL

Gingival cysts of infants

Gingival cysts of infants, also referred to as Bohn's nodules, occur commonly on the alveolar processes of newborn infants (Figure 1). They soon disappear through involution and are seldom seen after three months of age. These cysts arise from the dental lamina and although rarely biopsied, are lined by keratinizing squamous epithelium.[2]

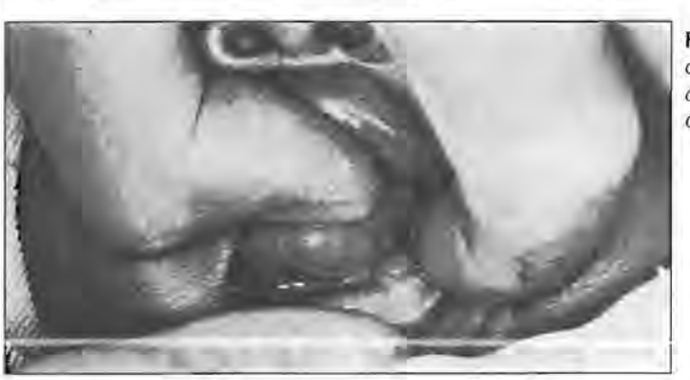


Figure 1. *Gingival cyst of the infant on the left mandibular alveolus.*

Odontogenic keratocysts

The term 'primordial cyst', which was often used synonomously with odontogenic keratocyst, has fallen in disuse because no convincing evidence for development from a tooth primordium has yet been forwarded. There is however, evidence supporting origin from primordial odontogenic epithelium, that is, dental lamina or its remnants[7,8]. Although other odontogenic cysts may exhibit foci of squamous metaplasia, odontogenic keratocysts are primarily recognised by their stretched and keratinized epithelial lining with a well defined, often palisaded basal cell layer. Basal cell budding, as well as daughter cyst formation are found in odontogenic keratocysts and are especially pronounced in patients with the naevoid basal cell carcinoma syndrome in which multiple keratocysts occur. These phenomena as well as the fragility of the cyst wall are the primary causes for incomplete surgical removal and the high recurrance rate. Odontogenic keratocysts .nay occur in the place of a tooth (replacement variety), around the crown of an impacted tooth (envelopmental variety) in the ramus of the mandible (extraneous variety) or between the roots of adjacent teeth (collateral variety)[9]. Although the majority present as unilocular radiolucencies (Figure 2), scalloped margins may be misinterpreted as multilocularity leading to an erroneous diagnosis of ameloblastoma[2]. The envelopmental variety is often indistinguishable radiologically from a dentigerous cyst and the collateral variety from a lateral periodontal or lateral placed radicular cyst.

Dentigerous (follicular) cysts

A dentigerous cyst is one which encloses the crown of an unerupted tooth by expansion of its follicle, and is attached to its neck[2] (Figure 3). It probably developes by accumulation of fluid between the reduced enamel epithelium and the enamel after formaton of the crown has been completed. The diagnosis of dentigerous cyst should not be made on radiographic evidence only, otherwise keratocysts of the envelopmental variety and unilocular ameloblastomas involving adjacent unerupted teeth, are liable to be misdiagnosed. The wall of a dentigerous cyst is lined by thin epithelium of two to three layers of undifferentiated cells derived from reduced enamel epithelium.

Figure 2. *Odontogenic keratocyst in the anterior mandible. Note the sclerotic margin surrounding the cyst.*

Figure 3. *Panoramic view of a dentigerous cyst surrounding the crown of an impacted maxillary central canine. Note the displacement of the permanent lateral incisor and canine.*

Eruption cyst

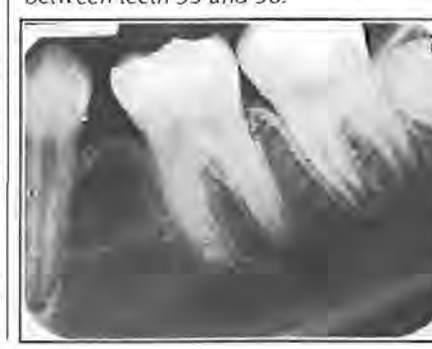
An eruption cyst is in effect a dentigerous cyst which occurs in the soft tissues. There is usually no radiographic evidence of bone involvement. The cyst is exposed to masticatory trauma and many eruption cysts burst spontaneously with only few requiring surgical exposure of the involved tooth.

Lateral periodontal cyst

The designation 'lateral periodontal cyst' is confined to those cysts which occur in the lateral periodontal position and in which an inflammatory aetiology and a diagnosis of collateral keratocyst have been excluded on clinical and histological grounds.[10] Radiographs show a round or oval, well circumscribed, radiolucent area somewhere between the apex and cervical margin of a vital tooth (Figure 4). Various theories on the histogenesis of this cyst type were forwarded, of which the proposal that it arises initially as a dentigerous cyst developing by expansion of the follicle along the lateral surface of the erupting tooth is an attractive one[11]. Most commonly, the lateral periodontal cyst is lined by a thin, non keratinized layer of squamous or cuboidal epithelium with small inconspicuous nucleii and convoluted epithelial plaques, which develop as a result of localized proliferation of cells[2].

The botryoid odontogenic cyst is a multilocular variant of the lateral periodontal cyst. This rare cyst has a

Figure 4. *Periapical radiograph showing a lateral periodontal cyst in the alveolus between teeth 35 and 36.*



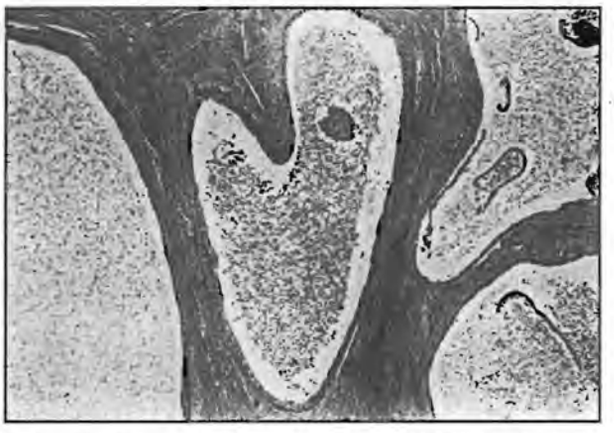


Figure 5. *Multicystic appearance of a botryoid odontogenic cyst (H&E stain X40).*

lining similar to the lateral periodontal cyst with thin connective tissue septae separating distinct cystic cavities (Figure 5).

Gingival cyst of adults

The gingival cysts of adults is located in the gingival soft tissue and presents as a gingival swelling without any radiographic signs of bone destruction. Although many theories have been proposed on its histogenesis, the most favoured is derivation from gingival odontogenic epithelial cell nests[2] or reduced enamel epithelium after the eruption of a tooth.[12,13] If the latter theory is accepted, gingival cysts in adults may represent the soft tissue counterpart of lateral periodontal cysts. This is supported by the numerous similarities both clinically and histologically between these two cyst types.

Glandular odontogenic cyst

A cyst with fairly typical histological features and which has some characteristics in common with lateral periodontal cyst has recently been reported[14,15]. Radiographically, some cases exhibit a unilocular radiolucency with either smooth or scalloped margins (Figure 6), while others are distinctly multilocular. The cyst may be lined in parts by a non-keratinized stratified squamous epithelium. The superficial layer of the epithelial lining consists of columnar or cubiodal cells with occasional cilia and the epithelium has a glandular or pseudo glandular structure, with intra-epithelial crypts lined by cells similar to those on the surface.

INFLAMMATORY

Radicular cyst

A radicular cyst is one which arises from epithelial residues in the periodontal ligament as a result of inflammation[2]. The inflammation usually follows necrosis of the dental pulp and the identification of a non vital tooth associated with the cyst is an important diagnostic parameter. Although these cysts are usually located around the apex of a tooth (Figure 7), they may also be found on the lateral surfaces of a root in association with the opening of an accessory pulpal canal. Radiographically these cysts are characterized by round or ovoid radiolucencies surrounded by a narrow radio-opaque margin which extends from the lamina dura of the involved tooth. The size of the lesion is not reliable in distinguishing it from a periapical granuloma, unless it is larger than 2 cm in diameter in which case the lesion is most likely a radicular cyst.[16] Almost all radicular cysts are lined

Figure 6. *Glandular odontogenic cyst presenting as an unilocular cyst in the maxilla. Note the displacement of the adjacent teeth.*

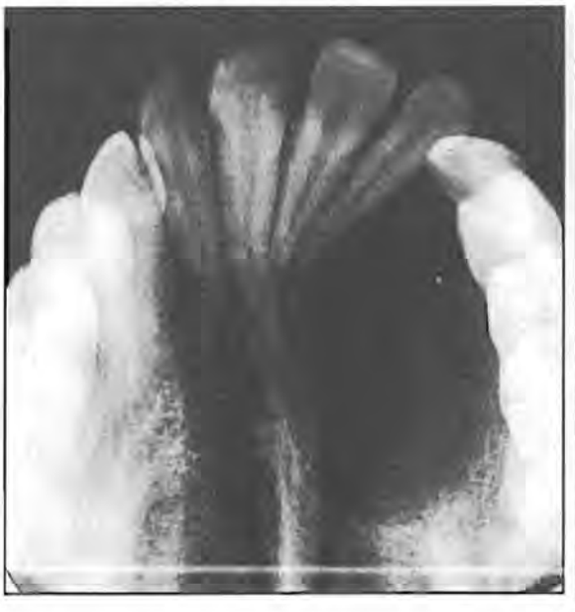
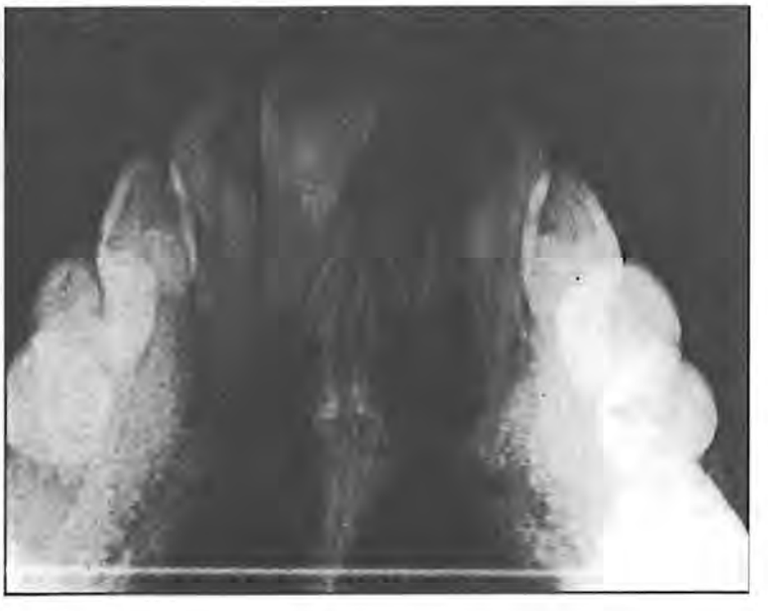


Figure 7. *Radiograph of a radicular cyst surrounding the apex of a maxillary incisor.*



wholly or in part by stratified squamous epithelium. The epithelial lining may proliferate and exhibits arcading and a considerable degree of spongiosis with an intense inflammatory infiltrate.

Residual cyst

A residual cyst can be described as a radicular cyst of which the associated tooth has been extracted. All the radiographic and histological features of radicular cysts except for the association with a non-vital tooth therefore apply to residual cysts.

Paradental cyst

Craig (1976) wrote the first detailed account of a cyst of inflammatory origin which occured on the lateral aspect of the roots of partially erupted mandibular third molars where there was an associated history of pericoronitis. In these cases the teeth are vital and radiographic examination shows a well demarcated radiolucency distally to a partially erupted tooth: Ackerman, Cohen and Altini[17] like Craig[18] favour origin from reduced enamel epithelium but suggested that cyst formation occurs as a result of unilateral expansion of the dental follicle secondary to inflammatory destruction of the periodontium and alveolar bone. This is different from the histogenesis of dentigerous cysts where expansion occurs primarily with consequent bone destruction.

Paradental cysts are microscopically indistinguishable from radicular cysts and a proper clinical history and radiograph must accompany the biopsy in order to facilitate a correct diagnosis.

Inflammatory collateral cyst

This rare cyst type occurs as a result of inflammatory process in the periodontal pocket[2]. The associated tooth is vital and the cyst is microscopically indistinguishable from radicular cysts. Microscopic diagnosis relies heavily on adequate clinical information. This cyst appears to favour developing buccally to the lower first or second molars.

CONCLUSION

Accurate diagnosis of cysts of odontogenic origin is important as various cyst types like odontogenic keratocysts and glandular odontogenic cysts are aggressive lesions and tend to recurr after incomplete removal. It is important that clinicians are aware of the unreliability of radiographic interpretations. On the other hand, a microscopic diagnosis of biopsies taken from densely inflamed cyst walls is often difficult, if not impossible, to interpret without clinical information and radiographs. A high degree of diagnostic accuracy, when dealing with jaw cysts, can only be achieved through communication between the clinician and resident pathologist.

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ition of South Africa, **48**, 557-559.

Differential diagnosis yst-like lesions: Clinico-pathologic features of 63 cases

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Keywords: odontogenic cyst; dentigerous cyst; unicystic ameloblastoma; keratocyst.

SUMMARY

A series of unilocular pathological conditions resembling dentigerous cysts was analyzed and the clinical and radiographic features correlated with the microscopic diagnosis. The most common lesions were found to be true dentigerous cysts followed by unicystic ameloblastomas and odontogenic keratocysts. Unicystic ameloblastomas with a dentigerous cyst-like appearance occurred most frequently in the mandibular third molar region and commonly caused expansion of the mandible. The adjacent teeth in these cases showed a high occurrence rate of root resorption. Unlike the site distribution of true dentigerous cysts reported in other series, 50 per cent of our cases occurred in the maxillary anterior and premolar regions. Our study emphasizes the importance of microscopic examination of all pericoronal cystic lesions.

OPSOMMING

Die kliniese- en radiologiese beeld van 63 gevalle van unilokulêre patologiese toestande wat tandhoudende siste naboots, is vergelyk met die mikroskopiese diagnose. Die mees algemene toestand wat voorgekom het, was tandhoudende siste, gevolg deur unisistiese ameloblastoom en odontogene keratosiste. Unisistiese ameloblastome met die radiologiese beeld van 'n tandhoudende sist, het mees algemeen in die mandibulêre derde molaar area presenteer en het dikwels beenekspansie veroorsaak. Die aangrensende tande het 'n hoë frekwensie van eksterne wortelresorpsie getoon. Anders as in ander reekse, het 50 persent van tandhoudende siste in die maksillêre anterior en premolaar areas voorgekom. Hierdie studie beklemtoon die belang van mikroskopiese ondersoek van alle perikoronale sisteuse letsels.

INTRODUCTION

A dentigerous cyst is defined as a unicystic cavity which encloses the crown of an unerupted tooth by expansion of its follicle and is attached to the neck of the tooth (Shear, 1992). In a radiographic context, a radiolucent area surrounding the crown of an unerupted tooth may be seen with odontogenic keratocysts of the envelopmental or follicular variety as well as unicystic ameloblastomas involving adjacent unerupted teeth, and these may be misinterpreted as dentigerous cysts. This could have prognostic consequences as the recurrence rates of the various pathologic lesions that envelop the crown of a tooth vary significantly. Simple enucleation is an adequate form of treatment for dentigerous cysts but more extensive surgery is required for unicystic ameloblastomas and odontogenic keratocysts. Even with adequate treatment, the recurrence rates of unicystic ameloblastomas and keratocysts are reported to be high (Vedtofte and Praetorius, 1979; Ueno *et al.*, 1986). Accurate diagnosis of jaw cysts is therefore essential for adequate treatment planning.

The purpose of this study was to appraise the clinico-pathologic features of pericoronal radiolucencies resembling dentigerous cysts.

MATERIAL AND METHODS

Sixty three lesions with a radiologic appearance of a dentigerous cyst were retrieved from the files of the department of Maxillo-Facial and Oral Surgery at the Medical University of Southern Africa. This hospital is a reference centre for the Northern Transvaal region and all patients in the study are Black and mostly of rural origin. The radiologic appearance with special reference to the size (longest axis measured on panoramic radiograph) and location of the lesion, the presence or absence of root resorption of adjacent teeth, expansion of cortical plates and displacement of the associated tooth, was compared with the age, sex and microscopic diagnosis of the lesion.

RESULTS

Sixty-three unilocular paracoronal cystic lesions resembled dentigerous cysts radiologically. The histological diagnosis of these lesions are listed in

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Fig. 1: Unicystic ameloblastoma with root resorption of the associated teeth.

Fig. 2: Adenomatoid odontogenic tumour causing tooth displacement and root resorption.

Fig. 3: Unicystic ameloblastoma of the left mandible showing enlargement in all dimensions.

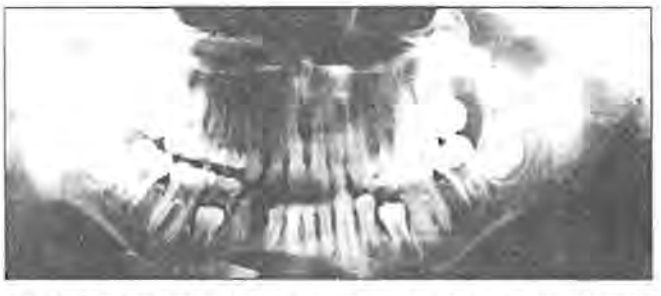

Fig. 4: Dentigerous cyst of the mandible showing enlargement along the medullary space.

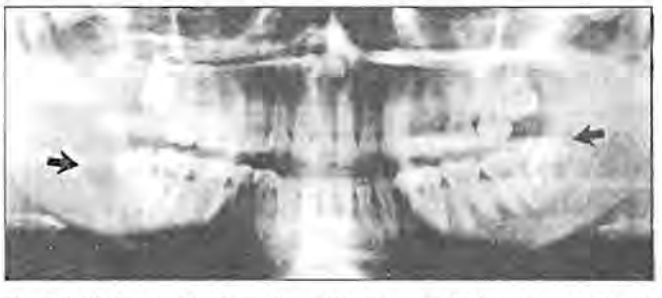

Fig. 5: Calcifying odontogenic cyst of the mandible associated with an impacted canine and exhibiting mural calcifications (arrows).

Table I. Three of the calcifying odontogenic cysts with a dentigerous cyst-like appearance were subclassified according to Praetorius *et al.*, (1981) as type IA, and one each as type IB and IC respectively. The sex distribution, mean age at presentation, average size of the cyst as measured on a panoramic radiograph and the presence of root resorption and tooth displacement are shown in Table I.

Fig. 6: Multiple keratocysts involving the left and right mandibular ramus and right globulomaxillary area in a patient with the naevoid basal cell carcinoma syndrome.

Unicystic ameloblastomas showed an equal sex distribution, while dentigerous cysts, odontogenic keratocysts and calcifying odontogenic cysts were more common in males. Adenomatoid odontogenic tumours were found in females only. The mean age at presentation of the six cyst types were not found to differ significantly. The mean size of the unicystic ameloblastomas were significantly larger than the odontogenic keratocysts and dentigerous cysts ($p<0,005$) while odontogenic keratocysts' mean size were significantly larger than that of dentigerous cysts ($p<0,05$). Root resorption was most frequently observed in unicystic ameloblastomas (64 per cent of cases) (Fig. 1) and calcifying odontogenic cysts (60 per cent of cases). Displacement of non-involved teeth was a constant finding in cystic adenomatoid odontogenic tumours (Fig. 2). The enlargement of unicystic ameloblastomas occurred in all dimensions and frequently caused bony expansion (Fig. 3). Enlargement of follicular and odontogenic keratocysts in the mandible appeared to follow the medullary space initially (Fig.4) with bony expansion seen only in the largest examples.

All paradental cysts were associated with partially erupted third molars. Six cystic adenomatoid odontogenic tumours occurred in the maxilla and one in the mandible. One very large lesion of the latter type extended across the maxillary midline. Three calcifying odontogenic cysts presented in the maxilla and two in the mandible. One cyst in each jaw showed radiographic evidence of calcifications (Fig. 5).

Thirteen dentigerous cysts were located in the maxilla, the majority of which were associated with impacted central incisors (4 cysts), canines (2 cysts) and premolars (4 cysts). In the mandible, only 3 dentigerous cysts involved third molars; one, a second molar, while two involved canines. One dentigerous cyst was associated with a primary maxillary canine. Eight odontogenic keratocysts were located in the mandibular (7 cases) or maxillary (one case) third molar areas and 5 presented in the canine region (3 maxillary and 2 mandibular). Two patients presenting with the basal cell nevus syndrome had multiple cysts

Table I: *Clinical data.*

	n	Sex		Mean Age (SD)	Mean Size in mm (SD)	Root Resorption	Tooth Displacement
		Male	Female				
Unicystic Ameloblastoma	14	7	7	15,5 ±6,3	80 ±22,7	9	5
Dentigerous Cyst	21	16	5	16 ±16,2	35 ±11,5	4	5
Odontogenic Keratocyst	13	10	3	15 ±12,5	45 ±20,1	1	3
Adenomatoid Odontogenic Tumour	7	0	7	12 ±3,9	45 ±15,7	1	7
Calcifying Odontogenic Cyst	5	4	1	23 ±7,0	40 ±6,5	2	3
Paradental Cyst	3	2	1	21 ±2,0	10 ±12,7	0	0

(Fig. 6). The unicystic ameloblastomas showed a predeliction for the mandibular third molar region (11 cysts) followed by the mandibular canine region (3 cysts). No unicystic ameloblastomas with a dentigerous cyst-like appearance occurred in the maxilla.

DISCUSSION

The importance of an accurate diagnosis of a lesion with a dentigerous cyst-like appearance, especially in a Black population sample in which dentigerous cysts are less common than in Whites (Shear, 1992), cannot be over emphasized. By the same token the presence of unicystic ameloblastomas must not be underestimated, being the second most common cystic lesion found in our patients. Outstanding characteristics of this potentially aggressive neoplasm is its large size when compared to the other cysts, its tendency to expand more symmetrically than other cystic lesion in the mandible as well as its common association with root resorption of adjacent teeth. Adenomatoid odontogenic tumours were found in females only but although the majority seem to affect the anterior maxilla, it also occurred in the mandible in one instance. Tooth displacement was more frequently observed in adenomatoid odontogenic tumours than in any of the other cystic lesions. Dentigerous cysts were more frequently encountered in the anterior maxilla and their most frequent association with impacted mandibular third molars (Shear, 1992) was not found in our study. The lower frequency of impacted third molars in Blacks (Brown *et al.*, 1982) may account for this observation in our exclusively Black sample. The attachment of the cyst wall to the impacted tooth is reported to extend more apically in ameloblastomas than dentigerous cysts (Ikeshima *et al.*, 1990). In large examples of dentigerous cysts the associated tooth is often rotated, making this measurement difficult to interpret on panoramic radiographs.

Our study does not support the report that there is a frequent occurrence of root resorption in association with dentigerous cysts (Struthers and Shear, 1976). The site distribution of odontogenic keratocysts in our study conform to that of another series (Shear, 1992). Forssell (1980) observed a relationship between the cyst and the crown of a tooth in 41 per cent of a series of 135 cases. McIvor (1972) however, demonstrated this relationship exclusively in the mandible. In our study, 4 maxillary odontogenic keratocysts presented in association with impacted teeth. The frequent association of odontogenic keratocysts with impacted teeth have led Altini and Cohen (1980) to introduce the term "follicular primordial cyst" for this group of lesions. They postulated that this association may arise following eruption of a tooth into a pre-existing cystic cavity in the same way as a tooth erupts into the oral cavity. Although we have no microscopic evidence, we believe that this hypothesis may be extended to all cysts in our series, except for the follicular and paradental cysts, in both of which types their association with an impacted tooth have been satisfactorily explained (Shear, 1992).

Although certain specific features seen on radiographs, such as the size of lesion, its location, the presence or absence of root resorption or tooth displacement and other factors such as age and sex of the patient may influence the clinical differential diagnosis, a thorough histological examination is essential in establishing an accurate diagnosis.

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GLANDULAR ODONTOGENIC CYST

Willem F.P. van Heerden, MChD, Erich J. Raubenheimer, MChD, and Martin L. Turner, DipTech(Med)

Two cases of glandular odontogenic cysts are reported. The unique histological features, eg, the intraepithelial glandular structure, papillary processes, and eosinophilic cuboidal and larger granular superficial cells are sufficient to warrant glandular odontogenic cyst as a distinct entity. Electron microscopic examination of the superficial eosinophilic cuboidal cells are suggestive of a process similar to apoptosis. Eroded cortical plates suggest an aggressive behavior.
HEAD & NECK 14:316–320

The glandular odontogenic cyst (GOC) is a rare cystic lesion that is not incorporated in classifications of jaw cysts. Only a few examples of this lesion have been described in the literature. Gardner et al[1] collected eight cases of GOC. Padayachee and van Wyk[2] reported two cases, which they described as "sialo-odontogenic cysts."

The GOC has an equal sex distribution and occurs in both the mandible and maxilla of adults.[1,2] These lesions, which can attain a large size, appear on radiographs as uni- or multilocular lytic lesions. The histologic features described by Gardner et al[1] include a cyst lining consisting of stratified squamous epithelium of varying thickness that contains pools of mucicarmine-positive material. The superficial layer consists of eosinophilic cubiodal and occasionally mucous- and ciliated cells. Spherical structures produced by swirling epithelium and lack of cell polarization are focally present in the epithelium lining. Irregular-shaped calcifications are occasionally found in the subepithelial connective tissue.

This report describes the clinical, histopathologic, and ultrastructural features of two cysts.

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CASE 1

A 27-year-old woman reported to the clinic complaining of a painless swelling in the anterior mandible of three years' duration. Intraoral examination revealed a 6 × 3 cm sized swelling extending from the left first mandibular molar to the right second premolar with buccal as well as lingual bone expansion. The mucosa was intact, but the bone was eroded in areas causing the swelling to fluctuate on palpation (Figure 1). No sensory nerve fallout was found. Radiography revealed a well-defined unilocular radiolucent lesion with a scalloped border. Displacement of the anterior teeth was present (Figure 2). During biopsy, a unicystic cavity containing yellow serous fluid was found. Differential diagnoses included a unicystic ameloblastoma and odontogenic keratocyst.

Histologic examination of the incisional biopsy revealed a cyst lining consisting of a nonkeratinized epithelium. The epithelium varied in thickness from double-layer cuboidal to

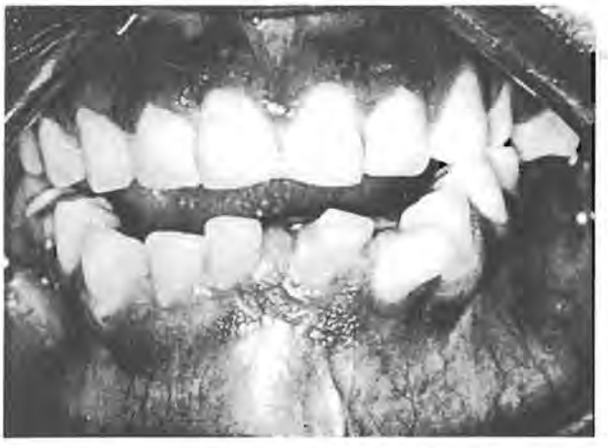

FIGURE 1. Mandibular lesion showing buccal and lingual expansion associated with tooth displacement.

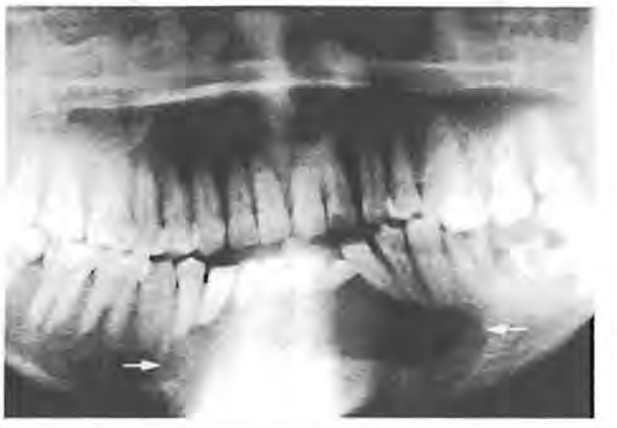

FIGURE 2. Pantomograph exhibiting an unilocular radiolucency (arrows) of the anterior mandible.

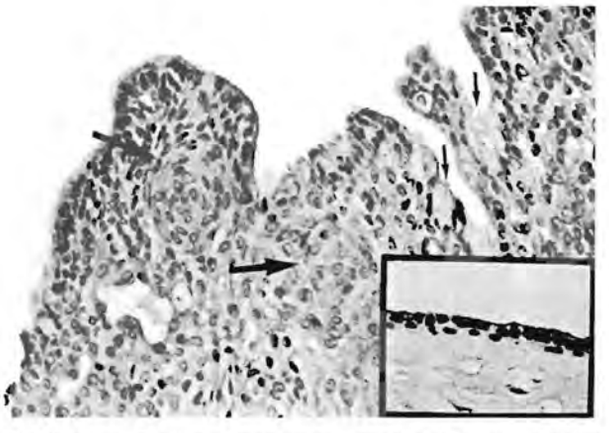

FIGURE 3. Papillary processes associated with epithelial spheres (bold arrows) and superficial mucous cells (fine arrows). Hematoxylin & eosin; original magnification, ×200. Inset: The cyst lining is partly composed of a double layer cuboidal epithelium. Hematoxylin & eosin; original magnification ×200.

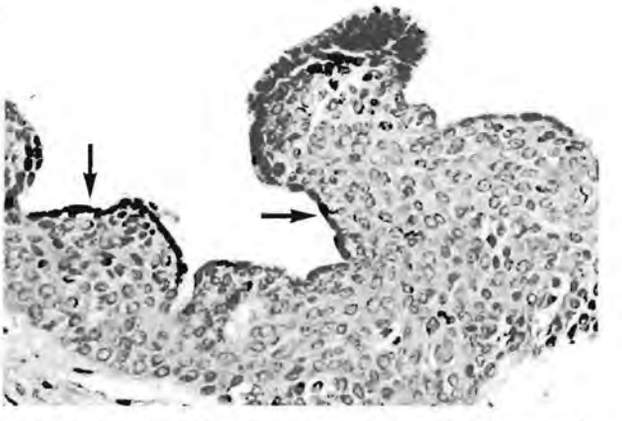

FIGURE 4. Superficial cell layer consisting of small cuboidal cells with hyperchromatic nucleii and eosinophilic cytoplasms (arrows). Note the papillary processes. Hematoxylin & eosin; original magnification, ×200.

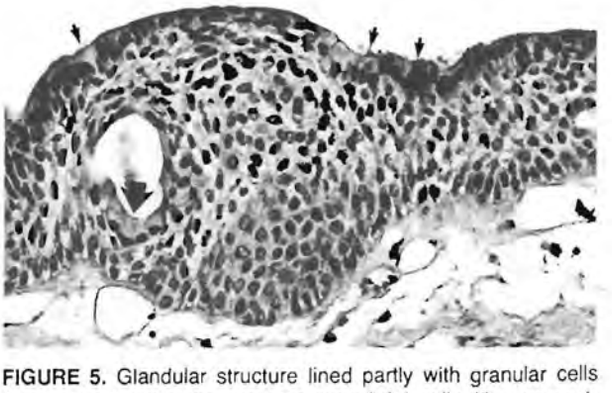

FIGURE 5. Glandular structure lined partly with granular cells (bold arrow). Note the granular superficial cells (fine arrows). Hematoxylin & eosin; original magnification, ×200.

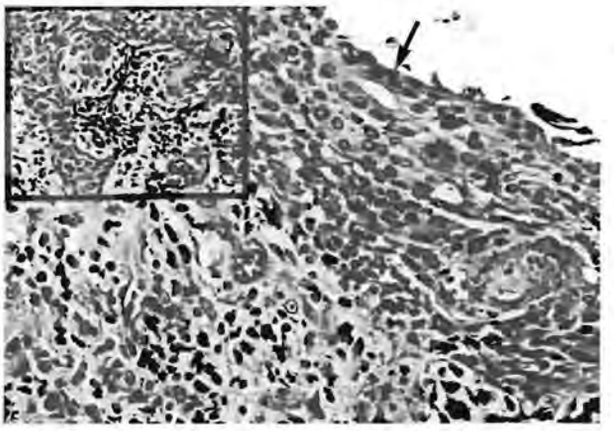

FIGURE 6. Inflammatory induced changes in lining of case 1. A glandular structure is visible (arrow). Hematoxylin & eosin; original magnification, ×200. Inset: Epithelial arcading associated with lymphocytes. Hematoxylin & eosin; original magnification ×100.

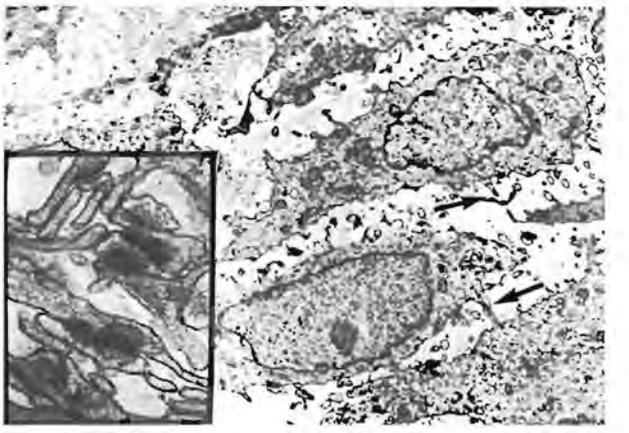

FIGURE 7. Transmission electron micrograph of the inflammed lining revealed widened intercellular spaces containing finger-like protrusions (arrows). Original magnification, ×2600. Inset: Well-formed desmosomes were present between the protrusions. Original magnification, ×8300.

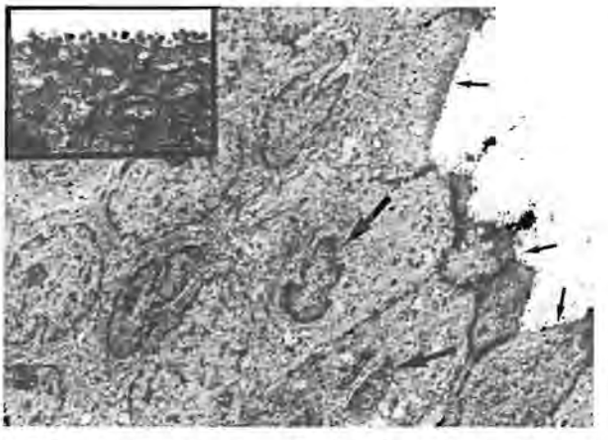

FIGURE 8. Electron micrograph of the lining of case 1 representing the superficial eosinophilic cuboidal cells. Note the smaller, denser nucleoli in the more superficial cells (bold arrows) and the absence of nuclear material in the remainder of the superficial cells (fine arrows). Original magnification, ×3300. Inset: Microvilli on the luminal aspect of the superficial cells. Original magnification, ×10,000.

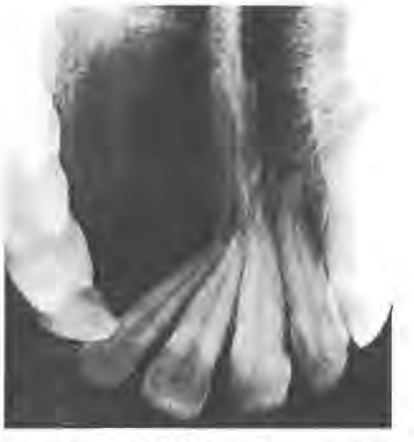

FIGURE 9. Occlusal radiograph revealed a well-circumscribed radiolucency causing root divergence of the lateral and incisor teeth.

stratified squamous. Papillary epithelial processes into the lumen were noted, especially where epithelial thickenings were present. Epithelial spheres consisting of swirled epithelial cells were found occasionally (Figure 3). The superficial cell layer consisted mainly of small cuboidal cells with scanty eosinophilic cytoplasm and hyperchromatic nuclei (Figure 4). Larger cells with an eosinophilic granular cytoplasm and a round nucleus, which was oriented away from the surface, as well as scattered mucous cells were also present in the superficial layer. Ciliated cells were focally seen.

Intra-epithelial glandular structures, filled with an eosinophilic, mucicarmine-positive material were present, the majority located in the superficial half of the epithelium (Figure 5). These glandular spaces were lined mainly by granular cells, although mucous cells were focally present. No mitotic figures were noted. Palisading of the basal cells were focally seen, and no maturation changes of the epithelial cells were noted. Cleaving between the epithelium and connective tissue was focally observed. The underlying connective tissue consisted of dense fibrous tissue with a few vascular spaces. No epithelial islands nor calcifications were noted.

A diagnosis of a glandular odontogenic cyst

was made, and the cyst lining was enucleated under general anesthesia. The wound was closed primarily and healing was uneventful. Small fragments of the lining were fixed separately in 3% gluteraldehyde for electron microscopic examination. Light microscopic examination of the enucleated material revealed a dense, chronic inflammatory cell infiltrate consisting mainly of lymphocytes in the subepithelial connective tissue and neutrophils in the epithelium. The epithelial lining had lost most of the features described in the incisional biopsy material. Epithelial hyperplasia and proliferation into the underlying connective tissue with an arcading effect were present. The eosinophilic cuboidal superficial cell layer as well as glandular structures in the epithelium were focally present (Figure 6).

Electron microscopic examination revealed widened intercellular spaces with numerous fingerlike protrusions that attached adjoining epithelial cells by well-formed desmosomes (Figure 7). As the biopsies taken for electron microscopy were not representative of all epithelial types as seen in the sections of the incision biopsy, a small fragment was then removed from the wax block of the noninflammed biopsy specimen and processed for electron microscopy. This epithelial lining consisted of tightly aggregated cells with well-formed desmosomes. Microvilli-like projections were present on the luminal aspect of the superficial cells, the majority of which contained no nucleii. Their cell volume seemed to be decreased, resulting in a closer association of the desmosomes (Figure 8). The cells immediately underneath the superficial cells contained a denser nucleus, and signs of nuclear fragmentation were present.

CASE 2

A 14-year-old boy presented with swelling of the right upper lip. Oral examination revealed a firm buccal and palatal swelling involving the right maxillary canine area. Radiographs showed a well circumscribed, unilocular lytic lesion in the globulo-maxillary area causing root divergence of the lateral and incisor teeth (Figure 9). A biopsy was taken, and microscopic examination showed a cyst lining with similar features as described in case 1 (Figure 10). A diagnosis of a glandular odontogenic cyst was made. The patient did not return for treatment.

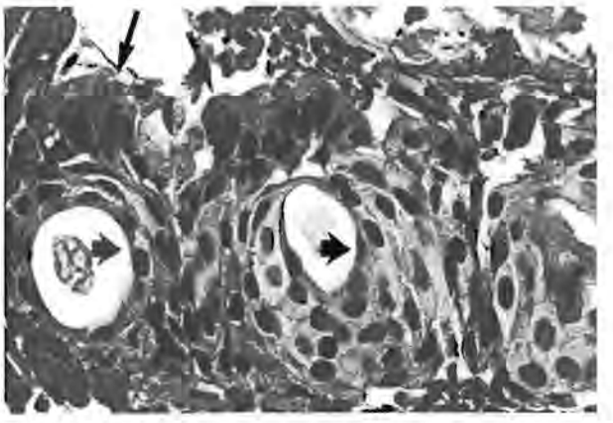

FIGURE 10. Glandular structures were present in the lining epithelium (short arrows). The superficial cells had a granular appearance (long arrows). Hematoxylin & eosin; original magnifications, ×400.

DISCUSSION

There are sufficient criteria to regard GOC as a distinct entity and not a variant of any other cyst. The unique features include the presence of eosinophilic cuboidal and larger granular superficial cells, intraepithelial glandular structures lined by granular and mucous cells, and papillary processes protruding into the lumen. Epithelial spheres are also found in both lateral periodontal cysts and dentigerous cysts.[3] The presence of numerous mucous cells alone does not warrant the diagnosis of GOC. Browne has shown that mucous metaplasia is fairly common in dentigerous cysts, and can be found in the majority of jaw cysts.[4]

The widened interepithelial cell spaces and the finger-like protrusions found in inflammed GOC tissue on electron microscopic examination are also present in inflammed as well as noninflammed radicular and follicular cysts.[5] The spinous cells of odontogenic keratocysts, however, show a close intercellular relationship with desmosomes rarely detected.[5] The tissue fragment removed from the wax block for electron microscopy study contained superficial eosinophilic cuboidal cells. It is tempting to speculate that the superficial cells undergo a process similar to apoptosis. This will explain the eosinophilic light microscopic appearance of the superficial cells with hyperchromatic nucleii, although the microvilli-like projections seen on electron microscopy are too small to represent apoptotic bodies.[6]

The prevalence of GOC is low. The two cases reported in this study are the only GOCs in our

collection of 152 jaw cysts that were diagnosed during an 8-year period. A contributory factor to this low prevalence may be the difficulty in identifying the characteristic features of a GOC in inflammed tissue, especially if only material from an incisional biopsy is available. The changes brought about by an inflammatory process were evident in the excised tissue in case 1.

Glandular odontogenic cysts are considered to be aggressive. One of the cases reported by Padayachee and van Wyk[2] recurred, and recurrences were present in two of the eight cases described by Gardner et al.[1] Although no recurrence was present in case 1, after 2 years, the eroded cortical plates suggested aggressive behavior.

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PIGMENTED NEUROECTODERMAL TUMOUR OF INFANCY

W F P van Heerden & E J Raubenheimer

Keywords: pigmented neuroectodermal tumour

Pigmented neuroectodermal tumour of infancy (PNTI) is an uncommon neoplasm occurring primarily in young children, 82% are 6 months or less in age while 92% are under 12 months[1]. The tumour has a predilection for the anterior maxilla but has also been reported in the mandible[1], epididymis[2], mediastinum and brain[1]. Clinically, PNTI presents as a soft tissue mass, 1-3 cm in size with a firm consistency. It is frequently associated with rapid growth and a stretched, non ulcerated overlying mucosa or skin. Radiographic features include an ill-defined radiolucent lesion causing local destruction and displacement of the developing teeth. Despite the tumour's rapid growth and tendency to invade bone the majority of cases are successfully treated with conservative therapy (local excision and curettage of underlying bone). Recurrences develop in about 15% of patients[1] while metastases and cellular malignant change have been documented in a few cases[3].

The purpose of this article is to present a case of PNTI with exceptional clinical features.

MATERIALS AND METHODS

A 7-month old female patient was referred to the Garankuwa Hospital with a large soft tissue of the right maxilla present since birth (Fig.1). The tumour had grown rapidly since then and caused severe disfigurement. Two areas of ulceration was present on the skin. An incisional biopsy was performed and diagnosed as a PNTI.

The tumour was excised and the post operative healing uneventful. The excised tumour measured 18 cm in the longest diameter. The consistency was firm and fibrous and the specimen had a blue-black colour on cut surface (Fig.2). It appeared to be well demarcated. Microscopically, it was composed of non encapsulated dense fibrovascular tissue with large epithelial-like melanin producing cells arranged either in strands or clusters and often forming the lining of small cleft-like and alveolar spaces. Smaller non pigmented cells resembling neuroblasts were present in the alveolar spaces or as isolated nests in the stroma (Fig.3). No mitotic figures were present. Immunohistochemical examinations using paraffin embedded tissue revealed focal positivity for neuron specific enolase (NSE) in both the pigmented and small cells. Vimentin was focally expressed in the pigmented cells. Both cell types were negative for S100-protein and cytokeratin.

DISCUSSION

This PNTI was the largest tumour of its kind described in the literature up to date. PNTI was first described in 1918 by Krompecher under the term congenital melanocarcinoma[4]. Difficulty in deciding the cellular origin has led to a variety of terms describing this lesion such as melanotic ameloblastoma, melanotic prognoma, retinal anlage tumour and pigmented epulis of infancy[5]. The concept of a congenital melanoma failed to explain the presence of the primitive neuroblast-like cells as well as the benign clinical course. The odontogenic theory was prompted by the predilection of this tumour for the maxilla but does not take into consideration the extragnatic sites where there are no odontogenic rests. The tumour cells also bear no resemblance to any cell involve in odontogenesis. The association between PNTI and the developing retina is highly unlikely because the retina is well developed in the embryo before the anlage of the maxilla and mandibles develops[6]. Electron microscopy and histochemical studies however have establish the neural crest as the most likely origin[7]. This will explain the presence of melanin and neuroblast cells, the distribution of the lesions as well as the few tumours associated with increased levels of vanillylmandelic acid[6].
The immunohistochemical findings in the present case was in large in agreement with similar studies in the literature[8]. Cytokeratin however is positive in the pig-

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mented cells in other studies. The absence of cytokeratin in our case was probably the result of different types of cytokeratin used as the primary antibody.

There was no evidence of the tumour recurring in the 6 months of follow-up examinations. It was found that incomplete surgical removal of this tumour is not necessarily associated with recurrences[9]. The debulking effect as well as the removal of stimulatory cells influencing the invading peripheral tumour cells are possible explanations.

Few other lesions would present in this age group and in the typical location. PNTI must be differentiated from congenital epulis as well as malignancies of early childhood such as neuroblastomas and rhabdomyosarcomas.

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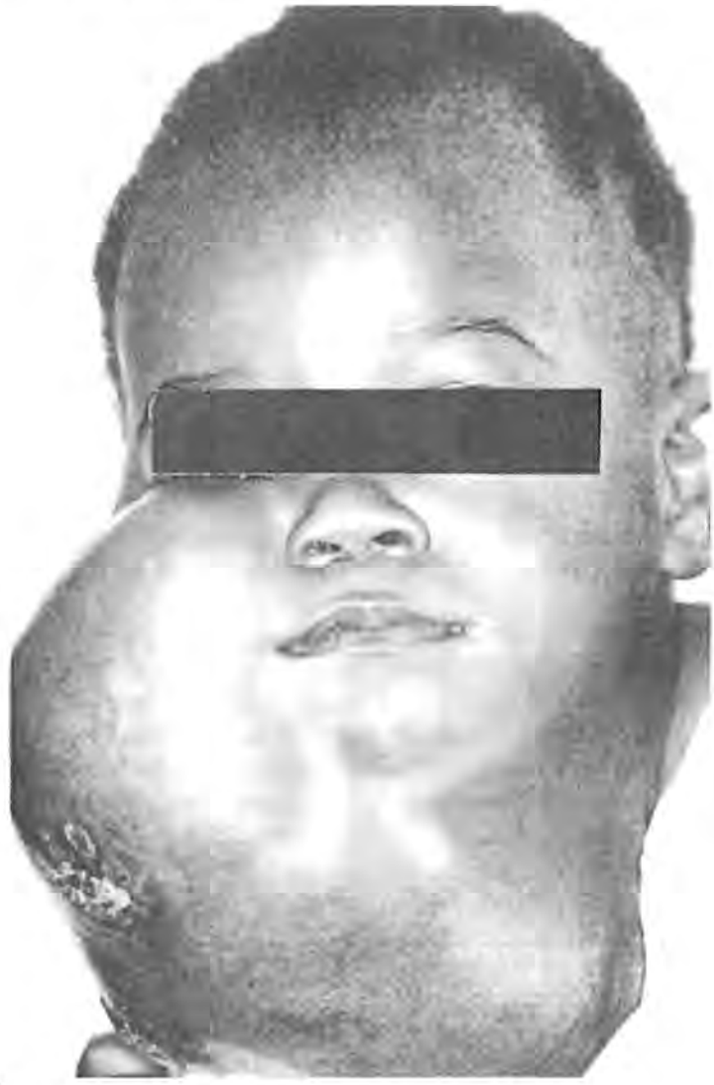

Fig. 1. The 7-month old female patient with the PNTI involving the whole right side of the face.

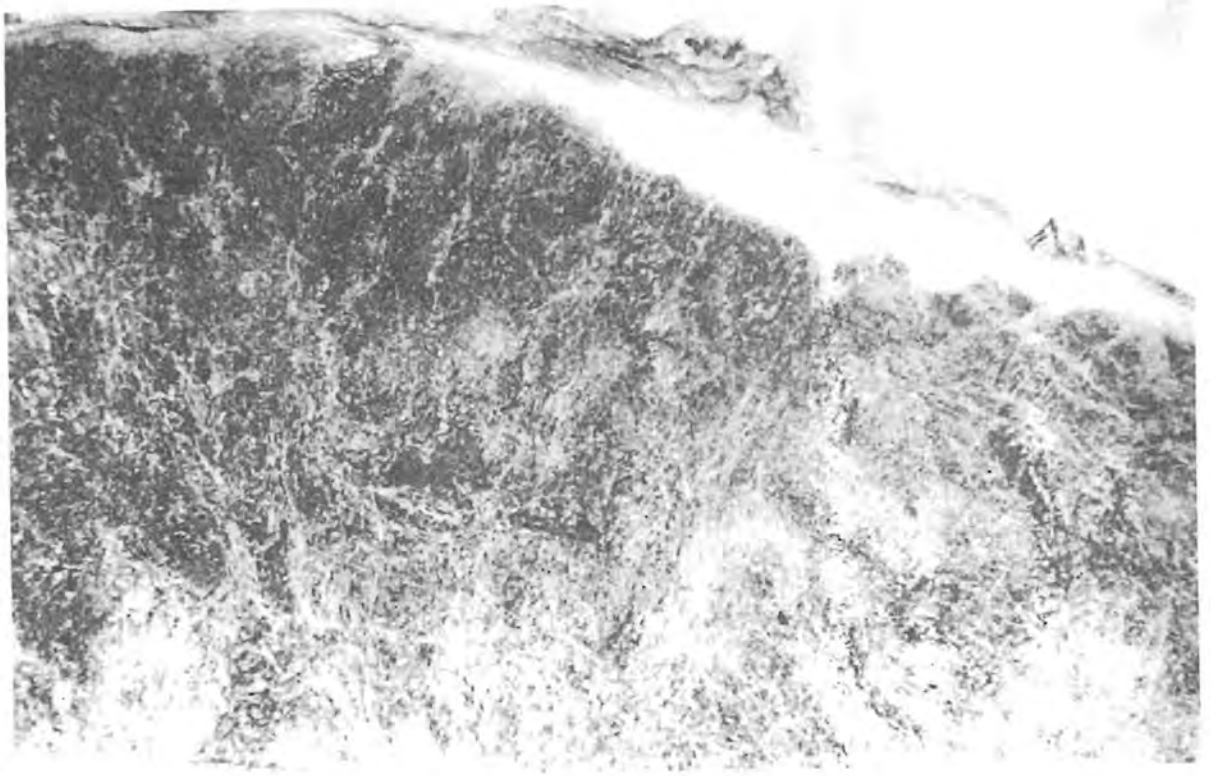

Fig. 2

Fig. 2. Cross section of the tumour showed a firm black fibrous tumour.

Fig. 3

Fig. 3. Microscopic examination showed cleft-like spaces lined by pigmented cells (thin arrows) as well as smaller cells resembling neuroblasts (bold arrows). (H & E x200).

Epstein-Barr virus prevalence in Burkitt's lymphomas in a South African population sample

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Running title: *EBV prevalence in Burkitt's lymphomas*

Abstract

The Epstein-Barr virus (EBV) is no longer thought to be the sole cause of Burkitt's lymphoma (BL) but is still accepted as a cofactor in the pathogenesis of this neoplasm. According to the National Cancer Registry of South Africa, BL represented less than 0,04% of all malignancies reported for the period of 1993-1995. No data is available on the association of EBV with BL in South Africa and it was decided to perform a pilot study in the Gauteng province of this country. Twenty-four cases of BL were retrieved from the archives of the Departments of Oral Pathology and Anatomical Pathology of Medunsa and the Universities of Pretoria and Witwatersrand. All cases were divided into two groups according to the primary site of the tumour: the Oral-Maxillo-Facial (OMF) group (14 cases) and the Non-Facial (NF) group (10 cases). *In situ* hybridisation for EBV encoded RNAs (EBERs) was performed on paraffin sections and the proliferation index (PI) of each case was determined by Ki-67. Fifty percent of the BL cases in this study were positive for the virus. The site of the primary tumour did not significantly influence the EBV status of the tumour ($p=0,88$). The mean PI was 87,5% and the EBV positive cases had a close to statistically significant higher PI than the negative ones ($p=0,05$). Larger studies should be conducted to evaluate the cytogenetics and possible role of HIV in this disease in South Africa.

Introduction

The role of viruses in oncogenesis has been a major research field during the last few decades, and it is now estimated that viruses cause approximately 15% of all human tumours[1]. The Epstein-Barr virus (EBV), a DNA virus, was first discovered in a line of explanted lymphoblasts from Burkitt's lymphoma in an Ugandian child[2], becoming the first virus to be related to human neoplasia. The virus appears to have at least two natural target cells, B-lymphocytes and epithelial cells[3]. EBV infection of B-lymphocytes results in the proliferation and transformation of some B-lymphocytes into immortal lymphoblastoid cells each carrying multiple copies of the EBV genome[4]. Once infected, individuals become life-long virus carriers[5]. The EBV genome encodes for more than a hundred genes, but only a restricted set of gene products, the six Epstein-Barr nuclear antigens (EBNA-1, -2, -3A, and –3B, -3C, and -LP), two latent membrane-associated oncoproteins (LMP-1 and -2) and two small non-polyadenylated nuclear RNA's, EBER-1 and EBER-2, are associated with latent infection[6]

Burkitt's lymphoma (BL) includes endemic BL (eBL) and sporadic BL (sBL)[7]. Of all the B-cell lymphomas EBV is most strongly associated with eBL in equatorial Africa[8]. This form of BL is recognised as the most common malignancy amongst children in tropical Africa[9, 10]. The clinical hallmark of eBL is a rapidly growing jaw tumour in a child[11]. The sporadic form of this disease has identical histological features to eBL[12], occurs worldwide, but is characteristically more prevalent in Europe and the USA[13]. Sporadic BL most commonly presents with a rapidly growing abdominal mass (70-90%), frequently involving the terminal ileum[14]. The occurrence of head and neck tumours is low in sBL[15], with jaw lesions reported in less than 9% of cases[14].

Although controversial[16], a third form of BL, Burkitt-like lymphoma, is described in the context of the acquired immunodeficiency syndrome (AIDS)[17]. Burkitt-like lymphoma involves peripheral lymph nodes, the central nervous system and bone marrow[18], with less frequent extranodal, gastrointestinal tract or jaw involvement[17]. Although these three types of

BL are morphologically indistinguishable from each other, there is sufficient clinical, immunophenotypic, genotypic and virological differences to consider them as distinct clinicopathological entities[19]. The most distinct differences are seen between eBL in equatorial Africa and sBL in the United States. Other geographic-regions are described to exhibit clinical and virological features intermediate between those of eBL and sBL[20].

The association of EBV with BL varies greatly in different parts of the world. Classic eBL is associated with EBV in more than 95% of cases[8] while sBL is less often associated with this virus, ranging from 5-15% in cases in Europe and the United States of America, 50-80% in the Middle East, South America and India, 13% in Japan and 28% in Hong Kong[21]. Interestingly, approximately 85% of tumours in North Africa contain EBV DNA, although the clinical characteristics of BL in this area are more in keeping with those of sBL[7]. The reasons for this are uncertain but might reflect the early EBV seroconversion in developing countries[22]. Despite the fact that most patients with AIDS carry a large burden of EBV, the EBV genome is detected in only 30-50% of lymphomas in patients with AIDS[23]. Although EBV is no longer thought to be the sole cause of BL, it is still accepted as a cofactor in the tumour pathogenesis by stimulating B-lymphocyte proliferation, which increases the likelihood of a selection of cells with mutation of the *c-myc* gene[24], an essential component of the pathogenesis of BL[25].

According to the National Cancer Registry of South Africa, forty cases of BL were reported during the period of 1993-1995, representing less than 0,04% of all malignancies[26]. Only one study on the possible nature or type of BL seen in South Africa could be found in the literature. In 1989, Hesseling[27] reported the clinical features, incidence, and seasonal occurrence of BL for the period 1977 to 1986, as consistent with typical eBL. The authors determined the serological EBV status in only two of the twenty-two patients in their study, but did not examine any of the tumours for the presence of the EBV-genome. Other than this study, which is not considered a true South African sample because it also included cases

from Namibia, no data is available on BL or its association with EBV in South African patients. Macdougall reported early EBV seroconversion in black South African children[28], results similar to those reported for other developing countries where eBL is strongly associated with EBV[22].

A pilot study was done in the Gauteng Province of South Africa in order to evaluate the prevalence of EBV in BL cases in a South African population sample. The possible effect of EBV on the proliferation index (PI) of the tumour cells and whether the anatomical site of the lymphoma influenced the presence of EBV was investigated.

Materials and Methods

Case selection

A retrospective study was done on BL cases diagnosed at the Departments of Oral Pathology and Anatomical Pathology of Medunsa, and the Universities of Pretoria and Witwatersrand, 1972 to 1998 inclusive. Criteria for inclusion in this study were a confirmed histological diagnosis of BL according to the criteria of the World Health Organisation[29], available clinical information including the site of the primary tumour as well as available paraffin-embedded tissue blocks for analysis by immunohistochemistry and RNA *in situ* hybridization. In order to determine if the anatomical site of the lymphoma influenced the presence of EBV, cases retrieved were arbitrarily divided into two groups according to the primary site of the tumour. The Oral-Maxillo-Facial (OMF) group included BL of the jawbones, Waldeyer's ring, supra-clavicular neck lymph nodes and oral soft tissues, and a Non-Facial (NF) group involving any infra-clavicular site. Patients with tumours at both sites were excluded from this study, as it was not possible to determine the primary site of the tumour as opposed to disease dissemination.

Immunohistochemistry

The immunophenotype of all tumours was confirmed by demonstration of B-lineage using a standard immunoperoxidase technique with L26, a pan-B-cell antigen (CD20, DAKO, Carpinteria, CA). The PI of each tumour was determined using prediluted antibody (DAKO) directed towards the Ki-67 nuclear antigen. Antigen enhancement was performed in a microwave using a pressure cooker and citric acid buffer. The lymphoid follicles in a normal reactive palatine tonsil were used as a positive control for the B-cell marker and the proliferation index of each tumour was determined by counting the number of positively stained nuclei per thousand tumour cells using a calibrated eyepiece. The number of Ki-67 positive nuclei was expressed as a percentage. Any degree of dark brown to black nuclear staining was considered positive for the Ki-67 antigen but whenever nuclear staining was doubtful, it was noted as negative.

In situ hybridisation (ISH).

To detect expression of the EBV-encoded small nuclear RNA's (EBER-1 and EBER-2), ISH with fluorescein-conjugated oligonucleotide probes was used. The probes were obtained commercially and consisted of a mixture of both EBER-1 and EBER-2 (Novocastra, Newcastle upon Tyne, UK). Probes were labeled with fluorescein isothiocyanate (FITC). Detection of hybridised probes was done with rabbit F(ab') anti-FITC conjugated to alkaline phosphatase. All glassware was treated with DEPC (di-ethyl pyrocarbonate) to prevent endogenous RNase activity. The ISH was done using the OmniSlide System (Hybaid, Teddington, Middlesex, United Kingdom). Sections of the EBV-infected cell line P3HR-1 processed to paraffin wax, was used as positive control and a section of human brain served as negative control. A dark brown to black granular stain within the nucleus of the cells was regarded a positive signal.

Statistical Analysis

Stringent testing of normality was determined by the chi^2-test. To determine the differences between the mean values of Ki-67 positivity the Student's t-test was used if the data was normally distributed, and the Mann-Whitney Test, if the data was not normally distributed. The differences between EBV positivity of the various groups were determined by the chi^2-test.

Results

Twenty-four cases complied with the inclusion criteria for this study. *Table I* demonstrates the number of patients, the EBV-positive cases and mean PI in each group.

Fifty percent of all BL cases in this study were EBV-positive and the mean PI of all twenty-four cases was 87,5%. When comparing the PI of EBV-positive cases with those of EBV-negative cases in both groups, a close to statistical significantly higher PI was found in the EBV-positive cases ($p = 0.055$). The primary site of the tumour, as arbitrary divided into the OMF and the NF groups, was also correlated with the EBV-status of the tumour tissue. The results showed that the site of the primary tumour did not significantly influence the EBV status ($p=0,88$) of such a tumour. Because of the retrospective nature of the study with some of the cases dating back to the early seventies, the HIV-status of only seventeen of the twenty-four cases was known. One of the patients in the OMF group was HIV-positive, ten were negative and four of the patients in this group had an unknown HIV-status. Two of the patients in the NF group were HIV-positive, four were negative and the HIV-status of six was unknown. The HIV-status did not significantly influence the EBV-status of the tumours in these groups.

Discussion

The three types of BL differ in their clinical presentations and EBV association, with the most distinct differences seen between eBL in Africa, and sBL in the United States of America. South Africa differs from equatorial Africa, both ethnically and environmentally. It has a divergent socio-economic status as well as several culturally different population groups living in geographical regions in which both eBL and sBL could occur. Burkitt's lymphoma accounted for approximately 0,04% of all malignancies in South Africa reported for the period 1993-1995[26]. Although EBV is no longer thought to be the sole cause of BL, the association of the virus with BL is still widely recognized. It was decided to perform a clinico-pathological study on a true South African population sample to determine the association of EBV with BL in this region.

To associate EBV with any tumour, identification of EBV genomes or gene products in the tissue is essential. Several monoclonal antibodies specific for EBNA-1, EBNA-2, LMP-1 and LMP-2A have become available for detection of latent EBV gene expression [30]. With the exception of EBNA-1, however, these viral proteins are not invariably expressed. EBER-1 and EBER-2 are consistently expressed in large numbers in all forms of EBV latency[31] and are ideal targets for ISH [32]. This method allows for the detection of viral nucleic acids in formalin-fixed paraffin-embedded tissue sections [3] and has become the most frequently used technique, accepted as the standard approach for diagnostic purposes[33]. Fifty percent of the BL's in our study showed nuclear staining as described in the literature[34, 35]. This degree of EBV positivity is at an intermediate level between the more than 95% EBV association in eBL cases in Africa [8], and 5-15% EBV association reported for sBL in the United States[21].

BL is one of the most rapidly growing tumours known, and will, when labeled with an appropriate proliferation marker, give a PI of close to 100%[16]. The Ki-67 protein is a nuclear protein required for DNA synthesis and is expressed in all phases of the cell cycle[36]. The

mean PI of tumour cells in this study was slightly lower than usually reported (87,5% vs. almost 100%). This can be ascribed to the probable sub-optimal tissue processing of the older tissue blocks with resultant poor antigen retrieval. The proliferation indices of EBV-positive tumours were compared with those of EBV-negative tumours and interestingly, the EBV-positive cases had a close to statistical significant higher proliferation index than the negative ones (p=0,055). This is in contradiction with other studies[37, 38] and it might be of value to investigate this phenomenon in larger study groups of BL.

The results from this study indicated that EBV still played an important role in the pathogenesis of BL in developing countries like South Africa. Our results also contradicted those of Hesseling *et al* who reported BL in South Africa and Namibia to be consistent with typical eBL[27]. Future research on the possible type of BL in South Africa should include cytogenetic studies as well as evaluation of the influence of HIV on the incidence, clinical features, EBV-status and molecular rearrangements of this lymphoma.

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Table I

	OMF Group	NF Group
Number of patients	14	10
EBV-positive cases	6	6
Mean PI (%)	88,6	86,1

THE ROLE OF SALIVARY GLANDS IN VERTEBRATES

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A salivary gland is defined as any organ that discharges a secretion (or saliva) into the upper part of the digestive tract. They are found not only in most vertebrates, but also in many invertebrates such as the Annelida (segmented worms), some Mollusca (snails) and Arthropoda (insects, crustacea, arachnids and related forms). In humans, salivary glands are grouped into a major (large) and minor (small) category. Three paired major salivary glands in man and are located in the neck and floor of mouth and produce 600-1500 ml saliva per day. Numerous small (or minor) salivary glands are present in the palate, tongue and lips.

In general terms, salivary glands in vertebrates can be considered as subserving many functions and are generally adapted to the environment of the animal. First and perhaps most important, saliva provide lubrication for the swallowing of food. The solution of food particles is important for the proper functioning of the taste receptors which are located in the lining of the oral cavity. The washing action of saliva as well as its antibacterial properties are essential for the maintenance of oral and dental health. In humans, lubrication evidently facilitates speech. In animals where ingested food are dry, the salivary glands are adapted to secrete large quantities of watery, sodium free and subsequently hypotonic saliva. The hypotonicity prevents dehydration of cells lining the digestive tract and increases the moisturising capacity of saliva, as both factors are dependant on osmotic principles. The paired parotid salivary glands of the African elephant is probably the largest in the animal kingdom with a combined mass of 15 kg and produce 50 liters saliva per hour during feeding. A normal feeding cycle, which extends over the greatest part of 24 hours, is characterized by the production of a large volume of saliva which moisturises and lubricates the daily diet of 200-300 kg leaves, bark and grass. Hippopotomi generally ingest lush vegetation on the banks of rivers and their salivary glands are subsequently smaller and the volume saliva produced significantly less than when compared to that of the elephant. Among aquatic animals, where lubrication of food is not necessary, salivary glands may be absent as in most Cetacea (dolphins and whales).

By secreting enzymes, salivary glands are capable of playing a role in digestion. Most animals and humans have relatively high concentrations of amylase (a starch digesting enzyme) in their saliva. The levels of this enzyme is however rather low in the saliva of the domestic cat and dog and absent from the saliva of the elephant. The significance of these findings are speculative. The high urea concentration in the saliva of the elephant is probably indicative of a recycling mechanism similar to that found in cattle where hindgut commensal protozoa, which aid in digestion, are dependant on a constant supply of urea for metabolic processes. Certain newborn animals, such as the suckling rat, produce large amounts of salivary lipase, a fat digesting enzyme which is partially responsible for the digestion of milk fats.

In fur-bearing animals such as the cat and rat, saliva plays an important role in the regulation of body temperature. By wetting their fur with saliva in response to heat, they obtain the same cooling capacity available to man by sweating.

Other less common functions of saliva is that of defence, paralysing or killing of prey. The only mammalian saliva known to be toxic is that of the Americans short-tailed shrew, Blarina brevicauda. The venom glands of snakes are modified salivary type glands which produce one of the most complex and highly evolved poisons known. Contraction of muscles surrounding the gland ejects the venom through openings on two or more teeth or fangs. In one group of sea snakes, Hydrophiidae, a large salivary type gland fulfills the functions of a salt excreting gland and plays an important role in maintaining the salt balance of the animal, making life possible in its saline environment.

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Salivary Immunoglobulin Related Proteins in 24 Patients with Multiple Myeloma

Erich Raubenheimer, Willie van Heerden, Joseph Dauth and Tracy van der Walt

Mixed saliva and blood of 24 cases of multiple myeloma (MM) were collected and the immunoglobulin and light chain concentrations compared with that found in the saliva and blood of 16 age matched control patients. The concentrations of salivary IgA, IgG and lambda light chains were significantly increased in IgA-, IgG- and lambda light chain producing MM respectively. Salivary IgA concentration in non-IgA MM and salivary IgG concentration in non-IgG MM were within normal ranges. Despite a significant decrease in circulating normal immunoglobulins, this study fails to support suppression of normal salivary immunoglobulin concentrations in patients suffering MM.
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INTRODUCTION

In the majority of patients with multiple myeloma (MM) serum protein electrophoresis will disclose the presence of a monoclonal paraprotein which may present as an increase in one of the immunoglobulin classes and/or immunoglobulin-related light chains (Bence-Jones proteins). MM are immunochemically typed according to the circulating monoclonal immunoglobulin and/or light chain type produced by the disseminated neoplastic plasma cells. This typing is helpful in predicting complications and prognosis of patients suffering MM [1]. The decrease in the concentrations of circulating normal immunoglobulins predispose to opportunistic infections, a serious and often terminal complication in MM [2].

Reports on the presence of abnormal immunoglobulin-related proteins in secretions of MM patients are infrequent in the literature. Analysis of saliva of 10 patients with MM [3], identified monoclonal IgA in 5 out of 7 patients with IgA MM and monoclonal IgG in both patients with IgG MM. No free light chains were detected in the saliva of the 1 patient with light chain producing MM. An increased concentration of IgG was present in the saliva of 1 case of IgG MM studied by Brandtzaeg [4].

The purpose of this study was to determine the concentrations of immunoglobulins and light chains in saliva and serum of 24 patients with MM and to compare the values obtained with that found in age matched, systemically healthy patients.

PATIENTS AND METHODS

Whole saliva and blood of 24 patients with MM and 16 age matched systemically healthy control patients were collected after a thorough clinical oral examination. The saliva was expressed with the aid of a sterile syringe from a cottonwool swab after it had been chewed for 3 min. Patients with overt signs of gingivitis or periodontitis were excluded from the control group of the study. Quantitation of IgA, IgG and IgM levels in serum were done with rate nephelometry (Auto ICS, Beckman Instruments Inc. Fullerton, U.S.A.). Immunochemical typing of the light chains in serum was carried out with immunofixation electrophoresis (Paragon™ IFE gels, Beckman Instruments Inc.).

Salivary immunoglobulins and light chains were quantitated with low concentration radial immunodiffusion plates (LC-Partigen® and M-Partigen®, Behringwerke AG, Marburg, West Germany). The concentrations were expressed in grams per litre (g/l), compared with the respective circulating concentrations and the findings were subjected to statistical analysis using Student's *t*-test for uncorrelated data.

RESULTS

Clinical examination of the MM patients revealed no signs of oral mucosal infections. 17 patients had IgG MM, 4 IgA MM and 3 light chain producing MM (two kappa- and one lambda MM). The mean concentrations and standard deviations of the major immunoglobulin classes in MM patients and the control group are expressed in Table 1 and the immunoglobulin light chain concentrations in Table 2. The circulating residual immunoglobulin concentrations in MM patients were generally below the normal ranges (Table 3) and that of the control group (Table 1). No significant differences were found between salivary IgA concentrations in non-IgA MM and the control group ($P > 0.05$) and salivary IgG levels in non-IgG MM and the control group ($P > 0.8$). In IgA MM, salivary IgA concentrations were found to be significantly higher than in the control group ($P < 0.01$). A significant increase in salivary IgG in IgG MM ($P < 0.01$ was also present. The concentration of lambda light chains in the saliva of lambda-producing MM was significantly higher than the control group ($P < 0.01$). Although salivary kappa light chain concentrations in kappa-producing MM showed great variations, with single values far above those of control patients, statistical analysis failed to prove a significantly higher concentration of kappa light chains in kappa-producing MM when compared to the control group ($P > 0.05$).

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Table 1. Concentrations of major immunoglobulin types in MM- and control patients

	Saliva g/l		Serum g/l		
	IgG	IgA	IgG	IgA	IgM
IgA MM (2 × IgA κ's 2 × IgA λ's)	0.04 ± 0.03	1.1 ± 0.9	5.85 ± 4.4	41.5 ± 25.3	2.0 ± 3.5
IgG MM (12 × IgG κ, 5 × IgG λ)	0.22 ± 0.16	0.05 ± 0.05	75.9 ± 32.4	0.63 ± 0.6	0.57 ± 0.42
Lambda MM ($n = 1$)	0	0.14	5.5	0.3	0.2
Kappa MM ($n = 2$)	0.7 ± 0.01	0.04	13.1 ± 1.5	0.65 ± 0.07	0.25 ± 0.07
Control ($n = 16$	0.047 ± 0.03	0.081 ± 0.03	20.0 ± 6.6	3.28 ± 1.3	2.16 ± 1.7

Table 2. Light chain concentrations in MM- and control patients

	Saliva g/l		Serum g/l	
	κ	λ	κ	λ
κ-producing MM ($n = 16$)	0.44 ± 1.0	0.006 ± 0.01	43.5 ± 24.5	2.2 ± 1.8
λ-producing MM ($n = 8$)	0.03 ± 0.06	0.16 ± 0.12	4.4 ± 2.9	55.1 ± 37.0
Control ($n = 16$)	0.03 ± 0.03	0.02 ± 0.01	13.4 ± 5.3	7.04 ± 1.5

Table 3. Normal ranges

Serum	
IgG	14.4—22.7 g/l
IgA	1.9—4.7 g/l
IgM	0.7—2.6 g/l
κ	5.66—13.0 g/l
λ	3.04—7.35 g/l
Saliva	
IgA	0.05—0.48 (mean 0.137) g/l*
IgG	0.007—0.037 (mean 0.016) g/l
κ	N/A
λ	N/A

*Gronblad 1981 [5].

DISCUSSION

This study represents the largest series in which the concentrations of immunoglobulin related proteins in saliva of patients with multiple myeloma were determined. Although changes in the circulating immunoglobulin concentrations are well documented [2], little is known of alterations in salivary immunoglobulins and immunoglobulin related proteins in this disease.

A study using immunoelectrophoresis to determine the presence of salivary immunoglobulins in 10 patients suffering MM [3] failed to express the concentrations and the findings can therefore not be compared directly to ours. These authors conclude that although the concentration of monoclonal immunoglobulin is low in saliva, its presence is adequate proof that circulating immunoglobulins can find their way into external secretions. The technique employed in our study is more sensitive and made accurate quantitation of the different immunoglobulin-related proteins possible. All our cases of IgA MM had significantly increased concentrations of IgA in saliva when compared to the salivary IgA concentrations found in the control group. The same applies to salivary IgG in IgG MM and lambda light chain concentrations in the saliva of lambda producing MM. Despite a few kappa producing MM that had high salivary kappa concentrations, statistical analysis failed to support a significant increase in salivary kappa concentrations in kappa producing MM when compared to control values. Although transmission of circulating immunoglobulin related proteins to saliva appears to be enhanced by elevated serum concentrations, no direct correlation could be found between these values.

The occurrence of systemic immune suppression in MM is well documented. This study supports the findings of Coelho *et al.* [3] which failed to identify salivary immunoglobulin impairment in MM. No statistical evidence of a decrease in the concentration of normal salivary IgA in non-IgA MM patients could be found in our study. This was confirmed in that no clinical evidence of an opportunistic infection was seen in the oral cavities of our MM patients. The mechanism by which normal immunoglobulin production is suppressed in MM, is not clearly understood [6]. It has been postulated that neoplastic plasma cells secrete a factor capable of activating suppressor macrophages which in turn inhibit normal B cell function [7]. The observation that salivary gland associated immunoglobulin production is not altered in MM, adds an interesting parameter to the debate on MM-induced immunoparesis.

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Intraoral salivary gland neoplasms: A retrospective study of seventy cases in an African population

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Intraoral salivary gland neoplasms diagnosed in the Department of Oral Pathology, Medical University of Southern Africa, Medunsa, were reassessed and revised with regard to histologic diagnosis. New entities and subclassifications that have been described in recent years were taken into account. Seventy cases were diagnosed during an 8-year period, and the sample consisted of black patients only. Benign mixed tumor was the most common entity and accounted for 48% of all tumors. Polymorphous low-grade adenocarcinoma comprised 15.7% of the sample and was the most frequent malignant tumor. The mean age of patients with benign and malignant tumors were 36.5 and 49.8 years, respectively ($p < 0.05$), and the palate was the most common site involved. Geographic differences do exist in the pattern and pathology of intraoral salivary gland neoplasms when compared with findings in other studies.
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The distribution and frequency of intraoral salivary gland neoplasms has been discussed in several published series,[1-5] in the majority of which the World Health Organization (WHO) classification[6] was used. However, various new entities and subclassifications that are not included in these articles have been described in recent years.[7, 8] This study was undertaken to determine the relative frequency and distribution of intraoral salivary gland neoplasms in a predominantly rural black African population and to provide data for comparison with findings in other geographic locations.

MATERIAL AND METHODS

All the intraoral salivary gland neoplasms diagnosed during the last 8 years were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa, Medunsa. Most patients seen at the hospitals served by the department are black and of rural southern African origin. Representative slides stained with hematoxylin and eosin were available for review, and, where necessary, appropriate special stains were used to establish a diagnosis. All cases were reassessed and revised with regard to histologic classification. Diagnosis was made with the WHO classification[6] as the basis. New entities such as polymorphous low-grade adenocarcinoma, and subclassifications that have been described in recent years, were taken into account. This includes the subclassification of mixed salivary gland tumors into types I to IV according to the proportion of the stroma in the tumor mass.[8] The polymorphous low-grade adenocarcinomas were divided into the terminal duct type and the papillary type according to the criteria of Slootweg and Müller.[9] The working classification used in this study is shown in Table I. Age, sex, and site were noted from the clinical records.

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Table I. Working classification

- Benign
  - Mixed tumor
    - Type I (stroma 30%-50%)
    - Type II (stroma >80%)
    - Type III (stroma <20%)
    - Type IV (myoepithelioma)
  - Monomorphic adenoma
- Malignant
  - Mucoepidermoid carcinoma
    - Low grade
    - Intermediate grade
    - High grade
  - Adenoid cystic carcinoma
    - Cribriform
    - Tubular/trabecular
    - Solid
  - Polymorphous low-grade adenocarcinoma
    - Terminal duct
    - Papillary
  - Acinic cell carcinoma
  - Carcinoma ex mixed tumor
  - Epidermoid carcinoma
  - Adenocarcinoma
  - Epithelial-myoepithelial carcinoma
  - Undifferentiated carcinoma

RESULTS

The sample consisted of a total of 70 cases of intraoral salivary gland neoplasms. Forty-three (62%) of the patients were female and 27 (38%) were male, yielding a female/male ratio of 1.6:1. The patients ranged in age from 10 to 85 years. Thirty-four cases (48%) were classified as benign; all these were mixed tumors in patients ranging in age from 10 to 64 years, with a mean age (±SD) of 36.5 ± 14.7 years. The female/male ratio was 2.4:1, with the mean age for females 34.9 ± 14.9 years and 40.3 ± 13.1 years for males. The palate was most commonly affected, accounting for 31 tumors (91%). The remaining mixed tumors were found on the upper lip. The location and subclassification of mixed tumors according to the criteria of Seifert et al.[8] are shown in Table II.

Thirty-six cases (52%) were classified as malignant. The patients ranged in age from 22 to 85 years, with a mean age of 49.8 ± 16.3 years. The difference in the mean age of patients with benign tumors and that of those with malignant tumors was statistically significant ($p < 0.05$). The female/male ratio was 1.1:1 for patients with malignant neoplasms. The distribution and location of the malignant tumors are shown in Table III.

Eleven tumors were diagnosed as polymorphous low-grade adenocarcinoma, accounting for 30% of the malignant neoplasms and 15.7% of all neoplasms. The patients' ages were between 32 and 70 years, with a mean age of 53 ± 12.6 years, and the female/male ratio was 1.2:1. Nine lesions (82%) occurred on the palate, and one each in the buccal mucosa and upper lip, respectively. Two tumors had a papillary-type growth pattern, and nine were either lobular or tubular in appearance. Nerve infiltration was present in one papillary-type and in two terminal duct–type tumors.

Table II. Location and subclassification of 34 mixed tumors

Type	*Palate*	*Upper lip*	*Total (%)*
I	25	1	26 (76)
II	2	1	2 (6)
III	5	1	6 (18)
IV			0
Total (%)	31 (91)	3 (9)	34 (100)

Nine cases of adenoid cystic carcinoma accounted for 12.8% of all tumors and 25% of the malignant tumors. The patients had a age range of 33 to 85 years with a mean of 54 ± 15.5 years, and the male/female ratio was 1.2:1. Seven patients had lesions on the palate, and one lesion each was located in the floor of the mouth and on the upper lip. Five tumors had a predominantly cribriform growth pattern, and two each had solid and tubular or trabecular growth, respectively.

Six patients with mucoepidermoid carcinoma accounted for 8.6% of all tumors and 16.7% of malignant tumors. The youngest patient was 22 years and the oldest 52 years of age, and the mean age at time of consultation was 39.8 ± 10.1 years. Sex distribution was equal, and the most common site of occurrence was the palate, with four tumors. One tumor was located on the buccal mucosa and mandibular gingiva, respectively. One mucoepidermoid carcinoma was classified microscopically as low grade, three as intermediate grade, and two as high grade.

Five carcinomas ex mixed tumor were diagnosed. Three of the patients were women and two were men; they ranged in age from 31 to 70 years, with a mean age of 48.2 ± 19.3 years. Four tumors occurred on the palate, and one in the retromolar area. The carcinomatous component in all five was classified as undifferentiated.

Three cases were diagnosed as adenocarcinomas, not otherwise specified. Two tumors were located in the buccal mucosa and one on the palate. Two cases occurred in females, and the mean age of this group was 57.3 ± 13.2 years. One tumor that occurred on the palate of a 49-year-old woman was diagnosed as an undifferentiated carcinoma.

One patient, a 65-year-old woman, had a epithelial-myoepithelial carcinoma of the palate. No case of monomorphic adenoma, acinic cell carcinoma, or

Table III. Distribution and location of malignant tumors

Tumor	*Palate*	*Upper lip*	*Buccal mucosa*	*Mouth floor*	*Retromolar*	*Mandibular gingiva*	*Total*	*% of total*	*% of malignant*
PLA	9	1	1				11	15.7	30
ACC	7	1		1			9	12.8	25
Mucoepidermoid CA	4		1			1	6	8.6	16.7
CA ex mixed tumor	4				1		5	7.1	14
Adenocarcinoma	1		2				3	4.3	8.3
Undifferentiated CA	1						1	1.4	2.8
E-M CA	1						1	1.4	2.8
Total (%)	27 (75)	2 (5.5)	4 (11)	1 (2.8)	1 (2.8)	1 (2.8)	36		

ACC, Adenoid cystic adenocarcinoma; *CA*, carcinoma; *E-M*, Epithelial-myoepithelial; *PLA*, polymorphous low-grade adenocarcinoma.

epidermoid carcinoma of the minor salivary glands occurred in this series.

DISCUSSION

In most studies benign mixed tumors constitute the majority of minor salivary gland neoplasms.[1-5] The frequency of benign mixed tumors is reported as 43% in the study of Eveson and Cawson,[4] 41% by Waldron et al.,[1] and 54% by Chau and Radden.[5] In Isacsson and Shear's series[2] 70% of the tumors were classified as benign mixed tumors. They postulated that the high frequency in their series was the result of the relative higher number of black than white patients, although 60% of their white patients had mixed tumors diagnosed. Schulenburg[10] reported that intraoral benign mixed tumors in his South African sample were 3.5 times more common in black than in white patients. In the present series, where the sample consisted of black patients only, 34 (48%) of the tumors were classified as benign mixed tumors, a frequency comparable to that reported in population samples in the United States and Europe.[1, 4]

The majority of tumors (52%) in the present study were malignant, a finding that does not support the ratio of benign to malignant tumors in recent reports. The proportion of benign tumors varied from 53%[11] to 72%[2] in recent studies. However, 80% of the cases reported by Spiro et al.[12] were classified as malignant. This high percentage of malignant tumors can be explained by the fact that their institution is a major cancer referring center.

The palate was the most common site of involvement of both malignant and benign tumors. The proportion of benign tumors occurring on the palate was larger than in the malignant group, although the difference is not statistically significant. Eighty-one percent of benign mixed tumors reported by Isacsson and Shear[2] occurred on the palate. This high frequency of palatal involvement might be due to the presence of black patients in both samples. The distribution of palatal tumors from several large series compared with our findings is reflected in Table IV.

Table IV. Reported frequency of intraoral salivary gland tumors of palate

Author	*Frequency (%)*	
	Mixed tumor	*Malignant tumor*
Present study	91	75
Thomas et al.[14]	65	63
Isacsson and Shear[2]	81	60
Eveson and Cawson[4]	60	55
Waldron et al.[1]	54	42
Regezi et al.[3]	55	49
Chau and Radden[5]	70	54
Chaudhry et al.[11]	65	35

The benign mixed tumors occurred at a significantly younger age than did the malignant tumors ($p < 0.05$), and a high percentage of the benign tumors affected female patients. These observations support the proposal by Isacsson and Shear[2] that in an African population a salivary gland tumor of the palate occurring in a relatively young patient is more likely to be benign than malignant. This appears to be especially true in women.

Seifert et al.[8] divided benign mixed tumors into four types according to the volume and properties of the stroma and the differentiation of the epithelial cells. Although types III and IV constituted 35% of minor salivary gland mixed tumors in their series, almost 50% of the carcinomas ex mixed tumor arose from tumors with these growth patterns.[8] We are unable to comment on the rate of malignant transformation of types III and IV because only small fragments of benign mixed tumor were present in the carcinomas ex mixed tumor in our series. The finding of Seifert et al.[8] could be related to the more common occurrence of mitotic activity in the solid areas. The majority of benign mixed tumors in the present series were classified as type I. Although mitotic activity, when present, was usually restricted to the solid parts of the tumor, the subclassification depended on the

amount of sections taken, because the growth pattern varied through the tumor.

The absence of monomorphic adenomas in the present study may be due to the fact that our sample consisted of black patients only. Isacsson and Shear[2] found three monomorphic adenomas (2.2%) in their sample of 136 black patients. Davies et al.,[13] in a study of salivary gland tumors in Uganda, found no monomorphic adenomas in 33 intraoral tumors. Thomas et al.,[14] who analyzed salivary gland tumors in Malawi, found one monomorphic adenoma (2%) in their total of 57 minor tumors. These frequencies are in contrast with the 10.7% reported by Waldron et al.,[1] 11% by Evenson and Cawson,[4] and 10% by Regezi et al.[3]

Polymorphous low-grade adenocarcinoma was the most common malignant tumor in the present series. Comparison of the frequency of polymorphous low-grade adenocarcinoma with that reported in other studies is difficult because the majority employed the WHO classification,[6] which does not recognize polymorphous low-grade adenocarcinomas as a separate entity. Polymorphous low-grade adenocarcinoma constituted 30% of the malignant tumors in the present study. Freedman and Lumerman[15] found polymorphous low-grade adenocarcinoma to constitute 7% of the 150 malignant intraoral tumors they examined. Aberle et al.[16] reviewed 109 cases of adenocarcinoma not otherwise specified, malignant mixed tumor, and adenoid cystic carcinoma, and found that 17% of their cases met the criteria of polymorphous low-grade adenocarcinoma. In the study of Waldron et al.[1] 26% of the malignant tumors were diagnosed as polymorphous low-grade adenocarcinoma. The differences among these findings are probably related to the criteria used for diagnosis of polymorphous low-grade adenocarcinoma, because overlapping histologic features with adenoid cystic carcinoma do exist.

The frequency of adenoid cystic carcinoma (12.8%) in our series is similar to that reported in the literature, for example, 13.1% by Eveson and Cawson,[4] 10.9% by Regezi et al.,[3] and 10.4% by Isacsson and Shear.[2] Adenoid cystic carcinoma accounted for 25% of the malignant tumors in our series, a figure lower than the 38% reported by Isacsson and Shear[2] and the 31% of Regezi et al.[3] Polymorphous low-grade adenocarcinoma was not classified as a separate entity in the previously mentioned series, and the reported frequencies of adenoid cystic carcinoma are probably too high.

In the majority of studies mucoepidermoid carcinoma was the most frequent type of malignant tumor, accounting for 15%[1] to 34%[11] of all intraoral salivary gland tumors. Mucoepidermoid carcinoma accounted for 8.6% of all tumors in the present series. This figure compares with the 6.5% reported by Isacsson and Shear,[2] also in a South African population. This corroborates the suggestion by Everson and Cawson[17] that a geographic variation in the frequency of mucoepidermoid carcinoma exists.

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Evaluation of the nucleolar organizer region associated proteins in minor salivary gland tumors

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Forty-three intraoral salivary gland tumors were studied to determine the value of the AgNOR technique in the assessment of these neoplasms. Well defined black dots were visible in the nucleii of all the specimens studied. The mean AgNOR count per nucleus for each tumor was calculated as follows: pleomorphic adenoma ($n=15$) 1.52; Polymorphous low-grade adenocarcinoma ($n=12$) 1.90; adenoid cystic carcinoma ($n=6$) 2.92; mucoepidermoid carcinoma ($n=4$) 1.93; carcinoma ex mixed tumor ($n=4$) 2.05; undifferentiated carcinoma ($n=1$) 3.13 and epithelial-myoepithelial carcinoma ($n=1$) 2.23. The difference between the means of benign and malignant tumors ($P<0.01$) and polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma ($P<0.01$) were highly significant. The overlapping of the AgNOR count betwen various tumors prohibited the use of this technique as an absolute criterion in establishing a final diagnosis. It could however be used as a diagnostic aid in differentiating between salivary gland neoplasms.

Key words: nucleolar organizer regions; salivary gland tumor.

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Nucleolar organizer regions (NORs) are loops of ribosomal DNA that transcribe to ribosomal RNA and thus ultimately to protein (1). NORs have been utilized by cytogeneticists for the evaluation of certain genetic disorders, notably trisomies and are located on the short arms of the five acrosentric chromosomes 13, 14, 15, 21, and 22 (2). The NORs can be demonstrated by means of a silver staining technique (AgNORs) that is performed at room temperature on paraffin embedded tissues (3). This technique is based on the argyrophylia of the NOR-associated proteins. The known NOR associated proteins are RNA polymerase I, C23 (nucleolin), B23, 100K and 80K protein (1). Their function is uncertain although a role in rDNA transcription is postulated (1).

AgNOR counts appear to relate to cell ploidy (4) as well as the rate of cellular proliferation of individual cells (5). Quantification of NORs by means of the AgNOR technique has been used to distinguish between high and low grade lymphomas (5) and between benign and malignant counterparts of various origins (6–9).

Small biopsy specimens from salivary gland tumors is often difficult to interpret and additional microscopic criteria can only benefit the diagnostic process. MORGAN *et al.* (10) and MATSUMURA *et al.* (11) have found a statistically significant difference between the numbers of AgNORs in the nuclei of benign versus malignant salivary gland neoplasms. For this technique to have an application in diagnostic histopathology, it's ease of interpretation and reproducibility between laboratories is important. This study was undertaken to evaluate the AgNOR staining technique as a diagnostic aid for salivary gland neoplasms.

Material and methods

Forty-three intraoral salivary gland tumors were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa. Fifteen were diagnosed as pleomorphic adenomas (PA) twelve as polymorphous low-grade adenocarcinomas (PLA), six as adenoid cystic carcinomas (ACC), four carcinomas ex pleomorphic adenoma, four as mucoepidermoid carcinomas (MEC), one as an undifferentiated carcinoma and one as an epithelial-myoepithelial carcinoma. The tissue samples had all been fixed in 10% formalin and processed to paraffin wax. Two 3 µm paraffin sections of each specimen were cut. One was stained with hematoxylin-eosin and the other with the AgNOR method as described by PLOTON *et al.* (3). The H&E sections were all reassessed and revised with regards to histologic classification. The AgNOR stained sections were examined under a 100X oil immersion lens by the two authors and intranuclear dots were counted in 200 randomly selected nuclei using an eyepiece graticule to prevent recounting. Nuclei of overlapping tumor cells were not included. Nucleolar clusters were counted as a single AgNOR and no attempt was made to resolve the clusters into their discernible number of discrete dots. The mean number of AgNOR dots per nucleus was determined for each specimen. The resulting data were analyzed by means of student's t-test for uncorrelated data.

Results

The NOR associated proteins were visible as well defined black dots inside and

Table 1. Mean number of AgNORs in the nuclei of salivary gland neoplasms

Specimen	PA (Type)	PLA	ACC (Growth Pattern)	MEC (Grade)	(Ca ex PA)	Undiff ca	EPI
1	1.11 (I)	1.30	3.06 (T)	1.19 (IG)	2.37	3.13	2.23
2	1.37 (I)	1.96	2.00 (T)	1.80 (IG)	1.40		
3	1.23 (III)	1.53	3.01 (T)	2.34 (HG)	2.63		
4	1.75 (I)	1.84	4.29 (C)	2.36 (HG)	1.79		
5	1.73 (I)	1.86	2.84 (C)				
6	1.71 (I)	1.88	1.78 (C)				
7	0.98 (I)	2.02					
8	1.52 (II)	1.93					
9	1.93 (I)	2.47					
10	1.41 (I)	2.09					
11	1.60 (I)	2.24					
12	1.48 (I)	1.64					
13	1.30 (I)						
14	2.25 (I)						
15	1.37 (III)						
Mean	1.52	1.90	2.83	1.93	2.05	3.13	2.23
SD	0.32	0.31	0.89	0.55	0.55		

PA = pleomorphic ademona; PLA = polymorphous low-grade adenocarcinoma; ACC = adenoid cystic carcinoma; MEC = mucoepidermoid carcinoma; CA ex PA = carcinoma ex pleomorphic adenoma; Undiff Ca = Undifferentiated carcinoma; EPI = epithelial-myoepithelial carcinoma; T = tubular/trabecular; S = solid; C = cribriform; IG = intermediate grade; HG = high grade

outside of the nucleolus of the tumor cells studied. Careful focussing was essential to clearly identify all the dots. The results were summarized in Table 1. The lowest mean of AgNOR dots per nucleus was found in PA (Fig. 1) and the highest in an ACC (Fig. 2). The dots in the malignant neoplasms had a greater variability in size and shape compared to those in the PA. The difference in the mean number of dots per nucleus between PA and PLA (Fig. 3) and between PLA and ACC were statistically highly significant ($P < 0.01$). The correlation coefficient between the two observers was 0.97.

Discussion

The reason for the varying quantities of AgNORs in nuclei of different tumors is uncertain. The NORs are located on the 5 acrocentric chromosomes resulting in 10 NOR bearing chromosomes during metaphase. These individual NORs are usually not discernible because they are tightly aggregated in the one or two nucleoli normally present in a cell (12). Active cell proliferation may be accompanied by nucleolar dissociation, resulting in dispersed AgNORs throughout the nucleus. This as well as an increase in transcriptional activity

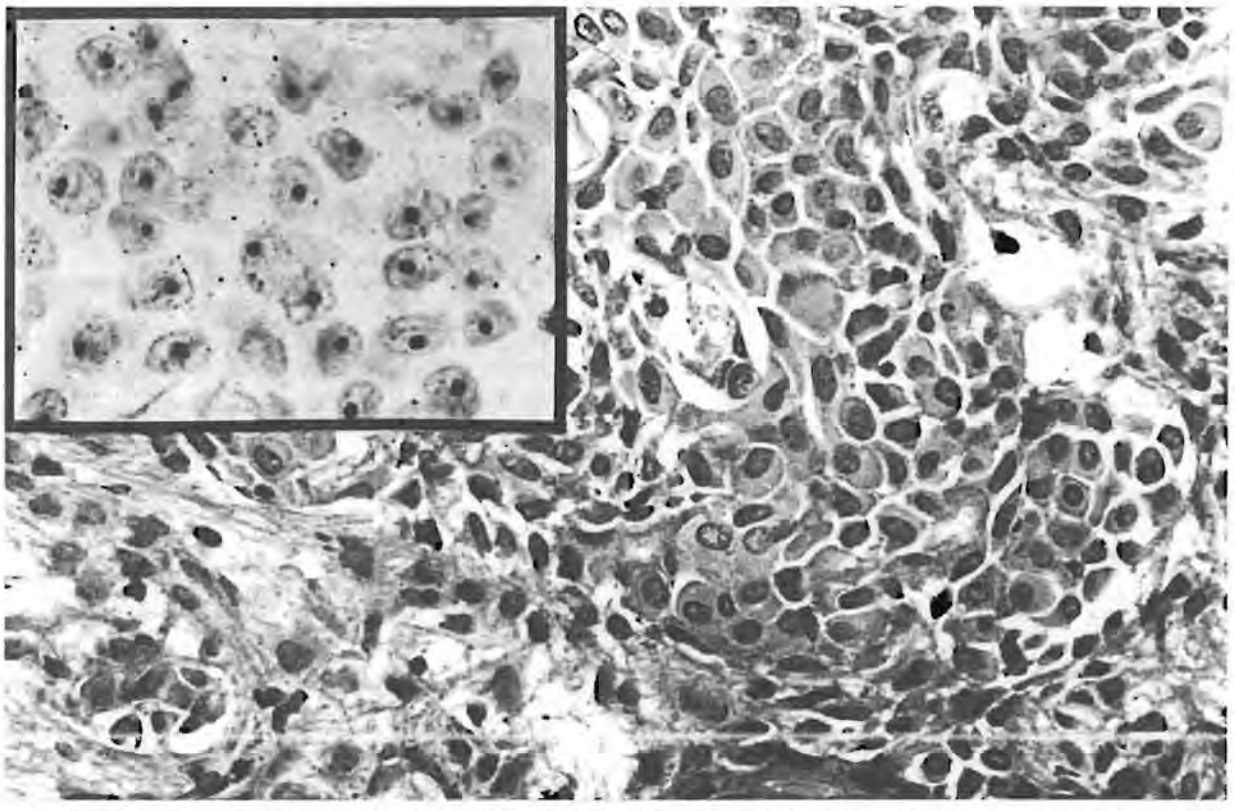

Fig. 1. Pleomorphic adenoma with plasmacytoid tumor cells. × 200. Inset: most cells contained one AgNOR dot per nucleus. × 400.

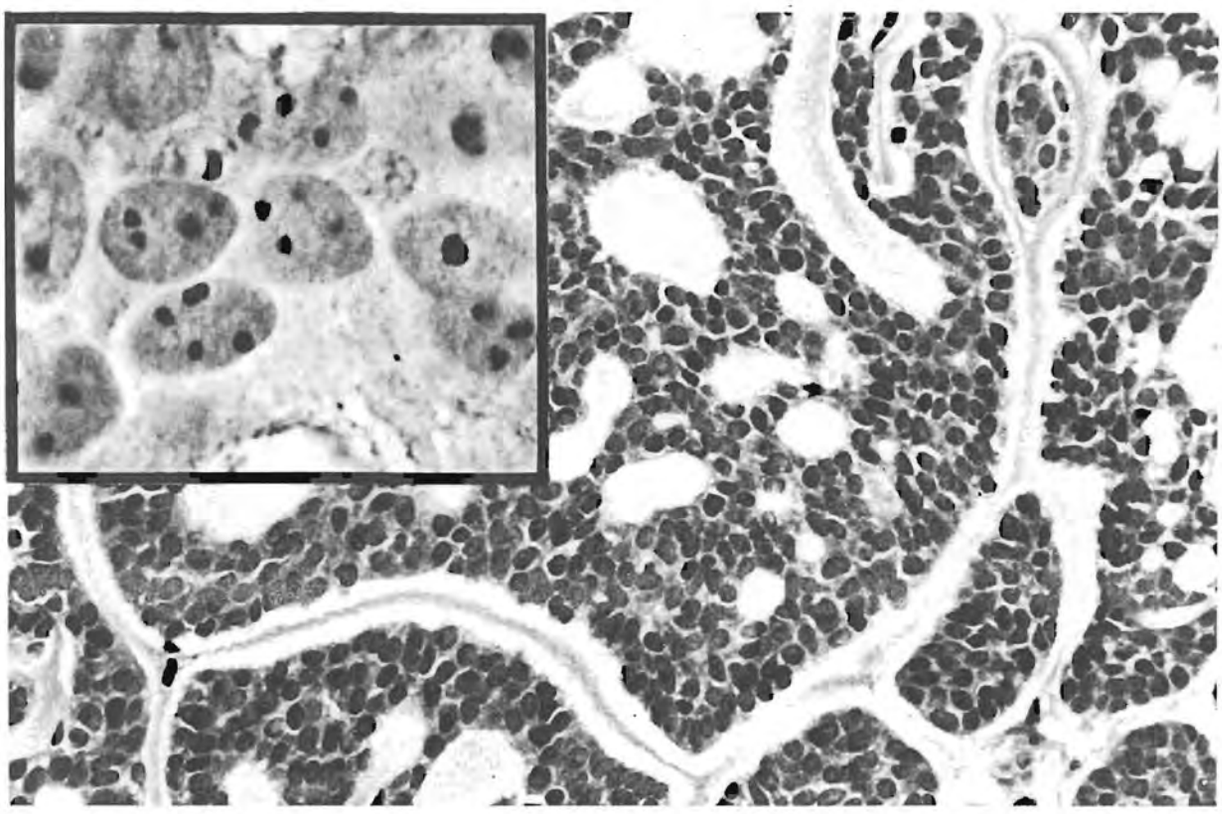

Fig. 2. Adenoid cystic carcinoma with cribriform growth pattern. × 200. Inset: multiple small AgNOR dots were present in nuclei. × 1000.

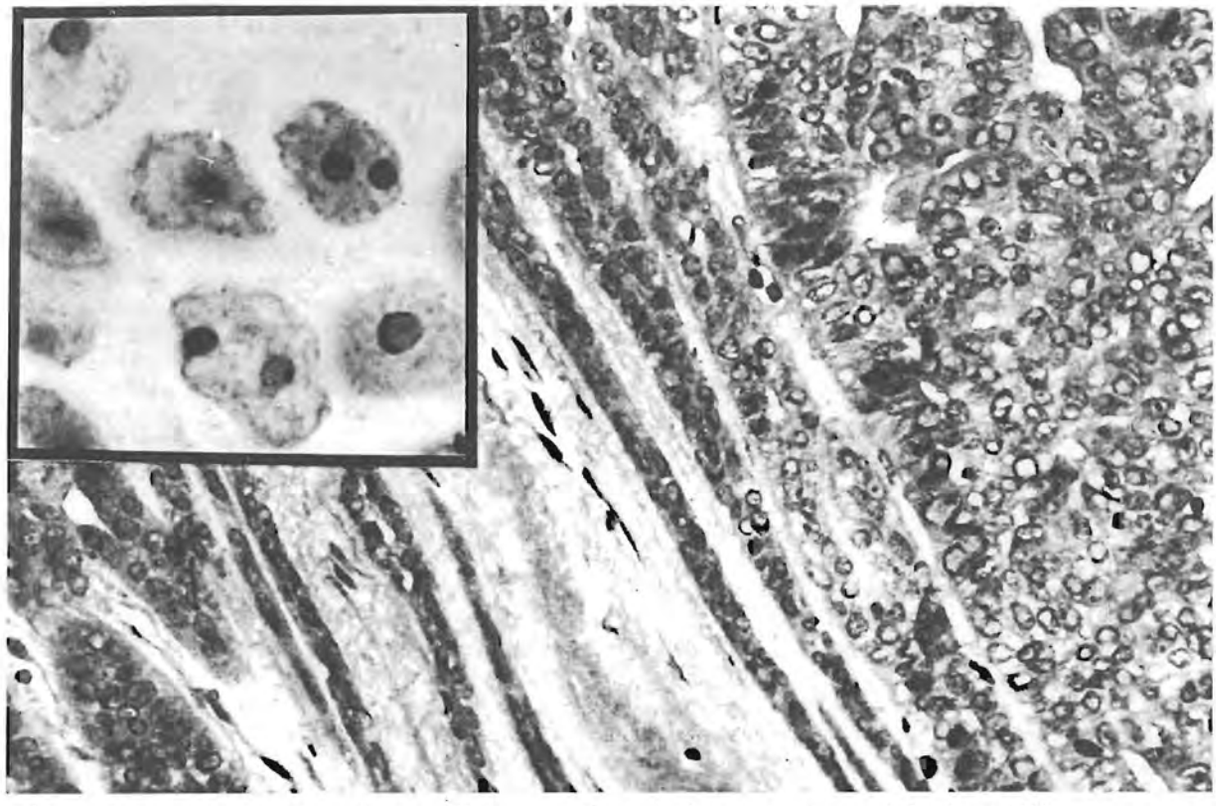

Fig. 3. Polymorphous low-grade adenocarcinoma. × 200. Inset: nuclei contained one or two large AgNOR dots. × 1000.

Table 2. Reported mean AgNOR counts in salivary gland neoplasms

Neoplasm	Present study	MORGAN *et al.*(10)	MATSUMURA *et al.* (11)
PA	1.52	1.47	1.62–1.68*
ACC	2.83	3.92	2.78
MEC	1.93	4.25	2.59

PA = pleomorphic adenoma; ACC = adenoid cystic carcinoma; MEC = mucoepidermoid carcinoma; * = different cell types in the same tumor were separately counted

will result in an increase in the mean AgNOR count of a cell population. In malignancy, the AgNORs tend to become more dispersed through the nucleus and thus more readibly discernable (12). HALL *et al.* (13) have shown that there is a significant correlation between the AgNOR count and presence of positive Ki-67 immunostaining in cells. Ki-67 is a monoclonal antibody that recognizes a nuclear antigen present only in proliferating cells (14). The possibility that the AgNOR count is related to cellular activity is also suggested by PLOTON *et al.* (3). SURESH *et al.* (4) have shown that AgNOR counts in non neoplastic trophoblastic tissue are a reflection of ploidy rather than cell proliferation. They suggested that the relationship between cell ploidy and AgNOR counts can be obscured in neoplastic lesions because of excessive proliferative activity of tumor cells.

Both ACC and PLA have an infiltrative growth pattern with an affinity for perineural spread. Cribriform, tubular and solid tumor cell arrangements can be found in ACC and PLA (15). Histologically, ACC differs from PLA in that the tumor cells have very little cytoplasm and contains hyperchromatic nucleii. Mitotic activity can be found in both tumors, although none of the PLA in our collection had a mitotic index of more than 5 mitotic figures per 10 high power fields (×400). Pleomorphism is absent in PLA whereas polymorphism is seldom seen in ACC. Despite these differences, it can be very difficult to distinguish between PLA and ACC, especially when only a small tissue fragment is submitted for histologic examination. ACC and PLA are thought to develop from the same precursor cell line (16) with the result that special staining techniques used as diagnostic procedures must be able to distinguish between cellular differentiation or activity of the two lesions. Various immunohistochemical techniques have shown potential with regards to the pathogenesis and differentiation of salivary gland tumors, although their reliability and diagnostic value is often unclear (10). A statistically significant difference between the mean AgNOR count in PLA and ACC was found in the present study. The higher count in ACC could probably be related to the more aggressive behaviour of this neoplasm when compared to PLA. The mean count in ACC did not correlate with the histologic growth pattern, a prognostic factor for tumor behavior in ACC. The highest count was present in a tumor with a predominantly cribriform growth pattern. In a study to evaluate the prognostic factors for ACC, HAMPER *et al.* (17) inter alia assessed DNA contents of the tumor cells using single cell scanning cytophotometry. They concluded that the shortest survival time was found in patients with tumors showing atypical histograms of nuclear contents of which 42% had a cribriform growth pattern. Previous studies (10, 11) evaluating the AgNOR technique in salivary gland neoplasms did not identify PLA as a separate entity, making comparison with the present study regarding PLA impossible. The overlapping of the AgNOR count between PLA and ACC prohibited the use of this technique as an absolute criterion to establish a final diagnosis but it could be used as a diagnostic aid to differentiate between these two neoplasms.

The mean value for MEC in the present study was lower than the value determined by MORGAN *et al.* (10) and MATSUMURA *et al.* (11) (Table 2). They do not specity the histologic grade of MEC included in their study. Although only four MEC's were examined in the present study, a substantial higher AgNOR count was found in the two high grade MEC. No significant difference in the AgNOR counts was found between the different types of BMT as classified using the criteria of SEIFERT *et al.* (18). This is supported by the findings of CHAU & RADDEN, (19) that there is no difference in the recurrence rate and frequency of capsular infiltration between the different subtypes of BMT.

The overlap between the AgNOR ranges in different tumors can be expected, since the absolute numbers of AgNORs in nucleii are not counted in 3 µm sections. Some AgNORs may have been missed in the 3 µm sections, especially in the malignant and high grade tumors where the nuclei were large and multiple small AgNORs were present.

The argyrophilic staining of AgNOR is not a method for demonstrating the nucleolus, but rather a technique to demonstrate its substructures in such a way as to allow study of their shape and number. Although the evaluation of AgNOR stains are time consuming, it appears to be of value in differentiating between salivary gland neoplasms.

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The relationship between Nucleolar Organiser Regions and DNA content in Salivary Gland Neoplasms

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Abstract

Thirty-three intraoral salivary gland neoplasms were evaluated to determine the proliferative index ($+G_2M$ fraction) and ploidy status and to correlate these findings with the nucleolar organiser regions (NOR) counts. Formalin fixed, paraffin-embedded tissue was used in all the cases. The mean proliferative index for each tumour was calculated as follows: pleomorphic adenoma (n = 11) 4.1; polymorphous low grade adenocarcinoma (n = 8) 6.8; adenoid cystic carcinoma (n = 3) 6.7; mucoepidermoid carcinoma (n = 4) 7.3; carcinoma ex pleomorphic adenoma (n = 3) 5.1; undifferentiated carcinoma (n = 1) 4.5 and epithelial-myoepithelial carcinoma (n = 1) 8.2. Three tumours, two adenoid cystic carcinomas and one carcinoma ex pleomorphic adenoma showed aneuploid stemlines.

Although a positive correlation between the AgNOR count and proliferative index of the salivary gland neoplasms was found, it was statistically not significant.

Introduction

Nucleolar organiser regions (NORs) are collections of nucleolar proteins associated with ribosomal genes that can be demonstrated in histologic sections using a silver staining technique (AgNOR)[1]. This technique is based on the argyrophylia of the NOR-associated proteins[2]. NORs are located on the short arms of the five acrocentric chromosomes 13, 14, 15, 21 and 22. The known NORs are RNA polymerase I, nucleolin, B23, 100K and 80K protein[1]. Their function is uncertain but a role in rDNA transcription is postulated. The quantification of AgNORs in histologic sections has been used as a diagnostic aid in distinguishing between benign and malignant tumours of various origins[3-6]

DNA content can be determined by flow cytometry by using fluorescent dyes that bind stoichiometrically to DNA[7]. The fluorescence intensity emitted by each nucleus through laser excitation is directly proportional to the DNA content of the cell[7]. The cell cycle is divided according to the amount of DNA in the nucleus at a particular time. Nuclei of cycling cells in the pre synthesis or G1 phase has a diploid or 2N amount of DNA. When the cells start to duplicate their DNA they have an intermediate amount of DNA between 2N and 4N. This phase is referred to as the synthesis phase (S-phase) and is of variable duration. After completion of the S-phase the cells enter the post synthesis phase (G2 phase) in which they have a 4N amount of DNA. The cells finally enter the mitotic phase (M-phase) and divide, whereafter they return to the G1 phase or enter a resting (G0 phase). In flow cytometry, cells in the G0 and G1 phases cannot be distinguished from each other, as they all have 2N DNA content. The same implies to cells in the G2 and M phases with a 4N DNA content (Figure 1).

The association between aneuploidy and aggressive tumour behaviour has been established for neoplasms from various sites[8-10]. The proliferation rate as defined by the S-phase fraction has also been used as a prognostic factor in adenoid cystic carcinomas of the head and neck[11].

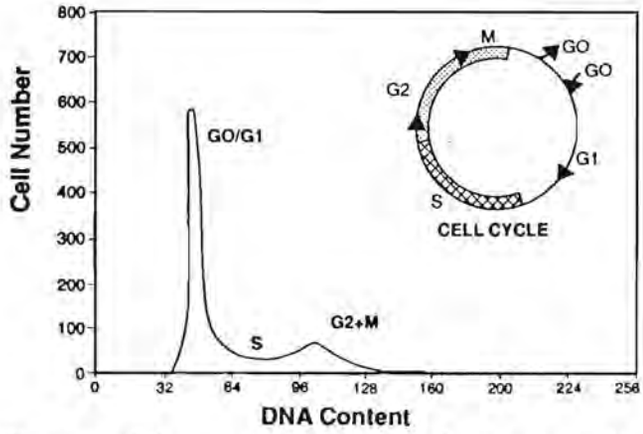

Figure 1: *Schematic representation of the relationship between DNA changes during the cell cycle and DNA histogram by flow cytometry. G0 represents the resting cells not taking part in the cell cycle.*

The reason for different quantities of AgNORs in nuclei is uncertain. The relationship between the AgNOR count and cellular activity has been demonstrated by Crocker and Nar[3]. Suresh *et al* however, have shown that AgNOR counts in non-neoplastic trophoblastic tissue are a reflection of ploidy rather than cell proliferation[12]. The purpose of this study was to determine the proliferative index and the ploidy status of minor salivary gland neoplasms and to correlate these findings with the AgNOR counts previously evaluated in the same tumours.

Materials and Method

Formalin fixed, paraffin embedded tissue from thirty-three intraoral salivary gland neoplasms, all included in a previous study where the AgNOR counts were evaluated[13] were retrieved. The tissue samples had all been fixed in 10% formalin and processed to paraffin wax. Sections were cut at 3cm thickness and dewaxed. The AgNOR solution comprised 2% gelatin in 1% formic acid that was mixed in a proportion of 1:2 volumes with 50% aqueous silver nitrate. This was immediately poured over the tissue sections and left for 30 min at room temperature. Counter staining was not performed. The AgNOR stained sections were examined under a 100x oil immersion lens and intranucleur dots were counted in 200 randomly selected nuclei using an eyepiece graticule to prevent recounting. Nuclei of overlapping tumour cells were not included. Nucleolar clusters were counted as a single AgNOR and no attempt was made to resolve the clusters into their discernible number of descrete dots. The mean number of AgNOR dots per nucleus was determined for each specimen. Eleven were diagnosed as pleomorphic adenomas (PA), eight as polymorphous low grade adenocarcinomas (PLA), five as adenoid cystic carcinomas (ACC), four as mucoepidermoid carcinomas (MEC), three as carcinoma ex pleomorphic adenoma, one as an undifferentiated carcinoma and one as an epithelial-myoepithelial carcinoma. Four 50μm sections from each

paraffin embedded block were cut and prepared for flow cytometry according to the Hedley method using a 0,5% pepsin solution[14]. The final cell suspension was passed through a 35mm mesh and the cell concentration established by means of a Coulter counter (Model FZ, Coulter Electronics, Hialeah, Fl). The cell concentration was adjusted to $\pm 2.0 \times 10^6$ cells/ml. The nuclei were stained with Propidium Iodide using a Coulter DNA Prep system, according to the manufacturers instructions. The cells were then analysed on an Epics Elite flow cytometer (Coulter Electronics, Hialeah, Fl) which had been calibrated with chicken red blood cells and DNA check beads. The Elite was operated at 15 mW and emitted an Argon ion laser at 488nm. The date rate varied between 20 – 200 events/second and 10 000 – 20 000 events were collected on a single parameter histogram. All data was collected in listmode fashion and the DNA histograms were analysed using Multicycle DNA analysis software program (Phoenix Flow Systems, San Diego, CA).

By convention, when using paraffin embedded tissue, the first peak was considered to be the normal DNA diploid peak representing the G0G1 phase of the cycle. DNA aneuploidy was reported when at least 2 separate G0/G1 peaks could be demonstrated. The coefficient of variation (CV) was calculated using the width of the peak (number of channels) at 61% of the maximum peak height divided by the peak height channel number, multiplied by a factor of 2.

The proliferative index (PI) was defined as the percentage of cells in the S + G2M phases combined. The correlation between the AgNOR count and PI were analysed using the Pearson's method while the Mann-Whitney Test was used to compare the PI between benign and malignant salivary gland neoplasms.

Results

Three tumours had aneuploid stemlines. Two were adenoid cystic carcinomas and the other was a carcinoma ex pleomorphic adenoma (**Figure 2**). The predominant growth pattern in the ACC were cribriform and tubular/trabecular respectively. Their AgNOR counts were 4.29 and 3.01 (**Figure 3**). The AgNOR count for the carcinoma ex PA was 2.37. Diploid stemlines were present in the remaining 30 neoplasms (**Figure 4**). The mean CV of the flow cytometry results were 3.96 ± 3.1 (SD). The proliferative index and AgNOR counts of the diploid tumours are summarised in **Table 1.**

A positive correlation between the mean PI and mean AgNOR counts in the various neoplasms were found. This

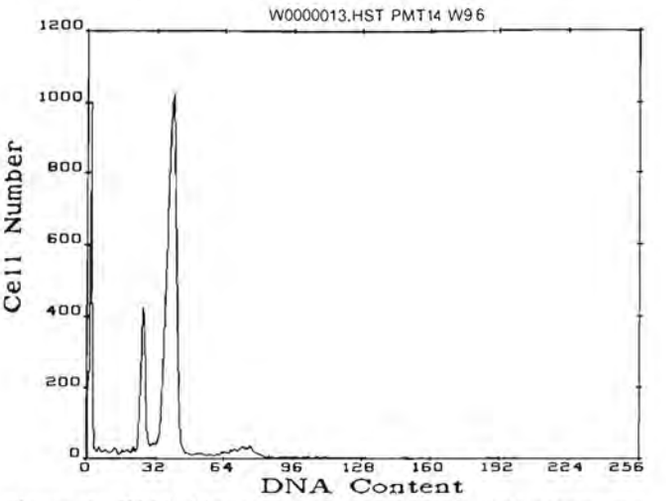

Figure 2: *DNA histogram of an adenoid cystic carcinoma showing aneuploidy.*

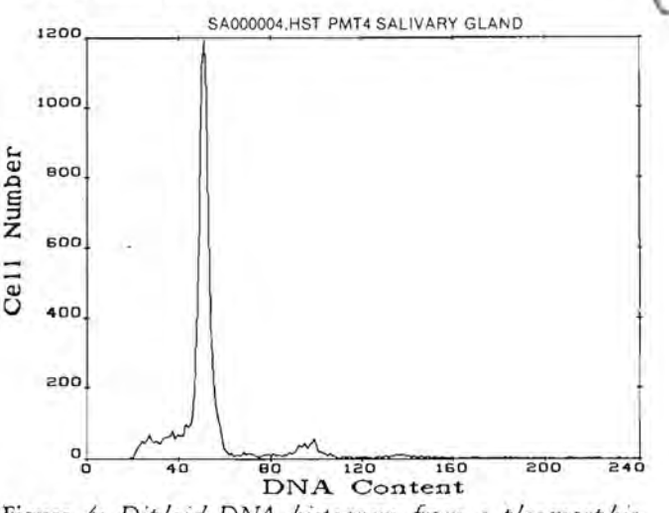

Figure 4: *Diploid DNA histogram from a pleomorphic adenoma.*

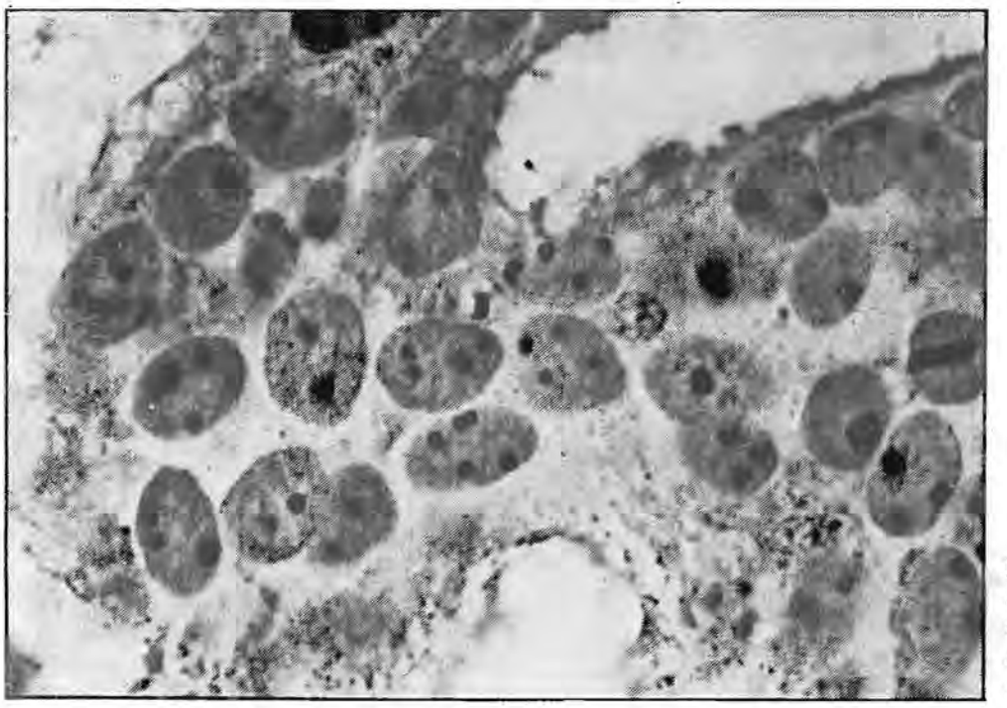

Figure 3: *AgNOR stain of a cribriform adenoid cystic carcinoma with an aneuploid DNA content. Original magnification, × 400.*

TABLE 1:
The PI and AgNOR count of the diploid salivary gland neoplasms

	PA	PLA	ACC	MEC	Ca ex PA	Undiff Ca	EPI
PI	4.1 ± 1.5	6.8 ± 5.3	6.7 ± 5.55	7.3 ± 2.3	5.1 ± 5.6	4.5	8.2
AgNOR	1.48 ± 0.3	1.85 ± 0.3	2.28 ± 0.7	1.95 ± 0.5	1.60 ± 0.3	5.13	2.23
n	11	8	3	4	2	1	1

PA = pleomorphic adenoma; PLA = polymorphous low grade adenocarcinoma;
ACC = adenoids cystic carcinoma; MEC = mucoepidermoid carcinoma;
Ca ex PA = carcinoma ex pleomorphic adenoma; Undiff Ca = undifferentiated carcinoma;
EPI = epithelial-myoepithelial carcinoma; PI = proliferative index

correlation however, was statistically not significant (P = 0.45). The difference between the mean PI of the benign salivary gland neoplasms and the diploid malignant salivary gland neoplasms was also not significant.

Discussion

The cell cycle distribution as determined by flow cytometry is usually calculated using commercially available mathematical software programs. Corrections are made to subtract background debri which intervene with the various phases of the cell cycle. Creation of debri by means of tissue preparation is a problem especially when using paraffin-embedded tissue for flow cytometric analysis. Although these corrections have shown to enhance the prognostic value of particularly the S-phase[15], it must always be borne in mind that neoplastic cells might be eliminated as debri. Expression of exact percentages of cells in the various stages of cell is therefore proliferation, a questionable practice. It is much more reliable to use the PI as a rough indicator of proliferative activity, especially when evaluating paraffin embedded tissues.

The number of aneuploid tumours (n = 3) in the present study was too small to make definite comments regarding its correlation with the AgNOR counts. It is interesting to note however, that the two AgNOR counts of the aneuploid ACC were the first and third highest count among the salivary gland neoplasms. In a study to evaluate the prognostic factors for ACC, Hamper *et al* concluded that the shortest survival time was found in patients with tumours showing aneuploid DNA contents[16]. Forty-two percent of these tumours had a predominant cribriform growth pattern. This fact correlates with our finding that the mean AgNOR count in ACC did not correspond with the histologic growth pattern, a prognostic factor for tumour behaviour in ACC. The highest count was present in a tumour with a predominant cribriform growth pattern. The same applied for the flow cytometric analysis. The two aneuploid ACC had more favourable cribriform and tubular growth patterns respectively. Luna *et al* however, found that aneuploidy is more frequently present in the solid pattern[17]. This is an indication that the growth pattern in ACC alone is not solely responsible for tumour behaviour.

The fact that only 3 tumours of the sample that included 22 malignant neoplasms were aneuploid is probably related to the phenomenon that malignant salivary gland neoplasms generally have a less aggressive behaviour compared to other malignancies.

A high AgNOR count in neoplasms may be related to an increase in cell ploidy due to a real increase in the number of chromosomes. Since the NORs are present only on the 5 acrocentric chromosomes, it may be possible that these chromosomes are not affected in a neoplastic transformation that is accompanied with hyperploidy. The NORs are usually tightly aggregated in one or two nucleoli in a cell[18]. Proliferative activity may be associated with nucleolar dissocation resulting in spreading of AgNORs through the nucleus. This, together with the transcriptional activity may result in an increase in the mean AgNOR count[18].

A variety of techniques are available to determine cellular proliferation in histological material. Visualisation of the NORs by means of a silver staining technique is frequently used. The percentage of cells in the S + G2M phases of the cell cycle can be determined with flow cytometry and immunohistochemical techniques using antibodies against proliferating cell nuclear antigen (PCNA) as well as Ki-67, a monoclonal antibody that recognises a nuclear antigen present only in proliferating cells, are some of the more advanced methods used.

This study failed to show a significant relationship between the mean PI and mean AgNOR count of the various neoplasms although they were positively correlated. Crocker *et al* found a significant linear correlation between the mean AgNOR count and S-phase fraction of high and low grade non Hodgkin's lymphomas, but not between the AgNOR count and ploidy status[19]. The difference between the mean PI of the benign and malignant tumours was not significant. This is in contrast to the highly significant difference between the same tumours when evaluating the mean AgNOR counts[13]. From this study it would appear that the AgNOR technique, which is fast and inexpensive, may be more suitable to accurately determine the proliferative activity when using paraffin embedded tissues

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A review of recent developments in the diagnosis of epithelial neoplasms of salivary gland origin

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Abstract The adoption by the World Health Organization of a revised classification for salivary gland neoplasms has introduced a new chapter in the diagnosis of these diverse growths. Universal acceptance of this proposal will contribute significantly to diagnostic uniformity. The introduction of an outline for the grading of malignant salivary gland neoplasms benefit preoperative prognostication and rationalize therapeutic regimes. The utilization of fine needle aspiration and frozen section for the establishment of a diagnosis are discouraged. Despite recent developments in histochemistry, immunohistochemistry and DNA content analyses of salivary gland neoplasms, the diagnosis still relies mainly on the growth pattern and cytologic features of a tumor. *(Eur J Lab Med 1995;1:107-112).*

Introduction

Although salivary glands share similar cellular phenotypes with sweat glands, mammary glands and the exocrine pancreas, neoplastic proliferations in the former are infinitely more complex and, from a cellular viewpoint, represent the most heterogenous group of proliferations in the human body. Despite recent developments in the understanding of the histogenesis of salivary gland neoplasms, the diagnostic process still relies mainly upon growth characteristics and cellular morphology. Special laboratory investigations like electron microscopy and cellular markers form a minor part of the diagnostic process and often only subtle microscopic differences distinguish neoplasms with diverse clinical outcomes. The subjectivity involved in the diagnosis of salivary gland neoplasms is highlighted in a recent study where 101 salivary gland neoplasms were reevaluated by a panel of senior pathologists. In a third there were minor disagreements, mostly related to subclassification, whereas major diagreements relating to benign versus malignant occurred in 7.9% of cases[1].

The purpose of this paper is to give an overview of recent developments in the diagnosis of salivary gland neoplasms.

Classification

For universal acceptance, a classification of pathologic proliferations should be based on patterns of differentiation that reflect the cell types of the parental tissue and simultaneously group neoplasms in prognostic categories. The most likable classification of salivary gland neoplasms is the morphologic working classification initially proposed by the Armed Forces Institute of Pathology[2] (Table 1) and later adopted by the World Health Organization's Committee on salivary gland tumors. Although the patterns of differentiation of salivary gland neoplasms is not addressed systematically in this classification, malignant growths are now for the first time prognostically grouped. The diagnostic refinement introduced by this new approach is clearly evident in a study which revised salivary gland neoplasms originally diagnosed according to the 1972 World Health Organizations classification[3]. In 29 cases the original diagnosis was changed and in 7 it resulted in a change from benign to malignant or vice versa[4]. Although the new approach to the classification has valuable clinical implications, it is by no means complete. Entities like the salivary gland anlage tumor[5], sialoblastoma[6,7] and hyalizing clear cell carcinoma[8] lack suffi-

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Table I. AFIP classification of primary epithelial neoplasms of salivary gland origin

Benign

- Mixed tumor (pleomorphic adenoma)
- Papillary cystadenoma lymphomatosum (Warthin's tumor)
- Oncocytoma
- Cystadenoma
- Basal cell adenoma
- Ductal papillomas
  - Sialadenoma papilliferum
  - Inverted ductal papilloma
  - Intraductal papilloma
- Myoepithelioma
- Sebaceous adenomas
  - sebaceous adenoma
  - sebaceous lymphadenoma
- Adenoma NOS

Malignant

Low-Grade

- Mucoepidermoid carcinoma (low grade)
- Acinic cell adenocarcinoma
- Polymorphous low grade adenocarcinoma
- Basal cell adenocarcinoma
- Adenocarcinoma (NOS) low grade
- Metastasizing mixed tumor

Intermediate-Grade

- Mucoepidermoid carcinoma (intermediate grade)
- Adenoid cystic carcinoma (cribriform-tubular types)
- Epithelial-myoepithelial carcinoma
- Adenocarcinoma NOS (intermediate grade)
- Clear cell carcinoma
- Cystadenocarcinoma
  - papillary
  - non papillary
- Sebaceous carcinomas
  - sebaceous carcinoma
  - sebaceous lymphadenocarcinoma
- Mucinous adenocarcinoma

High-Grade

- Mucoepidermoid carcinoma (high grade)
- Adenoid cystic carcinoma (solid)
- Malignant mixed tumor
  - carcinoma ex mixed tumor
  - carcinosarcoma
- Adenocarcinoma NOS (high grade)
- Squamous cell carcinoma
- Undifferentiated carcinoma
- Oncocytic carcinoma
- Adenosquamous carcinoma
- Salivary duct carcinoma
- Myoepithelial carcinoma

cient numbers for the establishment of behavioral patterns and may only find their way into future reappraisals of this classification.

Terminology

Arguments on the preferentiability of the designation "mixed tumor" or the term "pleomorphic adenoma" are unproductive and both are now accepted. "Adenolymphoma" as a synonym for papillary cystadenoma lymphomatosum should fall in disuse because any implication that this benign tumor is linked to lymphoma is misleading. Oxyphil cell adenoma and oncocytoma are used interchangebly. The ambiguous term "monomorphic adenoma" has fallen into disuse and all unclassifiable adenomas are now proposed to be designated as "adenoma, not otherwise specified". Although myoepithelioma is classified as a separate entity, no counter argument exists that this neoplasm is in fact an extreme differentiation on the diverse spectrum of pleomorphic adenoma. Separate categorization of myoepithelioma may however, prevent confusion with benign mesenchymal neoplasms, many of which resemble myoepitheliomas microscopically.

The term malignant mixed tumor (or malignant pleomorphic adenoma) should not be used as a specific diagnosis as it includes three different entities: carcinoma ex mixed tumor (or a carcinoma arising in a mixed tumor), carcinosarcoma (true malignant mixed tumor) and metastasizing mixed tumor. The suffix-tumor is now replaced by "carcinoma" in two neoplasms which are now known to be malignant: acinic cell carcinoma and mucoepidermoid carcinoma. As refinements in classifications proceed, the utilization of terms like "adenocarcinoma not otherwise specified" decrease. Although new clinico-pathological entities such as salivary duct carcinoma, terminal duct carcinoma and epithelial-myoepithelial carcinoma reduce the frequency by which this category is used, there still remain those adenocarcinomas which cannot be accommodated in other categories.

Frozen sections and fine needle aspirations

Frozen sections (FS) and fine needle aspirations (FNA) are increasingly accepted as cost effective and time saving techniques for the diagnosis of abnormal body masses. The cellular diversity which may be experienced within a salivary gland neoplasm decreases the potential accuracy of all techniques which suffer the disadvantage of not representing all cell types in a neoplastic proliferation. The status of invasion is one of the most important parameters in predicting the biologic behavior of salivary gland neoplasms[2]. The small sample obtained through FNA precludes the disclosure of this important parameter. Studies investigating the sensitivity and specificity of FNA frequently compare its diagnostic accuracy with histological diagnoses

based on dated classification systems, most of which do not recognize modern refinements in the diagnosis of salivary gland neoplasms. FNA appears to have a high success rate in distinguishing between benign and malignant salivary gland neoplasms[9-11]. The distinction between benign and malignant in a diagnosis on which the therapeutic approach is decided, is probably equally important to the grading of a specific malignant growth. In this respect, the limited sample obtained through FNA is often inadequate and its results cannot be compared with those obtained through incision biopsy. The cytological atypia frequently present in benign salivary gland neoplasms[9,12,13] and potential confusion with non-epithelial stromal neoplasms[11] are further pitfalls in the interpretation of FNA. Although there are unquestionable clinical indications for FNA, none merit its inclusion as part of the systematic evaluation on which the therapeutic approach is based[13].

In a series of 310 patients subjected to FS, the correct type of malignancy was diagnosed in only 51% of cases and in four patients, a false positive diagnosis of malignancy was made. The authors of this study conclude that FS is no more accurate in the evaluation of salivary gland tumors than FNA[16]. Although there are no indications against the utilization of FS for determining clear margins during excision, a primary diagnosis should not be established on FS alone.

Grading of salivary gland malignancies

This aspect of the diagnosis of malignant neoplasms is important particularly in the case of mucoepidermoid carcinomas, adenoid cystic carcinoma and adenocarcinoma which may be classified in more than one grade of malignant behavior. The microscopic criteria applied for grading are controversial and often highly subjective. Auclair, Goode and Ellis[17] proposed a point scoring system for the objective grading of muco-epidermoid carcinomas. The histopathologic features that indicate high grade behavior are an intracystic component of less than 20%, four or more mitoses per 10 high-power fields, neural invasion, necrosis and cellular anaplasia. Most differences of opinion involve the distinction between low and intermediate grades and their proposed point system may provide a basis for an objective solution. Factors that indicate a poor prognosis in adenoid cystic carcinomas encompass failure of local disease control at the initial surgical procedure, a solid pattern histologically, recurrent disease and distant metastases[2]. Despite the description of new clinicopathological entities like the salivary duct carcinoma and epithelial myoepithelial carcinoma which were formerly grouped in the adenocarcinoma "not otherwise specified" category, there still remain a group of adenocarcinomas that cannot be accommodated in conventional classifications. These malignancies are divided into low-, intermediate- and high grade categories on growth patterns and cytologic features[2]. Although histopathologic grading of acinic cell adenocarcinomas is possible, the influence of the different grades on the prognosis is debateable[18,19]. The limited malignant potential and excellent survival of patients with polymorphous low-grade adenocarcinoma is little affected by patterns of differentiation[20].

Histochemistry and immunohistochemistry

Although histochemical and immunohistochemical techniques have played an important role in investigations of the histogenesis of salivary gland neoplasms, their diagnostic applications are limited. This is mainly due to the wide spectrum of differentiation which may occur within a single salivary gland neoplasm, with each growth pattern exhibiting its own immunohistochemical characteristics[21-23]. Salivary gland neoplasms furthermore often share immunohistochemical staining characteristics with other neoplasms. Positive staining for prostate-specific antigen and prostate-specific acid phosphatase are frequently found in benign and malignant salivary neoplasms[24], a pitfall in the microscopic distinction between salivary gland carcinomas and metastatic deposits of prostatic carcinoma. Alpha 1-antitrypsin is a useful marker of basement membrane-like material[25] and can be helpful in distinguishing this product from myxoid interstitial deposits. A potential distraction to the diagnosis of myoepithelial tumors of salivary glands (i.e. myoepithelioma and myoepithelial carcinoma) is confusion with spindle cell mesenchymal proliferations. Demonstration of myoepithelial differentiation requires careful evaluation of immunohistochemical stains. The identification of S100 protein, actin and keratin either focally or diffusely, is helpful in confirming myoepithelial differentiation[26].

Microscopically, myoepitheliomas differentiate into three distinct cellular patterns: a spindle cell-, plasmacytoid- or a combination of plasmacytoid and spindle shaped cellular patterns[2]. If immunohistochemical criteria had to be applied rigorously, it is debateable whether the plasmacytoid variety, which is reported to stain negative for muscle specific actin, does represent true myoepithelial differentiation[27].

Confusion between the microscopic appearances of polymorphous low grade adenocarcinoma and benign pleomorphic adenoma may be avoided by employing stains for glial fibrillary acidic protein (GFAP). The former does not stain for this antigen

whereas its positivity is common in pleomorphic adenomas[28]. A greater diagnostic dilemma is the distinction between polymorphous low grade adenocarcinoma and adenoid cystic carcinoma. The immunochemical reactions of these two tumors are not sufficiently dissimilar to be of any practical value[29] and differences are mainly cytological and to a lesser extent morphological in nature. The presence of both sex steroids and the receptor for progesterone in adenoid cystic carcinomas[30] suggests a good possibility that some tumors in this group may respond to endocrine therapy.

Various reports propose a useful place for the counting of nucleolar organizer regions (NOR's) in order to predict the proliferative activity and prognosis of malignant salivary gland neoplasms[31-34] and distinguish between benign and malignant growths[41]. Our experience with this technique[35] as well as those of other researchers[36] were less rewarding and we believe this technique provides nothing but redundant information.

Research into the use of cellular markers to predict the behavior of salivary gland tumors is in its infancy. An association is reported between the expression of erbb2 oncoprotein and aggressiveness of malignant salivary gland tumours[37,38]. Loss of cellular differentiation appears to be linked with under expression of the c-fos oncogene[39] and evaluation of Ki-67 expression[40], immunoreactivity for PCNA[41] and c-myc, ras p21 and p53 expression[42] may become important determinants for malignant behavior.

DNA content analysis

The positive correlation between prognosis and ploidy status of malignant neoplasms is well established. Despite the presence of atypical cells in benign pleomorphic adenomas, all benign salivary gland tumours have diploid DNA contents and the malignant ones frequently display an aneuploid pattern[43,44]. A statistically significant correlation was found between DNA content and tumor size, histological grade, lymph node metastasis and lethality of 55 salivary gland carcinomas[45]. Flow cytometry was however, unable to predict the development of metastasis in cases of proven metastasizing mixed tumor[46]. DNA ploidy was shown to correlate with the prognosis of epithelial-myoepithelial carcinoma[47] myoepithelioma[48] and muco-epidermoid carcinoma[49]. The value of this technique in prognosticating adenoid cystic carcinomas is debateable[50,51] whereas no prognostic correlation could be found between DNA ploidy and the course of acinic cell adenocarcinomas[52,53]. Larger series will shed more light on the usefulness of DNA content analysis in the prediction of the behavior of salivary gland tumors.

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High-resolution DNA flow cytometry in papillary cystadenoma lymphomatosum (Warthin's tumour)

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Hemmer J, van Heerden WFP, Polackova J, Kraft K: High-resolution DNA flow cytometry in papillary cystadenoma lymphomatosum (Warthin's tumour). J Oral Pathol Med 1998; 27: 405–6. © Munksgaard, 1998.

Twenty-eight examples of papillary cystadenoma lymphomatosum (Warthin's tumour) of the parotid gland were analysed by high-resolution DNA flow cytometry. The mean coefficient of variation was found to be 1.19% (SD: 0.41). All tumours were DNA diploid. These results did not correspond with expected deviations based on published chromosomal studies. Also, the homogeneously low S-phase fractions (mean: 4.8%; SD: 2.7) found did not support the hypothesis of etiologically distinctive subgroups in these tumours.

Key words: cystadenoma lymphomatosum; cytogenetics; DNA flow cytometry; salivary gland tumours; Warthin's tumour

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Papillary cystadenoma lymphomatosum (PCL) is a benign neoplastic tumour involving mainly the parotid gland of elderly patients. The lesion is characterised by a combination of lymphoid tissue with double-layered epithelial cells consisting of outer columnar cells with an eosinophilic cytoplasm and a more cuboidal inner layer, lining the cystic spaces (1). The pathogenesis of PCL is controversial concerning the origin of both the lymphoid tissue and epithelium (1).

Cytogenetic studies on PCLs have identified three groups of epithelial cells with different karyotypic patterns: namely, one without any changes, a second group characterised by gains and losses of chromosomes, and a third showing structural chromosomal aberrations (2–4). These results suggest the existence of etiologically different subgroups of PCL. This study was undertaken to evaluate the contribution of high-resolution DNA flow cytometry to the cytogenetic analysis of PCL.

Material and methods

Fresh surgical samples of 28 PCLs were immediately processed for DNA flow cytometry. The tumour samples were homogenised in 0.9% NaCl. The nuclei were extracted by incubation in acid pepsin solution (0.5 g pepsin dissolved in 100 ml of 0.05 N HCl) at room temperature with careful stirring for 5 min. Remaining tissue fragments were removed using a 50 µm nylon mesh. The nuclei were fixed with 70% ethanol and stored at −20°C. For DNA-specific staining, the pelleted nuclei were resuspended in 0.5 ml acid pepsin solution. After 10 min incubation at room temperature, 4.5 ml of 5 µM DAPI solution (4',6-diamidino-phenylindole) containing 0.2 M trisodium citrate dihydrate was added. DNA flow cytometry was carried out using a PAS III flow cytometer equipped with a high-pressure 100 W mercury lamp (Partec; Muenster, Germany). The filter combination used was a UG 1 excitation filter, a TK 420 dichroic mirror and a GG 435 barrier filter. Human lymphocytes were added in a control measurement to confirm the DNA ploidy status of the sample cells. The cell cycle phase distribution was analysed using the MultiCycle software package (Phoenix Flow Systems, San Diego, CA, USA).

Results

Twelve patients were men and sixteen were women; their ages ranged between 34 and 89 years, with a median of 67 years. All 28 PCLs were localised unilaterally. High-resolution DNA flow cytometry showed that all cases of PCL in this study consisted exclusively of flow cytometrically diploid cells (Fig. 1). The coefficient of variation, a measure of the sensitivity of DNA measurements, varied between 0.72% and 2.14%, with a mean value of 1.31% (SD: 0.42). The S-phase fractions ranged between 0.2% and 8.7%, with a mean value of 4.8% (SD: 2.7).

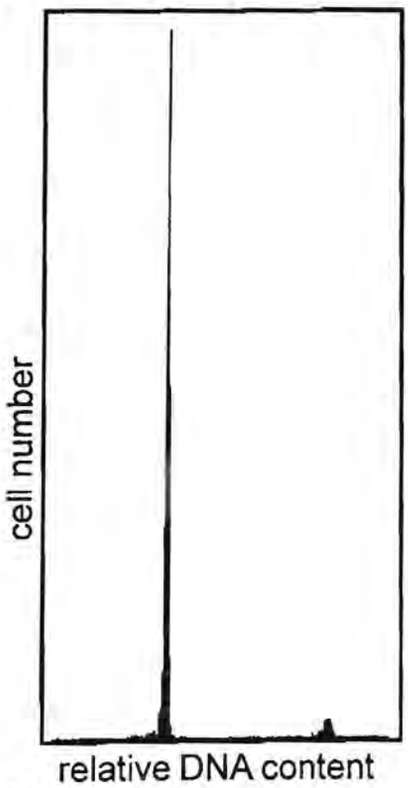

Fig. 1. Representative DNA histogram of a PCL. The diploid G1 and G0 cells are reflected by the first peak. The second peak with just double the G1–G0 DNA content contains the cells in the G2 phase and mitosis. Cells in the S phase characterised by intermediate DNA values are displayed between both peaks.

Discussion

DNA flow cytometry allows rapid and reliable identification of tumour cell lines with karyotype aberrations, provided the cytogenetic abnormalities result in a measurable change of the nuclear DNA content. A 3% difference in DNA content as reflected by the discrimination of x- and y-chromosome-bearing sperm cells is attainable with high-resolution flow cytometry (5). The sensitivity achieved through this technique in the present series of PCLs allows for the detection of tumour cell lines with karyotype aberrations resulting in a 2–4% deviation of the clonal DNA content from the normal diploid DNA value. Thus, even the gain or loss of a single chromosome 5, as was recently reported for a number of PCLs (2, 4), should be detectable by high-resolution flow cytometry.

No evidence of aneuploid tumour cell populations that correspond to expected deviations based on published chromosomal studies was found in the present series. This result agrees with two other studies on five and four PCLs, respectively (6, 7). DNA content heterogeneity could not be demonstrated by flow cytometry of multiple samples collected from 4 PCLs (7). Reports on numerical chromosome changes in PCL may therefore reflect the well-known problem of karyotypic changes due to cell culture artefacts rather than the existence of gross karyotype aberrations in PCL *in vivo* (8).

Even if translocations have successfully been identified by high-resolution DNA flow cytometry (9), structural chromosome rearrangements that have been reported in a significant number of PCLs (2–4, 10) remain unproved in the present flow cytometric study. Although highly specific rearrangements involving a few chromosomal regions have been reported in pleomorphic adenomas (3, 11), the limited cytogenetic data available on PCLs is not supportive enough for such events in these tumours. However, a homogeneously low proliferative activity as assessed in the present series, reduced p53 protein expression reported for five cases (12), and lack of c-erbB-2 oncoprotein expression in 31 other tumours (13) do not support the hypothesis of the existence of cytogenetically distinctive subgroups of PCL.

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Warthin's Tumour is not an Epstein-Barr Virus Related Disease

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Abstract. *Background: The histogenesis of Warthin's tumour (WT) is controversial. A possible role for Epstein-Barr virus (EBV) has been suggested. Materials and Methods: Twenty formaldehyde-fixed, paraffin-embedded blocks of WT from the parotid gland were examined for the presence of EBV. In situ hybridisation was performed using EBV encoded small nuclear RNAs (EBER1/2) probes labelled with fluorescein isothiocyanate. An EBV-positive P3HR-1 cell line processed to paraffin wax was used as a positive control and a brain section as negative control. Results: EBER1/2 could not be found in the neoplastic epithelial cells in any of the tumours nor in the adjacent normal parotid tissues. Individual positive lymphocytes were present in 7 tumours. Conclusions: These results indicated that EBV is not involved in the pathogenesis of WT.*

Warthin's tumour (WT) is a benign epithelial neoplasm of major salivary glands with a characteristically male preponderance. WT's are almost exclusively located in the parotid glands and may present as single, bilateral or multiple lesions (1). Histologically, WT consists of a double layer oncocytic epithelium with papillary structures lining cystic spaces, associated with a lymphoid stroma. The ratio of lymphoid stroma to epithelium varies widely and has been used by Seifert *et al* (2) to classify these tumours into subtypes.

The histogenesis of WT is controversial. The heterotopic theory suggests that the epithelial component of these tumours represents entrapped salivary gland epithelium in salivary gland associated lymph nodes (3). According to the immune theory, the lymphoid cells represent a secondary lymphocytic response to epithelial changes or stimuli (4). Epstein-Barr virus (EBV) has been implicated in the pathogenesis of WT, especially the lymphoid component (5). It has been suggested that release of EBV gene products or cytokines, particularly interferon-gamma, by infected cells, may activate lymphoid tissue to result in a polyclonal B-cell response (6).

Epstein Barr virus (EBV) is a double stranded DNA virus. It causes widespread infection and was found to be the aetiologic agent of infectious mononucleosis (7), endemic Burkitt's lymphoma (8), undifferentiated nasopharyngeal carcinoma (9) and EBV-induced disorders in immunodeficient patients (10). An association of other epithelial tumours with EBV has recently been suggested on the basis of molecular biological techniques. The presence of EBV DNA in tonsilar carcinomas (11), gastric carcinomas (12) epithelial thymic carcinomas (13) and undifferentiated salivary gland carcinomas (14) has been reported.

In the present study, biopsy specimens of 20 patients with solitary WT were screened for the presence of EBV using RNA *in situ* hybridisation (ISH).

Materials and Methods

Twenty cases diagnosed as WT were retrieved from the files of the Department of Pathology, Military Hospital of Ulm, Germany. Sections were cut from these formaldehyde-fixed, paraffin-embedded blocks and reviewed to subclassify the tumours in the different groups defined by Seifert *et al* (2): predominanty epithelial (< 30% lymphoid component); predominantly lymphoid (> 70% lymphoid component); typical (50% lymphoid component) and metaplastic (extensive squamous metaplasia).

To detect expression of the EBV-encoded small nuclear RNAs (EBER-1 and EBER-2), ISH with fluorescein-conjugated oligonucleotide probes was used. The probes were obtained commercially and consisted of a mixture of both EBER-1 and EBER-2 (Novocostra, Newcastle upon Tyne, UK). Probes were labelled with fluorescein isothiocyanate (FITC). Detection of hybridised probe was done with rabbit F(ab') anti-FITC conjugated to alkaline phosphatase. All glassware was treated with DEPC (di-

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Key Words: Warthin's tumour, Epstein-Barr virus, salivary gland neoplasms.

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ethyl pyrocarbonate) to prevent RNAase activity. The ISH was done using the OmniSlide System (Hybaid, Teddington, Middlesex, United Kingdom).

Formaldehyde-fixed tissue sections were cut 4μm thick and twice dewaxed in xylene for 3 min. The sections were dehydrated in 99% alcohol for 2 min followed by dehydration in 95% alcohol for 2 min whereafter they were twice immersed in pure water for 3 min and air dried. Slides were then placed on an incubation tray and covered with Proteinase K (Dako Corporation, Glostrup, Denmark). Eighty ml Proteinase K was diluted with 2ml 0.05M Tris-HCl pH 7.5 to 7.7 and digestion was performed for 6min at room temperature. Sections were washed in distilled H_2O, followed by DEPC treated water and then dehydrated in graded alcohols. The sections were put in the Omnislide slide rack and air-dried. Twenty μl of probe hybridisation solution was added to the sections on the slides and the sections covered with coverslips. The slides were then incubated at 37°C for two hours. The coverslips were removed in a 0.1% Triton X-100 solution and the slides were then immersed in post hybridisation solution for 10 min. The post hybridisation solution was tipped off and sections incubated with sufficient anti-FITC AP detection solution for 30 min at room temperature in the dark.

Slides were then washed twice in TBS buffer for 3 min each and followed by a 5min wash in alkaline phosphate substrate buffer. The slides were again placed in an incubation tray and the alkaline phosphates activity was demonstrated by covering the sections with 100ml of substrate solution. The sections were coverslipped and incubated overnight at room temperature in the dark. Slides were then washed in running tap water for 5 min, counterstained with Mayer's heamatoxylin for 1 min, blued in tap water for 5 min and mounted in an aqueous mountant (Dako Corporation, Glostrup, Denmark)

Sections of an EBV-positive P3HR-1 cell line processed to paraffin wax were used as positive control. A brain section served as negative control. Positive signals were regarded as dark brown to black staining concentrated in the nuclear area of the tumour cells with nucleolar sparing.

Results

All the tumours were in the parotid gland. Eighteen patients were male and the mean age of all the patients were 61.2 years (± 11.2). Based on the classification of Seifert *et al* (2), there were 11 cases (55%) classified as typical, 5 (25%) as predominantly epithelial and 4 (20%) as predominantly lymphoid.

The EBV-positive P3HR-1 cell line that was used as positive control stained intensely positive in all cases. Brain blocks used as a negative control demonstrated no hybridisation signal. EBER1/2 could not be found in the neoplastic epithelial cells in any of the tumours. Adjacent normal parotid tissues were also negative in all the cases. Individual positive lymphocytes were present in 7 tumours. No background staining could be seen on any slide.

Discussion

EBV DNA sequences have been detected by Santucci and co-workers in almost all epithelial tumour cells in 13/15 (86.7%) multiple or bilateral and in 1/6 (16.7%) solitary WT's (15). The same detection methods were used by Wolvius *et al* (16) who found similar signals, but suggested that the positive cytoplasmic staining may be the result of non-specific binding of the labelled probe, because by using irrelevant probes, similar staining results were found. Similar to the present study, Wolvius *et al* (16) were also unable to demonstrate any positive EBER-1/2 epithelial cells in their 10 cases of WT studied. Ogata *et al* (17), likewise, found neoplastic epithelial cells of WT negative for EBER1 probes using ISH. Similar to the present study, isolated stromal lymphocytes were positive in some of their tumours.

EBV DNA has been demonstrated in both the epithelial and lymphoid component of WT. Demonstration of EBV DNA on its own is, however, not sufficient to distinguish between latent and lytic viral infection. Demonstration of EBV DNA in WT using polymerase chain reaction (PCR) cannot be used as an indication of viral infection because circulating, EBV-carrying lymphocytes are found in non-neoplastic lymphoid tissues (18). Furthermore, the source of viral DNA detected with PCR cannot be determined unequivocally, because DNA extracts from heterogenous cell populations are analysed.

In situ hybridisation is the method of choice to evaluate EBV involvement in WT, because the distribution and nature of positive cells can be determined. This is important, especially in tumours consisting of different cell types.

EBERs are the most abundantly expressed viral transcripts found in EBV infected cells and may be present at 10^7 copies per cell (19). Despite their abundance, they do not code for protein and their function is unknown, although it has been proposed that they may be active during lytic replication. EBERs are expressed early after infection and reach substantial levels 70 hr after infection. (20). EBER transcripts are appropriate targets for ISH. Firstly, they exist as ribonucleoproteins complexed with the cellular protein La with extensive intramolecular base pairing and stable secondary structure (21, 22). As such, they may be more resistant to nuclease degradation than other transcripts and thus may especially useful in the investigation of routinely prepared formalin-fixed paraffin-embedded clinical specimens (23). Secondly, their abundance in latently infected cells suggests that their high copy number might compensate for any loss of sensitivity because of their small size.

Based on morphometric analysis evaluating the lymphoid component and cystic spaces together with the clinicopathologic data, Aguirre and co-workers (24) suggested a model of progression based on the subtypes of WT used by Seifert *et al* (2). According to their model, most cases of WT originate as a consequence of an unknown stimulus within the parotid lymph nodes resulting in a predominantly epithelial component as initial presentation. They further suggest that growth of

WT's occur because of lymphoid proliferation with subsequent development of the classical subtype to the predominantly lymphoid subtype. Tobacco or viruses have been suggested as potential initial stimuli (24).

Although this study did not prove any theory regarding the histogenesis of WT to be correct, it has demonstrated that EBV is not involved in the pathogenenesis of WT in the population sample studied. Other authors using the same methodology reached similar conclusions (16, 17).

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Intraoral Salivary Duct Carcinoma: A Report of 5 Cases

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Salivary duct carcinoma (SDC) is a high-grade malignant epithelial tumor of salivary glands first described by Kleinsasser et al.[1] It has also been termed cribriform salivary carcinoma of excretory ducts,[2] infiltrating salivary duct carcinoma,[3] and intraductal carcinoma.[4] SDC has a poor prognosis,[2,5,6] although patients with prolonged disease-free survival have been reported,[2,5] Low-grade variants of SDC have also been described.[7,8]

The peak incidence of SDCs is in the sixth and seventh decades of life, and it has a male predominance.[6,9] This neoplasm has a striking resemblance to ductal breast carcinoma and is characterized by the presence of intraductal, circumscribed tumor islands with a papillary, cribriform, or solid growth pattern associated with an infiltrative component. Comedonecrosis is frequently present.

SDCs occur almost exclusively in the major salivary glands with the parotid gland predominantly affected.[10] Only isolated cases involving minor salivary glands have been reported.[1,8,11-18] In this study, we report the clinicopathologic and immunohistochemical features of 5 cases of intraoral SDC. The DNA ploidy status of these tumors was also studied.

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Materials and Methods

Malignant intraoral salivary gland tumors diagnosed at the Departments of Oral Pathology at the University of Pretoria and Medical University of Southern Africa were reviewed. Four cases of SDC were included while an additional case was reclassified as an SDC according to the criteria of the World Health Organization.[19] Clinical and follow-up information was obtained from the patients' files and supplemented by communication with the referring practitioners or clinics and immediate family members. All the specimens were fixed in 10% buffered formalin, and the histologic features were evaluated by reviewing all the sections stained with hematoxylin and eosin. Additional slides of paraffin blocks were prepared for immunohistochemical analysis using the standard avidin-biotin peroxidase method. A panel of commercially available antibodies with appropriate controls was used (Table 1). The extent of immunohistochemical staining was evaluated and scored as + (1% to 9% of tumor cells), ++ (10% to 50% of tumor cells), and +++ (>50% of tumor cells). Staining intensity was not evaluated.

Flow cytometry was performed on 50 μm sections of the formalin-fixed paraffin-embedded tumor blocks. Tissue was processed according to the modified method described by Heiden et al.[20] The sections were enfolded with 50-μm nylon mesh and deparaffinized in xylene, hydrated in graded alcohols, and digested with Carlsberg solution. The nuclei were stained with DAPI solution (4′,6-diamidino-phenylindole) containing 0.2 M trisodium citrate dihydrate, and at least 10,000 events from each case were analyzed using a PAS III flow cytometer equipped with a high-pressure 100-W mercury lamp (Partec, Münster, Germany).

Results

The patients ranged in age from 47 to 71 years (mean age, 58.2 years). Two were female and 3 were male. All of the tumors were located in the palate and ranged in size from 5 to 14 cm. The tumors presented

Table 1. SPECIFICATIONS OF ANTIBODIES USED

Antibody	Source	Dilution	Antigen Retrieval	Detection
High-molecular-weight cytokeratin	DAKO (34BE12)	Prediluted	*	DAKO LSAB2
α-Smooth muscle actin	DAKO (1A4)	Prediluted	None	DAKO LSAB2
Vimentin	DAKO (V9)	Prediluted	None	DAKO LSAB2
Anti-S-100A	DAKO	Prediluted	*	DAKO LSAB2

*Microwave pressure cooker in citric buffer, pH 6.0.

as painful masses (Figs 1 through 3). No association with the parotid gland could be shown in any case using computed tomography investigation (Fig 4). The possibility of metastasis from an intraductal breast carcinoma was also excluded. Clinical evidence of regional lymph node metastasis was present in 2 cases. Three patients refused any form of treatment and were subsequently lost to follow-up. No information could be obtained from the referring clinics. One patient was treated with radical resection and is currently receiving postoperative radiotherapy. The clinicopathologic findings are summarized in Table 2.

Microscopically, all tumors consisted of an intraductal component with a predominantly cribriform pattern and central comedonecrosis (Fig 5). Infiltrating tumor islands, in a trabecular and cribriform pattern, in a stroma that varied from cellular to regions of hyalinization were also present in all 4 cases (Fig 6). The tumor cells had well-defined cell borders with eosinophilic cytoplasm and vesicular nuclei. Mitotic activity varied from moderate to high (Fig 7). The immunohistochemical results are shown in Table 3.

Flow cytometry analysis showed 4 tumors to be aneuploid (Fig 8) and one diploid (case 2) (Fig 9). The coefficient of variance (CV) of all the measurements was less than 3%.

Discussion

The possibility of metastases should be eliminated before a final diagnosis of SDC is made. Metastatic ductal carcinoma from the breast could be excluded with careful clinical examination and mammography. The histologic features of these tumors are very similar, although the presence of estrogen receptor protein and absence of carcinoembryonic antigen in breast carcinomas have been used to differentiate between SDC and ductal carcinoma of the breast.[21] Metastatic prostatic carcinoma can in the majority of cases be excluded by the absence of both prostate-specific antigen and prostate-specific acid phosphatase in the tumor cells.

The histologic differential diagnosis of SDC includes high-grade mucoepidermoid carcinoma, undifferentiated carcinoma, adenocarcinoma (not otherwise specified), dedifferentiated acinic cell carcinoma, and adenoid cystic carcinoma. High-grade mucoepidermoid carcinomas have epidermoid and intermediate basaloid cells as well as cells with mucicarmine demonstrable intracellular mucin, whereas only luminal mucin is found in SDC. Cribriform and papillary-cystic growth patterns are not found in mucoepidermoid carcinomas. Undifferentiated carcinoma lacks the eosinophilic cytoplasm of SDC and does not form glandular structures. Adenocarcinoma,

FIGURE 1. Introoral view of case 1 showing a massive tumor destroying the right maxilla, extending across the midline.

FIGURE 2. A tumor located in the left maxilla with buccal and palatal expansion from case 2.

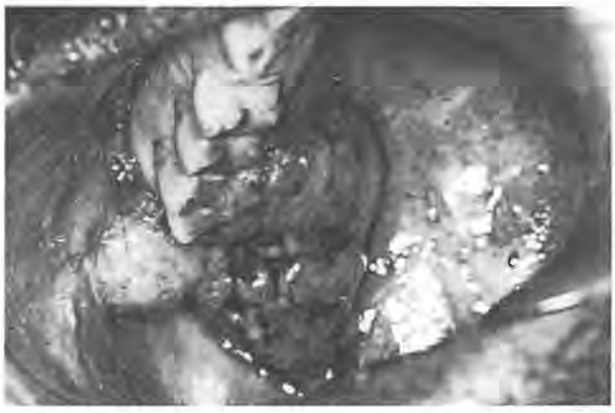

FIGURE 3. Case 3 presenting with an ulceroted tumor in the right palate involving the alveolar ridge.

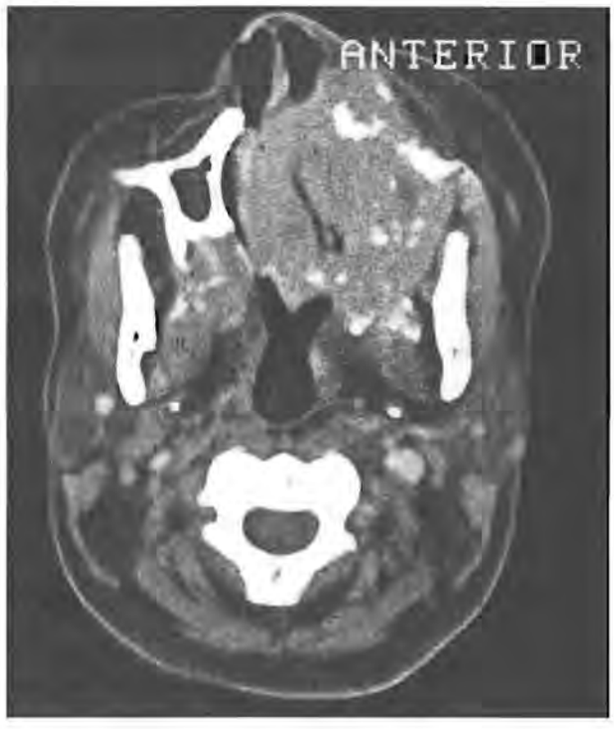

FIGURE 4. Computed tomography scan of patient described in case 2 showing a tumor in the left maxilla and palate with no parotid involvement.

not otherwise specified, shows glandular and ductal differentiation but lacks the distinctive features of SDC and is basically a diagnosis of exclusion.[10] Dedifferentiated acinic cell carcinoma may present as a poorly differentiated adenocarcinoma or undifferentiated adenocarcinoma but always in association with a usual-type low-grade acinic cell carcinoma.[22] Adenoid cystic carcinoma cells usually contain little cytoplasm and have angulated, basophilic nuclei. Comedonecrosis is also not a feature of adenoid cystic carcinoma.

The diagnosis of primary intraoral SDC necessitates exclusion of direct spread from one of the major salivary glands, especially the parotid, where most SDC arise. Computed tomography scans and other imaging techniques should not show any association with the parotid or any other major salivary gland. This is especially true when SDC of the cheek is diagnosed, which is not a common site for minor salivary gland tumors.[23]

Table 2. CLINICOPATHOGIC FEATURES OF 5 PATIENTS WITH INTRAORAL SDCs

Patient	Age (yr)	Gender	Site	Clinical Presentation	Tumor Size (cm)	Treatment	Follow-Up
1	53	F	Right palate and right alveolar ridge	Fungating mass; difficulty in breathing and eating; lymphadenopathy	±14	Biopsy	Patient refused treatment, lost to follow-up
2	71	M	Left palate and buccal sulcus	Pain, nerve fallout of II, III, V_b, and VII; lymphadenopathy	±5	Biopsy	Patient refused treatment, lost to follow-up
3	57	M	Right palate	Ulcerated tumor	±6	Biopsy	Patient refused treatment, lost to follow-up
4	63	F	Left palate	Painful, ulcerated tumor	±7	Maxillectomy, left neck dissection, postoperative radiotherapy	No recurrences after 10 months
5	47	M	Left palate	Fungating, nonulcerated tumor	±5	Biopsy	Patient still considering surgical treatment

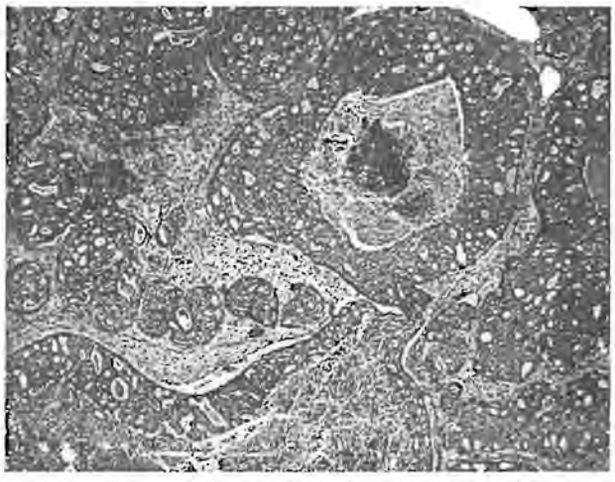

FIGURE 5. Photomicrograph of an SDC showing introductal growth pattern with a cribriform appearance and comedonecrosis in the larger tumor islands (hematoxylin-eosin stain, original magnification × 25)

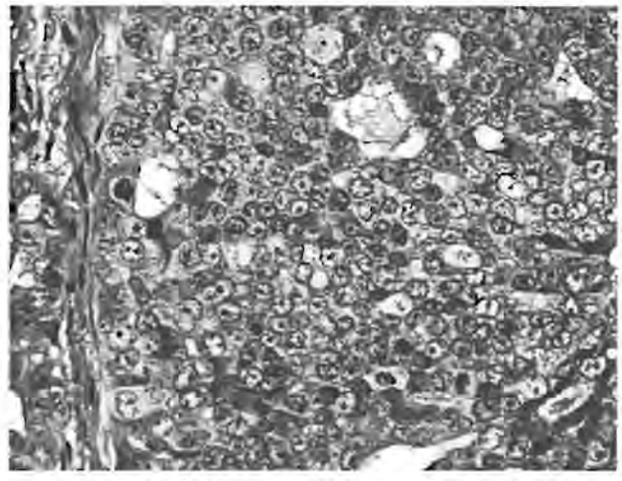

FIGURE 7. The tumor cells had vesicular nuclei with prominent nucleoli. Mitotic figures were prominent (hematoxylin-eosin stain, original magnification ×200).

Carcinoma ex pleomorphic adenoma is not uncommon in minor salivary glands[23] and SDC have been reported as the malignant component of these malignancies.[6,15,24] None of our cases had any histologic evidence of a preexisting pleomorphic adenoma, nor did a longstanding history suggest such an association. SDC has also been reported as a hybrid carcinoma of the minor salivary glands combined with an adenoid cystic carcinoma[25] and Warthin's tumor.[13]

SDC of the major salivary glands is an aggressive, high-grade malignancy. Comparison of the behavioral quality of SDC originating from minor salivary glands with that of the major glands is difficult, as only isolated cases of SDC have been reported. The clinical characteristics of our cases are similar to other studies reported for SDC of the major glands in that predominantly older male patients were involved. It was not possible to determine the clinical behavior of our 5 cases due to lack of follow-up information. However, the clinical appearance (large size and ulceration) of these tumors together with detectable peripheral neuropathy (ie, paresthesia, paralysis) and presence of fixed lymph nodes suggesting metastatic spread was supportive of an aggressive clinical behavior. The size of primary SDC was found to correlate with malignant potential. Hui et al[26] reported that tumors smaller than 3 cm correlate with a lower malignant potential, whereas Delgado et al[15] found a similar correlation with tumors smaller than 2 cm.

Immunohistochemical evaluation of some intermediate filaments in the tumor cells indicated that SDC is composed of predominantly ductal cells with little or no myoepithelial cell involvement. The strong expression of cytokeratin in the tumor cells is supported by the majority of studies on SDCs.[2,6,15,27] The tumor cells were negative for vimentin and smooth muscle actin, whereas positive staining with S-100 antibody was found in less than 10% of tumor cells in all 5 cases. Diffuse positive staining of S-100 was found by Brandwein et al[2] in 7 of 9 cases as well as in a single case of SDC reported in the palate.[25] Most studies, however, reported no immunoreactivity with S-100

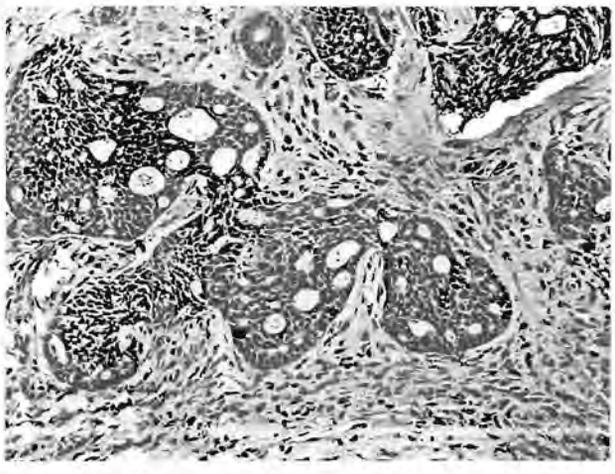

FIGURE 6. Infiltrating tumor islands in a cellular stroma (hematoxylin-eosin stain, original magnification ×100).

Table 3. IMMUNOHISTOCHEMICAL FINDINGS IN THE 5 INTRAORAL SDCs

Antibody	Patient 1	2	3	4
Keratin	+++	++	+++	++
α-Smooth muscle actin	−	−	−	−
Vimentin	−	−	−	−
S100	+	+	+	+

NOTE. −, Negative; +, 1% to 9% of tumor cells; ++, 10% to 50% of tumor cells; +++, more than 50% of tumor cells.

antibody.[15,17,27] The isolated (1% to 10%) positive staining of S-100 in our 4 cases may be due to the presence of Langerhans cells between the tumor cells, although tumor cells with a ductal differentiation may express S-100 protein.[28] Ultrastructurally, SDCs are composed of cuboidal to polygonal cells with interdigitations and cells forming ductlike structures with microvilli and apical vesicles; myoepithelial cells are absent. These findings support the ductal origin of SDC.[29]

Four of the SDCs in the present study displayed DNA aneuploidy. Several studies have measured the DNA content of SDC using flow cytometry with varied results. The majority found no correlation between the ploidy status and prognosis,[6,24,30] whereas Martinez-Barba et al[27] found a positive correlation between aneuploidy and the presence of distant metastases and fatal clinical outcome. Nuclear suspensions for flow cytometry analyses were obtained from paraffin-embedded sections in all of the above-mentioned studies, but none of these studies mentioned the CV obtained for the flow cytometry measurements. The CV of DNA measurements using paraffin-embedded tissue will invariably be higher than when using fresh tissue from the same tumor. It is possible that the reported diploid cases were in fact false diploid as tumor cells with a near diploid peak, implying small deviations of their DNA content from normal diploid cells, could not be distinguished due to the relatively high CV.

SDC is a distinct tumor that can originate from minor salivary glands. The histologic features are similar to those of tumors originating in the major salivary glands. The palate appeared to be the most common intraoral site for SDC. Although clinical features were suggestive of an aggressive behavior, more reported cases are required to determine the behavior in SDCs of minor salivary glands.

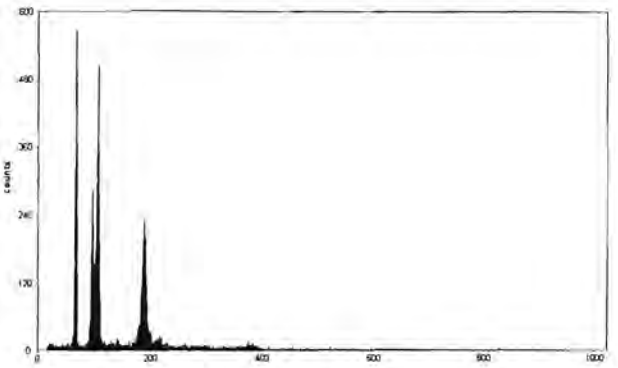

FIGURE 8. DNA histogram of case 1 showing the normal diploid peak at channel 100. Hypodiploid and hyperdiploid tumor cells were present.

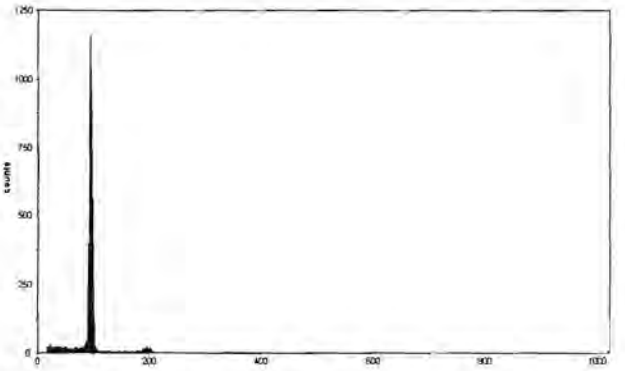

FIGURE 9. Diploid DNA histogram with diploid cells at channel 100 and the small peak at channel 200 representing the cells at G_2M phase.

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Tyrosine-rich crystalloids in a polymorphous low-grade adenocarcinoma

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A polymorphous low-grade adenocarcinoma with tyrosine-rich crystalloid deposits is reported. The literature is reviewed, and diagnostic and histogenetic implications of this finding are discussed.
(ORAL SURG ORAL MED ORAL PATHOL 1990;70:480-2)

Tyrosine-rich crystalloids occur mainly in salivary gland mixed tumors where they are reported in between 1.5% and 21% of cases.[1-4] The incidence of these deposits appears higher in mixed tumors involving black patients than white patients.[2,5] Salivary gland carcinomas that have been reported to contain tyrosine-rich crystalloids include one terminal duct adenocarcinoma,[6] an adenoid cystic carcinoma,[7] and a malignant mixed tumor.[8]

The origin of tyrosine-rich crystalloids in salivary gland neoplasms is speculative. The principal tumor cell associated with these deposits is the modified neoplastic myoepithelial cell,[6] which is also believed to be the source of the stromal matrix deposits in mixed tumors.[9]

CASE REPORT

A 36-year-old black female patient had a 3 × 2 cm, firm midline swelling at the junction of the hard and soft palate. No ulceration was present. A clinical diagnosis of benign mixed tumor was made and an incisional biopsy taken. Although tyrosine crystals were observed, perineural invasion prompted a provisional diagnosis of a polymorphous low-grade adenocarcinoma and wide excision was recommended. Microscopic examination of the surgical specimen showed an infiltrative neoplastic growth with a lobular architecture. Solid masses of epithelial cells, areas exhibiting ductlike differentiation, and cells arranged in long, single-layered strands were observed. The neoplasm was further characterized by a low mitotic activity, histologic diversity with cylindric, clear cell, and mucus cell differentiation, and a lack of pleomorphism. Evidence of perineural invasion was present (Fig. 1). Extensive crystalloid deposits were present in the connective tissue stroma and between the cells in the solid epithelial masses (Fig. 2). These crystals showed distinct brown staining with the Millon reaction and were nonbirefringent under polarized light. A diagnosis of polymorphous low-grade adenocarcinoma of minor salivary gland origin with tyrosine-rich crystalloid deposits was made.

DISCUSSION

Polymorphous low-grade adenocarcinoma, also referred to as *terminal duct adenocarcinoma* or *lobular carcinoma of minor salivary gland origin*,[9,10] is a recently described entity occurring most commonly in the palate and is characterized by a favorable prognosis. Histologically, the lesion is distinguished from other malignant tumors of salivary gland origin by its frequent lobular growth pattern, low mitotic rate, and cytologic uniformity. Although extensive nerve invasion and a cribriform growth pattern may resemble adenoid cystic carcinoma, polymorphous low-grade adenocarcinomas are characterized by histologic diversity, with cells exhibiting cuboidal to low columnar differentiation and an eosinophilic cytoplasm. The stroma, furthermore, often exhibits mucohyaline change in contrast to the bland basement membrane–like deposits of adenoid cystic carcinoma.[10-12] If these criteria are to be applied, it appears as if the adenoid cystic carcinoma containing tyrosine-rich crystalloids reported by Gould and coworkers[7] might possibly have been a polymorphous low-grade adenocarcinoma. If this case were to be accepted as a polymorphous low-grade adenocarcinoma, it would bring the total

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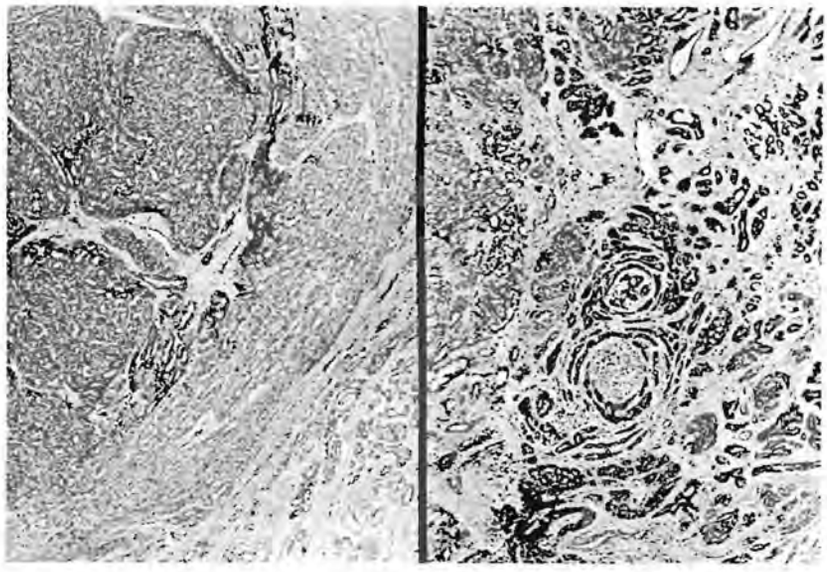

Fig. 1. Distinctly lobular arrangement and infiltrative growth of tumor *(left)* with perineural infiltration *(right)*. (Hematoxylin-eosin stain; original magnification, ×40.)

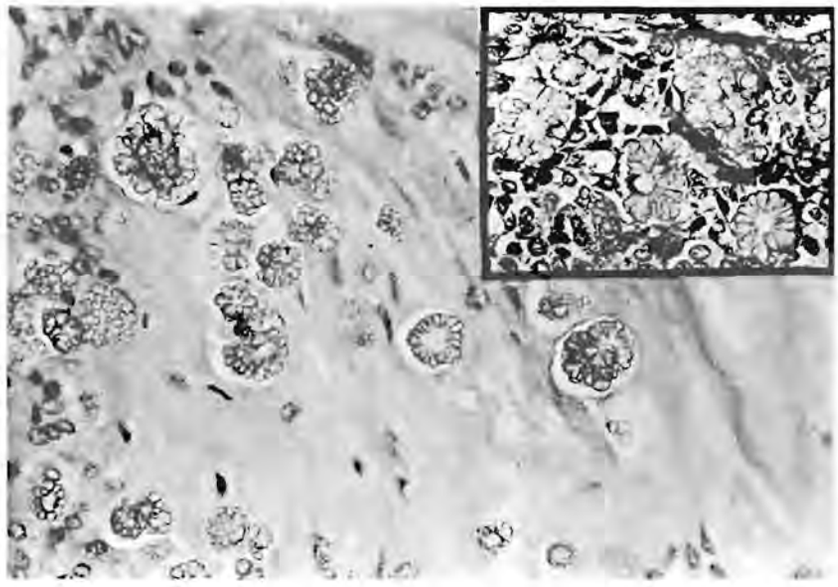

Fig. 2. Stromal and interepithelial *(inset)* deposits of tyrosine-rich crystalloids. (Hematoxylin-eosin stain; original magnification, ×200.)

number with tyrosine-rich crystalloids to three, including the case described by Harris and Shipkey[6] as a "terminal duct adenocarcinoma."

The identification of tyrosine-rich crystalloids in a neoplasm other than benign mixed tumor has important diagnostic implications, as many recent publications regard these crystalloids as a unique microscopic feature of salivary gland mixed tumors.[13-15] It is speculated that the formation of tyrosine-rich crystalloids in polymorphous low-grade adenocarcinomas may place these lesions on a level of cytodifferentiation closer to that of benign mixed tumors than to the other more malignant tumors of salivary gland origin.

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