

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 HI 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 \pm 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 \pm 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 μ m sections were cut from each block, using a new disposable blade after each block. To evaluate the possibility of contamination at this stage, 10 μ m 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 μ l of lysis buffer containing proteinase K. The samples were then heated to inactivate the proteinase K, centrifuged to pellet the debris and 10 μ l 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 μ l. 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'-CTTTAAAACCTCTAAAAATCAAAACTTTAGA (+) 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 μ l of DNA; 200 μ M of each nucleotide; 0,4 μ M of each primer; 1U of Taq DNA polymerase (Promega); 10 μ l of Promega 10 x buffer and made up to a final volume of 100 μ l. Each reaction mixture was covered with two drops of mineral oil. Template DNA for the second amplification round consisted of 10 μ l 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.

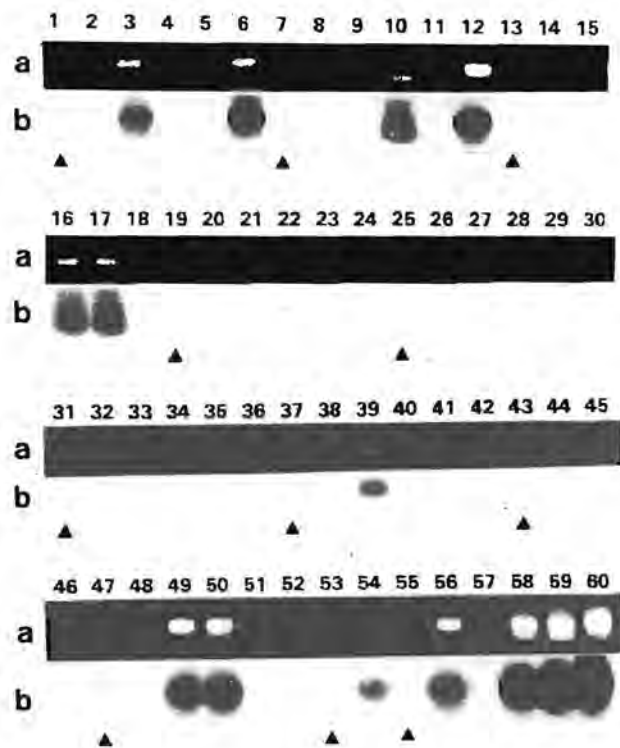


Figure 1. Oral squamous cell carcinomas of group 1. Numbers 58, 59 and 60 represent 1, 10 and 100 genome copies respectively; number 57 designate the negative control. (a)-gel electrophoreses; (b)-hybridisation; (\blacktriangle)-brain samples.

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 μ g/ μ l salmon sperm DNA. The dilutions containing 1, 10, 100 and 1 000 copies of plasmid/ μ l were included in each run. After the last round of amplification, 10 μ l 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 μ g/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

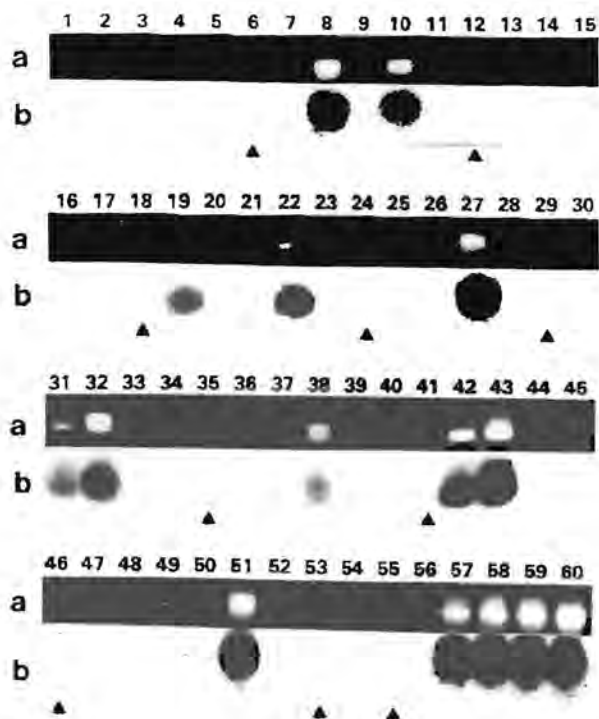


Figure 2. Squamous cell carcinomas of patients 40 years and younger. Numbers 57, 58, 59 and 60 represent 1, 10, 100 and 1000 genome copies respectively, while number 56 designate the negative control. (a)-gel electrophoreses; (b)-hybridisation; (▲)-brain samples.

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

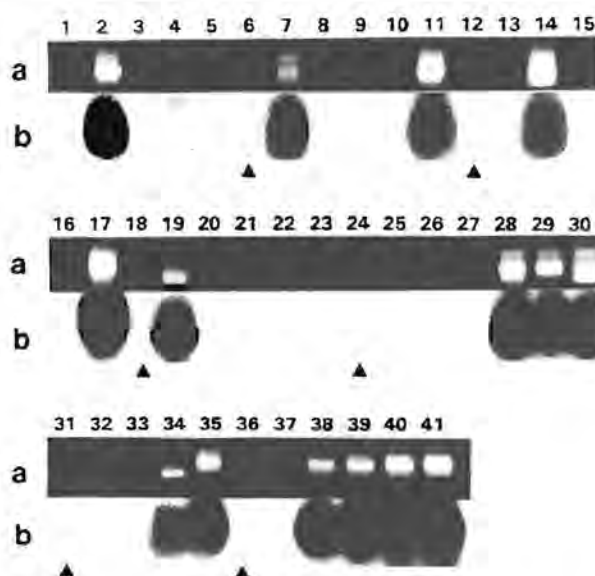


Figure 3. Control group. Numbers 38, 39, 40 and 41 represent 1, 10, 100, and 1000 genome copies respectively and number 37 the negative control. (a)-gel electrophoreses; (b)-hybridisation; (▲)-brain samples.

epithelial tumors with EBV has been suggested recently on the basis of molecular biological techniques. The presence of EBV DNA in tonsillar 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 infiltration 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 ubiquitous viruses such as EBV. Corroborative 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.

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Prevalence of Epstein-Barr virus in nasopharyngeal carcinoma in a South African 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 occurring 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,

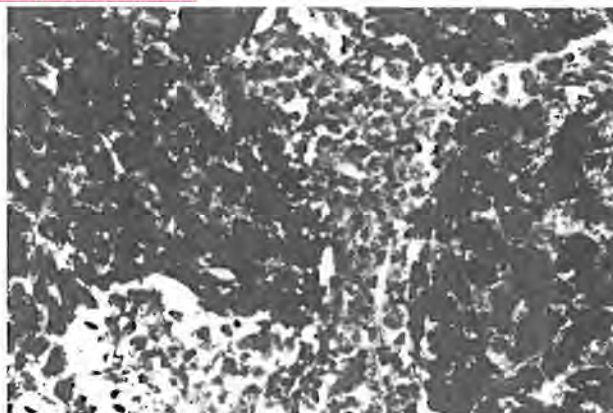


Figure 1: EBV *in situ* hybridisation of undifferentiated NPC. Hybridisation signals are restricted to tumour cells with no staining of surrounding lymphoid stroma. (Original magnification $\times 75$)

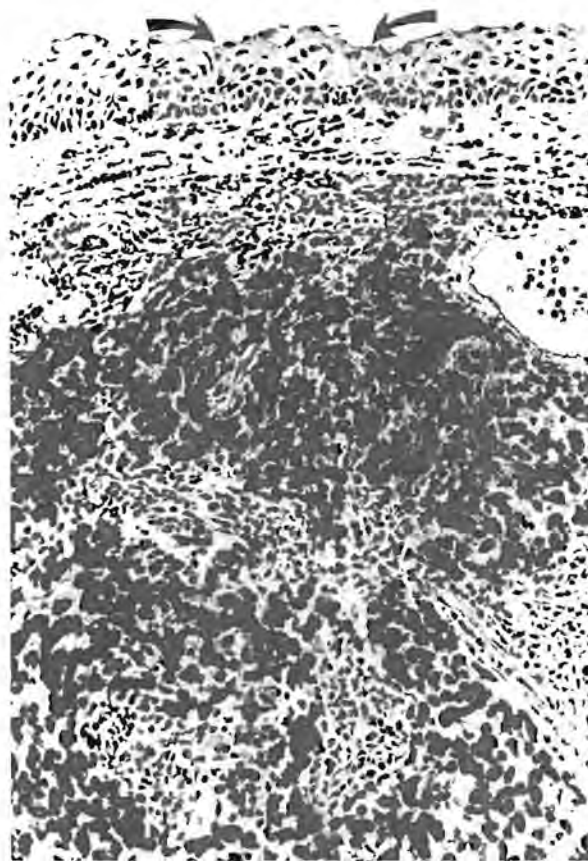


Figure 2: EBV *in situ* hybridisation of undifferentiated NPC. Sheet of positive staining malignant cells with normal overlying epithelium (arrows). (Original magnification $\times 50$)

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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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 HI 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 immunosuppressed individuals (4). Based on the organisation of the Bam HI 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 nasopharyngeal carcinomas (NPC), by specifically investigating the EBNA-2A and the 190 bp EBER fragments.

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. Haematoxylin 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 HI 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.

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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 MgCl₂; 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 and 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).

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)

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

Primer/Probe name	Sequence
EBV-1	CITTTAMACTCTAAAAATCAAAACTTTAGA
EBV-2	ACCAGAAATAGCTGCAGGACCACTTTATAC
EBV-3	MTGGGCGCCATTTTGT
EBV-4	TCCCTAGMCTGACMTT
PROBE-5	TATCTTTAGAGGGGAAAAGAGGMTMG
EBNA2 Gen1	AGGGATGCCTGGACACMG
EBNA2 Gen2	GTGCTGGTGTCTGTGGTGG
EBNA2 A-1	TCTTGATAGGGATCCGCTAGGATA
EBNA2 A-2	ACCGTGGTTCTGGACTATCTGGATC
PROBE A	CTCTGTACMCCGAGGCTTACC
EBNA2 B-1	CATGGTAGCCITAGGACATA
EBNA2 B-2	AGACTTAGTTGATGCCCTAG
PROBE B	AGGCCTACTCTTCCTCMCCCAG
EBER P1	GTGGTCCGCATGTTTTGATC
EBER P2	GCMCGGCTGTCTGTTTGA
EBER P3	GTGTCTACCTGMCTMGAC
EBER P4	CCTAGTGGTTTCGGACACAC
PROBE P5	MCGGGGCITMCGTTGCAT

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).

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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 positivity could be found if fresh tissue were investigated, because paraffin 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.

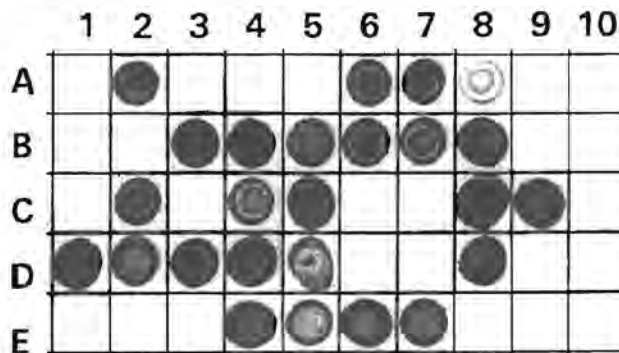


Figure 1. Hybridisation results on EBER PCR results. Row A1-9 correspond to patient samples 1-9; B1-9 to samples 10-18; C1-9 to samples 19-27; D1-9 to samples 28-36; E1-2 to samples 37 and 38; E3 is a negative reagent control; E4, 5, 6 and 7 correspond to 1, 10, 100 and a 1000 plasmid copies/ μ l and 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 Pa-

thologists 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 tumor¹. 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 differentiation⁶, 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 tumors⁸.

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 study⁵. 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 μ m 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 method⁹. 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 varia-

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

tion (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.

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% \pm 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 potential³. 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.

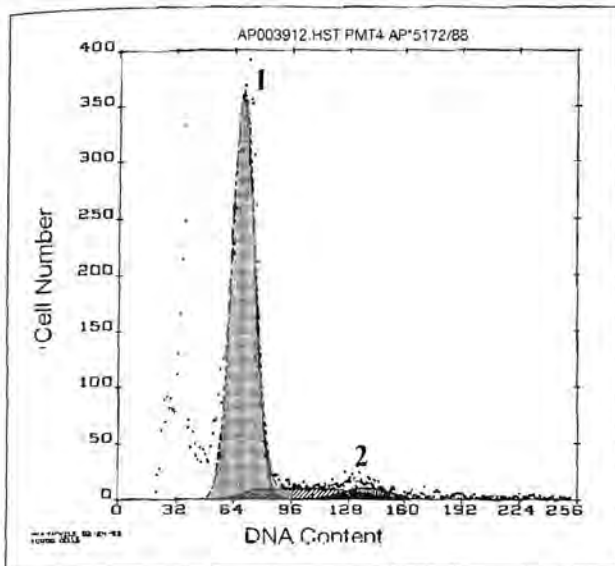


Figure 1. DNA histogram of a diploid oral squamous cell carcinoma. Peak 1 represents the diploid G_0/G_1 cells while peak 2 represents the tetraploid G_2 and mitosis cells.

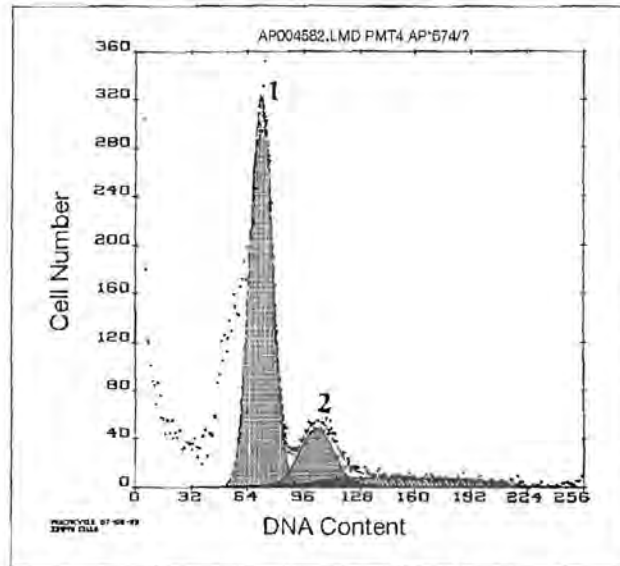


Figure 2. DNA histogram of an aneuploid oral squamous cell carcinoma. The two G_0/G_1 peaks (1 and 2) are clearly visible.

sive behavior and grading thereof may not give an accurate assessment of the prognosis. Increased labelling of bromodeoxyuridine¹⁰ as well as Ki-67¹¹ and H-ras mRNA¹² 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 significantly⁴. 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 method⁵. This omission resulted in an improved inter-observer reproducibility without influencing the prognostic value of the total malignancy score⁵. Less subjective methods of determining the proliferative activity of tumors exist, e.g. in vitro bromodeoxyuridine¹³ and PCNA¹¹ 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 method⁵. 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 progression⁸. Aneuploidy has also been linked to a decrease in histologic differentiation of oral squamous cell carcinoma⁶.

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 tissue¹⁶. Although acceptable CVs are possible with paraffin-embedded tissues⁹, 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.

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

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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 histocompatibility 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-

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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 laser 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, Liphaw) 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 mm² 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			Total
	5–8	9–12	13–20	
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.

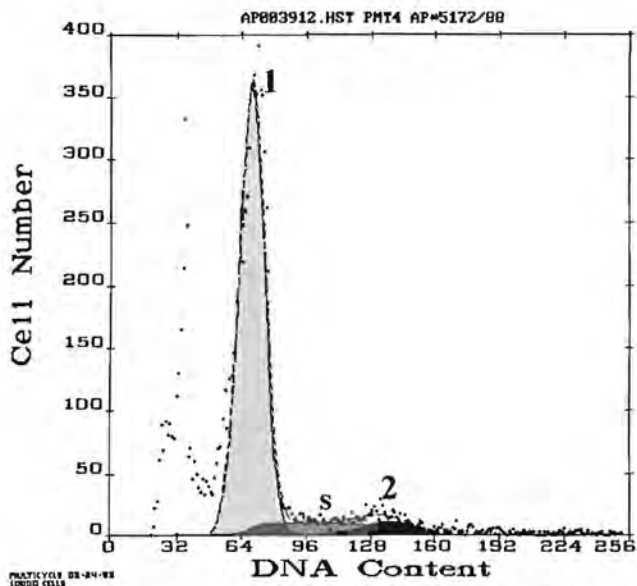


Fig. 1. DNA histogram of a diploid oral squamous cell carcinoma. Peak 1 represents the diploid G_0/G_1 cells, while peak 2 represents the tetraploid G_2 and mitosis cells. Cells in S-phase with intermediate DNA content are displayed between the two peaks.

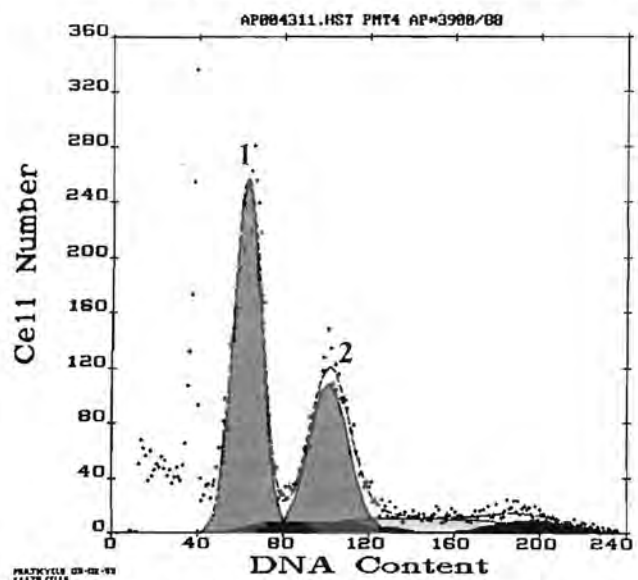


Fig. 2. DNA histogram of an aneuploid oral squamous cell carcinoma. The two G_0/G_1 peaks (1 and 2) are clearly visible.

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 carci-

nomas with an average lymphoplasmacytic score of 3 and 4 (Table 5). These differences however, were statistically not significant.

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.

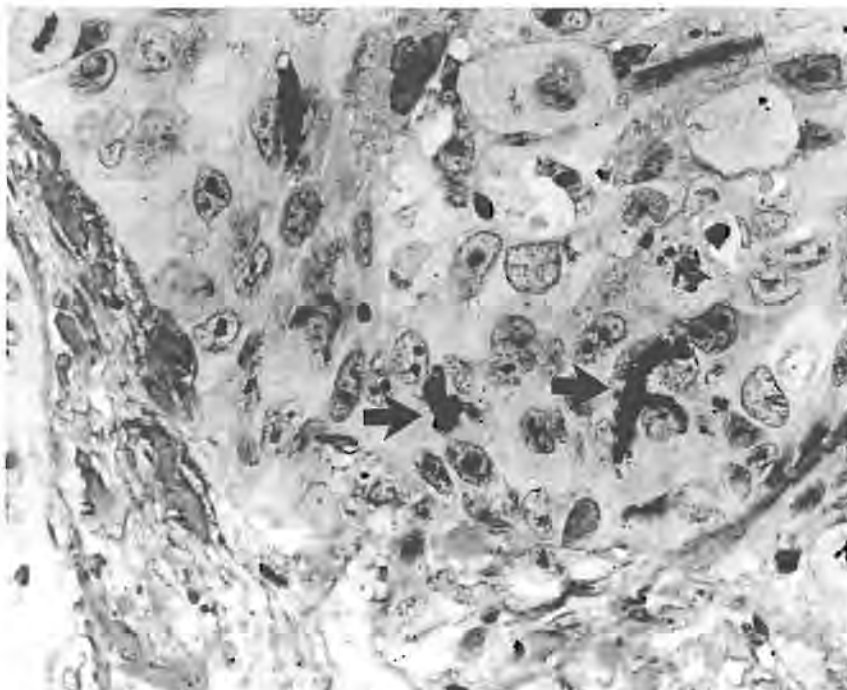


Fig. 3. S-100 protein-positive cells in a squamous carcinoma (arrows) $\times 200$.

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

	S-100 (/mm ² tumor)	HLA DR (/mm ² 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 co-workers 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.

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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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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		total
	N0	N ⁺	N0	N ⁺	
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

^apalliative treatment or treatment refused

with 5-fluorouracil and cisplatin while 23 received epirubicin and cisplatin preoperatively. Forty-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 (MgCl₂) 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 calculation 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 appearance 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

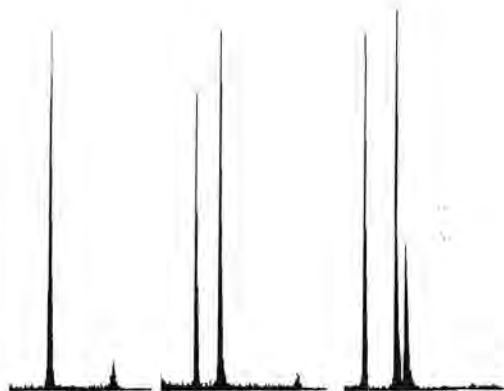


Figure 1. Representative DNA histograms of oral carcinomas. Diploid tumors (left) are characterized by a prominent peak representing the cells in the G1/G0-phase of the cell cycle. The much smaller peak with exactly double the DNA content of the first one contains cells in G2-phase and mitosis. The counts 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.

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

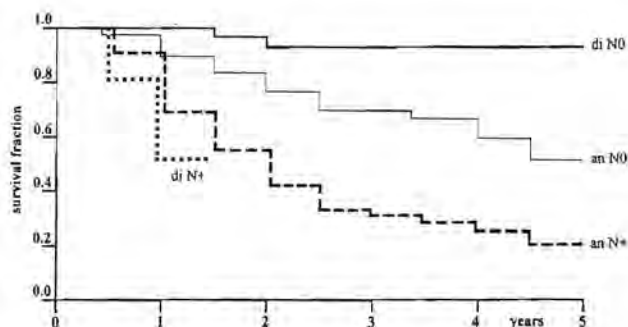


Figure 2. Overall survival of 279 oral carcinoma patients who underwent initial treatment by radical primary tumor surgery accompanied with a neck dissection if indicated with respect to lymph node involvement at presentation and DNA ploidy status of the primary tumor.

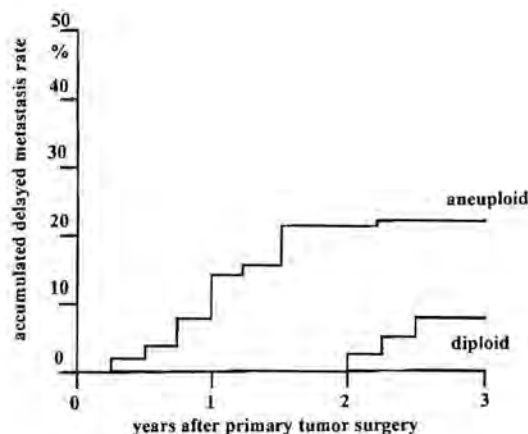


Figure 3. Time-adjusted delayed metastasis rate in patients without clinical or histological evidence of lymph node involvement at primary tumor surgery with respect to the DNA ploidy status of the primary tumor. Patients who showed lymph node involvement after or simultaneously with the development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

(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 ⁺		sum	p-value
	d	a	d	a		
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

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

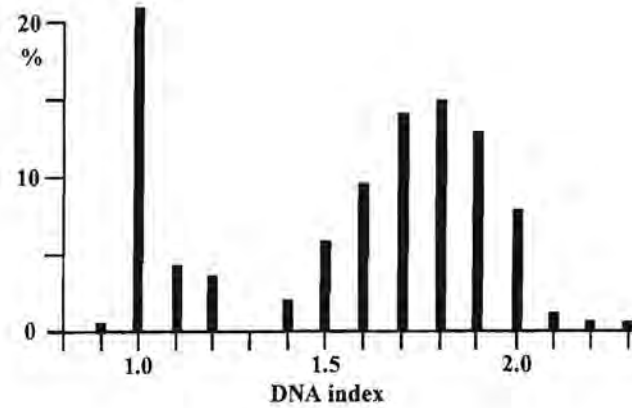


Figure 4. DNA index frequency distribution pattern of all monoclonal oral carcinoma cases.

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 in spite 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

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 provides 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 carcinomas 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 appearance of metastasis from diploid primary tumors is delayed by two years in comparison to the aneuploid group.

In conclusion, the present study demonstrates 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.

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 (MgCl₂) 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).

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

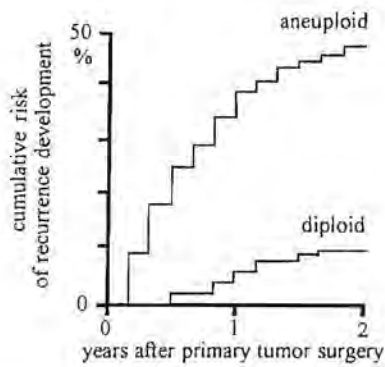


Figure 1. Recurrence development with respect to the DNA ploidy status of the primary tumor.

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 juxtprimary 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).

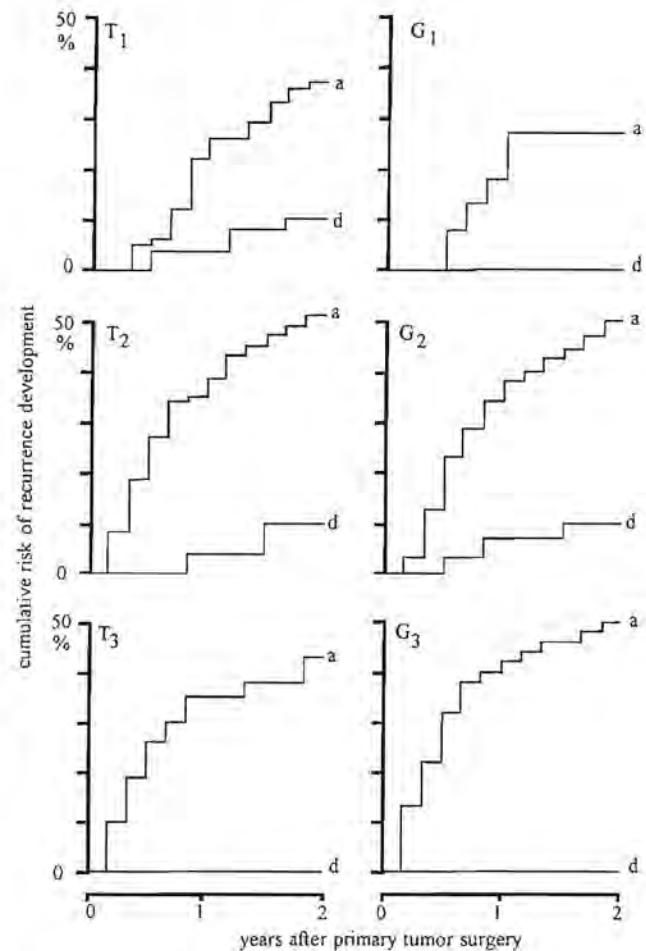


Figure 2. Recurrence development with respect to the DNA ploidy status of the primary tumor as well as with respect to tumor stage and histological grade.

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

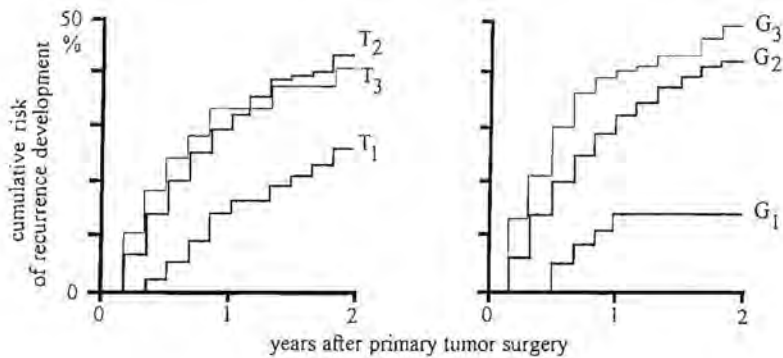


Figure 3. Recurrence development with respect to tumor stage and histological grade.

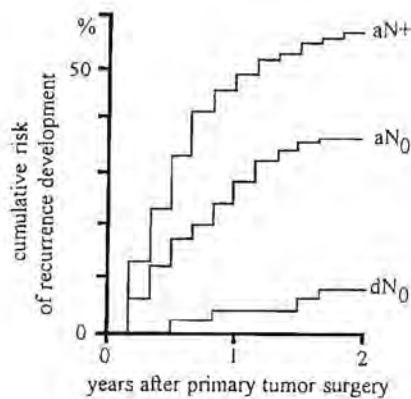


Figure 4. Recurrence development with respect to the DNA ploidy status of the primary tumor as well as with respect to lymph node involvement at primary tumor surgery.

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 juxtprimary 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

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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 tu-

mor. 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- μ m 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

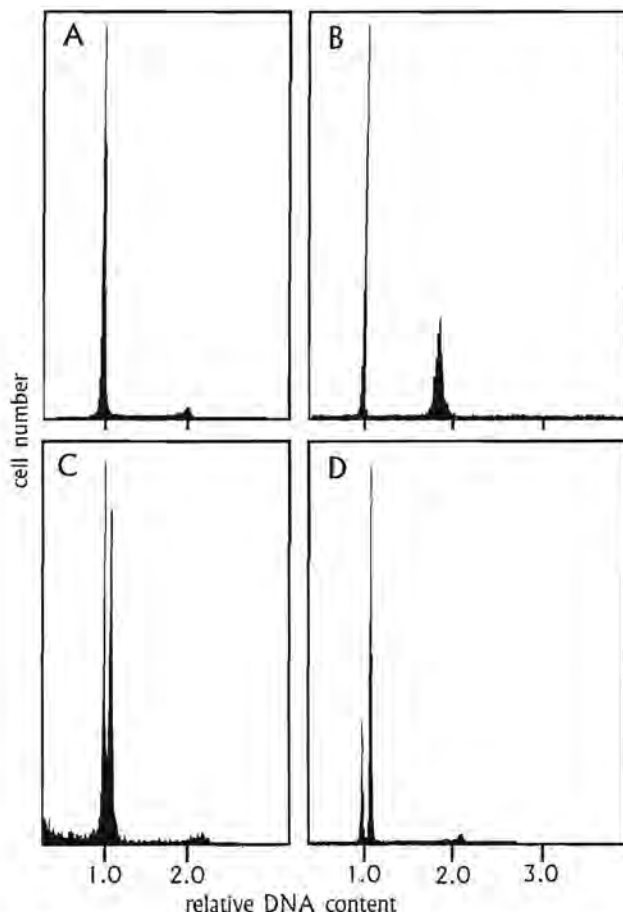


FIGURE 1. DNA histograms of primary oral carcinomas and their subsequent recurrences are shown. A diploid primary tumor (A) is shown, which developed a local recurrence characterized by an aneuploid tumor cell line with a DNA index of 1.85 (B). The lower panels demonstrate a primary tumor (C) and its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

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.

mors 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.

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

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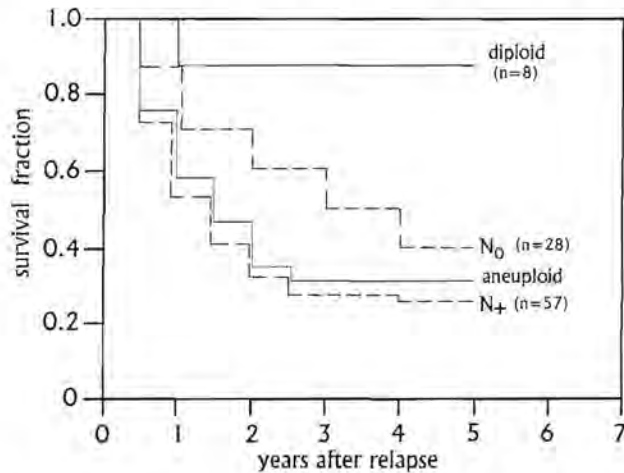


FIGURE 2. Overall survival rates of patients with diploid and aneuploid locally recurrent oral carcinomas are shown. All patients of the diploid group were lymph node negative. The aneuploid lymph node negative group (N₀) had no evidence of lymph node involvement at initial surgery or at second surgery. 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 surgery¹⁻³ 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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