THE ROLE OF SALIVARY GLANDS IN VERTEBRATES

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A salivary gland is defined as any organ that discharges a secretion (or saliva) into the upper part of the digestive tract. They are found not only in most vertebrates, but also in many invertebrates such as the Annelida (segmented worms), some Mollusca (snails) and Arthropoda (insects, crustacea, arachnids and related forms). In humans, salivary glands are grouped into a major (large) and minor (small) category. Three paired major salivary glands in man and are located in the neck and floor of mouth and produce 600-1500 ml saliva per day. Numerous small (or minor) salivary glands are present in the palate, tongue and lips.

In general terms, salivary glands in vertebrates can be considered as subserving many functions and are generally adapted to the environment of the animal. First and perhaps most important, saliva provide lubrication for the swallowing of food. The solution of food particles is important for the proper functioning of the taste receptors which are located in the lining of the oral cavity. The washing action of saliva as well as its antibacterial properties are essential for the maintenance of oral and dental health. In humans, lubrication evidently facilitates speech. In animals where ingested food are dry, the salivary glands are adapted to secrete large quantities of watery, sodium free and subsequently hypotonic saliva. The hypotonicity prevents dehydration of cells lining the digestive tract and increases the moisturising capacity of saliva, as both factors are dependant on osmotic principles. The paired parotid salivary glands of the African elephant is probably the largest in the animal kingdom with a combined mass of 15 kg and produce 50 liters saliva per hour during feeding. A normal feeding cycle, which extends over the greatest part of 24 hours, is characterized by the production of a large volume of saliva which moisturises and lubricates the daily diet of 200-300 kg leaves, bark and grass. Hippopotomi generally ingest lush vegetation on the banks of rivers and their salivary glands are subsequently smaller and the volume saliva produced significantly less than when compared to that of the elephant. Among aquatic animals, where lubrication of food is not necessary, salivary glands may be absent as in most Cetacea (dolphins and whales).

By secreting enzymes, salivary glands are capable of playing a role in digestion. Most animals and humans have relatively high concentrations of amylase (a starch digesting enzyme) in their saliva. The levels of this enzyme is however rather low in the saliva of the domestic cat and dog and absent from the saliva of the elephant. The significance of these findings are speculative. The high urea concentration in the saliva of the elephant is probably indicative of a recycling mechanism similar to that found in cattle where hindgut commensal protozoa, which aid in digestion, are dependant on a constant supply of urea for metabolic processes. Certain newborn animals, such as the suckling rat, produce large amounts of salivary lipase, a fat digesting enzyme which is partially responsible for the digestion of milk fats.

In fur-bearing animals such as the cat and rat, saliva plays an important role in the regulation of body temperature. By wetting their fur with saliva in response to heat, they obtain the same cooling capacity available to man by sweating.

Other less common functions of saliva is that of defence, paralysing or killing of prey. The only mammalian saliva known to be toxic is that of the Americans short-tailed shrew, Blarina brevicauda. The venom glands of snakes are modified salivary type glands which produce one of the most complex and highly evolved poisons known. Contraction of muscles surrounding the gland ejects the venom through openings on two or more teeth or fangs. In one group of sea snakes, Hydrophiidae, a large salivary type gland fulfills the functions of a salt excreting gland and plays an important role in maintaining the salt balance of the animal, making life possible in its saline environment.

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Salivary Immunoglobulin Related Proteins in 24 Patients with Multiple Myeloma

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Mixed saliva and blood of 24 cases of multiple myeloma (MM) were collected and the immunoglobulin and light chain concentrations compared with that found in the saliva and blood of 16 age matched control patients. The concentrations of salivary IgA, IgG and lambda light chains were significantly increased in IgA-, IgG- and lambda light chain producing MM respectively. Salivary IgA concentration in non-IgA MM and salivary IgG concentration in non-IgG MM were within normal ranges. Despite a significant decrease in circulating normal immunoglobulins, this study fails to support suppression of normal salivary immunoglobulin concentrations in patients suffering MM.
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INTRODUCTION

In the majority of patients with multiple myeloma (MM) serum protein electrophoresis will disclose the presence of a monoclonal paraprotein which may present as an increase in one of the immunoglobulin classes and/or immunoglobulin-related light chains (Bence-Jones proteins). MM are immunochemically typed according to the circulating monoclonal immunoglobulin and/or light chain type produced by the disseminated neoplastic plasma cells. This typing is helpful in predicting complications and prognosis of patients suffering MM [1]. The decrease in the concentrations of circulating normal immunoglobulins predispose to opportunistic infections, a serious and often terminal complication in MM [2].

Reports on the presence of abnormal immunoglobulin-related proteins in secretions of MM patients are infrequent in the literature. Analysis of saliva of 10 patients with MM [3], identified monoclonal IgA in 5 out of 7 patients with IgA MM and monoclonal IgG in both patients with IgG MM. No free light chains were detected in the saliva of the 1 patient with light chain producing MM. An increased concentration of IgG was present in the saliva of 1 case of IgG MM studied by Brandtzaeg [4].

The purpose of this study was to determine the concentrations of immunoglobulins and light chains in saliva and serum of 24 patients with MM and to compare the values obtained with that found in age matched, systemically healthy patients.

PATIENTS AND METHODS

Whole saliva and blood of 24 patients with MM and 16 age matched systemically healthy control patients were collected after a thorough clinical oral examination. The saliva was expressed with the aid of a sterile syringe from a cottonwool swab after it had been chewed for 3 min. Patients with overt signs of gingivitis or periodontitis were excluded from the control group of the study. Quantitation of IgA, IgG and IgM levels in serum were done with rate nephelometry (Auto ICS, Beckman Instruments Inc. Fullerton, U.S.A.). Immunochemical typing of the light chains in serum was carried out with immunofixation electrophoresis (Paragon™ IFE gels, Beckman Instruments Inc.).

Salivary immunoglobulins and light chains were quantitated with low concentration radial immunodiffusion plates (LC-Partigen® and M-Partigen®, Behringwerke AG, Marburg, West Germany). The concentrations were expressed in grams per litre (g/l), compared with the respective circulating concentrations and the findings were subjected to statistical analysis using Student's *t*-test for uncorrelated data.

RESULTS

Clinical examination of the MM patients revealed no signs of oral mucosal infections. 17 patients had IgG MM, 4 IgA MM and 3 light chain producing MM (two kappa- and one lambda MM). The mean concentrations and standard deviations of the major immunoglobulin classes in MM patients and the control group are expressed in Table 1 and the immunoglobulin light chain concentrations in Table 2. The circulating residual immunoglobulin concentrations in MM patients were generally below the normal ranges (Table 3) and that of the control group (Table 1). No significant differences were found between salivary IgA concentrations in non-IgA MM and the control group ($P > 0.05$) and salivary IgG levels in non-IgG MM and the control group ($P > 0.8$). In IgA MM, salivary IgA concentrations were found to be significantly higher than in the control group ($P < 0.01$). A significant increase in salivary IgG in IgG MM ($P < 0.01$ was also present. The concentration of lambda light chains in the saliva of lambda-producing MM was significantly higher than the control group ($P < 0.01$). Although salivary kappa light chain concentrations in kappa-producing MM showed great variations, with single values far above those of control patients, statistical analysis failed to prove a significantly higher concentration of kappa light chains in kappa-producing MM when compared to the control group ($P > 0.05$).

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Table 1. Concentrations of major immunoglobulin types in MM- and control patients

	Saliva g/l		Serum g/l		
	IgG	IgA	IgG	IgA	IgM
IgA MM (2 × IgA κ's 2 × IgA λ's)	0.04 ± 0.03	1.1 ± 0.9	5.85 ± 4.4	41.5 ± 25.3	2.0 ± 3.5
IgG MM (12 × IgG κ, 5 × IgG λ)	0.22 ± 0.16	0.05 ± 0.05	75.9 ± 32.4	0.63 ± 0.6	0.57 ± 0.42
Lambda MM ($n = 1$)	0	0.14	5.5	0.3	0.2
Kappa MM ($n = 2$)	0.7 ± 0.01	0.04	13.1 ± 1.5	0.65 ± 0.07	0.25 ± 0.07
Control ($n = 16$	0.047 ± 0.03	0.081 ± 0.03	20.0 ± 6.6	3.28 ± 1.3	2.16 ± 1.7

Table 2. Light chain concentrations in MM- and control patients

	Saliva g/l		Serum g/l	
	κ	λ	κ	λ
κ-producing MM ($n = 16$)	0.44 ± 1.0	0.006 ± 0.01	43.5 ± 24.5	2.2 ± 1.8
λ-producing MM ($n = 8$)	0.03 ± 0.06	0.16 ± 0.12	4.4 ± 2.9	55.1 ± 37.0
Control ($n = 16$)	0.03 ± 0.03	0.02 ± 0.01	13.4 ± 5.3	7.04 ± 1.5

Table 3. Normal ranges

Serum	
IgG	14.4—22.7 g/l
IgA	1.9—4.7 g/l
IgM	0.7—2.6 g/l
κ	5.66—13.0 g/l
λ	3.04—7.35 g/l
Saliva	
IgA	0.05—0.48 (mean 0.137) g/l*
IgG	0.007—0.037 (mean 0.016) g/l
κ	N/A
λ	N/A

*Gronblad 1981 [5].

DISCUSSION

This study represents the largest series in which the concentrations of immunoglobulin related proteins in saliva of patients with multiple myeloma were determined. Although changes in the circulating immunoglobulin concentrations are well documented [2], little is known of alterations in salivary immunoglobulins and immunoglobulin related proteins in this disease.

A study using immunoelectrophoresis to determine the presence of salivary immunoglobulins in 10 patients suffering MM [3] failed to express the concentrations and the findings can therefore not be compared directly to ours. These authors conclude that although the concentration of monoclonal immunoglobulin is low in saliva, its presence is adequate proof that circulating immunoglobulins can find their way into external secretions. The technique employed in our study is more sensitive and made accurate quantitation of the different immunoglobulin-related proteins possible. All our cases of IgA MM had significantly increased concentrations of IgA in saliva when compared to the salivary IgA concentrations found in the control group. The same applies to salivary IgG in IgG MM and lambda light chain concentrations in the saliva of lambda producing MM. Despite a few kappa producing MM that had high salivary kappa concentrations, statistical analysis failed to support a significant increase in salivary kappa concentrations in kappa producing MM when compared to control values. Although transmission of circulating immunoglobulin related proteins to saliva appears to be enhanced by elevated serum concentrations, no direct correlation could be found between these values.

The occurrence of systemic immune suppression in MM is well documented. This study supports the findings of Coelho *et al.* [3] which failed to identify salivary immunoglobulin impairment in MM. No statistical evidence of a decrease in the concentration of normal salivary IgA in non-IgA MM patients could be found in our study. This was confirmed in that no clinical evidence of an opportunistic infection was seen in the oral cavities of our MM patients. The mechanism by which normal immunoglobulin production is suppressed in MM, is not clearly understood [6]. It has been postulated that neoplastic plasma cells secrete a factor capable of activating suppressor macrophages which in turn inhibit normal B cell function [7]. The observation that salivary gland associated immunoglobulin production is not altered in MM, adds an interesting parameter to the debate on MM-induced immunoparesis.

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Intraoral salivary gland neoplasms: A retrospective study of seventy cases in an African population

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Intraoral salivary gland neoplasms diagnosed in the Department of Oral Pathology, Medical University of Southern Africa, Medunsa, were reassessed and revised with regard to histologic diagnosis. New entities and subclassifications that have been described in recent years were taken into account. Seventy cases were diagnosed during an 8-year period, and the sample consisted of black patients only. Benign mixed tumor was the most common entity and accounted for 48% of all tumors. Polymorphous low-grade adenocarcinoma comprised 15.7% of the sample and was the most frequent malignant tumor. The mean age of patients with benign and malignant tumors were 36.5 and 49.8 years, respectively ($p < 0.05$), and the palate was the most common site involved. Geographic differences do exist in the pattern and pathology of intraoral salivary gland neoplasms when compared with findings in other studies.
(ORAL SURG ORAL MED ORAL PATHOL 1991;71:579-82)

The distribution and frequency of intraoral salivary gland neoplasms has been discussed in several published series,[1-5] in the majority of which the World Health Organization (WHO) classification[6] was used. However, various new entities and subclassifications that are not included in these articles have been described in recent years.[7, 8] This study was undertaken to determine the relative frequency and distribution of intraoral salivary gland neoplasms in a predominantly rural black African population and to provide data for comparison with findings in other geographic locations.

MATERIAL AND METHODS

All the intraoral salivary gland neoplasms diagnosed during the last 8 years were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa, Medunsa. Most patients seen at the hospitals served by the department are black and of rural southern African origin. Representative slides stained with hematoxylin and eosin were available for review, and, where necessary, appropriate special stains were used to establish a diagnosis. All cases were reassessed and revised with regard to histologic classification. Diagnosis was made with the WHO classification[6] as the basis. New entities such as polymorphous low-grade adenocarcinoma, and subclassifications that have been described in recent years, were taken into account. This includes the subclassification of mixed salivary gland tumors into types I to IV according to the proportion of the stroma in the tumor mass.[8] The polymorphous low-grade adenocarcinomas were divided into the terminal duct type and the papillary type according to the criteria of Slootweg and Müller.[9] The working classification used in this study is shown in Table I. Age, sex, and site were noted from the clinical records.

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Table I. Working classification

- Benign
  - Mixed tumor
    - Type I (stroma 30%-50%)
    - Type II (stroma >80%)
    - Type III (stroma <20%)
    - Type IV (myoepithelioma)
  - Monomorphic adenoma
- Malignant
  - Mucoepidermoid carcinoma
    - Low grade
    - Intermediate grade
    - High grade
  - Adenoid cystic carcinoma
    - Cribriform
    - Tubular/trabecular
    - Solid
  - Polymorphous low-grade adenocarcinoma
    - Terminal duct
    - Papillary
  - Acinic cell carcinoma
  - Carcinoma ex mixed tumor
  - Epidermoid carcinoma
  - Adenocarcinoma
  - Epithelial-myoepithelial carcinoma
  - Undifferentiated carcinoma

RESULTS

The sample consisted of a total of 70 cases of intraoral salivary gland neoplasms. Forty-three (62%) of the patients were female and 27 (38%) were male, yielding a female/male ratio of 1.6:1. The patients ranged in age from 10 to 85 years. Thirty-four cases (48%) were classified as benign; all these were mixed tumors in patients ranging in age from 10 to 64 years, with a mean age (±SD) of 36.5 ± 14.7 years. The female/male ratio was 2.4:1, with the mean age for females 34.9 ± 14.9 years and 40.3 ± 13.1 years for males. The palate was most commonly affected, accounting for 31 tumors (91%). The remaining mixed tumors were found on the upper lip. The location and subclassification of mixed tumors according to the criteria of Seifert et al.[8] are shown in Table II.

Thirty-six cases (52%) were classified as malignant. The patients ranged in age from 22 to 85 years, with a mean age of 49.8 ± 16.3 years. The difference in the mean age of patients with benign tumors and that of those with malignant tumors was statistically significant ($p < 0.05$). The female/male ratio was 1.1:1 for patients with malignant neoplasms. The distribution and location of the malignant tumors are shown in Table III.

Eleven tumors were diagnosed as polymorphous low-grade adenocarcinoma, accounting for 30% of the malignant neoplasms and 15.7% of all neoplasms. The patients' ages were between 32 and 70 years, with a mean age of 53 ± 12.6 years, and the female/male ratio was 1.2:1. Nine lesions (82%) occurred on the palate, and one each in the buccal mucosa and upper lip, respectively. Two tumors had a papillary-type growth pattern, and nine were either lobular or tubular in appearance. Nerve infiltration was present in one papillary-type and in two terminal duct–type tumors.

Table II. Location and subclassification of 34 mixed tumors

Type	*Palate*	*Upper lip*	*Total (%)*
I	25	1	26 (76)
II	2	1	2 (6)
III	5	1	6 (18)
IV			0
Total (%)	31 (91)	3 (9)	34 (100)

Nine cases of adenoid cystic carcinoma accounted for 12.8% of all tumors and 25% of the malignant tumors. The patients had a age range of 33 to 85 years with a mean of 54 ± 15.5 years, and the male/female ratio was 1.2:1. Seven patients had lesions on the palate, and one lesion each was located in the floor of the mouth and on the upper lip. Five tumors had a predominantly cribriform growth pattern, and two each had solid and tubular or trabecular growth, respectively.

Six patients with mucoepidermoid carcinoma accounted for 8.6% of all tumors and 16.7% of malignant tumors. The youngest patient was 22 years and the oldest 52 years of age, and the mean age at time of consultation was 39.8 ± 10.1 years. Sex distribution was equal, and the most common site of occurrence was the palate, with four tumors. One tumor was located on the buccal mucosa and mandibular gingiva, respectively. One mucoepidermoid carcinoma was classified microscopically as low grade, three as intermediate grade, and two as high grade.

Five carcinomas ex mixed tumor were diagnosed. Three of the patients were women and two were men; they ranged in age from 31 to 70 years, with a mean age of 48.2 ± 19.3 years. Four tumors occurred on the palate, and one in the retromolar area. The carcinomatous component in all five was classified as undifferentiated.

Three cases were diagnosed as adenocarcinomas, not otherwise specified. Two tumors were located in the buccal mucosa and one on the palate. Two cases occurred in females, and the mean age of this group was 57.3 ± 13.2 years. One tumor that occurred on the palate of a 49-year-old woman was diagnosed as an undifferentiated carcinoma.

One patient, a 65-year-old woman, had a epithelial-myoepithelial carcinoma of the palate. No case of monomorphic adenoma, acinic cell carcinoma, or

Table III. Distribution and location of malignant tumors

Tumor	*Palate*	*Upper lip*	*Buccal mucosa*	*Mouth floor*	*Retromolar*	*Mandibular gingiva*	*Total*	*% of total*	*% of malignant*
PLA	9	1	1				11	15.7	30
ACC	7	1		1			9	12.8	25
Mucoepidermoid CA	4		1			1	6	8.6	16.7
CA ex mixed tumor	4				1		5	7.1	14
Adenocarcinoma	1		2				3	4.3	8.3
Undifferentiated CA	1						1	1.4	2.8
E-M CA	1						1	1.4	2.8
Total (%)	27 (75)	2 (5.5)	4 (11)	1 (2.8)	1 (2.8)	1 (2.8)	36		

ACC, Adenoid cystic adenocarcinoma; *CA*, carcinoma; *E-M*, Epithelial-myoepithelial; *PLA*, polymorphous low-grade adenocarcinoma.

epidermoid carcinoma of the minor salivary glands occurred in this series.

DISCUSSION

In most studies benign mixed tumors constitute the majority of minor salivary gland neoplasms.[1-5] The frequency of benign mixed tumors is reported as 43% in the study of Eveson and Cawson,[4] 41% by Waldron et al.,[1] and 54% by Chau and Radden.[5] In Isacsson and Shear's series[2] 70% of the tumors were classified as benign mixed tumors. They postulated that the high frequency in their series was the result of the relative higher number of black than white patients, although 60% of their white patients had mixed tumors diagnosed. Schulenburg[10] reported that intraoral benign mixed tumors in his South African sample were 3.5 times more common in black than in white patients. In the present series, where the sample consisted of black patients only, 34 (48%) of the tumors were classified as benign mixed tumors, a frequency comparable to that reported in population samples in the United States and Europe.[1, 4]

The majority of tumors (52%) in the present study were malignant, a finding that does not support the ratio of benign to malignant tumors in recent reports. The proportion of benign tumors varied from 53%[11] to 72%[2] in recent studies. However, 80% of the cases reported by Spiro et al.[12] were classified as malignant. This high percentage of malignant tumors can be explained by the fact that their institution is a major cancer referring center.

The palate was the most common site of involvement of both malignant and benign tumors. The proportion of benign tumors occurring on the palate was larger than in the malignant group, although the difference is not statistically significant. Eighty-one percent of benign mixed tumors reported by Isacsson and Shear[2] occurred on the palate. This high frequency of palatal involvement might be due to the presence of black patients in both samples. The distribution of palatal tumors from several large series compared with our findings is reflected in Table IV.

Table IV. Reported frequency of intraoral salivary gland tumors of palate

Author	*Frequency (%)*	
	Mixed tumor	*Malignant tumor*
Present study	91	75
Thomas et al.[14]	65	63
Isacsson and Shear[2]	81	60
Eveson and Cawson[4]	60	55
Waldron et al.[1]	54	42
Regezi et al.[3]	55	49
Chau and Radden[5]	70	54
Chaudhry et al.[11]	65	35

The benign mixed tumors occurred at a significantly younger age than did the malignant tumors ($p < 0.05$), and a high percentage of the benign tumors affected female patients. These observations support the proposal by Isacsson and Shear[2] that in an African population a salivary gland tumor of the palate occurring in a relatively young patient is more likely to be benign than malignant. This appears to be especially true in women.

Seifert et al.[8] divided benign mixed tumors into four types according to the volume and properties of the stroma and the differentiation of the epithelial cells. Although types III and IV constituted 35% of minor salivary gland mixed tumors in their series, almost 50% of the carcinomas ex mixed tumor arose from tumors with these growth patterns.[8] We are unable to comment on the rate of malignant transformation of types III and IV because only small fragments of benign mixed tumor were present in the carcinomas ex mixed tumor in our series. The finding of Seifert et al.[8] could be related to the more common occurrence of mitotic activity in the solid areas. The majority of benign mixed tumors in the present series were classified as type I. Although mitotic activity, when present, was usually restricted to the solid parts of the tumor, the subclassification depended on the

amount of sections taken, because the growth pattern varied through the tumor.

The absence of monomorphic adenomas in the present study may be due to the fact that our sample consisted of black patients only. Isacsson and Shear[2] found three monomorphic adenomas (2.2%) in their sample of 136 black patients. Davies et al.,[13] in a study of salivary gland tumors in Uganda, found no monomorphic adenomas in 33 intraoral tumors. Thomas et al.,[14] who analyzed salivary gland tumors in Malawi, found one monomorphic adenoma (2%) in their total of 57 minor tumors. These frequencies are in contrast with the 10.7% reported by Waldron et al.,[1] 11% by Evenson and Cawson,[4] and 10% by Regezi et al.[3]

Polymorphous low-grade adenocarcinoma was the most common malignant tumor in the present series. Comparison of the frequency of polymorphous low-grade adenocarcinoma with that reported in other studies is difficult because the majority employed the WHO classification,[6] which does not recognize polymorphous low-grade adenocarcinomas as a separate entity. Polymorphous low-grade adenocarcinoma constituted 30% of the malignant tumors in the present study. Freedman and Lumerman[15] found polymorphous low-grade adenocarcinoma to constitute 7% of the 150 malignant intraoral tumors they examined. Aberle et al.[16] reviewed 109 cases of adenocarcinoma not otherwise specified, malignant mixed tumor, and adenoid cystic carcinoma, and found that 17% of their cases met the criteria of polymorphous low-grade adenocarcinoma. In the study of Waldron et al.[1] 26% of the malignant tumors were diagnosed as polymorphous low-grade adenocarcinoma. The differences among these findings are probably related to the criteria used for diagnosis of polymorphous low-grade adenocarcinoma, because overlapping histologic features with adenoid cystic carcinoma do exist.

The frequency of adenoid cystic carcinoma (12.8%) in our series is similar to that reported in the literature, for example, 13.1% by Eveson and Cawson,[4] 10.9% by Regezi et al.,[3] and 10.4% by Isacsson and Shear.[2] Adenoid cystic carcinoma accounted for 25% of the malignant tumors in our series, a figure lower than the 38% reported by Isacsson and Shear[2] and the 31% of Regezi et al.[3] Polymorphous low-grade adenocarcinoma was not classified as a separate entity in the previously mentioned series, and the reported frequencies of adenoid cystic carcinoma are probably too high.

In the majority of studies mucoepidermoid carcinoma was the most frequent type of malignant tumor, accounting for 15%[1] to 34%[11] of all intraoral salivary gland tumors. Mucoepidermoid carcinoma accounted for 8.6% of all tumors in the present series. This figure compares with the 6.5% reported by Isacsson and Shear,[2] also in a South African population. This corroborates the suggestion by Everson and Cawson[17] that a geographic variation in the frequency of mucoepidermoid carcinoma exists.

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Evaluation of the nucleolar organizer region associated proteins in minor salivary gland tumors

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Forty-three intraoral salivary gland tumors were studied to determine the value of the AgNOR technique in the assessment of these neoplasms. Well defined black dots were visible in the nucleii of all the specimens studied. The mean AgNOR count per nucleus for each tumor was calculated as follows: pleomorphic adenoma ($n=15$) 1.52; Polymorphous low-grade adenocarcinoma ($n=12$) 1.90; adenoid cystic carcinoma ($n=6$) 2.92; mucoepidermoid carcinoma ($n=4$) 1.93; carcinoma ex mixed tumor ($n=4$) 2.05; undifferentiated carcinoma ($n=1$) 3.13 and epithelial-myoepithelial carcinoma ($n=1$) 2.23. The difference between the means of benign and malignant tumors ($P<0.01$) and polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma ($P<0.01$) were highly significant. The overlapping of the AgNOR count betwen various tumors prohibited the use of this technique as an absolute criterion in establishing a final diagnosis. It could however be used as a diagnostic aid in differentiating between salivary gland neoplasms.

Key words: nucleolar organizer regions; salivary gland tumor.

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Nucleolar organizer regions (NORs) are loops of ribosomal DNA that transcribe to ribosomal RNA and thus ultimately to protein (1). NORs have been utilized by cytogeneticists for the evaluation of certain genetic disorders, notably trisomies and are located on the short arms of the five acrosentric chromosomes 13, 14, 15, 21, and 22 (2). The NORs can be demonstrated by means of a silver staining technique (AgNORs) that is performed at room temperature on paraffin embedded tissues (3). This technique is based on the argyrophylia of the NOR-associated proteins. The known NOR associated proteins are RNA polymerase I, C23 (nucleolin), B23, 100K and 80K protein (1). Their function is uncertain although a role in rDNA transcription is postulated (1).

AgNOR counts appear to relate to cell ploidy (4) as well as the rate of cellular proliferation of individual cells (5). Quantification of NORs by means of the AgNOR technique has been used to distinguish between high and low grade lymphomas (5) and between benign and malignant counterparts of various origins (6–9).

Small biopsy specimens from salivary gland tumors is often difficult to interpret and additional microscopic criteria can only benefit the diagnostic process. MORGAN *et al.* (10) and MATSUMURA *et al.* (11) have found a statistically significant difference between the numbers of AgNORs in the nuclei of benign versus malignant salivary gland neoplasms. For this technique to have an application in diagnostic histopathology, it's ease of interpretation and reproducibility between laboratories is important. This study was undertaken to evaluate the AgNOR staining technique as a diagnostic aid for salivary gland neoplasms.

Material and methods

Forty-three intraoral salivary gland tumors were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa. Fifteen were diagnosed as pleomorphic adenomas (PA) twelve as polymorphous low-grade adenocarcinomas (PLA), six as adenoid cystic carcinomas (ACC), four carcinomas ex pleomorphic adenoma, four as mucoepidermoid carcinomas (MEC), one as an undifferentiated carcinoma and one as an epithelial-myoepithelial carcinoma. The tissue samples had all been fixed in 10% formalin and processed to paraffin wax. Two 3 µm paraffin sections of each specimen were cut. One was stained with hematoxylin-eosin and the other with the AgNOR method as described by PLOTON *et al.* (3). The H&E sections were all reassessed and revised with regards to histologic classification. The AgNOR stained sections were examined under a 100X oil immersion lens by the two authors and intranuclear dots were counted in 200 randomly selected nuclei using an eyepiece graticule to prevent recounting. Nuclei of overlapping tumor cells were not included. Nucleolar clusters were counted as a single AgNOR and no attempt was made to resolve the clusters into their discernible number of discrete dots. The mean number of AgNOR dots per nucleus was determined for each specimen. The resulting data were analyzed by means of student's t-test for uncorrelated data.

Results

The NOR associated proteins were visible as well defined black dots inside and

Table 1. Mean number of AgNORs in the nuclei of salivary gland neoplasms

Specimen	PA (Type)	PLA	ACC (Growth Pattern)	MEC (Grade)	(Ca ex PA)	Undiff ca	EPI
1	1.11 (I)	1.30	3.06 (T)	1.19 (IG)	2.37	3.13	2.23
2	1.37 (I)	1.96	2.00 (T)	1.80 (IG)	1.40		
3	1.23 (III)	1.53	3.01 (T)	2.34 (HG)	2.63		
4	1.75 (I)	1.84	4.29 (C)	2.36 (HG)	1.79		
5	1.73 (I)	1.86	2.84 (C)				
6	1.71 (I)	1.88	1.78 (C)				
7	0.98 (I)	2.02					
8	1.52 (II)	1.93					
9	1.93 (I)	2.47					
10	1.41 (I)	2.09					
11	1.60 (I)	2.24					
12	1.48 (I)	1.64					
13	1.30 (I)						
14	2.25 (I)						
15	1.37 (III)						
Mean	1.52	1.90	2.83	1.93	2.05	3.13	2.23
SD	0.32	0.31	0.89	0.55	0.55		

PA = pleomorphic ademona; PLA = polymorphous low-grade adenocarcinoma; ACC = adenoid cystic carcinoma; MEC = mucoepidermoid carcinoma; CA ex PA = carcinoma ex pleomorphic adenoma; Undiff Ca = Undifferentiated carcinoma; EPI = epithelial-myoepithelial carcinoma; T = tubular/trabecular; S = solid; C = cribriform; IG = intermediate grade; HG = high grade

outside of the nucleolus of the tumor cells studied. Careful focussing was essential to clearly identify all the dots. The results were summarized in Table 1. The lowest mean of AgNOR dots per nucleus was found in PA (Fig. 1) and the highest in an ACC (Fig. 2). The dots in the malignant neoplasms had a greater variability in size and shape compared to those in the PA. The difference in the mean number of dots per nucleus between PA and PLA (Fig. 3) and between PLA and ACC were statistically highly significant ($P < 0.01$). The correlation coefficient between the two observers was 0.97.

Discussion

The reason for the varying quantities of AgNORs in nuclei of different tumors is uncertain. The NORs are located on the 5 acrocentric chromosomes resulting in 10 NOR bearing chromosomes during metaphase. These individual NORs are usually not discernible because they are tightly aggregated in the one or two nucleoli normally present in a cell (12). Active cell proliferation may be accompanied by nucleolar dissociation, resulting in dispersed AgNORs throughout the nucleus. This as well as an increase in transcriptional activity

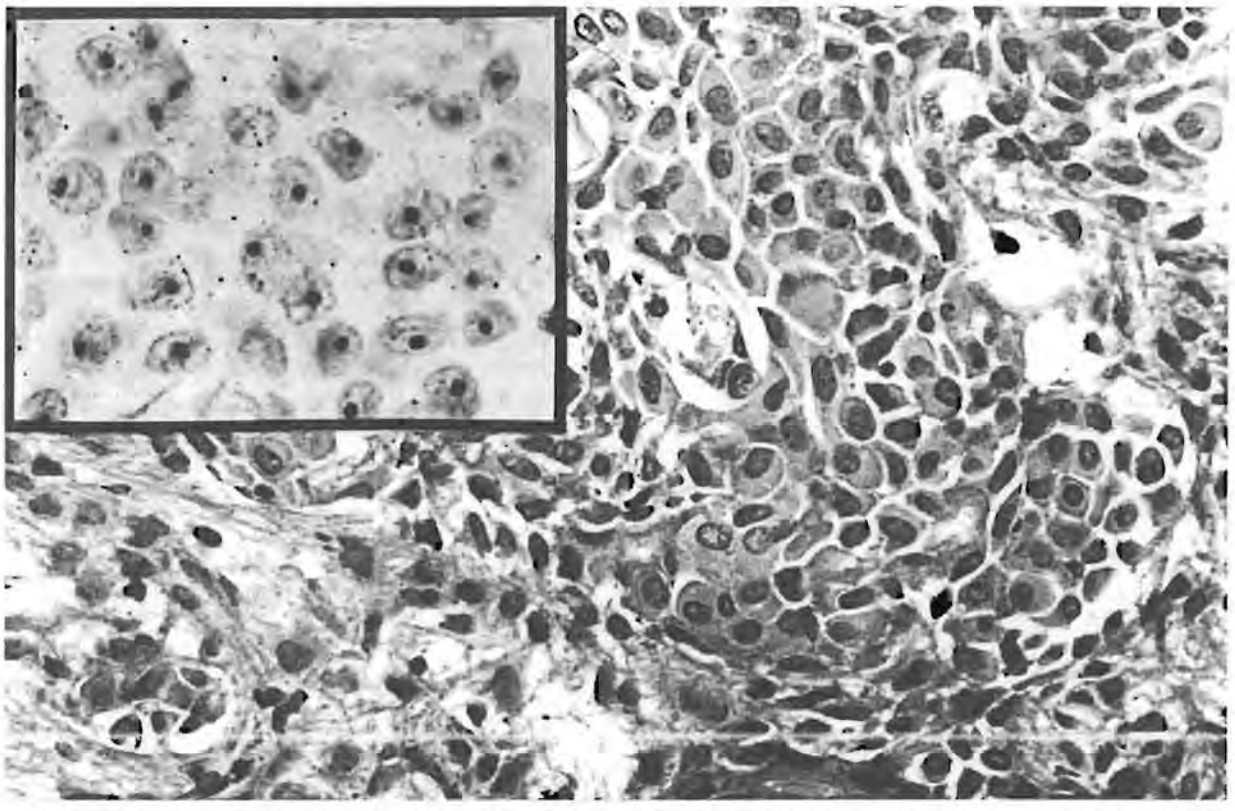

Fig. 1. Pleomorphic adenoma with plasmacytoid tumor cells. × 200. Inset: most cells contained one AgNOR dot per nucleus. × 400.

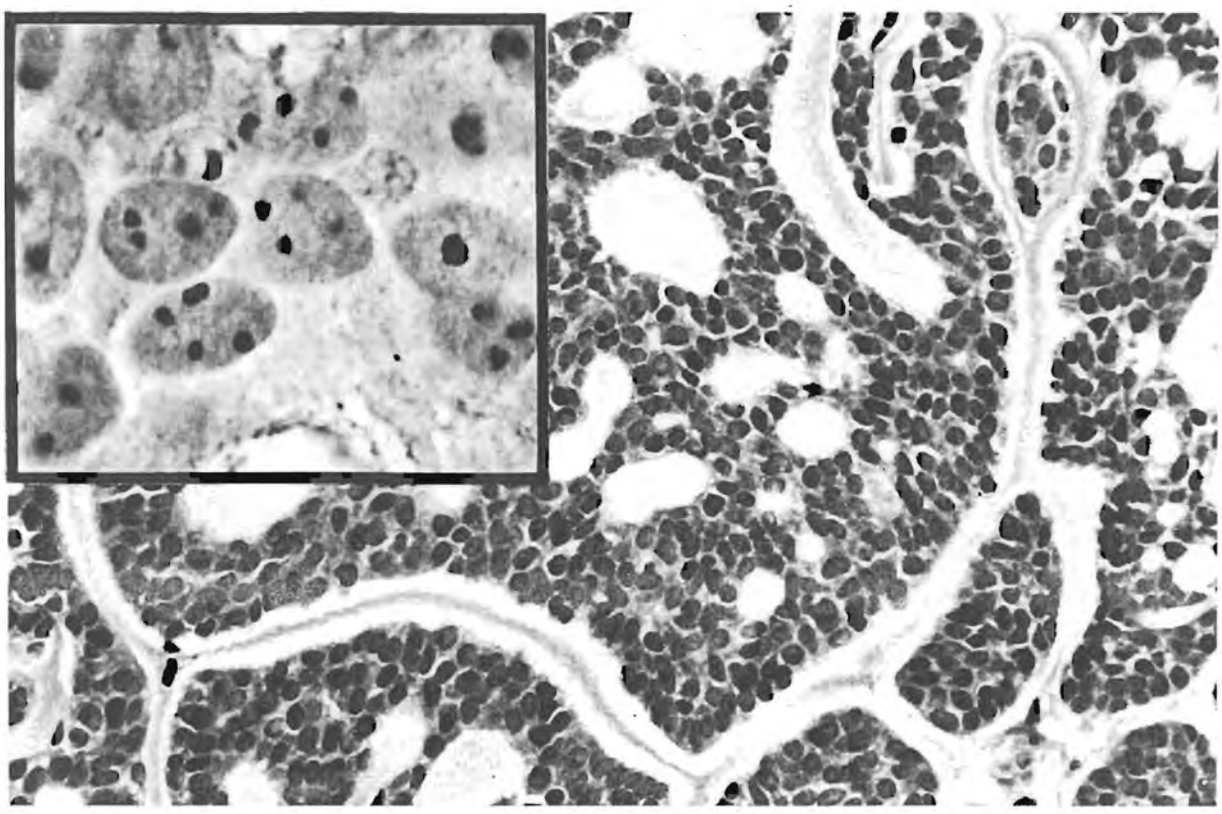

Fig. 2. Adenoid cystic carcinoma with cribriform growth pattern. × 200. Inset: multiple small AgNOR dots were present in nuclei. × 1000.

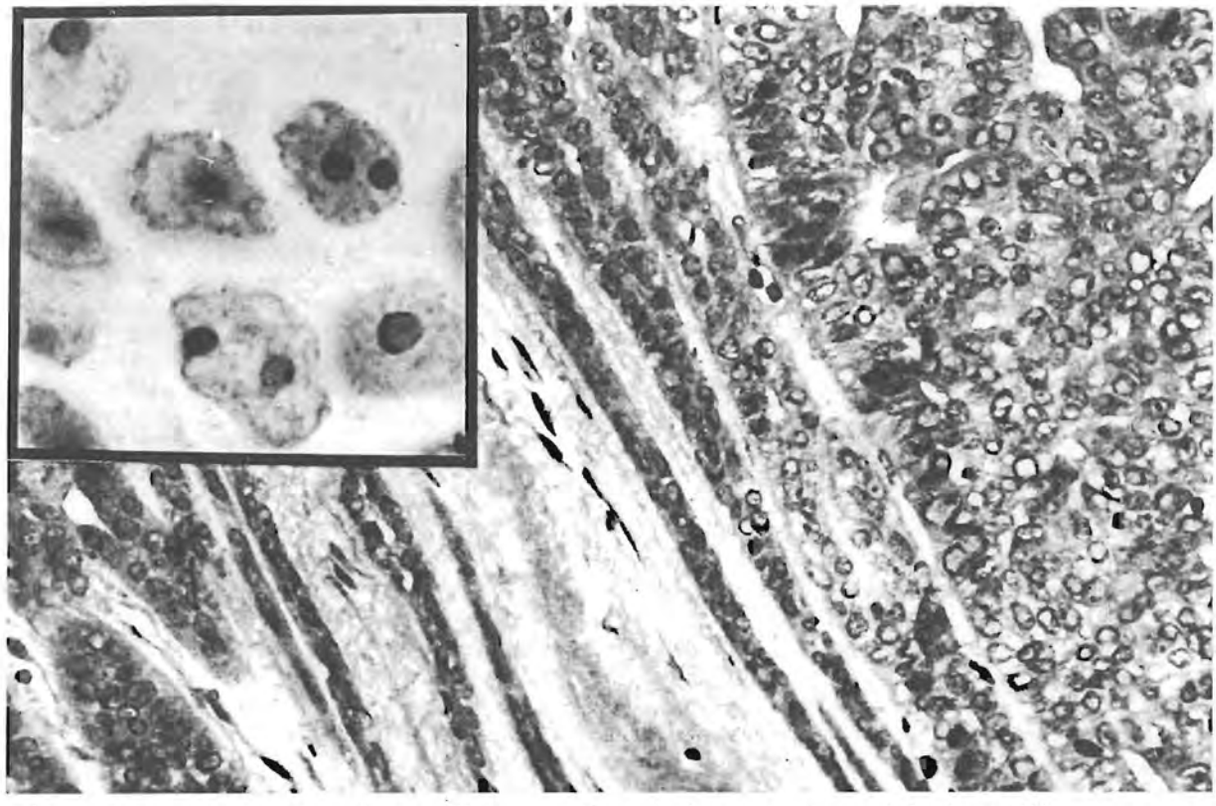

Fig. 3. Polymorphous low-grade adenocarcinoma. × 200. Inset: nuclei contained one or two large AgNOR dots. × 1000.

Table 2. Reported mean AgNOR counts in salivary gland neoplasms

Neoplasm	Present study	MORGAN *et al.*(10)	MATSUMURA *et al.* (11)
PA	1.52	1.47	1.62–1.68*
ACC	2.83	3.92	2.78
MEC	1.93	4.25	2.59

PA = pleomorphic adenoma; ACC = adenoid cystic carcinoma; MEC = mucoepidermoid carcinoma; * = different cell types in the same tumor were separately counted

will result in an increase in the mean AgNOR count of a cell population. In malignancy, the AgNORs tend to become more dispersed through the nucleus and thus more readibly discernable (12). HALL *et al.* (13) have shown that there is a significant correlation between the AgNOR count and presence of positive Ki-67 immunostaining in cells. Ki-67 is a monoclonal antibody that recognizes a nuclear antigen present only in proliferating cells (14). The possibility that the AgNOR count is related to cellular activity is also suggested by PLOTON *et al.* (3). SURESH *et al.* (4) have shown that AgNOR counts in non neoplastic trophoblastic tissue are a reflection of ploidy rather than cell proliferation. They suggested that the relationship between cell ploidy and AgNOR counts can be obscured in neoplastic lesions because of excessive proliferative activity of tumor cells.

Both ACC and PLA have an infiltrative growth pattern with an affinity for perineural spread. Cribriform, tubular and solid tumor cell arrangements can be found in ACC and PLA (15). Histologically, ACC differs from PLA in that the tumor cells have very little cytoplasm and contains hyperchromatic nucleii. Mitotic activity can be found in both tumors, although none of the PLA in our collection had a mitotic index of more than 5 mitotic figures per 10 high power fields (×400). Pleomorphism is absent in PLA whereas polymorphism is seldom seen in ACC. Despite these differences, it can be very difficult to distinguish between PLA and ACC, especially when only a small tissue fragment is submitted for histologic examination. ACC and PLA are thought to develop from the same precursor cell line (16) with the result that special staining techniques used as diagnostic procedures must be able to distinguish between cellular differentiation or activity of the two lesions. Various immunohistochemical techniques have shown potential with regards to the pathogenesis and differentiation of salivary gland tumors, although their reliability and diagnostic value is often unclear (10). A statistically significant difference between the mean AgNOR count in PLA and ACC was found in the present study. The higher count in ACC could probably be related to the more aggressive behaviour of this neoplasm when compared to PLA. The mean count in ACC did not correlate with the histologic growth pattern, a prognostic factor for tumor behavior in ACC. The highest count was present in a tumor with a predominantly cribriform growth pattern. In a study to evaluate the prognostic factors for ACC, HAMPER *et al.* (17) inter alia assessed DNA contents of the tumor cells using single cell scanning cytophotometry. They concluded that the shortest survival time was found in patients with tumors showing atypical histograms of nuclear contents of which 42% had a cribriform growth pattern. Previous studies (10, 11) evaluating the AgNOR technique in salivary gland neoplasms did not identify PLA as a separate entity, making comparison with the present study regarding PLA impossible. The overlapping of the AgNOR count between PLA and ACC prohibited the use of this technique as an absolute criterion to establish a final diagnosis but it could be used as a diagnostic aid to differentiate between these two neoplasms.

The mean value for MEC in the present study was lower than the value determined by MORGAN *et al.* (10) and MATSUMURA *et al.* (11) (Table 2). They do not specity the histologic grade of MEC included in their study. Although only four MEC's were examined in the present study, a substantial higher AgNOR count was found in the two high grade MEC. No significant difference in the AgNOR counts was found between the different types of BMT as classified using the criteria of SEIFERT *et al.* (18). This is supported by the findings of CHAU & RADDEN, (19) that there is no difference in the recurrence rate and frequency of capsular infiltration between the different subtypes of BMT.

The overlap between the AgNOR ranges in different tumors can be expected, since the absolute numbers of AgNORs in nucleii are not counted in 3 µm sections. Some AgNORs may have been missed in the 3 µm sections, especially in the malignant and high grade tumors where the nuclei were large and multiple small AgNORs were present.

The argyrophilic staining of AgNOR is not a method for demonstrating the nucleolus, but rather a technique to demonstrate its substructures in such a way as to allow study of their shape and number. Although the evaluation of AgNOR stains are time consuming, it appears to be of value in differentiating between salivary gland neoplasms.

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The relationship between Nucleolar Organiser Regions and DNA content in Salivary Gland Neoplasms

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Abstract

Thirty-three intraoral salivary gland neoplasms were evaluated to determine the proliferative index ($+G_2M$ fraction) and ploidy status and to correlate these findings with the nucleolar organiser regions (NOR) counts. Formalin fixed, paraffin-embedded tissue was used in all the cases. The mean proliferative index for each tumour was calculated as follows: pleomorphic adenoma (n = 11) 4.1; polymorphous low grade adenocarcinoma (n = 8) 6.8; adenoid cystic carcinoma (n = 3) 6.7; mucoepidermoid carcinoma (n = 4) 7.3; carcinoma ex pleomorphic adenoma (n = 3) 5.1; undifferentiated carcinoma (n = 1) 4.5 and epithelial-myoepithelial carcinoma (n = 1) 8.2. Three tumours, two adenoid cystic carcinomas and one carcinoma ex pleomorphic adenoma showed aneuploid stemlines.

Although a positive correlation between the AgNOR count and proliferative index of the salivary gland neoplasms was found, it was statistically not significant.

Introduction

Nucleolar organiser regions (NORs) are collections of nucleolar proteins associated with ribosomal genes that can be demonstrated in histologic sections using a silver staining technique (AgNOR)[1]. This technique is based on the argyrophylia of the NOR-associated proteins[2]. NORs are located on the short arms of the five acrocentric chromosomes 13, 14, 15, 21 and 22. The known NORs are RNA polymerase I, nucleolin, B23, 100K and 80K protein[1]. Their function is uncertain but a role in rDNA transcription is postulated. The quantification of AgNORs in histologic sections has been used as a diagnostic aid in distinguishing between benign and malignant tumours of various origins[3-6]

DNA content can be determined by flow cytometry by using fluorescent dyes that bind stoichiometrically to DNA[7]. The fluorescence intensity emitted by each nucleus through laser excitation is directly proportional to the DNA content of the cell[7]. The cell cycle is divided according to the amount of DNA in the nucleus at a particular time. Nuclei of cycling cells in the pre synthesis or G1 phase has a diploid or 2N amount of DNA. When the cells start to duplicate their DNA they have an intermediate amount of DNA between 2N and 4N. This phase is referred to as the synthesis phase (S-phase) and is of variable duration. After completion of the S-phase the cells enter the post synthesis phase (G2 phase) in which they have a 4N amount of DNA. The cells finally enter the mitotic phase (M-phase) and divide, whereafter they return to the G1 phase or enter a resting (G0 phase). In flow cytometry, cells in the G0 and G1 phases cannot be distinguished from each other, as they all have 2N DNA content. The same implies to cells in the G2 and M phases with a 4N DNA content (Figure 1).

The association between aneuploidy and aggressive tumour behaviour has been established for neoplasms from various sites[8-10]. The proliferation rate as defined by the S-phase fraction has also been used as a prognostic factor in adenoid cystic carcinomas of the head and neck[11].

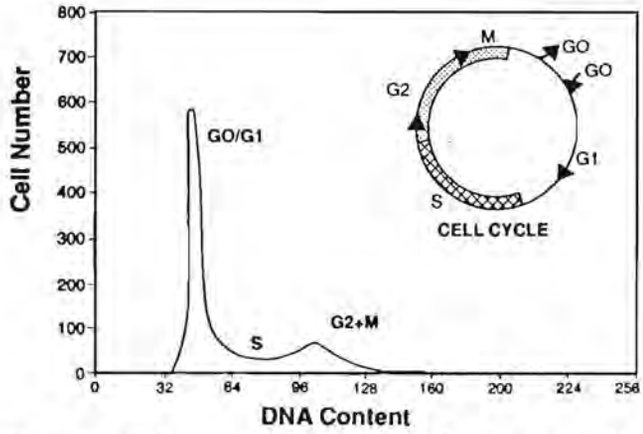

Figure 1: *Schematic representation of the relationship between DNA changes during the cell cycle and DNA histogram by flow cytometry. G0 represents the resting cells not taking part in the cell cycle.*

The reason for different quantities of AgNORs in nuclei is uncertain. The relationship between the AgNOR count and cellular activity has been demonstrated by Crocker and Nar[3]. Suresh *et al* however, have shown that AgNOR counts in non-neoplastic trophoblastic tissue are a reflection of ploidy rather than cell proliferation[12]. The purpose of this study was to determine the proliferative index and the ploidy status of minor salivary gland neoplasms and to correlate these findings with the AgNOR counts previously evaluated in the same tumours.

Materials and Method

Formalin fixed, paraffin embedded tissue from thirty-three intraoral salivary gland neoplasms, all included in a previous study where the AgNOR counts were evaluated[13] were retrieved. The tissue samples had all been fixed in 10% formalin and processed to paraffin wax. Sections were cut at 3cm thickness and dewaxed. The AgNOR solution comprised 2% gelatin in 1% formic acid that was mixed in a proportion of 1:2 volumes with 50% aqueous silver nitrate. This was immediately poured over the tissue sections and left for 30 min at room temperature. Counter staining was not performed. The AgNOR stained sections were examined under a 100x oil immersion lens and intranucleur dots were counted in 200 randomly selected nuclei using an eyepiece graticule to prevent recounting. Nuclei of overlapping tumour cells were not included. Nucleolar clusters were counted as a single AgNOR and no attempt was made to resolve the clusters into their discernible number of descrete dots. The mean number of AgNOR dots per nucleus was determined for each specimen. Eleven were diagnosed as pleomorphic adenomas (PA), eight as polymorphous low grade adenocarcinomas (PLA), five as adenoid cystic carcinomas (ACC), four as mucoepidermoid carcinomas (MEC), three as carcinoma ex pleomorphic adenoma, one as an undifferentiated carcinoma and one as an epithelial-myoepithelial carcinoma. Four 50μm sections from each

paraffin embedded block were cut and prepared for flow cytometry according to the Hedley method using a 0,5% pepsin solution[14]. The final cell suspension was passed through a 35mm mesh and the cell concentration established by means of a Coulter counter (Model FZ, Coulter Electronics, Hialeah, Fl). The cell concentration was adjusted to $\pm 2.0 \times 10^6$ cells/ml. The nuclei were stained with Propidium Iodide using a Coulter DNA Prep system, according to the manufacturers instructions. The cells were then analysed on an Epics Elite flow cytometer (Coulter Electronics, Hialeah, Fl) which had been calibrated with chicken red blood cells and DNA check beads. The Elite was operated at 15 mW and emitted an Argon ion laser at 488nm. The date rate varied between 20 – 200 events/second and 10 000 – 20 000 events were collected on a single parameter histogram. All data was collected in listmode fashion and the DNA histograms were analysed using Multicycle DNA analysis software program (Phoenix Flow Systems, San Diego, CA).

By convention, when using paraffin embedded tissue, the first peak was considered to be the normal DNA diploid peak representing the G0G1 phase of the cycle. DNA aneuploidy was reported when at least 2 separate G0/G1 peaks could be demonstrated. The coefficient of variation (CV) was calculated using the width of the peak (number of channels) at 61% of the maximum peak height divided by the peak height channel number, multiplied by a factor of 2.

The proliferative index (PI) was defined as the percentage of cells in the S + G2M phases combined. The correlation between the AgNOR count and PI were analysed using the Pearson's method while the Mann-Whitney Test was used to compare the PI between benign and malignant salivary gland neoplasms.

Results

Three tumours had aneuploid stemlines. Two were adenoid cystic carcinomas and the other was a carcinoma ex pleomorphic adenoma (**Figure 2**). The predominant growth pattern in the ACC were cribriform and tubular/trabecular respectively. Their AgNOR counts were 4.29 and 3.01 (**Figure 3**). The AgNOR count for the carcinoma ex PA was 2.37. Diploid stemlines were present in the remaining 30 neoplasms (**Figure 4**). The mean CV of the flow cytometry results were 3.96 ± 3.1 (SD). The proliferative index and AgNOR counts of the diploid tumours are summarised in **Table 1.**

A positive correlation between the mean PI and mean AgNOR counts in the various neoplasms were found. This

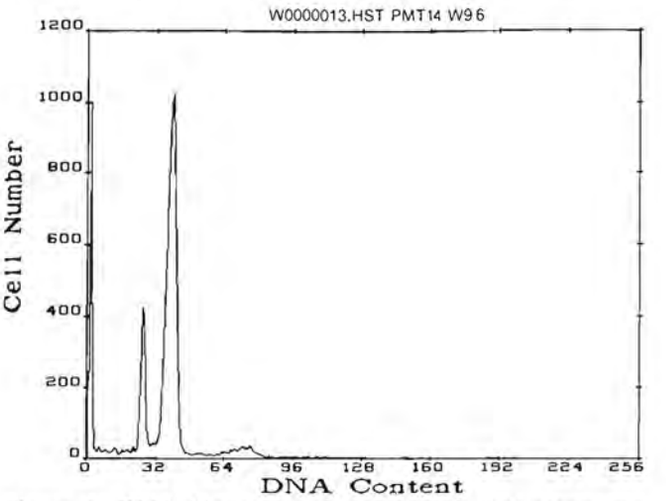

Figure 2: *DNA histogram of an adenoid cystic carcinoma showing aneuploidy.*

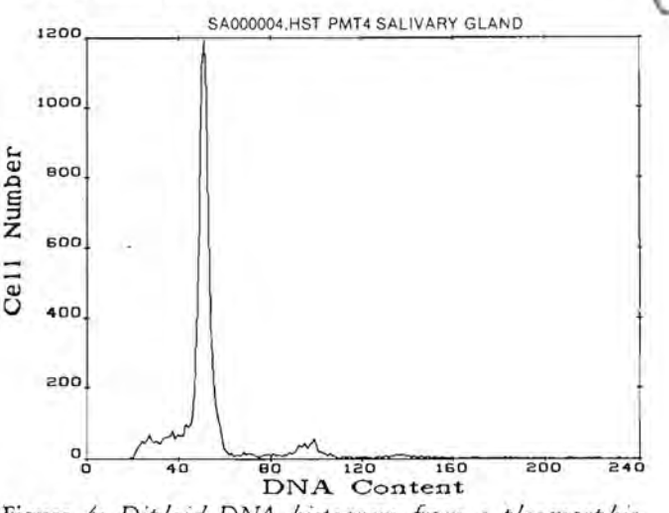

Figure 4: *Diploid DNA histogram from a pleomorphic adenoma.*

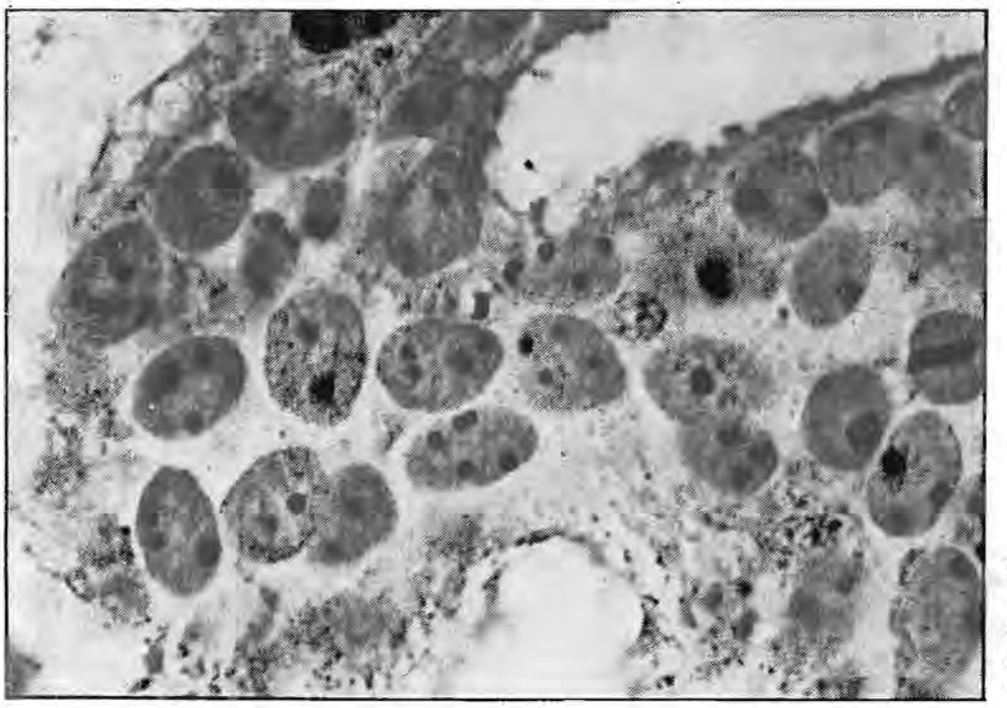

Figure 3: *AgNOR stain of a cribriform adenoid cystic carcinoma with an aneuploid DNA content. Original magnification, × 400.*

TABLE 1:
The PI and AgNOR count of the diploid salivary gland neoplasms

	PA	PLA	ACC	MEC	Ca ex PA	Undiff Ca	EPI
PI	4.1 ± 1.5	6.8 ± 5.3	6.7 ± 5.55	7.3 ± 2.3	5.1 ± 5.6	4.5	8.2
AgNOR	1.48 ± 0.3	1.85 ± 0.3	2.28 ± 0.7	1.95 ± 0.5	1.60 ± 0.3	5.13	2.23
n	11	8	3	4	2	1	1

PA = pleomorphic adenoma; PLA = polymorphous low grade adenocarcinoma;
ACC = adenoids cystic carcinoma; MEC = mucoepidermoid carcinoma;
Ca ex PA = carcinoma ex pleomorphic adenoma; Undiff Ca = undifferentiated carcinoma;
EPI = epithelial-myoepithelial carcinoma; PI = proliferative index

correlation however, was statistically not significant (P = 0.45). The difference between the mean PI of the benign salivary gland neoplasms and the diploid malignant salivary gland neoplasms was also not significant.

Discussion

The cell cycle distribution as determined by flow cytometry is usually calculated using commercially available mathematical software programs. Corrections are made to subtract background debri which intervene with the various phases of the cell cycle. Creation of debri by means of tissue preparation is a problem especially when using paraffin-embedded tissue for flow cytometric analysis. Although these corrections have shown to enhance the prognostic value of particularly the S-phase[15], it must always be borne in mind that neoplastic cells might be eliminated as debri. Expression of exact percentages of cells in the various stages of cell is therefore proliferation, a questionable practice. It is much more reliable to use the PI as a rough indicator of proliferative activity, especially when evaluating paraffin embedded tissues.

The number of aneuploid tumours (n = 3) in the present study was too small to make definite comments regarding its correlation with the AgNOR counts. It is interesting to note however, that the two AgNOR counts of the aneuploid ACC were the first and third highest count among the salivary gland neoplasms. In a study to evaluate the prognostic factors for ACC, Hamper *et al* concluded that the shortest survival time was found in patients with tumours showing aneuploid DNA contents[16]. Forty-two percent of these tumours had a predominant cribriform growth pattern. This fact correlates with our finding that the mean AgNOR count in ACC did not correspond with the histologic growth pattern, a prognostic factor for tumour behaviour in ACC. The highest count was present in a tumour with a predominant cribriform growth pattern. The same applied for the flow cytometric analysis. The two aneuploid ACC had more favourable cribriform and tubular growth patterns respectively. Luna *et al* however, found that aneuploidy is more frequently present in the solid pattern[17]. This is an indication that the growth pattern in ACC alone is not solely responsible for tumour behaviour.

The fact that only 3 tumours of the sample that included 22 malignant neoplasms were aneuploid is probably related to the phenomenon that malignant salivary gland neoplasms generally have a less aggressive behaviour compared to other malignancies.

A high AgNOR count in neoplasms may be related to an increase in cell ploidy due to a real increase in the number of chromosomes. Since the NORs are present only on the 5 acrocentric chromosomes, it may be possible that these chromosomes are not affected in a neoplastic transformation that is accompanied with hyperploidy. The NORs are usually tightly aggregated in one or two nucleoli in a cell[18]. Proliferative activity may be associated with nucleolar dissocation resulting in spreading of AgNORs through the nucleus. This, together with the transcriptional activity may result in an increase in the mean AgNOR count[18].

A variety of techniques are available to determine cellular proliferation in histological material. Visualisation of the NORs by means of a silver staining technique is frequently used. The percentage of cells in the S + G2M phases of the cell cycle can be determined with flow cytometry and immunohistochemical techniques using antibodies against proliferating cell nuclear antigen (PCNA) as well as Ki-67, a monoclonal antibody that recognises a nuclear antigen present only in proliferating cells, are some of the more advanced methods used.

This study failed to show a significant relationship between the mean PI and mean AgNOR count of the various neoplasms although they were positively correlated. Crocker *et al* found a significant linear correlation between the mean AgNOR count and S-phase fraction of high and low grade non Hodgkin's lymphomas, but not between the AgNOR count and ploidy status[19]. The difference between the mean PI of the benign and malignant tumours was not significant. This is in contrast to the highly significant difference between the same tumours when evaluating the mean AgNOR counts[13]. From this study it would appear that the AgNOR technique, which is fast and inexpensive, may be more suitable to accurately determine the proliferative activity when using paraffin embedded tissues

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A review of recent developments in the diagnosis of epithelial neoplasms of salivary gland origin

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Abstract The adoption by the World Health Organization of a revised classification for salivary gland neoplasms has introduced a new chapter in the diagnosis of these diverse growths. Universal acceptance of this proposal will contribute significantly to diagnostic uniformity. The introduction of an outline for the grading of malignant salivary gland neoplasms benefit preoperative prognostication and rationalize therapeutic regimes. The utilization of fine needle aspiration and frozen section for the establishment of a diagnosis are discouraged. Despite recent developments in histochemistry, immunohistochemistry and DNA content analyses of salivary gland neoplasms, the diagnosis still relies mainly on the growth pattern and cytologic features of a tumor. *(Eur J Lab Med 1995;1:107-112).*

Introduction

Although salivary glands share similar cellular phenotypes with sweat glands, mammary glands and the exocrine pancreas, neoplastic proliferations in the former are infinitely more complex and, from a cellular viewpoint, represent the most heterogenous group of proliferations in the human body. Despite recent developments in the understanding of the histogenesis of salivary gland neoplasms, the diagnostic process still relies mainly upon growth characteristics and cellular morphology. Special laboratory investigations like electron microscopy and cellular markers form a minor part of the diagnostic process and often only subtle microscopic differences distinguish neoplasms with diverse clinical outcomes. The subjectivity involved in the diagnosis of salivary gland neoplasms is highlighted in a recent study where 101 salivary gland neoplasms were reevaluated by a panel of senior pathologists. In a third there were minor disagreements, mostly related to subclassification, whereas major diagreements relating to benign versus malignant occurred in 7.9% of cases[1].

The purpose of this paper is to give an overview of recent developments in the diagnosis of salivary gland neoplasms.

Classification

For universal acceptance, a classification of pathologic proliferations should be based on patterns of differentiation that reflect the cell types of the parental tissue and simultaneously group neoplasms in prognostic categories. The most likable classification of salivary gland neoplasms is the morphologic working classification initially proposed by the Armed Forces Institute of Pathology[2] (Table 1) and later adopted by the World Health Organization's Committee on salivary gland tumors. Although the patterns of differentiation of salivary gland neoplasms is not addressed systematically in this classification, malignant growths are now for the first time prognostically grouped. The diagnostic refinement introduced by this new approach is clearly evident in a study which revised salivary gland neoplasms originally diagnosed according to the 1972 World Health Organizations classification[3]. In 29 cases the original diagnosis was changed and in 7 it resulted in a change from benign to malignant or vice versa[4]. Although the new approach to the classification has valuable clinical implications, it is by no means complete. Entities like the salivary gland anlage tumor[5], sialoblastoma[6,7] and hyalizing clear cell carcinoma[8] lack suffi-

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Table I. AFIP classification of primary epithelial neoplasms of salivary gland origin

Benign

- Mixed tumor (pleomorphic adenoma)
- Papillary cystadenoma lymphomatosum (Warthin's tumor)
- Oncocytoma
- Cystadenoma
- Basal cell adenoma
- Ductal papillomas
  - Sialadenoma papilliferum
  - Inverted ductal papilloma
  - Intraductal papilloma
- Myoepithelioma
- Sebaceous adenomas
  - sebaceous adenoma
  - sebaceous lymphadenoma
- Adenoma NOS

Malignant

Low-Grade

- Mucoepidermoid carcinoma (low grade)
- Acinic cell adenocarcinoma
- Polymorphous low grade adenocarcinoma
- Basal cell adenocarcinoma
- Adenocarcinoma (NOS) low grade
- Metastasizing mixed tumor

Intermediate-Grade

- Mucoepidermoid carcinoma (intermediate grade)
- Adenoid cystic carcinoma (cribriform-tubular types)
- Epithelial-myoepithelial carcinoma
- Adenocarcinoma NOS (intermediate grade)
- Clear cell carcinoma
- Cystadenocarcinoma
  - papillary
  - non papillary
- Sebaceous carcinomas
  - sebaceous carcinoma
  - sebaceous lymphadenocarcinoma
- Mucinous adenocarcinoma

High-Grade

- Mucoepidermoid carcinoma (high grade)
- Adenoid cystic carcinoma (solid)
- Malignant mixed tumor
  - carcinoma ex mixed tumor
  - carcinosarcoma
- Adenocarcinoma NOS (high grade)
- Squamous cell carcinoma
- Undifferentiated carcinoma
- Oncocytic carcinoma
- Adenosquamous carcinoma
- Salivary duct carcinoma
- Myoepithelial carcinoma

cient numbers for the establishment of behavioral patterns and may only find their way into future reappraisals of this classification.

Terminology

Arguments on the preferentiability of the designation "mixed tumor" or the term "pleomorphic adenoma" are unproductive and both are now accepted. "Adenolymphoma" as a synonym for papillary cystadenoma lymphomatosum should fall in disuse because any implication that this benign tumor is linked to lymphoma is misleading. Oxyphil cell adenoma and oncocytoma are used interchangebly. The ambiguous term "monomorphic adenoma" has fallen into disuse and all unclassifiable adenomas are now proposed to be designated as "adenoma, not otherwise specified". Although myoepithelioma is classified as a separate entity, no counter argument exists that this neoplasm is in fact an extreme differentiation on the diverse spectrum of pleomorphic adenoma. Separate categorization of myoepithelioma may however, prevent confusion with benign mesenchymal neoplasms, many of which resemble myoepitheliomas microscopically.

The term malignant mixed tumor (or malignant pleomorphic adenoma) should not be used as a specific diagnosis as it includes three different entities: carcinoma ex mixed tumor (or a carcinoma arising in a mixed tumor), carcinosarcoma (true malignant mixed tumor) and metastasizing mixed tumor. The suffix-tumor is now replaced by "carcinoma" in two neoplasms which are now known to be malignant: acinic cell carcinoma and mucoepidermoid carcinoma. As refinements in classifications proceed, the utilization of terms like "adenocarcinoma not otherwise specified" decrease. Although new clinico-pathological entities such as salivary duct carcinoma, terminal duct carcinoma and epithelial-myoepithelial carcinoma reduce the frequency by which this category is used, there still remain those adenocarcinomas which cannot be accommodated in other categories.

Frozen sections and fine needle aspirations

Frozen sections (FS) and fine needle aspirations (FNA) are increasingly accepted as cost effective and time saving techniques for the diagnosis of abnormal body masses. The cellular diversity which may be experienced within a salivary gland neoplasm decreases the potential accuracy of all techniques which suffer the disadvantage of not representing all cell types in a neoplastic proliferation. The status of invasion is one of the most important parameters in predicting the biologic behavior of salivary gland neoplasms[2]. The small sample obtained through FNA precludes the disclosure of this important parameter. Studies investigating the sensitivity and specificity of FNA frequently compare its diagnostic accuracy with histological diagnoses

based on dated classification systems, most of which do not recognize modern refinements in the diagnosis of salivary gland neoplasms. FNA appears to have a high success rate in distinguishing between benign and malignant salivary gland neoplasms[9-11]. The distinction between benign and malignant in a diagnosis on which the therapeutic approach is decided, is probably equally important to the grading of a specific malignant growth. In this respect, the limited sample obtained through FNA is often inadequate and its results cannot be compared with those obtained through incision biopsy. The cytological atypia frequently present in benign salivary gland neoplasms[9,12,13] and potential confusion with non-epithelial stromal neoplasms[11] are further pitfalls in the interpretation of FNA. Although there are unquestionable clinical indications for FNA, none merit its inclusion as part of the systematic evaluation on which the therapeutic approach is based[13].

In a series of 310 patients subjected to FS, the correct type of malignancy was diagnosed in only 51% of cases and in four patients, a false positive diagnosis of malignancy was made. The authors of this study conclude that FS is no more accurate in the evaluation of salivary gland tumors than FNA[16]. Although there are no indications against the utilization of FS for determining clear margins during excision, a primary diagnosis should not be established on FS alone.

Grading of salivary gland malignancies

This aspect of the diagnosis of malignant neoplasms is important particularly in the case of mucoepidermoid carcinomas, adenoid cystic carcinoma and adenocarcinoma which may be classified in more than one grade of malignant behavior. The microscopic criteria applied for grading are controversial and often highly subjective. Auclair, Goode and Ellis[17] proposed a point scoring system for the objective grading of muco-epidermoid carcinomas. The histopathologic features that indicate high grade behavior are an intracystic component of less than 20%, four or more mitoses per 10 high-power fields, neural invasion, necrosis and cellular anaplasia. Most differences of opinion involve the distinction between low and intermediate grades and their proposed point system may provide a basis for an objective solution. Factors that indicate a poor prognosis in adenoid cystic carcinomas encompass failure of local disease control at the initial surgical procedure, a solid pattern histologically, recurrent disease and distant metastases[2]. Despite the description of new clinicopathological entities like the salivary duct carcinoma and epithelial myoepithelial carcinoma which were formerly grouped in the adenocarcinoma "not otherwise specified" category, there still remain a group of adenocarcinomas that cannot be accommodated in conventional classifications. These malignancies are divided into low-, intermediate- and high grade categories on growth patterns and cytologic features[2]. Although histopathologic grading of acinic cell adenocarcinomas is possible, the influence of the different grades on the prognosis is debateable[18,19]. The limited malignant potential and excellent survival of patients with polymorphous low-grade adenocarcinoma is little affected by patterns of differentiation[20].

Histochemistry and immunohistochemistry

Although histochemical and immunohistochemical techniques have played an important role in investigations of the histogenesis of salivary gland neoplasms, their diagnostic applications are limited. This is mainly due to the wide spectrum of differentiation which may occur within a single salivary gland neoplasm, with each growth pattern exhibiting its own immunohistochemical characteristics[21-23]. Salivary gland neoplasms furthermore often share immunohistochemical staining characteristics with other neoplasms. Positive staining for prostate-specific antigen and prostate-specific acid phosphatase are frequently found in benign and malignant salivary neoplasms[24], a pitfall in the microscopic distinction between salivary gland carcinomas and metastatic deposits of prostatic carcinoma. Alpha 1-antitrypsin is a useful marker of basement membrane-like material[25] and can be helpful in distinguishing this product from myxoid interstitial deposits. A potential distraction to the diagnosis of myoepithelial tumors of salivary glands (i.e. myoepithelioma and myoepithelial carcinoma) is confusion with spindle cell mesenchymal proliferations. Demonstration of myoepithelial differentiation requires careful evaluation of immunohistochemical stains. The identification of S100 protein, actin and keratin either focally or diffusely, is helpful in confirming myoepithelial differentiation[26].

Microscopically, myoepitheliomas differentiate into three distinct cellular patterns: a spindle cell-, plasmacytoid- or a combination of plasmacytoid and spindle shaped cellular patterns[2]. If immunohistochemical criteria had to be applied rigorously, it is debateable whether the plasmacytoid variety, which is reported to stain negative for muscle specific actin, does represent true myoepithelial differentiation[27].

Confusion between the microscopic appearances of polymorphous low grade adenocarcinoma and benign pleomorphic adenoma may be avoided by employing stains for glial fibrillary acidic protein (GFAP). The former does not stain for this antigen

whereas its positivity is common in pleomorphic adenomas[28]. A greater diagnostic dilemma is the distinction between polymorphous low grade adenocarcinoma and adenoid cystic carcinoma. The immunochemical reactions of these two tumors are not sufficiently dissimilar to be of any practical value[29] and differences are mainly cytological and to a lesser extent morphological in nature. The presence of both sex steroids and the receptor for progesterone in adenoid cystic carcinomas[30] suggests a good possibility that some tumors in this group may respond to endocrine therapy.

Various reports propose a useful place for the counting of nucleolar organizer regions (NOR's) in order to predict the proliferative activity and prognosis of malignant salivary gland neoplasms[31-34] and distinguish between benign and malignant growths[41]. Our experience with this technique[35] as well as those of other researchers[36] were less rewarding and we believe this technique provides nothing but redundant information.

Research into the use of cellular markers to predict the behavior of salivary gland tumors is in its infancy. An association is reported between the expression of erbb2 oncoprotein and aggressiveness of malignant salivary gland tumours[37,38]. Loss of cellular differentiation appears to be linked with under expression of the c-fos oncogene[39] and evaluation of Ki-67 expression[40], immunoreactivity for PCNA[41] and c-myc, ras p21 and p53 expression[42] may become important determinants for malignant behavior.

DNA content analysis

The positive correlation between prognosis and ploidy status of malignant neoplasms is well established. Despite the presence of atypical cells in benign pleomorphic adenomas, all benign salivary gland tumours have diploid DNA contents and the malignant ones frequently display an aneuploid pattern[43,44]. A statistically significant correlation was found between DNA content and tumor size, histological grade, lymph node metastasis and lethality of 55 salivary gland carcinomas[45]. Flow cytometry was however, unable to predict the development of metastasis in cases of proven metastasizing mixed tumor[46]. DNA ploidy was shown to correlate with the prognosis of epithelial-myoepithelial carcinoma[47] myoepithelioma[48] and muco-epidermoid carcinoma[49]. The value of this technique in prognosticating adenoid cystic carcinomas is debateable[50,51] whereas no prognostic correlation could be found between DNA ploidy and the course of acinic cell adenocarcinomas[52,53]. Larger series will shed more light on the usefulness of DNA content analysis in the prediction of the behavior of salivary gland tumors.

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High-resolution DNA flow cytometry in papillary cystadenoma lymphomatosum (Warthin's tumour)

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Hemmer J, van Heerden WFP, Polackova J, Kraft K: High-resolution DNA flow cytometry in papillary cystadenoma lymphomatosum (Warthin's tumour). J Oral Pathol Med 1998; 27: 405–6. © Munksgaard, 1998.

Twenty-eight examples of papillary cystadenoma lymphomatosum (Warthin's tumour) of the parotid gland were analysed by high-resolution DNA flow cytometry. The mean coefficient of variation was found to be 1.19% (SD: 0.41). All tumours were DNA diploid. These results did not correspond with expected deviations based on published chromosomal studies. Also, the homogeneously low S-phase fractions (mean: 4.8%; SD: 2.7) found did not support the hypothesis of etiologically distinctive subgroups in these tumours.

Key words: cystadenoma lymphomatosum; cytogenetics; DNA flow cytometry; salivary gland tumours; Warthin's tumour

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Papillary cystadenoma lymphomatosum (PCL) is a benign neoplastic tumour involving mainly the parotid gland of elderly patients. The lesion is characterised by a combination of lymphoid tissue with double-layered epithelial cells consisting of outer columnar cells with an eosinophilic cytoplasm and a more cuboidal inner layer, lining the cystic spaces (1). The pathogenesis of PCL is controversial concerning the origin of both the lymphoid tissue and epithelium (1).

Cytogenetic studies on PCLs have identified three groups of epithelial cells with different karyotypic patterns: namely, one without any changes, a second group characterised by gains and losses of chromosomes, and a third showing structural chromosomal aberrations (2–4). These results suggest the existence of etiologically different subgroups of PCL. This study was undertaken to evaluate the contribution of high-resolution DNA flow cytometry to the cytogenetic analysis of PCL.

Material and methods

Fresh surgical samples of 28 PCLs were immediately processed for DNA flow cytometry. The tumour samples were homogenised in 0.9% NaCl. The nuclei were extracted by incubation in acid pepsin solution (0.5 g pepsin dissolved in 100 ml of 0.05 N HCl) at room temperature with careful stirring for 5 min. Remaining tissue fragments were removed using a 50 µm nylon mesh. The nuclei were fixed with 70% ethanol and stored at −20°C. For DNA-specific staining, the pelleted nuclei were resuspended in 0.5 ml acid pepsin solution. After 10 min incubation at room temperature, 4.5 ml of 5 µM DAPI solution (4',6-diamidino-phenylindole) containing 0.2 M trisodium citrate dihydrate was added. DNA flow cytometry was carried out using a PAS III flow cytometer equipped with a high-pressure 100 W mercury lamp (Partec; Muenster, Germany). The filter combination used was a UG 1 excitation filter, a TK 420 dichroic mirror and a GG 435 barrier filter. Human lymphocytes were added in a control measurement to confirm the DNA ploidy status of the sample cells. The cell cycle phase distribution was analysed using the MultiCycle software package (Phoenix Flow Systems, San Diego, CA, USA).

Results

Twelve patients were men and sixteen were women; their ages ranged between 34 and 89 years, with a median of 67 years. All 28 PCLs were localised unilaterally. High-resolution DNA flow cytometry showed that all cases of PCL in this study consisted exclusively of flow cytometrically diploid cells (Fig. 1). The coefficient of variation, a measure of the sensitivity of DNA measurements, varied between 0.72% and 2.14%, with a mean value of 1.31% (SD: 0.42). The S-phase fractions ranged between 0.2% and 8.7%, with a mean value of 4.8% (SD: 2.7).

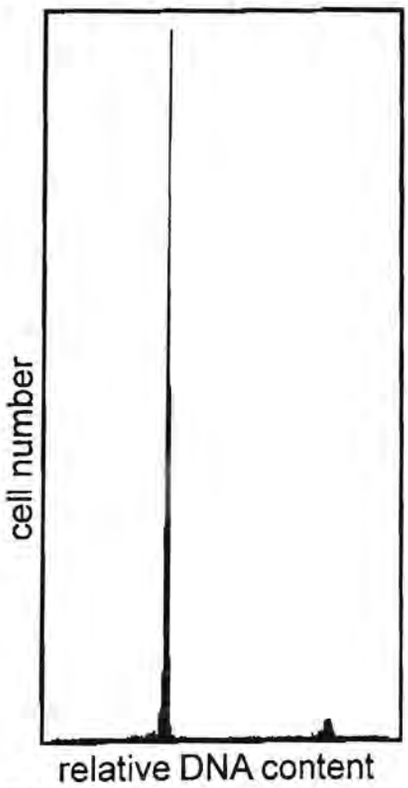

Fig. 1. Representative DNA histogram of a PCL. The diploid G1 and G0 cells are reflected by the first peak. The second peak with just double the G1–G0 DNA content contains the cells in the G2 phase and mitosis. Cells in the S phase characterised by intermediate DNA values are displayed between both peaks.

Discussion

DNA flow cytometry allows rapid and reliable identification of tumour cell lines with karyotype aberrations, provided the cytogenetic abnormalities result in a measurable change of the nuclear DNA content. A 3% difference in DNA content as reflected by the discrimination of x- and y-chromosome-bearing sperm cells is attainable with high-resolution flow cytometry (5). The sensitivity achieved through this technique in the present series of PCLs allows for the detection of tumour cell lines with karyotype aberrations resulting in a 2–4% deviation of the clonal DNA content from the normal diploid DNA value. Thus, even the gain or loss of a single chromosome 5, as was recently reported for a number of PCLs (2, 4), should be detectable by high-resolution flow cytometry.

No evidence of aneuploid tumour cell populations that correspond to expected deviations based on published chromosomal studies was found in the present series. This result agrees with two other studies on five and four PCLs, respectively (6, 7). DNA content heterogeneity could not be demonstrated by flow cytometry of multiple samples collected from 4 PCLs (7). Reports on numerical chromosome changes in PCL may therefore reflect the well-known problem of karyotypic changes due to cell culture artefacts rather than the existence of gross karyotype aberrations in PCL *in vivo* (8).

Even if translocations have successfully been identified by high-resolution DNA flow cytometry (9), structural chromosome rearrangements that have been reported in a significant number of PCLs (2–4, 10) remain unproved in the present flow cytometric study. Although highly specific rearrangements involving a few chromosomal regions have been reported in pleomorphic adenomas (3, 11), the limited cytogenetic data available on PCLs is not supportive enough for such events in these tumours. However, a homogeneously low proliferative activity as assessed in the present series, reduced p53 protein expression reported for five cases (12), and lack of c-erbB-2 oncoprotein expression in 31 other tumours (13) do not support the hypothesis of the existence of cytogenetically distinctive subgroups of PCL.

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Warthin's Tumour is not an Epstein-Barr Virus Related Disease

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Abstract. *Background: The histogenesis of Warthin's tumour (WT) is controversial. A possible role for Epstein-Barr virus (EBV) has been suggested. Materials and Methods: Twenty formaldehyde-fixed, paraffin-embedded blocks of WT from the parotid gland were examined for the presence of EBV. In situ hybridisation was performed using EBV encoded small nuclear RNAs (EBER1/2) probes labelled with fluorescein isothiocyanate. An EBV-positive P3HR-1 cell line processed to paraffin wax was used as a positive control and a brain section as negative control. Results: EBER1/2 could not be found in the neoplastic epithelial cells in any of the tumours nor in the adjacent normal parotid tissues. Individual positive lymphocytes were present in 7 tumours. Conclusions: These results indicated that EBV is not involved in the pathogenesis of WT.*

Warthin's tumour (WT) is a benign epithelial neoplasm of major salivary glands with a characteristically male preponderance. WT's are almost exclusively located in the parotid glands and may present as single, bilateral or multiple lesions (1). Histologically, WT consists of a double layer oncocytic epithelium with papillary structures lining cystic spaces, associated with a lymphoid stroma. The ratio of lymphoid stroma to epithelium varies widely and has been used by Seifert *et al* (2) to classify these tumours into subtypes.

The histogenesis of WT is controversial. The heterotopic theory suggests that the epithelial component of these tumours represents entrapped salivary gland epithelium in salivary gland associated lymph nodes (3). According to the immune theory, the lymphoid cells represent a secondary lymphocytic response to epithelial changes or stimuli (4). Epstein-Barr virus (EBV) has been implicated in the pathogenesis of WT, especially the lymphoid component (5). It has been suggested that release of EBV gene products or cytokines, particularly interferon-gamma, by infected cells, may activate lymphoid tissue to result in a polyclonal B-cell response (6).

Epstein Barr virus (EBV) is a double stranded DNA virus. It causes widespread infection and was found to be the aetiologic agent of infectious mononucleosis (7), endemic Burkitt's lymphoma (8), undifferentiated nasopharyngeal carcinoma (9) and EBV-induced disorders in immunodeficient patients (10). An association of other epithelial tumours with EBV has recently been suggested on the basis of molecular biological techniques. The presence of EBV DNA in tonsilar carcinomas (11), gastric carcinomas (12) epithelial thymic carcinomas (13) and undifferentiated salivary gland carcinomas (14) has been reported.

In the present study, biopsy specimens of 20 patients with solitary WT were screened for the presence of EBV using RNA *in situ* hybridisation (ISH).

Materials and Methods

Twenty cases diagnosed as WT were retrieved from the files of the Department of Pathology, Military Hospital of Ulm, Germany. Sections were cut from these formaldehyde-fixed, paraffin-embedded blocks and reviewed to subclassify the tumours in the different groups defined by Seifert *et al* (2): predominanty epithelial (< 30% lymphoid component); predominantly lymphoid (> 70% lymphoid component); typical (50% lymphoid component) and metaplastic (extensive squamous metaplasia).

To detect expression of the EBV-encoded small nuclear RNAs (EBER-1 and EBER-2), ISH with fluorescein-conjugated oligonucleotide probes was used. The probes were obtained commercially and consisted of a mixture of both EBER-1 and EBER-2 (Novocostra, Newcastle upon Tyne, UK). Probes were labelled with fluorescein isothiocyanate (FITC). Detection of hybridised probe was done with rabbit F(ab') anti-FITC conjugated to alkaline phosphatase. All glassware was treated with DEPC (di-

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ethyl pyrocarbonate) to prevent RNAase activity. The ISH was done using the OmniSlide System (Hybaid, Teddington, Middlesex, United Kingdom).

Formaldehyde-fixed tissue sections were cut 4μm thick and twice dewaxed in xylene for 3 min. The sections were dehydrated in 99% alcohol for 2 min followed by dehydration in 95% alcohol for 2 min whereafter they were twice immersed in pure water for 3 min and air dried. Slides were then placed on an incubation tray and covered with Proteinase K (Dako Corporation, Glostrup, Denmark). Eighty ml Proteinase K was diluted with 2ml 0.05M Tris-HCl pH 7.5 to 7.7 and digestion was performed for 6min at room temperature. Sections were washed in distilled H_2O, followed by DEPC treated water and then dehydrated in graded alcohols. The sections were put in the Omnislide slide rack and air-dried. Twenty μl of probe hybridisation solution was added to the sections on the slides and the sections covered with coverslips. The slides were then incubated at 37°C for two hours. The coverslips were removed in a 0.1% Triton X-100 solution and the slides were then immersed in post hybridisation solution for 10 min. The post hybridisation solution was tipped off and sections incubated with sufficient anti-FITC AP detection solution for 30 min at room temperature in the dark.

Slides were then washed twice in TBS buffer for 3 min each and followed by a 5min wash in alkaline phosphate substrate buffer. The slides were again placed in an incubation tray and the alkaline phosphates activity was demonstrated by covering the sections with 100ml of substrate solution. The sections were coverslipped and incubated overnight at room temperature in the dark. Slides were then washed in running tap water for 5 min, counterstained with Mayer's heamatoxylin for 1 min, blued in tap water for 5 min and mounted in an aqueous mountant (Dako Corporation, Glostrup, Denmark)

Sections of an EBV-positive P3HR-1 cell line processed to paraffin wax were used as positive control. A brain section served as negative control. Positive signals were regarded as dark brown to black staining concentrated in the nuclear area of the tumour cells with nucleolar sparing.

Results

All the tumours were in the parotid gland. Eighteen patients were male and the mean age of all the patients were 61.2 years (± 11.2). Based on the classification of Seifert *et al* (2), there were 11 cases (55%) classified as typical, 5 (25%) as predominantly epithelial and 4 (20%) as predominantly lymphoid.

The EBV-positive P3HR-1 cell line that was used as positive control stained intensely positive in all cases. Brain blocks used as a negative control demonstrated no hybridisation signal. EBER1/2 could not be found in the neoplastic epithelial cells in any of the tumours. Adjacent normal parotid tissues were also negative in all the cases. Individual positive lymphocytes were present in 7 tumours. No background staining could be seen on any slide.

Discussion

EBV DNA sequences have been detected by Santucci and co-workers in almost all epithelial tumour cells in 13/15 (86.7%) multiple or bilateral and in 1/6 (16.7%) solitary WT's (15). The same detection methods were used by Wolvius *et al* (16) who found similar signals, but suggested that the positive cytoplasmic staining may be the result of non-specific binding of the labelled probe, because by using irrelevant probes, similar staining results were found. Similar to the present study, Wolvius *et al* (16) were also unable to demonstrate any positive EBER-1/2 epithelial cells in their 10 cases of WT studied. Ogata *et al* (17), likewise, found neoplastic epithelial cells of WT negative for EBER1 probes using ISH. Similar to the present study, isolated stromal lymphocytes were positive in some of their tumours.

EBV DNA has been demonstrated in both the epithelial and lymphoid component of WT. Demonstration of EBV DNA on its own is, however, not sufficient to distinguish between latent and lytic viral infection. Demonstration of EBV DNA in WT using polymerase chain reaction (PCR) cannot be used as an indication of viral infection because circulating, EBV-carrying lymphocytes are found in non-neoplastic lymphoid tissues (18). Furthermore, the source of viral DNA detected with PCR cannot be determined unequivocally, because DNA extracts from heterogenous cell populations are analysed.

In situ hybridisation is the method of choice to evaluate EBV involvement in WT, because the distribution and nature of positive cells can be determined. This is important, especially in tumours consisting of different cell types.

EBERs are the most abundantly expressed viral transcripts found in EBV infected cells and may be present at 10^7 copies per cell (19). Despite their abundance, they do not code for protein and their function is unknown, although it has been proposed that they may be active during lytic replication. EBERs are expressed early after infection and reach substantial levels 70 hr after infection. (20). EBER transcripts are appropriate targets for ISH. Firstly, they exist as ribonucleoproteins complexed with the cellular protein La with extensive intramolecular base pairing and stable secondary structure (21, 22). As such, they may be more resistant to nuclease degradation than other transcripts and thus may especially useful in the investigation of routinely prepared formalin-fixed paraffin-embedded clinical specimens (23). Secondly, their abundance in latently infected cells suggests that their high copy number might compensate for any loss of sensitivity because of their small size.

Based on morphometric analysis evaluating the lymphoid component and cystic spaces together with the clinicopathologic data, Aguirre and co-workers (24) suggested a model of progression based on the subtypes of WT used by Seifert *et al* (2). According to their model, most cases of WT originate as a consequence of an unknown stimulus within the parotid lymph nodes resulting in a predominantly epithelial component as initial presentation. They further suggest that growth of

WT's occur because of lymphoid proliferation with subsequent development of the classical subtype to the predominantly lymphoid subtype. Tobacco or viruses have been suggested as potential initial stimuli (24).

Although this study did not prove any theory regarding the histogenesis of WT to be correct, it has demonstrated that EBV is not involved in the pathogenenesis of WT in the population sample studied. Other authors using the same methodology reached similar conclusions (16, 17).

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Intraoral Salivary Duct Carcinoma: A Report of 5 Cases

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Salivary duct carcinoma (SDC) is a high-grade malignant epithelial tumor of salivary glands first described by Kleinsasser et al.[1] It has also been termed cribriform salivary carcinoma of excretory ducts,[2] infiltrating salivary duct carcinoma,[3] and intraductal carcinoma.[4] SDC has a poor prognosis,[2,5,6] although patients with prolonged disease-free survival have been reported,[2,5] Low-grade variants of SDC have also been described.[7,8]

The peak incidence of SDCs is in the sixth and seventh decades of life, and it has a male predominance.[6,9] This neoplasm has a striking resemblance to ductal breast carcinoma and is characterized by the presence of intraductal, circumscribed tumor islands with a papillary, cribriform, or solid growth pattern associated with an infiltrative component. Comedonecrosis is frequently present.

SDCs occur almost exclusively in the major salivary glands with the parotid gland predominantly affected.[10] Only isolated cases involving minor salivary glands have been reported.[1,8,11-18] In this study, we report the clinicopathologic and immunohistochemical features of 5 cases of intraoral SDC. The DNA ploidy status of these tumors was also studied.

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Materials and Methods

Malignant intraoral salivary gland tumors diagnosed at the Departments of Oral Pathology at the University of Pretoria and Medical University of Southern Africa were reviewed. Four cases of SDC were included while an additional case was reclassified as an SDC according to the criteria of the World Health Organization.[19] Clinical and follow-up information was obtained from the patients' files and supplemented by communication with the referring practitioners or clinics and immediate family members. All the specimens were fixed in 10% buffered formalin, and the histologic features were evaluated by reviewing all the sections stained with hematoxylin and eosin. Additional slides of paraffin blocks were prepared for immunohistochemical analysis using the standard avidin-biotin peroxidase method. A panel of commercially available antibodies with appropriate controls was used (Table 1). The extent of immunohistochemical staining was evaluated and scored as + (1% to 9% of tumor cells), ++ (10% to 50% of tumor cells), and +++ (>50% of tumor cells). Staining intensity was not evaluated.

Flow cytometry was performed on 50 μm sections of the formalin-fixed paraffin-embedded tumor blocks. Tissue was processed according to the modified method described by Heiden et al.[20] The sections were enfolded with 50-μm nylon mesh and deparaffinized in xylene, hydrated in graded alcohols, and digested with Carlsberg solution. The nuclei were stained with DAPI solution (4′,6-diamidino-phenylindole) containing 0.2 M trisodium citrate dihydrate, and at least 10,000 events from each case were analyzed using a PAS III flow cytometer equipped with a high-pressure 100-W mercury lamp (Partec, Münster, Germany).

Results

The patients ranged in age from 47 to 71 years (mean age, 58.2 years). Two were female and 3 were male. All of the tumors were located in the palate and ranged in size from 5 to 14 cm. The tumors presented

Table 1. SPECIFICATIONS OF ANTIBODIES USED

Antibody	Source	Dilution	Antigen Retrieval	Detection
High-molecular-weight cytokeratin	DAKO (34BE12)	Prediluted	*	DAKO LSAB2
α-Smooth muscle actin	DAKO (1A4)	Prediluted	None	DAKO LSAB2
Vimentin	DAKO (V9)	Prediluted	None	DAKO LSAB2
Anti-S-100A	DAKO	Prediluted	*	DAKO LSAB2

*Microwave pressure cooker in citric buffer, pH 6.0.

as painful masses (Figs 1 through 3). No association with the parotid gland could be shown in any case using computed tomography investigation (Fig 4). The possibility of metastasis from an intraductal breast carcinoma was also excluded. Clinical evidence of regional lymph node metastasis was present in 2 cases. Three patients refused any form of treatment and were subsequently lost to follow-up. No information could be obtained from the referring clinics. One patient was treated with radical resection and is currently receiving postoperative radiotherapy. The clinicopathologic findings are summarized in Table 2.

Microscopically, all tumors consisted of an intraductal component with a predominantly cribriform pattern and central comedonecrosis (Fig 5). Infiltrating tumor islands, in a trabecular and cribriform pattern, in a stroma that varied from cellular to regions of hyalinization were also present in all 4 cases (Fig 6). The tumor cells had well-defined cell borders with eosinophilic cytoplasm and vesicular nuclei. Mitotic activity varied from moderate to high (Fig 7). The immunohistochemical results are shown in Table 3.

Flow cytometry analysis showed 4 tumors to be aneuploid (Fig 8) and one diploid (case 2) (Fig 9). The coefficient of variance (CV) of all the measurements was less than 3%.

Discussion

The possibility of metastases should be eliminated before a final diagnosis of SDC is made. Metastatic ductal carcinoma from the breast could be excluded with careful clinical examination and mammography. The histologic features of these tumors are very similar, although the presence of estrogen receptor protein and absence of carcinoembryonic antigen in breast carcinomas have been used to differentiate between SDC and ductal carcinoma of the breast.[21] Metastatic prostatic carcinoma can in the majority of cases be excluded by the absence of both prostate-specific antigen and prostate-specific acid phosphatase in the tumor cells.

The histologic differential diagnosis of SDC includes high-grade mucoepidermoid carcinoma, undifferentiated carcinoma, adenocarcinoma (not otherwise specified), dedifferentiated acinic cell carcinoma, and adenoid cystic carcinoma. High-grade mucoepidermoid carcinomas have epidermoid and intermediate basaloid cells as well as cells with mucicarmine demonstrable intracellular mucin, whereas only luminal mucin is found in SDC. Cribriform and papillary-cystic growth patterns are not found in mucoepidermoid carcinomas. Undifferentiated carcinoma lacks the eosinophilic cytoplasm of SDC and does not form glandular structures. Adenocarcinoma,

FIGURE 1. Introoral view of case 1 showing a massive tumor destroying the right maxilla, extending across the midline.

FIGURE 2. A tumor located in the left maxilla with buccal and palatal expansion from case 2.

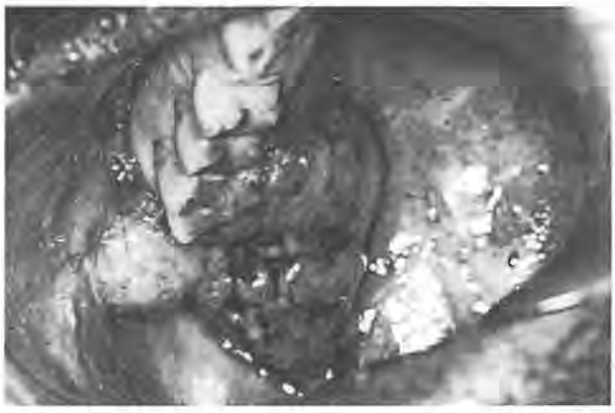

FIGURE 3. Case 3 presenting with an ulceroted tumor in the right palate involving the alveolar ridge.

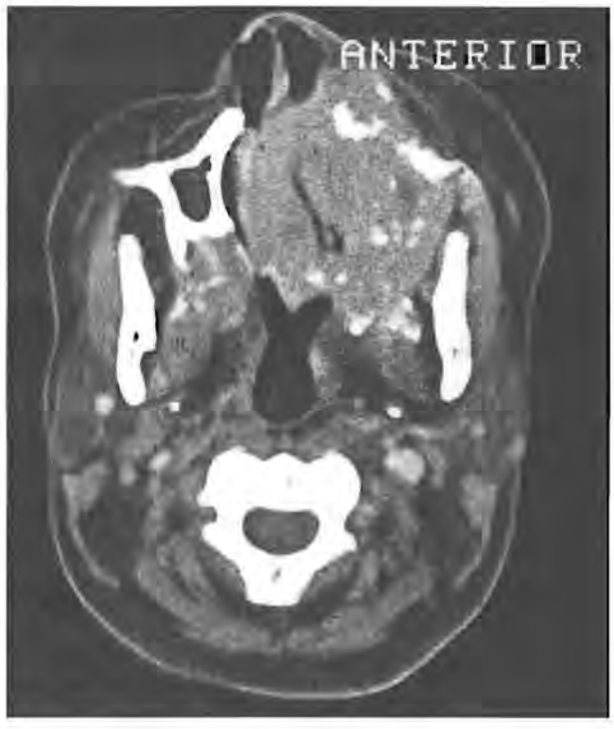

FIGURE 4. Computed tomography scan of patient described in case 2 showing a tumor in the left maxilla and palate with no parotid involvement.

not otherwise specified, shows glandular and ductal differentiation but lacks the distinctive features of SDC and is basically a diagnosis of exclusion.[10] Dedifferentiated acinic cell carcinoma may present as a poorly differentiated adenocarcinoma or undifferentiated adenocarcinoma but always in association with a usual-type low-grade acinic cell carcinoma.[22] Adenoid cystic carcinoma cells usually contain little cytoplasm and have angulated, basophilic nuclei. Comedonecrosis is also not a feature of adenoid cystic carcinoma.

The diagnosis of primary intraoral SDC necessitates exclusion of direct spread from one of the major salivary glands, especially the parotid, where most SDC arise. Computed tomography scans and other imaging techniques should not show any association with the parotid or any other major salivary gland. This is especially true when SDC of the cheek is diagnosed, which is not a common site for minor salivary gland tumors.[23]

Table 2. CLINICOPATHOGIC FEATURES OF 5 PATIENTS WITH INTRAORAL SDCs

Patient	Age (yr)	Gender	Site	Clinical Presentation	Tumor Size (cm)	Treatment	Follow-Up
1	53	F	Right palate and right alveolar ridge	Fungating mass; difficulty in breathing and eating; lymphadenopathy	±14	Biopsy	Patient refused treatment, lost to follow-up
2	71	M	Left palate and buccal sulcus	Pain, nerve fallout of II, III, V_b, and VII; lymphadenopathy	±5	Biopsy	Patient refused treatment, lost to follow-up
3	57	M	Right palate	Ulcerated tumor	±6	Biopsy	Patient refused treatment, lost to follow-up
4	63	F	Left palate	Painful, ulcerated tumor	±7	Maxillectomy, left neck dissection, postoperative radiotherapy	No recurrences after 10 months
5	47	M	Left palate	Fungating, nonulcerated tumor	±5	Biopsy	Patient still considering surgical treatment

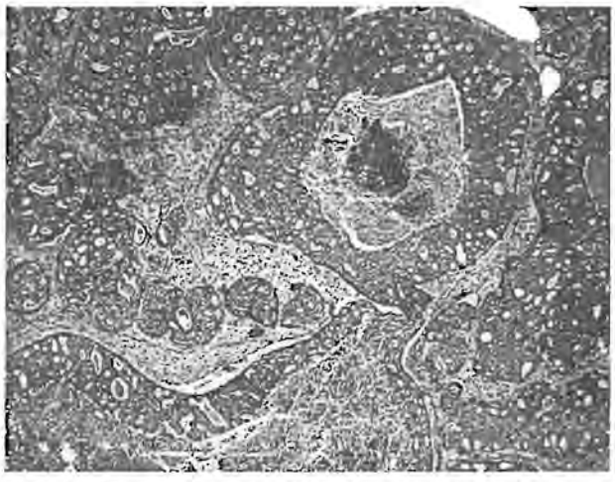

FIGURE 5. Photomicrograph of an SDC showing introductal growth pattern with a cribriform appearance and comedonecrosis in the larger tumor islands (hematoxylin-eosin stain, original magnification × 25)

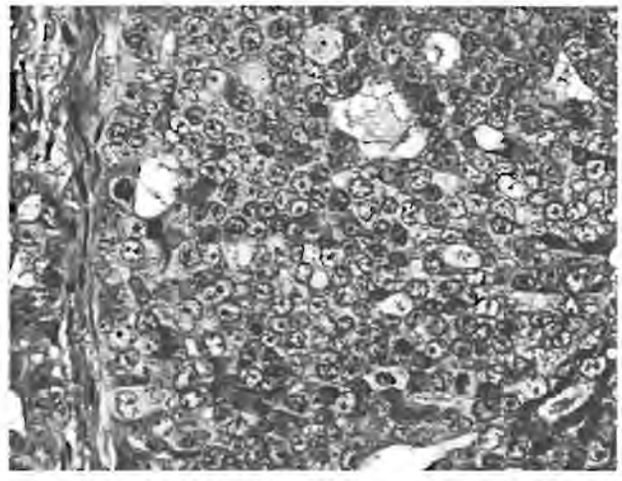

FIGURE 7. The tumor cells had vesicular nuclei with prominent nucleoli. Mitotic figures were prominent (hematoxylin-eosin stain, original magnification ×200).

Carcinoma ex pleomorphic adenoma is not uncommon in minor salivary glands[23] and SDC have been reported as the malignant component of these malignancies.[6,15,24] None of our cases had any histologic evidence of a preexisting pleomorphic adenoma, nor did a longstanding history suggest such an association. SDC has also been reported as a hybrid carcinoma of the minor salivary glands combined with an adenoid cystic carcinoma[25] and Warthin's tumor.[13]

SDC of the major salivary glands is an aggressive, high-grade malignancy. Comparison of the behavioral quality of SDC originating from minor salivary glands with that of the major glands is difficult, as only isolated cases of SDC have been reported. The clinical characteristics of our cases are similar to other studies reported for SDC of the major glands in that predominantly older male patients were involved. It was not possible to determine the clinical behavior of our 5 cases due to lack of follow-up information. However, the clinical appearance (large size and ulceration) of these tumors together with detectable peripheral neuropathy (ie, paresthesia, paralysis) and presence of fixed lymph nodes suggesting metastatic spread was supportive of an aggressive clinical behavior. The size of primary SDC was found to correlate with malignant potential. Hui et al[26] reported that tumors smaller than 3 cm correlate with a lower malignant potential, whereas Delgado et al[15] found a similar correlation with tumors smaller than 2 cm.

Immunohistochemical evaluation of some intermediate filaments in the tumor cells indicated that SDC is composed of predominantly ductal cells with little or no myoepithelial cell involvement. The strong expression of cytokeratin in the tumor cells is supported by the majority of studies on SDCs.[2,6,15,27] The tumor cells were negative for vimentin and smooth muscle actin, whereas positive staining with S-100 antibody was found in less than 10% of tumor cells in all 5 cases. Diffuse positive staining of S-100 was found by Brandwein et al[2] in 7 of 9 cases as well as in a single case of SDC reported in the palate.[25] Most studies, however, reported no immunoreactivity with S-100

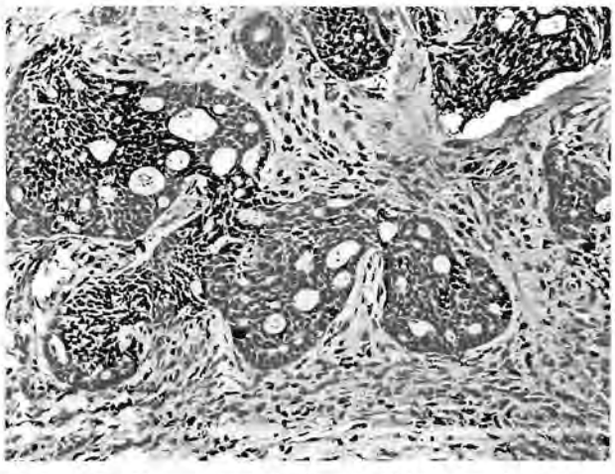

FIGURE 6. Infiltrating tumor islands in a cellular stroma (hematoxylin-eosin stain, original magnification ×100).

Table 3. IMMUNOHISTOCHEMICAL FINDINGS IN THE 5 INTRAORAL SDCs

Antibody	Patient 1	2	3	4
Keratin	+++	++	+++	++
α-Smooth muscle actin	−	−	−	−
Vimentin	−	−	−	−
S100	+	+	+	+

NOTE. −, Negative; +, 1% to 9% of tumor cells; ++, 10% to 50% of tumor cells; +++, more than 50% of tumor cells.

antibody.[15,17,27] The isolated (1% to 10%) positive staining of S-100 in our 4 cases may be due to the presence of Langerhans cells between the tumor cells, although tumor cells with a ductal differentiation may express S-100 protein.[28] Ultrastructurally, SDCs are composed of cuboidal to polygonal cells with interdigitations and cells forming ductlike structures with microvilli and apical vesicles; myoepithelial cells are absent. These findings support the ductal origin of SDC.[29]

Four of the SDCs in the present study displayed DNA aneuploidy. Several studies have measured the DNA content of SDC using flow cytometry with varied results. The majority found no correlation between the ploidy status and prognosis,[6,24,30] whereas Martinez-Barba et al[27] found a positive correlation between aneuploidy and the presence of distant metastases and fatal clinical outcome. Nuclear suspensions for flow cytometry analyses were obtained from paraffin-embedded sections in all of the above-mentioned studies, but none of these studies mentioned the CV obtained for the flow cytometry measurements. The CV of DNA measurements using paraffin-embedded tissue will invariably be higher than when using fresh tissue from the same tumor. It is possible that the reported diploid cases were in fact false diploid as tumor cells with a near diploid peak, implying small deviations of their DNA content from normal diploid cells, could not be distinguished due to the relatively high CV.

SDC is a distinct tumor that can originate from minor salivary glands. The histologic features are similar to those of tumors originating in the major salivary glands. The palate appeared to be the most common intraoral site for SDC. Although clinical features were suggestive of an aggressive behavior, more reported cases are required to determine the behavior in SDCs of minor salivary glands.

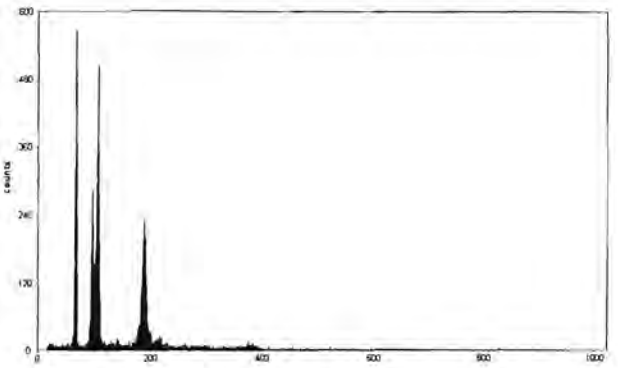

FIGURE 8. DNA histogram of case 1 showing the normal diploid peak at channel 100. Hypodiploid and hyperdiploid tumor cells were present.

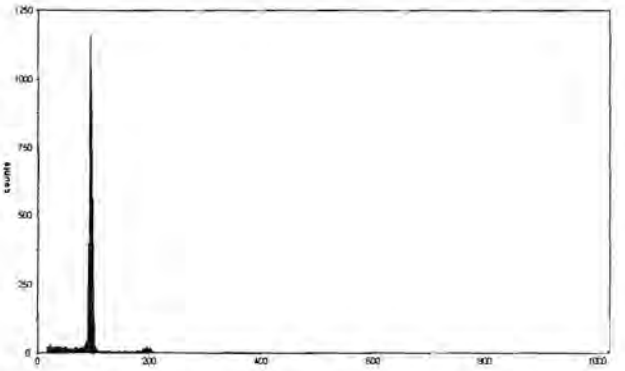

FIGURE 9. Diploid DNA histogram with diploid cells at channel 100 and the small peak at channel 200 representing the cells at G_2M phase.

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Tyrosine-rich crystalloids in a polymorphous low-grade adenocarcinoma

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A polymorphous low-grade adenocarcinoma with tyrosine-rich crystalloid deposits is reported. The literature is reviewed, and diagnostic and histogenetic implications of this finding are discussed.
(ORAL SURG ORAL MED ORAL PATHOL 1990;70:480-2)

Tyrosine-rich crystalloids occur mainly in salivary gland mixed tumors where they are reported in between 1.5% and 21% of cases.[1-4] The incidence of these deposits appears higher in mixed tumors involving black patients than white patients.[2,5] Salivary gland carcinomas that have been reported to contain tyrosine-rich crystalloids include one terminal duct adenocarcinoma,[6] an adenoid cystic carcinoma,[7] and a malignant mixed tumor.[8]

The origin of tyrosine-rich crystalloids in salivary gland neoplasms is speculative. The principal tumor cell associated with these deposits is the modified neoplastic myoepithelial cell,[6] which is also believed to be the source of the stromal matrix deposits in mixed tumors.[9]

CASE REPORT

A 36-year-old black female patient had a 3 × 2 cm, firm midline swelling at the junction of the hard and soft palate. No ulceration was present. A clinical diagnosis of benign mixed tumor was made and an incisional biopsy taken. Although tyrosine crystals were observed, perineural invasion prompted a provisional diagnosis of a polymorphous low-grade adenocarcinoma and wide excision was recommended. Microscopic examination of the surgical specimen showed an infiltrative neoplastic growth with a lobular architecture. Solid masses of epithelial cells, areas exhibiting ductlike differentiation, and cells arranged in long, single-layered strands were observed. The neoplasm was further characterized by a low mitotic activity, histologic diversity with cylindric, clear cell, and mucus cell differentiation, and a lack of pleomorphism. Evidence of perineural invasion was present (Fig. 1). Extensive crystalloid deposits were present in the connective tissue stroma and between the cells in the solid epithelial masses (Fig. 2). These crystals showed distinct brown staining with the Millon reaction and were nonbirefringent under polarized light. A diagnosis of polymorphous low-grade adenocarcinoma of minor salivary gland origin with tyrosine-rich crystalloid deposits was made.

DISCUSSION

Polymorphous low-grade adenocarcinoma, also referred to as *terminal duct adenocarcinoma* or *lobular carcinoma of minor salivary gland origin*,[9,10] is a recently described entity occurring most commonly in the palate and is characterized by a favorable prognosis. Histologically, the lesion is distinguished from other malignant tumors of salivary gland origin by its frequent lobular growth pattern, low mitotic rate, and cytologic uniformity. Although extensive nerve invasion and a cribriform growth pattern may resemble adenoid cystic carcinoma, polymorphous low-grade adenocarcinomas are characterized by histologic diversity, with cells exhibiting cuboidal to low columnar differentiation and an eosinophilic cytoplasm. The stroma, furthermore, often exhibits mucohyaline change in contrast to the bland basement membrane–like deposits of adenoid cystic carcinoma.[10-12] If these criteria are to be applied, it appears as if the adenoid cystic carcinoma containing tyrosine-rich crystalloids reported by Gould and coworkers[7] might possibly have been a polymorphous low-grade adenocarcinoma. If this case were to be accepted as a polymorphous low-grade adenocarcinoma, it would bring the total

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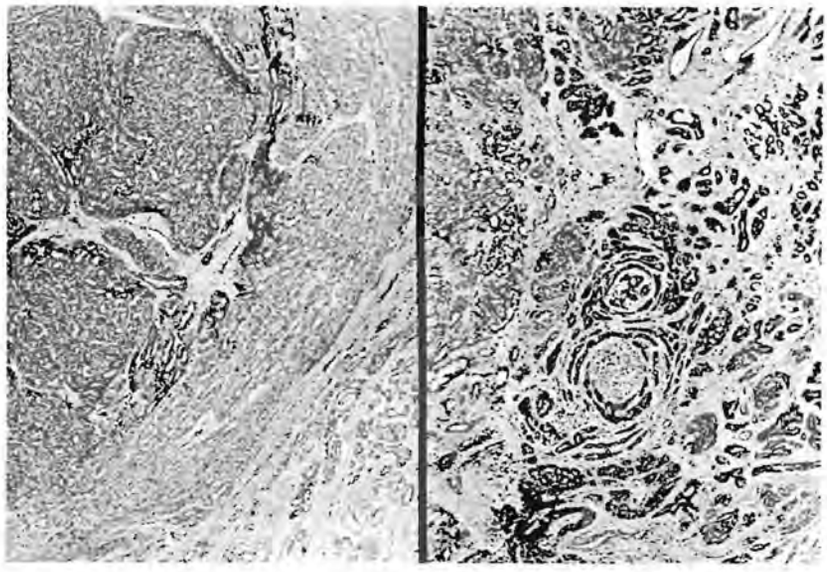

Fig. 1. Distinctly lobular arrangement and infiltrative growth of tumor *(left)* with perineural infiltration *(right)*. (Hematoxylin-eosin stain; original magnification, ×40.)

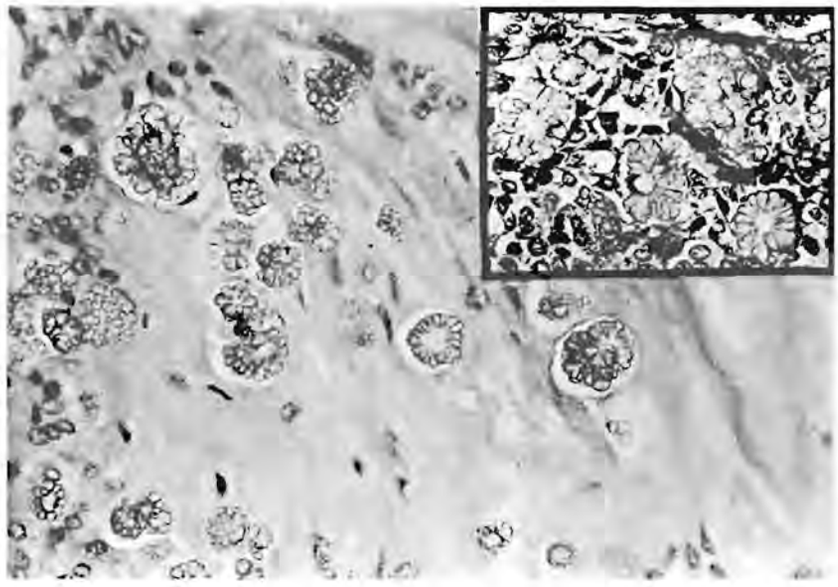

Fig. 2. Stromal and interepithelial *(inset)* deposits of tyrosine-rich crystalloids. (Hematoxylin-eosin stain; original magnification, ×200.)

number with tyrosine-rich crystalloids to three, including the case described by Harris and Shipkey[6] as a "terminal duct adenocarcinoma."

The identification of tyrosine-rich crystalloids in a neoplasm other than benign mixed tumor has important diagnostic implications, as many recent publications regard these crystalloids as a unique microscopic feature of salivary gland mixed tumors.[13-15] It is speculated that the formation of tyrosine-rich crystalloids in polymorphous low-grade adenocarcinomas may place these lesions on a level of cytodifferentiation closer to that of benign mixed tumors than to the other more malignant tumors of salivary gland origin.

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