Keratin profiles of normal and malignant oral mucosa using exfoliative cytology

G R Ogden, S McQueen, D M Chisholm, E B Lane

Abstract
Aims—To assess keratin profiles from smears of malignant and contralateral normal oral mucosa as part of the development of a screening procedure for oral cancer based on exfoliative cytology.

Methods—Smears were taken from oral cancers (confirmed by biopsy) and from the contralateral site of 20 patients. Using a panel of antikeratin antibodies, the keratins expressed by these cells were identified using a standard immunocyto-chemical technique (Vectastain) and assessed on a 3 point scale.

Results—Using \( \chi^2 \) analysis, noticeable differences between the keratin profiles for malignant mucosal smears compared with the contralateral mucosal smears were found. This was particularly evident for the simple epithelial keratins.

Conclusion—Individual keratins can be identified in smears from oral cancers. The identification of simple epithelial keratins seems to be the best keratin markers associated with malignancy. Their detection within smears from oral lesions could be valuable in the early diagnosis of oral cancer.

Worldwide, oral cancer is the 6th most common cancer.\(^1\) In the United Kingdom about 1900 new cases and 960 deaths occur every year.\(^2\) Ideally disease prevention is what is required. But there seems little evidence to suggest that exposure to the two factors most frequently associated with oral cancer (alcohol and tobacco) is decreasing.\(^3,4\) Even then total reliance on prevention is not possible because all the aetiological factors for oral cancer are not known.\(^5,6\)

Despite the fact that oral cancer can be cured if treated early enough,\(^7\) the 5 year survival (about 35%)\(^8\) has not really improved with advances in surgery, radiotherapy, and chemotherapy.\(^9\) The main reason is probably the late presentation of these tumours.\(^10,11\) In turn this may be due to: (i) the asymptomatic nature of the early lesion;\(^12\) (ii) lack of self examination by patients;\(^13,14\) (iii) misdiagnosis by clinician;\(^15,16\) and (iv) the patient’s fear.\(^17,18\) These obstacles have to be overcome if the prognosis is to improve.

Recent calls for a screening procedure for oral cancer still rely on subjective assessment by the clinician as to whether the mucosa appears clinically malignant or not.\(^19,20\) Recent advances in the quantitative assessment of oral cytology\(^21-23\) may offer a suitable technique for such screening, analogous to the screening procedure based on exfoliative cytology for cervical cancer, a disease which may have a similar incidence to oral cancer.\(^24\)

Heavily keratinised oral lesions make it difficult to gain a representative sample of the underlying lesion, but this is offset by their relatively low malignant transformation rate—about 2%.\(^25\) Far more worrying are the speckled leukoplakias and erythroplakias which are reported to undergo malignant change more frequently. These lesions are amenable to sampling using exfoliative cytology. Not all general practitioners would biopsy the oral mucosa and they may be more inclined to smear an abnormality of the mucosa. Furthermore, in patients who are under review following treatment of an oral squamous cell carcinoma or in patients who have widespread instability of the oral mucosa, repeat biopsies at every review are not practicable. Such patients would soon fail to attend for review. Exfoliative cytology permits the frequent sampling of such sites with minimal inconvenience to the patient. This forms part of the overall management of our patients treated in Tayside.

As well as assessment of nuclear status, exfoliative cytology samples can be assessed for differentiation status. In epithelial cells differential expression of keratin filament proteins is a well established indicator of differentiation, against which many well characterised antibodies are available.\(^26,27\) Keratins are for the most part conserved during malignant transformation when all other identifying criteria of the cell’s origin may have been lost. Because cytokeratin profiles are useful in tumour diagnosis\(^28\) their identification in oral smears may detect subtle changes in tissues undergoing malignant change.

Keratin expression has been studied in normal oral epithelia,\(^29\) but keratin antibodies had not previously been applied to smears from oral cancers. Knowledge of the normal keratin profile determined the range of keratins studied.

Methods
Paired smears were taken from biopsy confirmed oral cancers and from the contralateral mucosal site of 20 patients, using a Cytobrush (MedScand, Colgate Medical Ltd,
Keratin expression in oral cytology

Table 1: Range of keratin monoclonal antibodies used for immunoperoxidase staining

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Reactivity</th>
<th>Reference</th>
<th>Source</th>
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<tbody>
<tr>
<td>LP34</td>
<td>1 in 10</td>
<td>+</td>
<td>Lane et al(^a)</td>
<td>EBL</td>
</tr>
<tr>
<td>AE8</td>
<td>1 in 50</td>
<td>+</td>
<td>Dhossayly et al(^a)</td>
<td>ICN</td>
</tr>
<tr>
<td>LH1</td>
<td>Undiluted</td>
<td>+</td>
<td>Lane et al(^a)</td>
<td>EBL</td>
</tr>
<tr>
<td>LP2K</td>
<td>1 in 5</td>
<td>+</td>
<td>Stasiak et al(^a)</td>
<td>EBL</td>
</tr>
<tr>
<td>CAM5-2</td>
<td>Undiluted</td>
<td>+</td>
<td>Makin et al(^a)</td>
<td>ICRF</td>
</tr>
<tr>
<td>LE41</td>
<td>Undiluted</td>
<td>+</td>
<td>Lane(^b)</td>
<td>EBL</td>
</tr>
<tr>
<td>LE61</td>
<td>Undiluted</td>
<td>+</td>
<td>Lane(^b)</td>
<td>EBL</td>
</tr>
</tbody>
</table>

ICN, High Wycombe, Bucks
ICRF, South Mimms, Herts
Dr I Leigh, Dept of Experimental Dermatology, The Royal London Hospital

Table 2: Keratin profiles for each patient for smears from normal (N) and malignant (M) oral mucosa, together with differentiation status

<table>
<thead>
<tr>
<th>Case number</th>
<th>Site</th>
<th>Differentiation status</th>
<th>AB8</th>
<th>LP34</th>
<th>LP2K</th>
<th>LH1</th>
<th>CAM5-2</th>
<th>LE41 + LE61</th>
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<td>1</td>
<td>LesBM</td>
<td>W</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
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<td>P</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>3</td>
<td>LesPal</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>LesPal</td>
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<td>LesBM</td>
<td>M</td>
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</tr>
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<td>6</td>
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<td>1</td>
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<tr>
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<td>1</td>
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<td>1</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
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<td>2</td>
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<td>1</td>
<td>1</td>
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<td>LesVT</td>
<td>W</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>M</td>
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<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>16</td>
<td>LesFOM</td>
<td>M</td>
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<td>17</td>
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<td>W</td>
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<td>2</td>
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<td>0</td>
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<td>0</td>
</tr>
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<td>M</td>
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<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
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</tr>
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<td>LesFOM</td>
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<td>2</td>
<td>1</td>
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<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Key: Site: BM = Buccal mucosa; Pal = Palate; VT = Ventral tongue; FOM = Floor of mouth; Alv = Alveola. Differentiation status of tumours: W = well differentiated; M = moderately differentiated; P = poorly differentiated. Keratin expression: 0 = absent, 1 = few cells positive; 2 = many cells positive.

England), and fixed using a commercially available spray fixative containing isopropyl alcohol and polyethylene glycol (Vale Smear Fix, Vale Laboratories, England). Smears were then stored at -70°C until required.

Table 1 shows the range of monoclonal antibodies used to identify the cytokeratin profile (together with dilutions, and source). Goat serum was used as the negative control. Four “wells” were created on each plain glass slide with the aid of a wax pen (PAP pen, Agar Scientific Ltd., England) thus allowing up to four antibodies to be used on each smear.

A standard protocol was followed using avidin biotin visualisation (Vilestain, Vector Labs., Peterborough, England). Briefly the smears were thawed, fixed in acetone (100%) for 5 minutes, then air dried at room temperature. After incubation with the antibodies, the peroxidase label distribution was visualised using diaminobenzidine tetrahydrochloride (DAB) substrate. The smears were then lightly counterstained with Mayer’s haematoxylin, dehydrated, and mounted in DPX.

The presence of a particular keratin was then assessed according to the number of brown (“positive”) cells present, on a 3 point scale: 0 = no cells positive; 1 = few cells positive; 2 = many positive cells. All smears were graded by one author (GRO). Intraobserver error was randomly checked to assess reproducibility. The entire “well” exposed to a particular antikeratin antibody was scanned for the presence of positively stained cells.

The criteria used for selection of patients reported in this study were that: all should have had histological evidence of carcinoma, smears had been taken from tumour and contralateral sites; and that cytokeratin profiles were available. From our cohort of patients, sequentially the first 20 satisfying these criteria were admitted to the series. By chance this resulted in roughly equal numbers from four different sites in the mouth, including the rare site of the soft palate.

Results

The results for expression of a particular keratin in both the lesonal smear (les) and the contralateral normal mucosal smear (N) are given in table 2 and include results for LP34, which acted as the positive control. The results for smears from these two sites in each patient were compared using χ² analysis. A significant difference between normal and malignant mucosal smears was found for keratin 8/18 (p < 0.01), keratin 19 (p < 0.01), and keratin 13 (p < 0.05). For keratin 8/18, the scores for CAM5-2 and LE41/LE61 were averaged for statistical comparison of normal and lesonal smears. Although 20 patients were assessed, cells were not always present within the “well” dedicated to that particular antikeratin antibody. Variations in cell density between individual “wells” in one smear and between cases were apparent. When there were insufficient cells to grade the smear no grade was given.

Figure 1 illustrates positive staining with LP34 of both normal cells (centre) and malignant cells (lower right), in a smear (graded 2) taken from a carcinoma of buccal mucosa.

Figure 2 shows positive staining with CAM5-2 together with larger negatively stained cells in a smear (graded 1) taken from a carcinoma of hard palate.

Discussion

This preliminary report establishes that different keratins can be identified in smears taken from biopsy confirmed oral cancers and that the consequent profile seen is different from that observed in the smears from clini-
Plain glass slides were used at first, with "wells" created by the use of a wax pen. These wells also had the added advantage of limiting the amount of reagent used, and thus the expense of the procedure. However, positive steps had to be taken to avoid bias caused by uneven cell spreading across the slide because the antibodies always followed the same order; those near the boundary of the slide (the marker for keratin 10, LH1) were more likely not to contain sufficient cells on which to grade the expression of that particular keratin. Sampling errors will prejudice the results and possibly limit the value of exfoliative cytology but must be accepted within the limits of the technique. Empty wells occurred in 29 of 240 wells and were excluded from statistical analysis. We have now modified this to use 4-well slides (Hendley Essex), instead of plain slides. Thus the sample instrument makes contact with all four sites that will subsequently receive an antikeratin antibody, which greatly increases the likelihood of cells being present. These smears were taken with the Cytobrush which we have found superior to the traditional wooden spatula. Another factor to consider with regard to cell yield is that of site. In our experience the hard palate is the site most frequently associated with a poor cell harvest. The keratins showing the most significant difference between smears from normal and malignant mucosa were the simple keratins 8, 18, and 19. This is perhaps not so surprising, given that for normal oral mucosa the other keratins assessed (keratins 10 and 13) are expressed throughout the suprabasal region of keratinised (K10) and non-keratinised (K13) regions in the oral cavity. In tissue biopsy specimens it has been claimed that well differentiated oral carcinomas express variable mixtures of keratins 10 and 13. However, as both K10 and K13 may be expressed in normal and malignant tissue, this makes them unsuitable as definitive markers of malignancy, particularly as most oral cancers are well differentiated. Simple keratins (K8 and K18) are not normally expressed in oral mucosa and keratin 19 is limited to the basal cells. It has been suggested that suprabasal expression of keratin 19 is associated with malignancy, and that keratins 8 and 18 do not seem to be found in association with benign mucosal disorders but are associated with many oral cancers. These simple keratins have also been identified in premalignant lesions. Thus their identification within a smear of a clinically suspicious lesion would warrant consideration of a biopsy to exclude malignancy.

Because the initial changes which give rise to a clinically visible lesion are thought to occur within the epithelium, the sampling of individual cells might offer a better chance of detecting malignant change, particularly as over 90% of all cancers in the oral cavity are squamous cell carcinomas. Oral exfoliative cytology received a great deal of attention in the 1960s, eventually falling from favour.
due largely to the subjective nature of its interpretation. There were also difficulties in obtaining a representative sample from hyperkeratotic lesions. However, their rate of malignant transformation is much less than that of speckled leukoplasia and erythroplasia which are amenable to sampling using exfoliative cytology. There is no doubt that histological assessment remains the accepted method of diagnosis. Although the diagnosis of frank oral carcinoma is relatively clear cut, the same is not necessarily true of epithelial dysplasia. Hence the development of a reliable test for use in the detection and follow up of malignant lesions, which is pain free, simple, and reproducible, remains the prime objective.

We have previously shown that the use of exfoliative cytology can detect malignant change, based on the assessment of nuclear and cytoplasmic areas of Papanicolaou stained cells and DNA profiles of Feulgen stained smears taken from the lesion and compared with those taken from the contralateral normal mucosa. However, continued refinements are required to improve the technique. Ideally, the identification of a marker present in malignant cells but not in normal cells is required.

This preliminary report suggests that further studies on keratin expression in oral exfoliative cytology for the diagnosis of oral cancer should concentrate on keratins 8, 18, and 19, given their association with malignant disease reported here and in published findings. The identification of a keratin marker that is lost during malignant transformation—for example, keratin 13—is less satisfactory because such keratins can be found in normal mucosa adjacent to the sampled lesion. Such cells might “contaminate” the smear, particularly if the lesion under investigation is small.

The identification of cytokeratin expression in oral exfoliative cytology is encouraging, particularly when such expression seems to be quite robust. Further studies are currently underway to evaluate these preliminary results and to address the slight variations in the normal group seen in table 2. However, their combination with quantitative cytomorphological assessment may well contribute towards the development of a screening programme to detect oral cancers at an early stage.

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