Pergamon

Oral Oncol, Eur J Cancer, Vol. 30B, No. 6, pp. 405–408, 1994

0964–1955/94 $7.00 + 0.00

0964–1955(94)00019–0

DNA and Keratin Analysis of Oral Exfoliative Cytology in the Detection of Oral Cancer

G.R. Ogden, J.G. Cowpe, D.M. Chisholm and E.B. Lane

Refinements in oral exfoliative cytology may make it a suitable screening technique for the early diagnosis of oral cancer. In this study DNA range profiles were combined with keratin expression in an attempt to improve the diagnostic accuracy of oral exfoliative cytology. Smears were taken from 33 biopsy-proven oral cancers and the contralateral normal site. For DNA range profiles the smears underwent Feulgen hydrolysis, with DNA distribution being assessed using the Vickers M85 microdensitometer. For keratin expression a panel of antikeratin antibodies were applied. The smears for keratin expression were then graded on a three-point scale. Abnormal DNA range profiles were observed in 23 of 33 smears taken from oral cancers and in two smears from normal oral mucosa (sensitivity 70%, specificity 90%, positive predictive value 90%). The simple epithelial keratins 8 and 19 were identified in the majority of oral cancer smears. The sensitivity of keratin 19 was greater (90%). However, keratin 8 was the most useful keratin marker associated with malignancy (sensitivity 62%, specificity 100%, positive predictive value 100%). The combination of simple keratin expression and DNA content improved the cancer detection rate beyond that obtainable with DNA range profile alone.

Keywords: oral cancer, DNA cytophotometry, cytokeratins, oral exfoliative cytology

Oral Oncol, Eur J Cancer, Vol. 30B, No. 6, pp. 405–408, 1994.

INTRODUCTION

ORAL EXFOLIATIVE cytology enjoyed much attention in the 1950s and 1960s eventually falling from favour due largely to the subjective nature of its interpretation [1]. It was found that the classification used by Papanicolaou for cervical smears could not be so easily transcribed to oral smears.

In the last decade interest in oral exfoliative cytology has once again emerged following the application of various quantitative techniques [2, 3]. Mean nuclear and cytoplasmic area values for a range of oral mucosal lesions have been plotted on a scattergram [4]. When a discriminant line was superimposed on the scattergrams it was found that smears from oral cancers frequently fell above the line, i.e. displayed a reduced cytoplasmic area and/or increased nuclear area [4]. This observation would be considered suspicious and promote biopsy.

Correspondence to G.R. Ogden.
G.R. Ogden and D.M. Chisholm are at the Department of Dental Surgery and Periodontology; E.B. Lane is at the Department of Anatomy and Physiology, Dental Hospital and School, University of Dundee; and J.G. Cowpe is at the Department of Oral Surgery, Medicine and Pathology, University of Wales College of Medicine, U.K.
Received 15 Feb. 1994, provisionally accepted 21 Mar. 1994; revised manuscript received 2 June 1994.

EJC 30:6-C

A further method of quantitation which has been applied to oral smears is DNA cytophotometry [5]. The integrated optical density of Feulgen-stained nuclei was assessed and DNA range profiles obtained using a microdensitometer. Abnormal DNA range profiles of oral cancers have frequently been obtained using this method [4, 6] although diploid DNA oral cancers are known to occur [7–9]. Thus, a DNA range profile alone is unlikely to detect every oral cancer. The combination of mean nuclear and cytoplasmic area values together with DNA range profiles has led to an increase in the diagnostic sensitivity of oral cytology. However, further refinements are necessary, not least in the search for a marker of malignancy that is readily identifiable and whose assessment is not too time consuming. If it is to become part of a screening programme then ideally a cytological smear should be easily processed using existing equipment and not require additional expenditure. Quantitative cytology, being a simple and acceptable diagnostic test both to clinicians and patients, can be repeatedly utilised each time a patient is seen and, therefore, provides an ideal method for screening patients for the early detection of oral malignancy.

In an attempt to improve the diagnostic sensitivity of cytology further we decided to combine assessment of DNA range profiles with an assessment of keratin expression. Keratins are intermediate filament proteins found in the

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

Name	Dilution	Keratin reactivity	Reference	Commercial source
LP34	1 in 10	K1, K5, K6, K18	Lane *et al.* [19]	Dako
AE8	1 in 50	K13	Dhouailly *et al.* [20]	ICN
LH1	Undiluted	K10	Lane *et al.* [19], Leigh *et al* [21]	Seralab
LP2K	1 in 5	K19	Stasiak *et al.* [22]	Amersham Int
CAM5.2	Undiluted	K8 (+K7)	Makin *et al.* [23]	Beckton-Dickinson

cytoplasm of all epithelial cells [10]. There are at least 30 different keratins whose expression alters with the state of tissue differentiation [11, 12].

In a preliminary communication, we reported the comparison of cytokeratin profiles in smears taken from oral cancers and the contralateral normal site. Keratins are usually conserved during malignant transformation when all other identifying criteria of the cell origin may have been lost [10, 13], hence the reason for using these markers. The application of immunocytochemical techniques to cytology has not been as widely used as in diagnostic histopathology [14, 15].

Although individual keratins could be identified there was no keratin that was expressed by *all* cancers when not expressed in normal cells [16]. Therefore, it was proposed to combine keratin profile with DNA range profiles as a potential screening procedure for oral cancer for those in high-risk communities [17].

MATERIALS AND METHODS

Smears were collected from 33 biopsy-proven oral cancers and the contralateral normal site of Caucasian patients attending the Dundee Dental Hospital and Dundee Royal Infirmary. Two smears were processed for DNA assessment and four smears for keratin expression as detailed below. All smears were collected using a cytobrush (Medscand, Colgate Medical, U.K.) and fixed immediately using a spray fixative (Vale Smear fix, Vale Labs, U.K.).

Assessment of nuclear Feulgen DNA content

Following the use of the spray fixative each smear was immersed in methanol: formalin: acetic acid (85:10:5) for 1 h, and was then washed in running tap water for a further hour [18]. Hydrolysis in 5 N HCl was then carried out at 25°C, in an agitated water bath for 80 min followed by rinsing in distilled water. Schiff's reagent was applied for 45 min (according to the method of de Tomasi) washing of the smear in running tap water for 5 min and dehydration through 95% to absolute alcohol and xylene completed the staining process.

The Feulgen reaction depends upon the prior hydrolysis of DNA by acid, to unmask aldehyde groups in the DNA, which in turn reacts with Schiff's base to produce an intensely coloured dye, the amount of dye present in the nuclei being proportional to the DNA content.

The DNA content was analysed using a Vickers M85 microdensitometer. A total of 50 randomly-selected cells were measured, with the result that 50 values, registered in arbitrary integrated optical density units, were recorded for each smear. These values were then displayed as a DNA distribution histogram (DNA range profile) for each specimen.

Cytokeratin expression

Smears for immunocytochemistry were stored at −70°C for up to 6 months until required. Table 1 displays the range of monoclonal antibodies used to characterise the cytokeratin profile (together with dilutions and source). Goat serum was used as the negative control. A standard protocol was followed using avidin-biotin visualisation (Vectastain, Vector Labs, Peterborough, U.K.). Briefly, the smears were thawed, fixed in acetone (100%) for 5 min, then air-dried at room temperature. After incubation with the antibodies shown, 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 assessed on a three-point scale according to the number of brown (positive) cells present: 0 = no cells positive; 1 = few cells positive among many negative cells; 2 = most cells positive. All smears were graded by one author. Intra-observer error was randomly checked to assess reproducibility. The entire area exposed to a particular antikeratin antibody was scanned for the presence of positively-stained cells.

RESULTS

The age range for the 33 cases was from 32 to 86 years (mean 68 years), with 19 males and 14 females. 6 patients admitted to smoking more than 10 cigarettes a day, and 18 patients admitted to both smoking and increased alcohol intake (greater than four units a day). 9 patients had never smoked nor taken excessive regular amounts of alcohol. The following intra-oral sites were involved: buccal mucosa 7 cases, floor of mouth (including ventral tongue) 17 cases, palate 7 cases and gingiva 2 cases.

The results of the DNA range profiles were expressed as either diploid or abnormal (signifying marked variation from the normal diploid state). Keratin expression was graded as described above. Smears from normal and malignant mucosa of all 33 individuals were tested with the antibodies listed in Table 1. In a few cases some smear samples had insufficient cells for the test to be scored, accounting for the lower total sample number in parts of Table 2. The results are shown in Table 2. The sensitivity, specificity and positive predictive value for each keratin and for DNA range profile alone are given in Table 3. Slight differences in total numbers of smears

Table 2. Analysis of keratin expression by DNA profile for smears of normal and malignant oral mucosa

	Keratin DNA content							
	K8		K19		K13		K10	
	N	A	N	A	N	A	N	A
Normal mucosa								
0	31	2	18	1	6	1	24	2
1	0	0	6	0	8	0	1	0
2	0	0	4	1	14	1	0	0
Malignant mucosa								
0	3	9	1	2	2	7	4	12
1	3	10	3	17	4	12	2	5
2	3	4	4	3	2	3	2	1

N, normal diploid; A, abnormal DNA range profile; 0, no cells positive to keratin; 1, few cells positive to keratin; 2, most cells positive to keratin.

occurred occasionally, when no cells were present for assessment.

It can be seen that whilst normal mucosal smears were invariably diploid, nine of the smears from the oral cancers were also diploid. No particular keratin profile was associated with an abnormal DNA profile, although most diploid smears from the oral cancers were found to have some cells expressing the simple epithelial keratins.

DISCUSSION

Previous results have indicated that clinically normal mucosa in healthy patients displays a diploid DNA range profile [2]. Furthermore, smoking, deficiency states and the menstrual cycle do not produce abnormal DNA range profiles [24, 25]. Of the various influences on normal oral mucosa studied so far only radiotherapy has been associated with a deviation from the diploid state of normal oral mucosa [25]. Even then values returned to the usual diploid state within 6 weeks of completion of treatment. Thus, abnormal DNA range profiles are almost invariably associated with malignant disease [26].

Abnormal DNA range profiles were observed in two smears collected from clinically-normal oral mucosa, whilst diploid DNA range profiles were observed in nine smears from biopsy-proven oral cancers. Thus these findings are at odds with our previous report [4]. Whilst two smears from clinically-normal oral mucosa displayed abnormal DNA range profiles, these were not the typical polyploid distributions that are seen in smears from malignant mucosa [4]. Approximately five cells out of the total of 50 appeared to be abnormal, hence an abnormal DNA range profile was recorded. Whether this is indicative of a field change is uncertain, but it is most interesting to note that both these cases expressed the keratin K19. This is matched by a smear graded 2 for K19 in 1 of the 2 cases, and could provide support for Lindberg and Rheinwald's hypothesis [27] that K19 is a marker of pre-malignancy. The other case, where an abnormal DNA range profile was detected in normal mucosa, did not express either keratins K8 or K19 but did get a recurrence, from which she did not survive. She was 84 years old and abstained from both alcohol and tobacco. Thus an abnormal DNA range profile from clinically normal oral mucosa, although extremely rare, may be very significant.

Diploid DNA range profiles, from cells in an oral cancer, are to be expected [7–9], yet until recently all our smears from oral cancers had displayed abnormal DNA range profiles. To ensure a successful diagnosis, the quality of the smear is extremely critical. If the lesions were small and situated posteriorly in the mouth then the combination of controlling salivary flow, gaining access and trying to avoid smearing the surrounding 'normal' mucosa, may have proved impossible. Thus, surrounding normal cells rather than tumour cells may have been harvested.

The majority of smears were processed for keratin expression within 2 weeks of collection. However, initial smears had been stored for up to 6 months. We have previously shown that such smears can be stored for at least 1 year, at $-70°C$, without a profound loss of keratin immunoreactivity [27].

The sensitivity of using DNA profile alone was found to be 70%. When keratin profiles were considered together with DNA range profiles, K8 and K19 were strongly associated with malignancy, particularly when combined with an abnormal DNA range profile (see Table 3 for statistical analysis). A surprising number of normal mucosal smears detected K19 (11 of 30) reflected in the lower specificity rate when compared to keratin 8 or DNA range profile alone. Although the potential for K19 to be a marker for malignancy is debatable [28–30], the combination of an abnormal DNA range profile and K19 expression using exfoliative cytology strongly suggests malignant disease (positive predictive value 95%). For K10 and K13 no obvious link was seen to a particular DNA state. The main disadvantage of K10 was its low sensitivity. However, there was a trend towards the identification of K10 in malignant tumours from non-cornifying sites (where K10 is rarely expressed).

The value of combining DNA range profiles with keratin expression is illustrated in Tables 2 and 3 where six of the nine diploid oral cancer smears were found to contain cells expressing K8, something which did not arise in the diploid normal mucosal smears. If one equates the expression of K8 in squamous epithelium as indicative of malignancy (since keratin 8 has been reported to be absent from keratinocytes in normal and benign oral mucosa of cancer-free individuals

Table 3. Statistical analysis of each keratin (K) by DNA profile (percentage values)

	K8			K19			K13			K10			DNA
	N+A	N	A	N+A	N	A	N+A	N	A	N+A	N	A	only
Sensitivity	62	66	60	90	87	90	70	75	68	38	50	50	70
Specificity	100	100	100	63	64	50	23	21	100	96	96	100	90
Positive predictive value	100	100	100	70	40	95	47	21	94	90	80	100	90

N, normal diploid DNA range profile; A, abnormal DNA range profile; N+A, DNA results combined.

[31–35], and yet has been identified in some oral cancers [11, 30, 32, 35, 36]), then its identification within oral smears of clinically suspicious lesions would strongly suggest malignant disease (positive predictive value 95%). In this study, combining the two parameters together, resulted in correct identification of a malignant tumour in 29 of 33 cases.

In conclusion, the results outlined in this study suggest that oral cancer detection rate could be substantially improved by combining the assessment of DNA range profiles and keratin expression in oral mucosal smears, and warrants continued investigation.

1. Dabelsteen E, Roed-Petersen B, Smith CJ, Pindborg JJ. The limitations of exfoliative cytology for the detection of epithelial atypia in oral leukoplakias. *Br J Cancer* 1971, **25**, 21–24.
2. Cowpe, JG, Longmore RB, Green MW. Quantitative exfoliative cytology of normal oral squames: an age, site and sex related survey. *J R Soc Med* 1985, **78**, 995–1004.
3. Ogden GR, Green MW, Cowpe JG. Quantitative assessment of various fixatives on nuclear and cytoplasmic area in oral cytology. *Histochem J* 1989, **21**, 703–706.
4. Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytology of abnormal oral mucosal smears. *J R Soc Med* 1988, **81**, 509–513.
5. Cowpe JG, Longmore RB. Nuclear area and Feulgen DNA content of normal buccal mucosal smears. *J Oral Pathol* 1982, **10**, 81–86.
6. Ogden GR, Cowpe JG. Quantitative cytophotometric analysis as an aid to the detection of recurrent oral cancer. *Br J Oral Maxillofacial Surg* 1989, **27**, 224–228.
7. Franzen G, Klitenberg C, Olofsson J, Risberg B. DNA measurements—an objective predictor of response to irradiation? A review of 24 squamous cell carcinomas of the oral cavity. *Br J Cancer* 1986, **53**, 643–651.
8. Tytor M, Franzen G, Olofsson J, Brunk U, Nordenskjold B. DNA content, malignancy grading and prognosis in T1 and T2 oral cavity carcinomas. *Br J Cancer* 1987, **56**, 647–652.
9. Kearsley JH, Furlong KL, Cooke RA, Waters MJ. An immunohistochemical assessment of cellular proliferation markers in head and neck squamous cell cancers. *Br J Cancer* 1990, **61**, 821–827.
10. Lane EB, Alexander CM. Use of keratin antibodies in tumor diagnosis. *Semin Cancer Biol* 1990, **1**, 165–179.
11. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelial tumors and cultured cells. *Cell* 1982, **32**, 11–24.
12. Moll R, Schiller DL, Franke WW. Identification of protein IT of the intestinal cytoskeleton as a noval type I cytokeratin with unusual properties and expression patterns. *J Cell Biol* 1990, **111**, 567–580.
13. Osborn M, Weber K. Tumor diagnosis by intermediate filament typing: a novel tool for surgical pathology. *Lab Invest* 1983, **48**, 372–394.
14. Schlimok G, Funke I, Holzmann B, *et al.* Micrometastatic cancer cells in bone marrow: *in vitro* detection with anti-cytokeratin and *in vivo* labeling with anti-1701A monoclonal antibodies. *Proc Natl Acad Sci USA* 1987, **84**, 8672–8676.
15. Nadji M, Ganjei P. Immunocytochemistry in diagnostic cytology: a 12-year perspective. *Am J Clin Pathol* 1990, **94**, 470–475.
16. Ogden GR, McQueen S, Chisholm DM, Lane EB. Keratin profiles of normal and malignant oral mucosa using exfoliative cytology. *J Clin Pathol* 1993, **46**, 352–356.
17. Johnson NW. Orofacial neoplasms: global epidemiology, risk factors and recommendations for research. F.D.I. Technical Report No. 36. London, 1990, 10.
18. Bedi KS, Goldstein DJ. Apparent anomalies in nuclear Feulgen DNA contents: the role of systematic microdensitometric errors. *J Cell Biol* 1976, **71**, 68–88.
19. Lane EB, Bartek J, Purkis PE, Leigh IM. Keratin antigens in differentiating skin. *Ann NY Acad Sci USA* 1985, **455**, 241–258.
20. Dhouailly D, Xun C, Monabe M, Schermer A, Sun T-T. Expression of hair-related keratins in a soft epithelium: sub populations of human and mouse dorsal tongue keratinocytes express keratin markers for hair, skin and oesophageal types of differentiation. *Exp Cell Res* 1989, **181**, 141–158.
21. Leigh, IM, Purkis, PE, Whitehead P, Lane EB. Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as markers of epidermal differentiation. *Br J Dermatol* 1993, **129**, 110–119.
22. Stasiak P, Purkis PE, Leigh IM, Lane EB. Keratin 19: predicted amino acid sequence and broad tissue distribution suggest it evolved from keratinocyte keratins. *J Invest Dermatol* 1989, **92**, 707–716.
23. Makin CA, Bobrow LG, Bodmer WF. Monoclonal antibody to cytokeratin for use in routine histopathology. *J Clin Pathol* 1984, **37**, 975–983.
24. Ogden GR, Cowpe JG, Green MW. Quantitative exfoliative cytology of normal buccal mucosa: effect of smoking. *J Oral Pathol Med* 1990, **19**, 53–55.
25. Connor E, Cowpe JG, Lewis MAO, Ogden GR. DNA-content, nuclear and cell size of buccal smears in iron deficient patients. *J Dent Res* 1994, **73**, (in press).
26. Tucker JH, Cowpe JG, Ogden GR. Nuclear DNA content and morphometric characteristics of normal, premalignant and malignant oral smears. *Anal Cell Pathol* 1994, **6**, 117–128.
27. Ogden GR, Chisholm DM, Green M, Cowpe JG, Lane EB. Influence of temperature on long term keratin immunoreactivity for oral exfoliative cytology. *Analyt Quart Cytol Histol*, in press.
28. Lindberg K, Rheinwald JG. Suprabasal 40 kd keratin (K19) expression as an immunohistologic marker of premalignancy in oral epithelium. *Am J Pathol* 1989, **134**, 89–98.
29. Ogden GR, Chrisholm DM, Leigh IM, Lane EB. Cytokeratin profiles in Dyskeratosis congenita: an immunocytochemical investigation of lingual hyperkeratosis. *J Oral Pathol Med* 1992, **21**, 353–357.
30. Ogden GR, Chisholm DM, Adi M, Lane EB. Cytokeratin expression in oral cancer and its relationship to tumour differentiation. *J Oral Pathol Med* 1993, **22**, 82–86.
31. Morgan PR, Leigh IM, Purkis PE, Gardiner ID, van Muijen GNP, Lane EB. Site variations in keratin expression in human oral epithelia—an immunocytochemical study of individual keratins. *Epithelia* 1987, **1**, 31–43.
32. Morgan PR, Shirlaw PJ, Johnson NW, Leigh IM, Lane EB. Potential applications of antikeratin antibodies in oral diagnosis. *J Oral Pathol* 1987, **16**, 212–222.
33. Sawaf MH, Ouhayoun JP, Shabana AHM, Forest N. Cytokeratin expression in human tongue epithelium. *Am J Anat* 1990, **189**, 155–166.
34. Williams DM, Leigh IM, Greenspan D, Greenspan JS. Altered patterns of keratin expression in oral hairy leukoplakia: prognostic implications. *J Oral Pathol Med* 1991, **20**, 167–171.
35. Sawaf MH, Ouhayoun JP, Forest N. Cytokeratin profiles in oral epithelia: a review and a new classification. *J Biol Buccale* 1991, **19**, 187–198.
36. Vaidya MM, Borges AM, Pradham SA, Raipal RM, Bhissey AN. Altered keratin expression in buccal mucosal squamous cell carcinoma. *J Oral Pathol Med* 1989, **18**, 282–286.

Acknowledgements—This research was in part funded by the Medical Research Council and Cunningham Trust. We thank Dr M. Green for statistical advice and Mrs D. Morrison for typing the manuscript.