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Diagnosis of oral precancer and cancer

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

Summary

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

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

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

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

Clinical diagnosis

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

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

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

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

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Stomatoscopy

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

Toluidine blue staining

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

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

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

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

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

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

Exfoliative cytology

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

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

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

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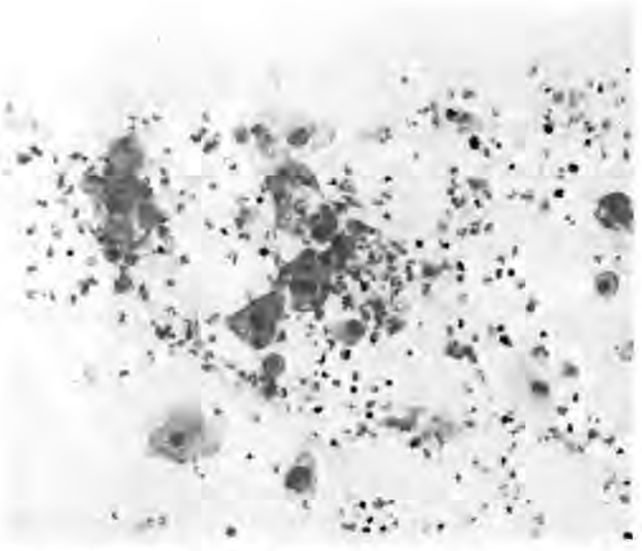

Fig. 1. Cytological smear of an oral lesion displaying variation in size and shape of the epithelial cells, nuclear hyperchromatism and pleomorphism, which are suggestive of malignancy (H and E x 126).

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

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

Radiography and computed tomography

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

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

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

Histological diagnosis

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

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

Biopsy procedures

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

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

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

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

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

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

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

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

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

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

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

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

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

Histopathological examination

Epithelial dysplasia and carcinoma in situ

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

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

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

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

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

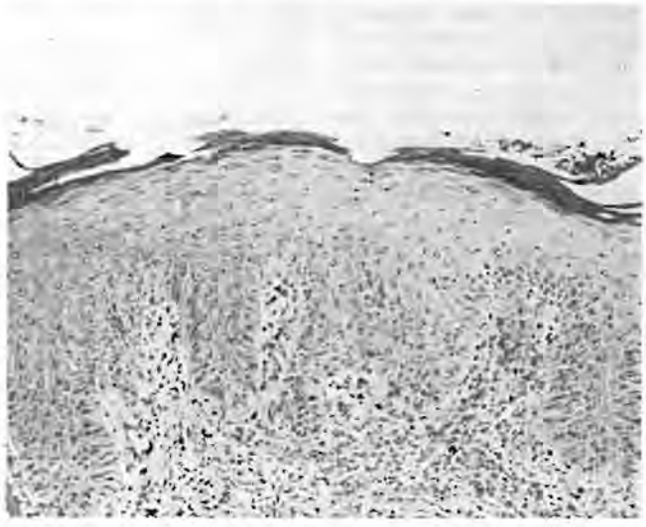

Fig. 2. Slight epithelial dysplasia. Drop-shaped rete ridges, irregular epithelial stratification and hyperkeratosis are illustrated (H and E x 200).

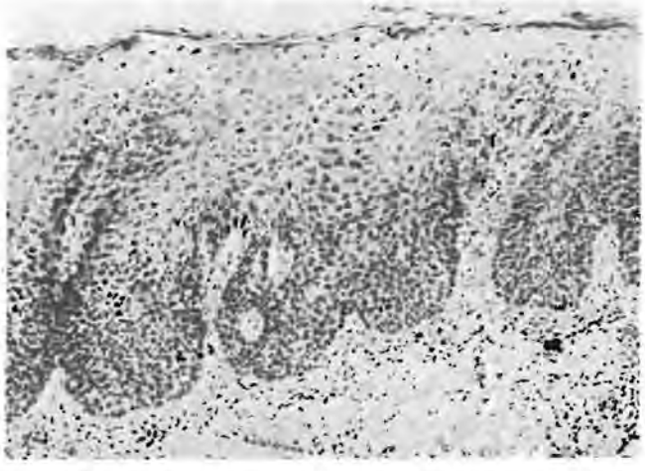

Fig. 3. Moderate epithelial dysplasia, characterised by an increased nuclear cytoplasmic ratio, drop-shaped rete ridges, cellular pleomorphism and nuclear hyperchromatism (H and E x 80).

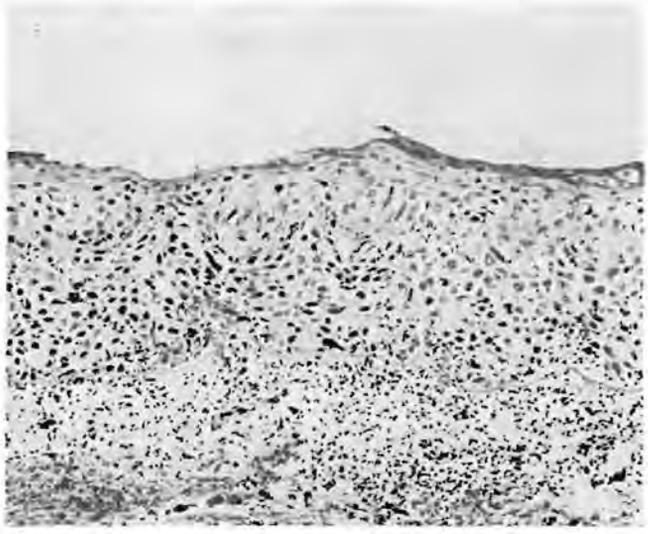

Fig. 4. Carcinoma *in situ* (severe epithelial dysplasia). The epithelial cells exhibit all the features of malignant cells, without infiltration into the underlying connective tissue (H and E x 80).

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

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

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

Squamous cell carcinoma

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

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

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

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

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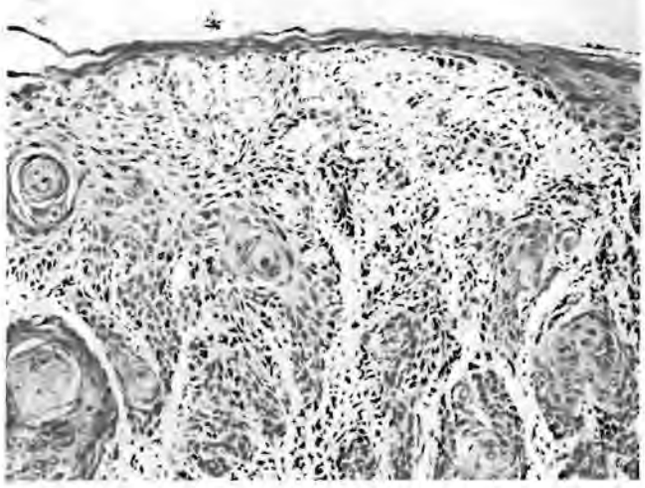

Fig. 5. Well-differentiated squamous cell carcinoma. Invasion of the submucosa and abundant keratinisation are present (H and E x 126).

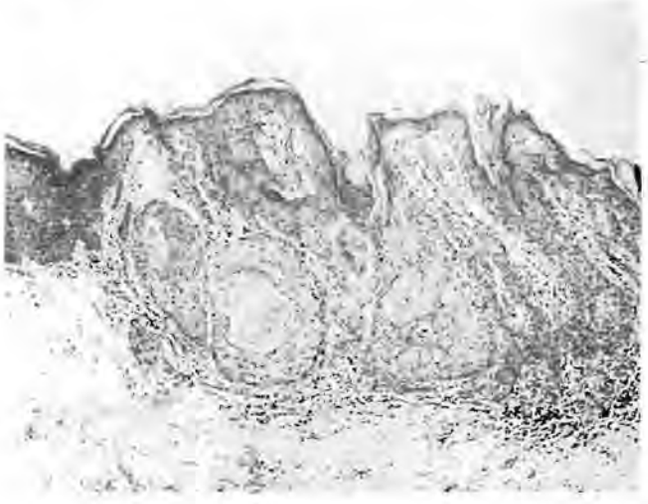

Fig. 6. Well-differentiated squamous cell carcinoma. There is localised invasion into the underlying submucosa. A chronic inflammatory reaction is present at the base of the tumour (H and E x 126).

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

Histological grading

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

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

Advanced diagnostic procedures

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

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

The authors thank Mr J. Nell for the production of the photographs and Mrs H. Pienaar for typing the manuscript.

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Oral cancer

W F P van Heerden and T J P Swart

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

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

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

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

Clinical features and diagnostic considerations

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

High risk patients

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

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

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

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

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

Premalignant lesions and conditions

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

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

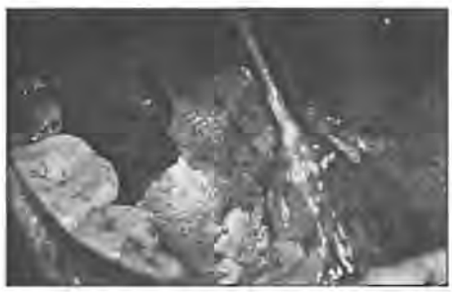

Fig. 1. *Leukoplakia lesion in the floor of mouth. Histologic examination showed a severe epithelial dysplasia.*

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

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

Early detection of cancerous lesions

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

Management principles

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

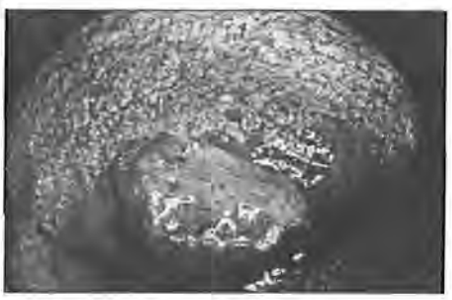

Fig. 3. *Squamous cell carcinoma on the lateral border of the tongue. Note the rolled indurated margins.*

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

Pharmacological agents available for treatment

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

Avoiding treatment errors

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

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Further reading

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

Keywords: Oral cancer, prevention, early detection

Clinical relevance

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

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

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

High risk patients

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

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

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

Premalignant lesions and conditions

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

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

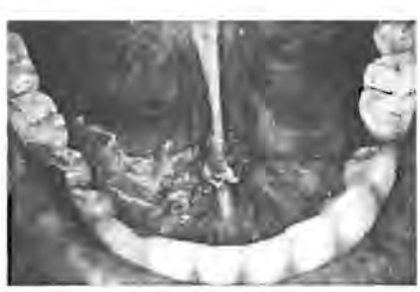

Fig. 1. Leukoplakia on the floor of mouth.

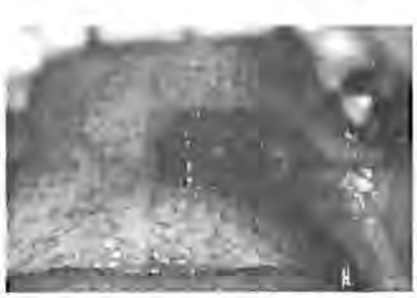

Fig. 2. Erythroplakia on the soft palate.

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

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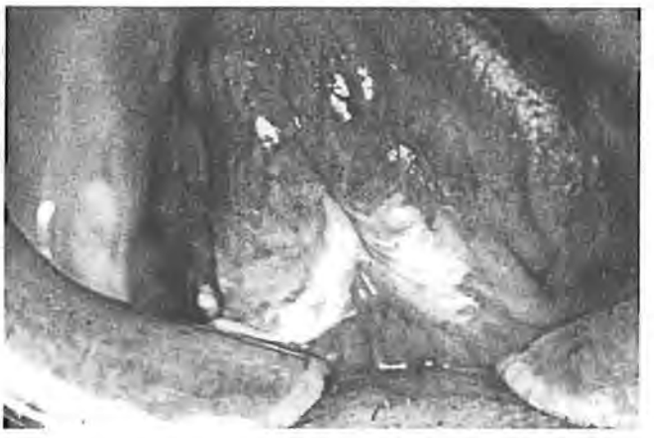

Fig. 3. Irregular/nodular leukoplakia on the ventral aspect of the tongue.

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

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

Early detection of cancerous lesions

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

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

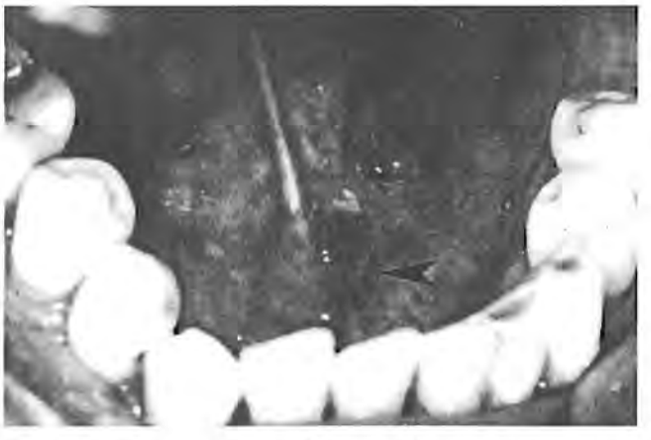

Fig. 4. Small leukoplakia on the floor of mouth. Biopsy (site indicated by arrow) showed an early invasive squamous cell carcinoma.

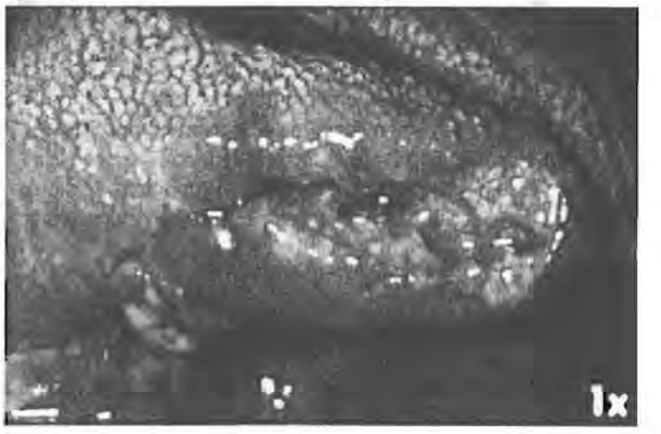

Fig. 5. An advanced oral squamous cell carcinoma with indurated rolled margins.

Management principles

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

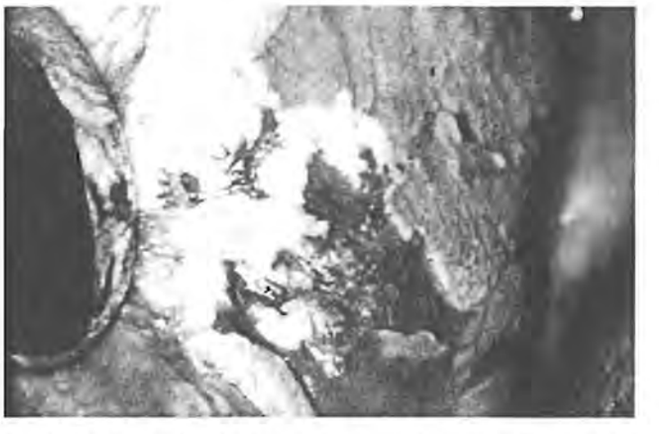

Fig. 6. Erythroleukoplakia on the buccal mucosa. The biopsy should always include a red area.

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

Excision biopsy

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

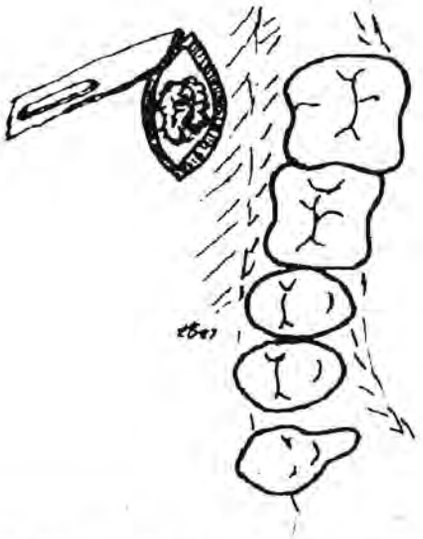

Fig. 7. Schematic drawing of an excision biopsy.

Incision biopsy

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

The surgical technique

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

Cytological curettage

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

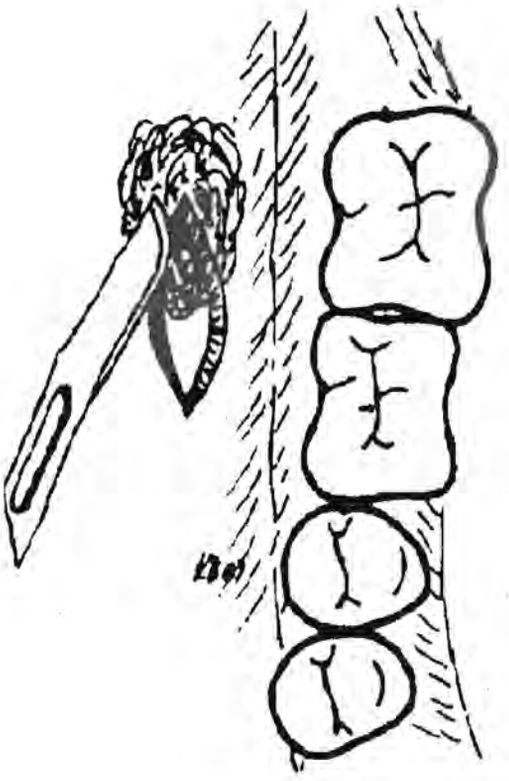

Fig. 8. Schematic drawing of an incision biopsy.

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

Conclusion

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

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in vivo 7: 229-232 (1993)

Langerhans Cells and Human Papillomaviruses in Oesophageal and Laryngeal Carcinomas

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

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

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

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

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

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Key Words: Langerhans cells, human papillomavirus, oesophageal carcinoma, laryngeal carcinoma.

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

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

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

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

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

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

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

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

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

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

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

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

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Received November 24, 1992
Accepted January 13, 1993

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

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

S Afr Med J 1995; 85: 894-896.

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

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

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

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

Material and methods

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

Immunocytochemistry

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

HPV DNA in situ *hybridisation (ISH)*

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

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

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

Results

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

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

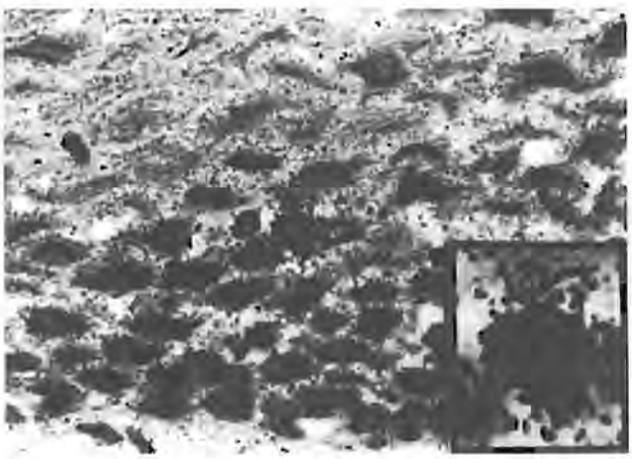

Fig. 1. Micrograph showing the presence of HPV-18 DNA as condensations of black-silver grains superimposed on the nucleus (x 200), with inset of high-power detail of positive cell (x 400).

Discussion

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

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

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

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

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

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

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

The authors wish to thank Mrs H. van der Walt for secretarial services and Dr A. Gunders for help in preparing the manuscript. The study was supported by grants from the Poliomyelitis Research Foundation, Sandringham, and the Medical Research Council of South Africa.

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Accepted 9 Nov 1993.

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

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

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

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

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

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

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

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

S Afr Med J 1995; **85**: 896-898.

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

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ANTICANCER RESEARCH *16*: 969-974 (1996)

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

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

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

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

Materials and Methods

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

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Key Words: Human papillomavirus, DNA, PCR, oral squamous cell carcinomas.

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

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

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

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

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

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

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

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

Results

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

Discussion

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

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

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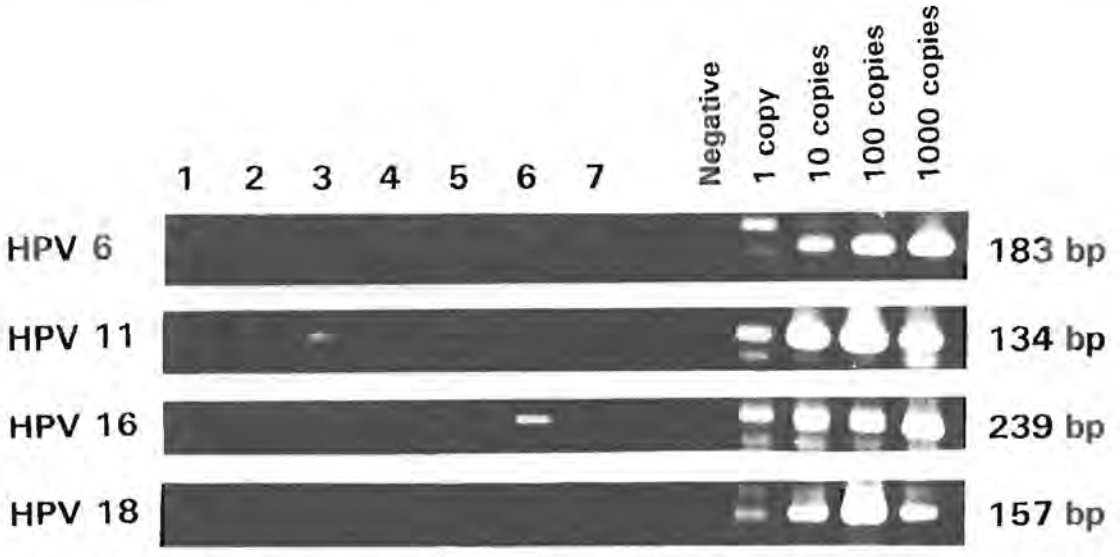

Figure 1. *Gel electrophoresis. Numbers 1-7 represent seven patients selected from groups 1-3. Patients 3 and 6 originated from groups 1 and 2 respectively. The negative reagent control included for each HPV type and the plasmid controls containing 1, 10, 100 and 1000 copies are indicated. The sizes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

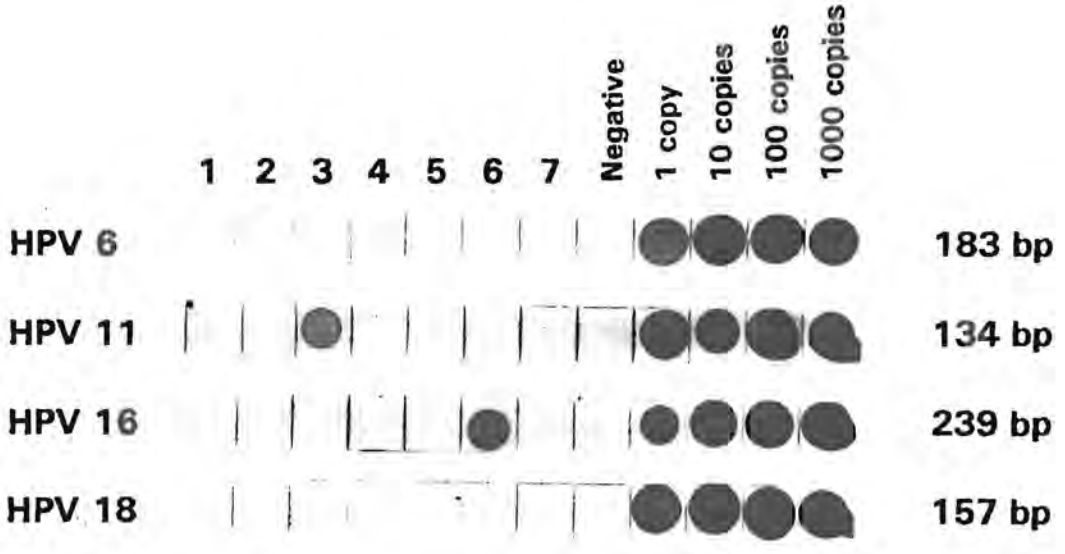

Figure 2. *Dot blot hybridisation. Numbers 1-7 represent the patients selected, as in figure 1. Negative controls, positive controls and PCR fragment sizes are shown.*

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

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

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

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

* Indicate normal oral mucosa.

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

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

Acknowledgements

The authors wish to thank Ms T. Stander for excellent technical assistance. This study was supported by a grant from the Poliomyelitis Research Foundation.

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Received October 23, 1995
Accepted November 24, 1995

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

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Key Words: Oral cancer, oral squamous cell carcinoma, cervical carcinoma, human papillomavirus, epidemiology.

Running title: HPV as mutual aetiological agent for oral and cervical cancer

Epidemiological study

Date of submission: 7 April 2003

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

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

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

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

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Material and Methods

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

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

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

Results

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

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

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

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

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

Discussion

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

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

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

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

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

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

Acknowledgements

This study was supported by a grant from the Medical Research Council of South Africa.

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

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

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

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

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

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

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in vivo 9: 199-202 (1995)

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

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

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

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

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

Materials and Methods

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

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

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

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

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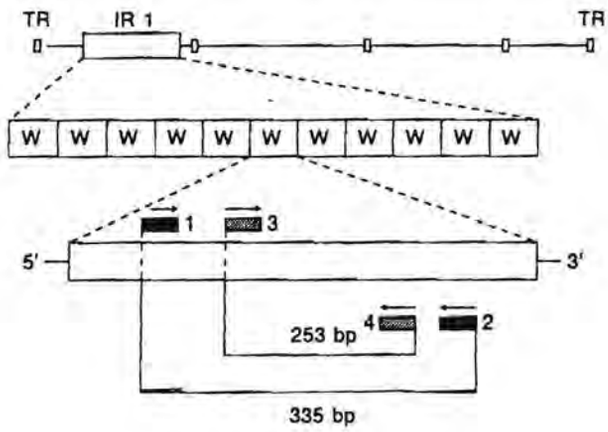

OLIGONUCLEOTIDE SEQUENCES

1 CTTTAAAACTCTAAAAATCAAAACTTTAGA
2 ACCAGAAATAGCTGCAGGACCACTTTATAC
3 AATGGGCGCCATTTTGT
4 TCCCTAGAACTGACAATT

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

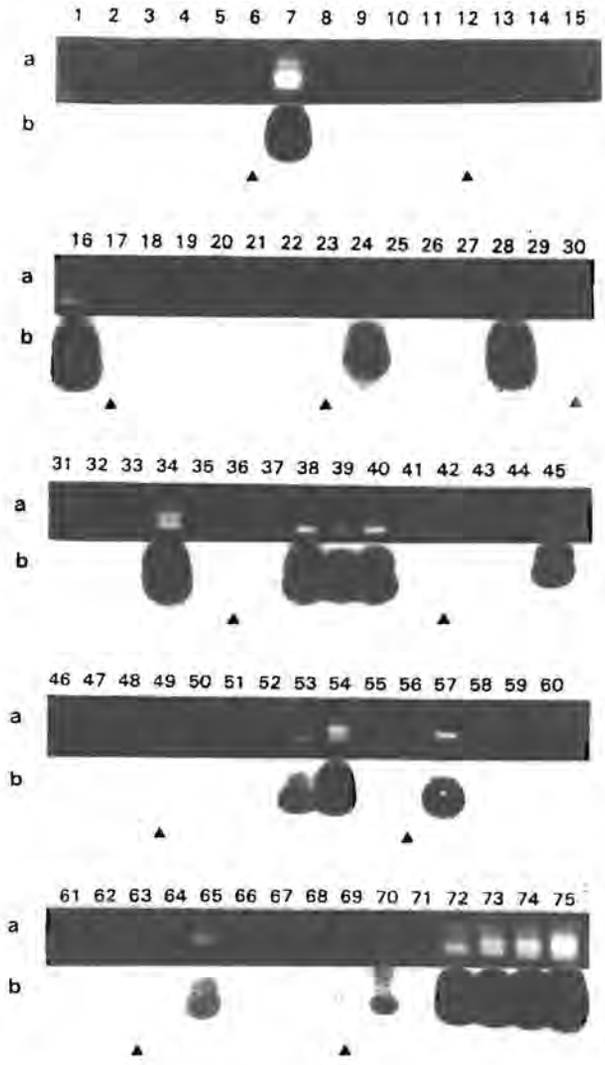

Figure 2. *Carcinomas with normal epithelium. Numbers (10, 11) and (47, 68) were patients tested in duplicate; (71): negative control; (72, 73, 74, 75) represent 1, 10, 100 and 1000 genome copies respectively; (a): gel electrophoresis; (b): hybridization; (▲): brain samples.*

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

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

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

Results

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

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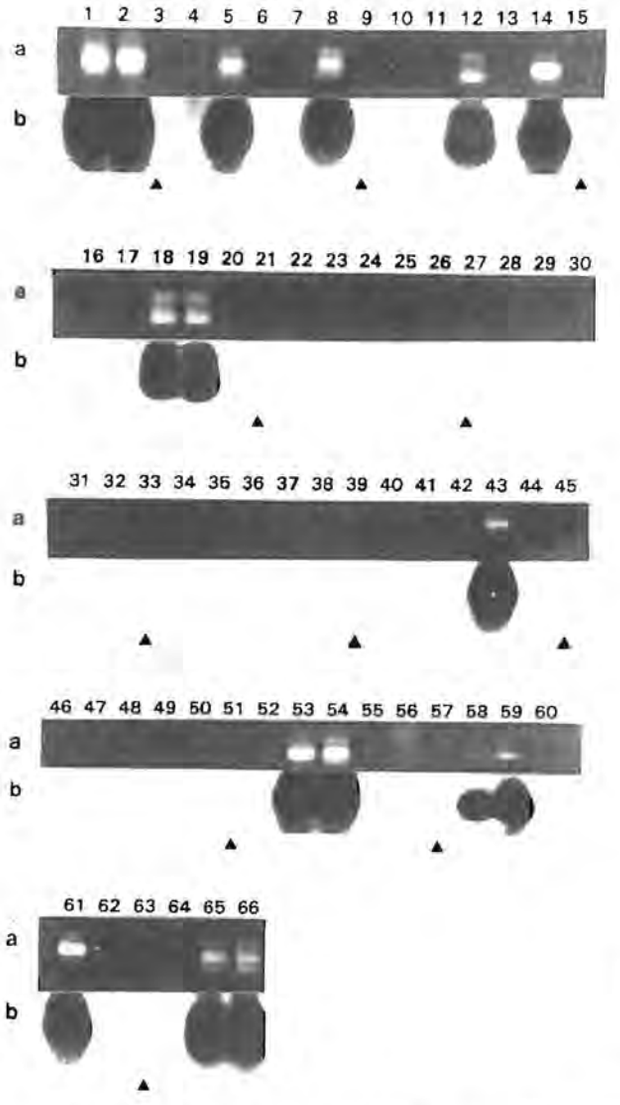

Figure 3. *Carcinomas without normal epithelium. Numbers (28, 32) and (58, 59) were patients tested in duplicate; (64): negative control; (65, 66, 1, 2) represent 1, 10, 100 and 1 000 genome copies respectively; (a): gel electrophoresis; (b): hybridization; (▲): brain samples.*

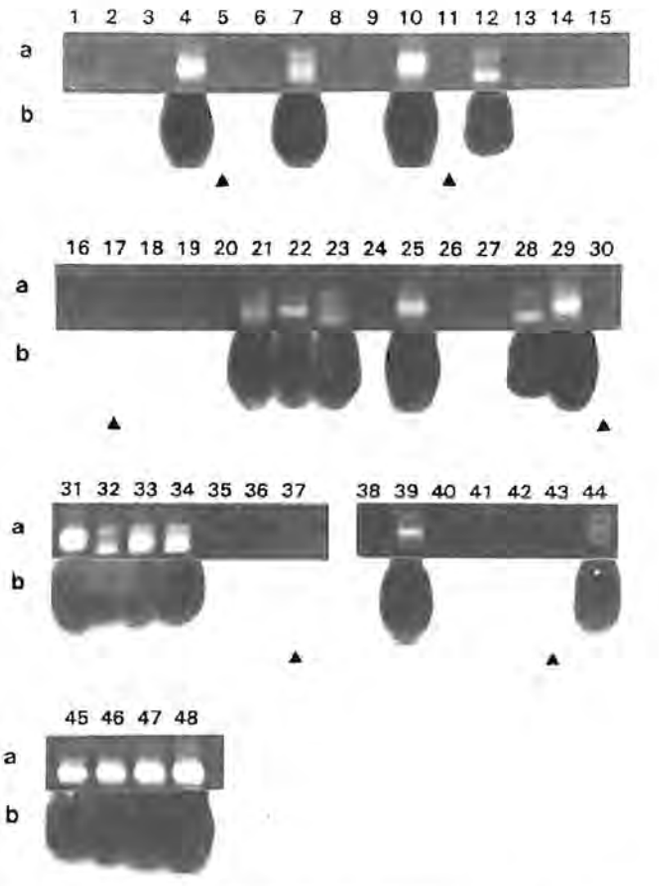

Figure 4. *Control group. (45, 46, 47, 48) represent 1, 10, 100 and 1 000 genome copies respectively; (a): gel electrophoresis; (b): hybridization; (▲): brain samples.*

Discussion

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

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

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

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

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

Acknowledgements

The authors wish to thank Ms T Stander, Ms T-L Smith and Mr R Lötter for excellent technical assistance. This study was supported by the Medical Research Council and Cancer Association of South Africa as well as the Chairman's Fund, De Beers Consolidated Mines Limited.

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Received March 3, 1995
Accepted March 24, 1995