An Overview of the Prevention of Oral Cancer and Diagnostic Markers of Malignant Change: 2. Markers of Value in Tumour Diagnosis

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Abstract: Earlier diagnosis of oral cancer should lead to an improvement in prognosis. This, the second part of a two-part overview, is concerned with the various cellular markers available for diagnosis of oral cancer Traditionally diagnosis has relied upon the histomorphological interpretation of a tissue section following biopsy The application of advances in various laboratory techniques for the identification of different cellular markers is briefly reviewed. In addition, although it is currently only a research tool, the potential role of oral exfoliative cytology in screening is considered.

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Clinical Relevance: Identification of various cell markers may provide valuable information of diagnostic and prognostic significance.

The most commonly applied classification used in clinical practice to assess the patient's prognosis is the TNM system developed by the International Union Against Cancer 1988 (Table 1). Modifications do exist (for example, the STNMP, which takes into account site (S) and pathology (P)). All of these systems highlight the importance of nodal involvement in assessing the prognosis, and are now well established. Unfortunately, inaccuracy still exists in measuring nodal status as preoperative diagnosis of metastasis by routine methods remains an inexact science. Examination under anaesthesia with computed tomography is reliable only in patients with bulky metastatic deposits. The clinical TNM staging of the disease (Table 1) can be different from what is found after excision and histopathological examination (termed pTNM). Despite this, the most important prognostic factor remains the stage of disease at presentation.

The standard method employed for the diagnosis of oral cancer is that of histological examination of a haematoxylin and eosin-stained tissue section. Biopsied tissue is usually fixed in formalin, although the epitopes for various cellular markers are generally better preserved in fresh tissue that has been frozen (e.g. in liquid nitrogen). In oral pathology laboratories the sections are routinely studied for evidence of cellular changes that equate to a diagnosis of dysplasia or carcinoma. These morphological changes may reflect abnormalities in proliferation, maturation and differentiation of the epithelial cells as well as providing evidence of invasion into adjacent tissues (Table 2; Figure 1). Reliance on subjective interpretation of morphology provides limited information for the prediction of the biological behaviour of the lesion. However, at present this remains the most reliable indicator of malignancy. The most important determinant of relative risk of malignant potential is the presence of dysplasia. Other factors that affect prognosis include the depth of the lesion (>8 mm carries a poorer prognosis) and the pattern of invasion. Various grading systems have been developed; these may be of prognostic value and some have been claimed to improve the reproducibility of the diagnosis—for example, Bryne's Malignancy Grading System of cells at the invading edge of the squamous cell carcinoma (Table 3) allows for a comparison between the Bryne score and prognosis. The histopathologist also plays an important role in assessing not only the primary tumour for ensuring complete excision but also in establishing the extent of disease by examining the neck nodes, which provide important prognostic information (e.g. extracapsular spread is associated with a poorer prognosis).

CARCINOGENESIS

As carcinogenesis is a multistep process, genetic damage may occur at any point in the initiation, promotion

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Stage	Tumour size	Nodal status	Metastasis
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IV	Any T	Any N	M1

T represents tumour size, where Tis = carcinoma *in situ*;
T 1 = < 2 cm in greatest diameter;
T 2 = 2-4 cm;
T 3 = > 4cm and
T 4 tumour with extension to involve adjacent structures, e.g. bone, extrinsic muscles of tongue.
N0 = no clinically detectable enlarged nodes;
N1 = enlarged ipsilateral nodes present </= 3 cm;
N2 = ipsilateral single >3-6 cm or ipsilateral multiple </= 6 cm;
N3 = any palpable node >6 cm.
M0 = absent metastasis;
M1 = detected distant (blood-borne) metastasis.

Table 1. *The UICC (1992) TNM staging system.*

and progression of the disease. The initiation probably involves mutational events in unknown genes caused by chemical carcinogens, radiation or viruses and is thought to be irreversible. The latter stages of promotion and progression involve further genetic alterations which lead to malignancy. The expansion of a malignant clone eventually leads to local invasion and possible distant metastasis. Mutations of tumour suppressor genes (genes that protect us from cancer) are also often identified, the most common being that of the p53 gene.

As carcinogenesis is a multistep process host factors (age, genetic inheritance, humoral and cellular immunity) may all play a role. Identification of genetic mutations may predict which lesions will become cancerous and may therefore allow screening in high-risk groups.

Genes may be studied at three levels: DNA, RNA and protein, either in prepared tissue sections (*in situ*) or in isolation. The molecular techniques available for the analysis of genes include:

- the polymerase chain reaction (PCR);
- Southern blotting (DNA analysis);
- Northern blotting (RNA analysis);
- Western blotting (protein);
- *in situ* hybridization; and
- immunohistochemistry.

The PCR is an *in vitro* method for amplifying specific DNA sequences. Starting with trace amounts of a particular nucleic acid sequence from any source, the PCR enzymatically generates millions of exact copies, thereby making genetic analysis of tiny samples a relatively simple process. It can also be used to amplify the DNA in a tissue specimen (*in situ* method). *In*

Dysplasia	Squamous cell carcinoma Degree of differentiation		
Nuclear and cellular pleomorphism	Well	Moderate	Poor
Altered nuclear/cytoplasmic ratio	***Recognizable squamous epithelium***	***Abundant mitotic figures/ atypical***	***Epithelial cells more irregular***
Nuclear hyperchromatism	***Masses of prickle cells***	***Prickle cells more pleomorphic***	***More atypical***
Abnormal keratinization (keratin pearls)	***Frequent keratin pearls***	***Keratin pearls sparse/absent***	***Keratin pearls absent***
Loss or reduction of intercellular adhesion	***Recognizable intercellular bridges***		
Disturbed polarity of basal cells or loss of cellular orientation	***Limiting layer of peripheral basal cells***		
Basal cell hyperplasia, Drop-shaped rete pegs, Irregular epithelial stratification or disturbed maturation, Prominent nucleoli and Increased and abnormal mitoses.	Variable lymphocytic and plasma cell infiltration in the stroma. Locally destructive with variable pattern of infiltration from a broad invasion front to islands or cords of epithelium lymphatic permeation, vascular invasion, sarcolemmal and perineural spread, facial spread and bony invasion.		

Table 2. *The histological features of haematoxylin and eosin stained sections of diseased oral mucosa* ***(after Soames and Southam, 1993).***

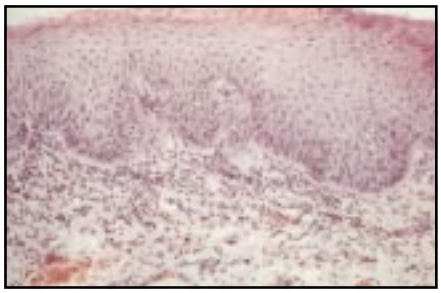

Figure 1. *Haematoxylin- and eosin-stained section of oral mucosa demonstrating features of mild dysplasia—hyperparakeratosis, acanthosis, basal cell hyperplasia, early formation of drop-shaped rete pegs, mild basal lamina replication and a patchy non-specific inflammatory cell infiltrate in the upper lamina propria.*

situ hybridization permits the direct analysis of DNA or RNA in tissues so that specific cells, populations of cells or chromosomes can be examined.

At present, immunohistochemistry is the most readily available technique. Protein expression can be identified by applying antibodies to tissue sections. However, it is worth noting that failure to detect the marker does not necessarily mean that the marker is not there. Anything that masks or prevents the antibody sticking to the antigen (such as tissue processing) will lead to a false-negative result.

IDENTIFICATION OF TUMOUR MARKERS

Some of the tumour markers that may aid the diagnosis of malignancy or provide information of relevance to the prognosis of the lesion are outlined below.

Oncogenes and Tumour Suppressor Genes

An oncogene is a mutated or over-expressed version of a normal gene (the proto-oncogene) that can release the cell from the normal constraints on growth and thus alone, or together with other changes, convert a normal cell into a tumour cell. For example, over-expression of *c-myc*, a gene normally involved in the control of growth and differentiation, or reduced expression of *bcl*, which normally plays a role in the control of apoptosis or programmed cell death, have both been implicated in oral cancer.

Studies using restriction fragment length polymorphisms and microsatellite assays have revealed a complex range of genetic abnormalities present in the oral mucosa of patients, with field change ranging from dysplasia to carcinoma. In some cases the 'genetic fingerprint' of the dysplastic and cancer cells are identical, suggesting that the dysplastic

Figure 2. *p53 immunoreactivity (CM1) of overlying dysplastic oral epithelium and deeply invading squamous carcinoma. Darkly stained positive cells are seen in the basal layer of the overlying mucosa and in the invading edge of the tumour.*

lesion could have been a precursor lesion or spread locally. Diagnosis of such field change may help predict patients at increased risk of developing another tumour.

p53

The tumour suppressor genes are a distinct class of genes which promote neoplasia upon loss of function, the most common of which is p53. This is normally switched on in response to damage to DNA, leading to the

Morphological feature	Score			
	1	2	3	4
Degree of keratinization	Highly keratinized (>50% of the cells)	Moderately keratinized (20-50% of the cells)	Minimal keratinization (5-20% of the cells)	No keratinization
Nuclear polymorphism	Little nuclear polymorphism (75% mature cells)	Moderately abundant nuclear polymorphism (50-75% mature cells)	Abundant nuclear polymorphism (25-50% mature cells)	Extreme nuclear polymorphism (0-25% mature cells)
Number of mitoses (high power field)	0-1	2-3	4-5	>5
Pattern of invasion	Pushing, well delineated infiltrating borders	Infiltrating, solid cords, bands and/or strands	Small groups of infiltrating cells (n>15)	Marked and widespread cellular dissociation in small groups and/or in single cells (n<15)
Lymphoplasmic infiltration	Marked	Moderate	Slight	None

Table 3. *The Bryne Malignancy Grading System (1992). The most anaplastic cells at the invasive front of the tumour are studied.*

Figure 3. *Ki67 immunoreactivity of the infiltrating front of an oral squamous carcinoma. Brown-staining positive cells are situated in the periphery.*

accumulation of p53 protein which stops the cell cycle and allows for DNA repair. If it cannot effect repair, then the cell will undergo apoptosis (cell death). Mutation of the tumour suppressor may alter its function and lead to a mutated cell surviving and developing into a cancer.

Detection of the p53 protein is not exclusively linked with mutation, as anything that leads to the stabilization of wild-type p53 (normally found in cells) or alters its detection threshold can lead to immunoreactivity (a positive staining reaction). No anti-p53 antibody is specific for all mutations of the p53 protein. However, its identification is frequently associated with mutation and thus its identification in a lesion may be suggestive of malignancy. (Figure 2).

Mutagen Sensitivity

By studying the sensitivity of peripheral blood lymphocytes to the clastogenic activity (induction of chromatid breaks) of bleomycin the mutagen sensitivity can be determined. This is purported to be indicative of latent or 'hidden' chromosomal instability. Individuals with a high mutagen sensitivity are potentially at greater risk of developing multiple primary tumours.

This simple assay may be of value in targeting patients who would benefit from more frequent and thorough follow-up, behavioural interventions and enrolment in chemoprevention studies.

Proliferation Markers

For many tumours, rapid growth is associated with poor prognosis. Immunohistochemical detection of proliferating cells indicates the percentage of cells in the DNA synthesis phase. Numbers can be estimated using several different methods (Ki67, PCNA, AgNORs, bromodeoxyuridine labelling, epidermal growth factor, transferrin receptor, etc.)

Ki67 (Figure 3) detects an antigen expressed by cells that are in G1, S1, G2 and M phases of the cell cycle. The original antibody recognized this antigen only in fresh tissue samples but recently a monoclonal antibody (MiB1) and polyclonal antibodies that recognize parts of the Ki67 antigen which survives the fixation process have been developed.

Another marker of cell proliferation is proliferating cell nuclear antigen (PCNA), and studies have shown a dual role for PCNA in cell replication and DNA repair. These two functions help to explain the conflicting data concerning the value of measuring proliferation with PCNA antibodies.

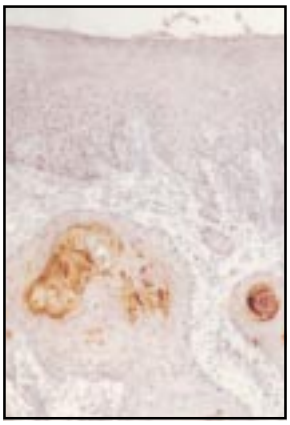

Figure 4. *Cytokeratin 10 immunoreactivity (LH1) of a biopsy of oral mucosa showing deeply invading squamous carcinoma. Brown central staining is seen in association with keratin pearls.*

Figure 5. *Angiogenic immunoreactivity (vWF) of supporting stromal tissues in an area of oral mucosa demonstrating severe dysplasia. Brown-stained endothelial cells of vessels are seen in the supporting stroma deep to the tumour.*

AgNORs are nucleolar organizing regions that take up silver. The number of nucleolar organizing regions present within the nucleus is thought to reflect the production of ribosomal components and hence protein synthesis within the cell, an increase in number being associated with increased metabolic/cellular activity. Unfortunately, as yet no simple relationship has been identified between tumour behaviour and proliferation rate using these markers.

DNA Content of Tumours

Almost all human cancers contain cells with an abnormal DNA distribution (an alteration in the number of chromosomes, known as aneuploidy). Ploidy (DNA content) measurements performed by flow cytometry, static cytometry or image cytometry offer relatively objective measurements of analysing the DNA content of cells. Studies in oral cancer have indicated that the DNA content of a tumour is an indicator of prognosis and aggressive behaviour, with a poorer prognosis associated with aneuploid tumours. A non-diploid DNA profile is frequently associated with oral cancers.

Cell Surface Antigens

These include the integrins (a family of cell-surface glycoproteins that are involved in binding to extracellular matrix components), cadherins (cell

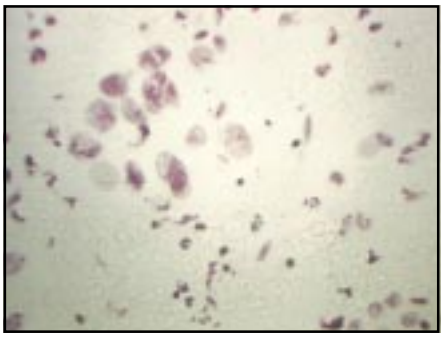

Figure 6. *Feulgen-stained nuclei of cells removed from an oral cancer following exfoliative cytology. Note the variation in nuclear size.*

adhesion molecules) and histo-blood group antigens.

The cadherin/catenin complex and the integrins are the prime mediators of cell adhesion in normal and transformed cells, cadherin/catenin being largely responsible for intercellular adhesion and integrins for cell/extracellular matrix interactions. Intercellular and cell matrix adhesion mediated by cadherin/catenin and integrins may influence both structural morphology and functional differentiation: loss of this control mechanism may promote neoplastic change.

Each cell surface antigen may be classified into various subtypes. Some are expressed in normal development (and lost in malignant transformation). Others may be found only in association with malignant disease and not in normal development (e.g. expression of blood group antigen precursors Leb and LEy may promote the adhesion of cancer cells to endothelial cells).

Cytokeratins

At least 20 different keratins are found within the cytoplasm of epithelial cells. These vary with the state of differentiation (Figure 4). Although the oral mucosa reflects a broad range of keratins, the so-called 'simple' epithelial keratins (e.g. K8, K18, K19) are the best markers of malignancy, along with the loss of the secondary differentiation markers, keratin 10 and 13.

It has been suggested that the expression of particular keratins (16 and 19) within the lining of the upper aerodigestive tract are predictive of an increased risk of developing a further tumour. The detection of keratin in cells from oral smears may also titrate the efficacy of chemoprevention treatment, although this is unproven. Keratins have been used to determine whether a poorly differentiated tumour is of epithelial (expresses keratin) or mesenchymal origin (no keratin expression).

Stromal Changes

The formation of new blood vessels is essential not only for carcinogenesis but also for metastasis. Studies have shown an increase in neovascularization (angiogenesis) with malignant change and tumour progression. Various methods have been used to study this phenomenon, such as measuring the density or volume of the microvasculature in histological sections with antibodies that recognize various antigens on endothelial cells (e.g. CD 31 and von Willebrand factor) (see Figure 5). Microvascular volume is closely related to tumour progression in oral cancer.

Exfoliative Cytology

This is the study of the morphology of suprabasal (superficial) mucosal cells using light microscopy. The quantitative assessment of changes in morphometry (e.g. nuclear and cell area) coupled with identification of various tumour markers like those described above (Figures 6 and 7) has increased the sensitivity and specificity of exfoliative cytology, although it is as yet only a research tool.

SUMMARY

The diagnosis of oral cancer remains firmly embedded in the histomorphological analysis of a haematoxylin and eosin-stained tissue section. This can be supplemented by the study of finer detail using

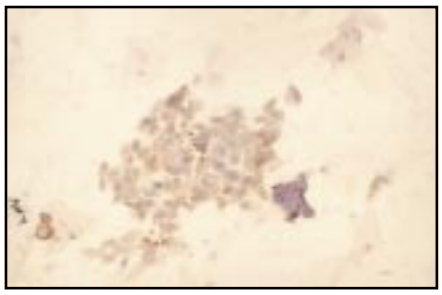

Figure 7. *Smear obtained from an oral cancer in which the cytoplasm of the cells was found to express keratin 8 (antibody LE41). The blue nuclei have been counter-stained with haematoxylin.*

immunohistochemical and other molecular biological techniques to study various markers associated with malignant change. The results using these techniques may also provide an insight into the prognosis of the lesion. However, the major drawback still remains that there is no single marker present in all oral cancers that is not present in normal or benign mucosal lesions. It remains to be seen whether a combination of markers can improve the diagnostic capability of exfoliative cytology in detecting oral cancer.

It is to be expected that diagnosis will use both morphological (including immunohistochemical detection of specific tumour markers) and molecular profiling (including assessment of genetic 'risk'), which will not only diagnose the tumour but will indicate the most appropriate form of therapy.

Further Reading

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