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# Expression of Human Cytokeratin 14 in Normal, Premalignant and Malignant Oral Tissue Following Isolation by Plaque Differential Hybridisation

**J. J. Marley, P. A. Robinson and W. J. Hume**

**Differences in gene transcription between RNA samples extracted from oral normal and squamous cell carcinoma (SCC) tissue were examined using the technique of cDNA library differential plaque screening. A differentially expressed transcript was selected on the basis of it being under-expressed in the cancer tissue and was identified, using DNA sequencing, as cytokeratin 14. The level of cytokeratin 14 transcription in RNA samples extracted from a range of oral SCC and normal tissue, as well as "white patch" lesions, was then investigated. Cytokeratin 14 appeared to be significantly under-expressed in oral cancer specimens studied compared to normal and white-patch tissue ($P < 0.01$). The trend for higher levels of cytokeratin 14 transcription in the dysplastic "white patch" samples compared to that observed for the malignant tissue ($P < 0.05$) suggests that the decrease in cytokeratin 14 transcription is a late event in the carcinogenic pathway.**

**Keywords: genes, RNA, cDNA, cytokeratins, oral cancer, differential hybridisation**
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## INTRODUCTION

PREVIOUS ATTEMPTS have been made to rationalise the prognostic assessment of both premalignant and malignant lesions of the mouth by combining histological (grading) and clinical (staging) observations. These factors have been demonstrated to have their limitations, especially where an attempt is made to predict malignant development from premalignant lesions [1]. Emphasis is often placed on morphological assessment of lesions by light microscopic histological evaluation. This may be too limiting, as it does not take into account other individual factors which affect the biological behaviour of a given lesion [2]. It is generally assumed that dysplastic lesions have a greater propensity for malignant conversion [3] and the more severe the dysplasia, the greater the risk [4]. However, the grade of dysplasia alone is not a reliable predictor of prognosis [1]. Not all cases in which dysplastic epithelium is present will progress to malignancy, and some may even regress [5].

There has, therefore, been increased interest in alternative diagnostic/prognostic methods, the aim of which must be to predict the true biological potential more accurately than conventional histology and, ultimately, to unmask those few lesions which present as histologically innocuous, but which ultimately develop into invasive carcinomas (for reviews, see [6, 7]). There remains an overwhelming need to identify probes which could be used to investigate molecular changes which occur earlier in the carcinogenic pathway than demonstrable histological changes. The techniques of molecular genetics present a means of relating the apparently disparate morphological features of premalignant and malignant lesions to the alterations of gene regulation and expression. Changes in the expression patterns of cellular genes are implicit in tumour progression [8] and may be manifested by altered levels of cytoplasmic messenger RNA (mRNA) or protein. Differential plaque hybridisation can be used to identify differences in the levels of individual mRNA moieties between two or more mRNA populations [9]. The approach is to construct complementary DNA (cDNA) libraries from at least one of the RNA populations under investigation, e.g. RNA extracted from oral squamous cell carcinoma (SCC). This produces a library of information relating to gene expression patterns in the tissue at the time of resection/biopsy; the population of cDNA should reflect the corresponding levels of mRNA in the tissue at that time. The libraries are then screened with cDNA probes prepared from RNA extracted from tissues under study, e.g. oral SCC and normal tissue. The resultant autoradiographic signals are examined and cDNA clones that are differentially expressed between the SCC and

Correspondence to J. J. Marley at the Division of Dental Surgery, School of Clinical Dentistry, Queen's University Belfast, Grosvenor Road, Belfast BT12 6BP, U.K.
P. A. Robinson and W. J. Hume are at the Division of Oral Surgery, Leeds Dental Institute, Clarendon Way, Leeds LS2 9LU, U.K.
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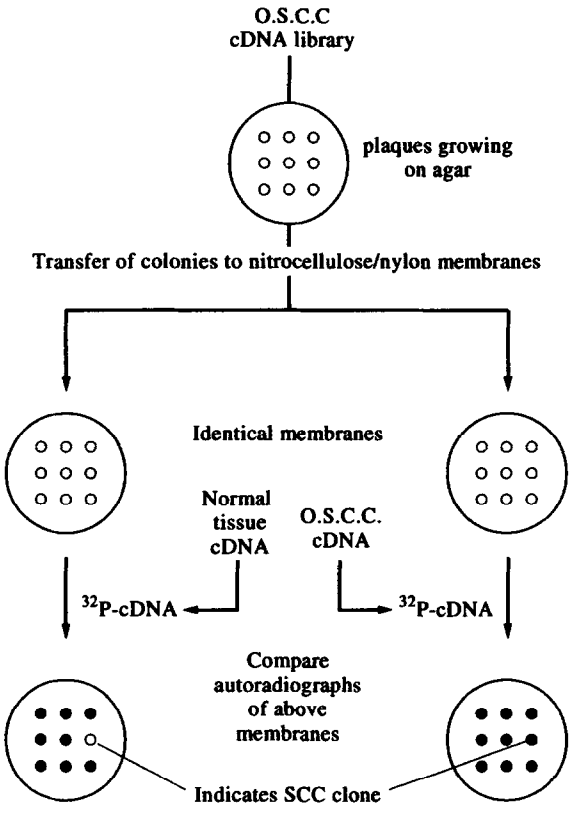


Fig. 1

*Table 1. Patient groups divided according to sex and age*

| | Normal | White patch | Squamous carcinoma |
|---|---|---|---|
| No. of males | 9 | 9 | 6 |
| Mean age (years) | 29 | 49 | 56 |
| Range (years) | 21–39 | 29–68 | 46–71 |
| No. of females | 12 | 17 | 3 |
| Mean age (years) | 26 | 57 | 71 |
| Range (years) | 18–40 | 24–74 | 64–72 |
| Total no. | 21 | 26 | 9 |

its normal counterpart identified and investigated for possible use as tumour markers (see Fig. 1).

We describe in this manuscript a comparison of gene expression between normal palatal mucosa and oral SCC using plaque differential hybridisation and the subsequent identification of cytokeratin 14 as being differentially expressed in other oral malignant tissue compared with normal and "white patch" tissue.

## PATIENTS AND METHODS

*Patient information*

Patients were divided into three groups depending on their presentation—normal, white patch and squamous carcinoma. These were further subdivided in terms of age and sex distribution, as described in Table 1.

*Table 2. Histological diagnosis and position of white patch lesions and staging and position of oral SCC lesions*

| | White patch | | | Oral SCC | |
|---|---|---|---|---|---|
| Sample | Diagnosis | Site | Sample | Stage | Site |
| 1 | FK | BM | 27 | 1 | ? |
| 2 | MOA | SL | 28 | 2 | L |
| 3 | FTH | SL | 29 | 4 | A |
| 4 | K | T | 30 | 4 | T |
| 5 | MA | SL | 31 | 4 | $2^0$ |
| 6 | MA | BM | 32 | 4 | P |
| 7 | MA | SL | 33 | 4 | T |
| 8 | MA | BM | 34 | 4 | SL |
| 9 | MOA | BM | 35 | 4 | M |
| 10 | SA | SL | | | |
| 11 | K | BM | | | |
| 12 | K | BM | | | |
| 13 | K | R | | | |
| 14 | K | R | | | |
| 15 | K | BM | | | |
| 16 | K | BM | | | |
| 17 | C | BM | | | |
| 18 | C | BM | | | |
| 19 | C | BM | | | |
| 20 | LP | BM | | | |
| 21 | FEP | T | | | |
| 22 | CIS | BM | | | |
| 23 | VH | BM | | | |
| 24 | SCP | T | | | |
| 25 | AK | L | | | |
| 26 | WSN | BM | | | |

FK, frictional keratosis; MOA, moderate atypia; FTH, fibrous tissue hyperplasia; K, hyperkeratosis; MA, mild atypia; SA, severe atypia; C, candidosis; LP, lichen planus; FEP, fibroepithelial polyp; CIS, carcinoma *in situ*; VH, verucous hyperplasia; SCP, squamous cell papilloma; AK, actinic keratosis; WSN, white sponge naevus; BM, buccal mucosa; SL, sub-lingual; L, lip; R, retromolar; T, tongue; A, antrum; P, pharynx; M, mandible; $2^0$, secondary; ?, unknown site.

*Sample information*

The abnormal tissue samples, i.e. "white patches" and squamous carcinoma, were further subdivided according to histopathology, site and staging where applicable and are described in Table 2. Normal and oral SCC tissue for cDNA library construction was obtained from M. Corrigan (Consultant Oral and Maxillofacial Surgeon, Division of Dental Surgery, Leeds Dental Institute, Leeds, U.K.). Normal oral tissue was derived from a palatal flap of a female 15-year-old non-smoker patient presenting for surgical exposure of an unerupted canine tooth. Oral SCC tissue was derived from a lesion of the right retromolar region of a 53-year-old, female smoker (20 cigarettes/day).

All other patients attended the Departments of Oral Surgery in either Sunderland General Hospital, Sunderland; Bradford Royal Infirmary and St. Lukes Hospital, Bradford; or the Leeds Dental Institute, Leeds.

Twenty-one biopsies were collected from patients attending clinics for removal of impacted wisdom teeth. Biopsies were taken from mucosal flaps raised during surgical removal at points free from visible signs of inflammation. These samples were taken as being normal samples.

26 patients presented for biopsies of white patches and 9 patients were diagnosed as having oral SCC from a previous incisional biopsy. Lesions were removed and divided for

histopathology assessment after paraffin embedding or storage in liquid nitrogen. Necrotic areas of tissue were avoided in all cases. Histopathology assessment was performed by either A. High or W. J. Hume (Consultants in Oral Pathology, Division of Dental Surgery, Leeds Dental Institute, Leeds, U.K.). The white-patch group was subdivided on the basis of their histopathology; mild, moderate and severe atypias were classified as potentially premalignant. Hyperkeratotic, chronic candidosis and other miscellaneous lesions are also within this clinical definition of white patch. Three of the non-dysplastic white patch samples; 3, 21 and 26, were included as controls.

Oral SCC was classified on the basis of the TNM system [10].

*RNA extraction and northern dot blot analysis*

Total cellular RNA used for library construction was extracted as described previously [11] and analysed by formaldehyde gel electrophoresis [12]. Transfer of RNA from agarose gels to Hybond $N^+$ membranes (Amersham International, Aylesbury, Bucks, U.K.) was performed as per manufacturer's instructions.

RNA dot blots were carried out using the Bio-Dot SF Blotting Apparatus (Bio-Rad Laboratories, Hemel Hempstead, Herts, U.K.) as per manufacturer's instructions. One microgram of total RNA was loaded into each well of the dot blot apparatus. Chick $\beta$-actin was used as a control probe in dot blot analysis.

*cDNA library construction*

cDNA libraries were constructed in lambdaGEM2 using total cellular RNA extracted from individual normal ($1 \times 10^6$ independent plaque forming units, pfu) and malignant oral mucosal ($1 \times 10^8$ pfu) biopsies as described previously [13].

*Probe construction*

*Differential screening.* As all the RNA for library construction was used, from the normal and oral SCC samples, cDNA probes for the differential screening had to be prepared directly from the cDNA libraries. Plate lysates of the normal and oral SCC libraries were prepared on 15-cm agar plates as described previously [14]. Bacteriophage particles were eluted into 5 ml SM buffer (10 mM tris/HCl, containing 10 mM magnesium chloride, 50 mM sodium chloride and 0.01% gelatin) per plate and phage DNA purified as described previously [15].

cDNA was amplified using the polymerase chain reaction (PCR) using vector-specific primers that flanked the cDNA insertion site. In brief, 100 ng of DNA were added to 100 µl 10 mM Tris/HCl buffer, pH 9.0 (25°C), containing 50 mM KCl, 2.0 mM $MgCl_2$, 200 µM dNTP, 0.05% Tween 20, 0.05% NP40, 0.01% gelatin, 50 pmoles lambdaGem2 SP6 (5′ ATTTAGGTGACACTATA 3′) and T7 (5′ TAATACGACTCACTAT 3′) promoter primers and 2.5 U Taq polymerase. Amplification conditions were 35 cycles of denaturation (1 min, 96°C), annealing (2 min, 55°C) and chain extension (10 min, 74°C). An extended elongation time was used due to the heterogeneous population of inserts being amplified [16]. Amplified cDNA was purified by phenol/chloroform extraction and ethanol precipitation. Sedimented cDNA was dissolved at approximately 1 mg/ml in double-distilled, autoclaved water (approximately 15 µl per 100 µl PCR). Radiolabelled cDNA probes were then prepared using 100 ng amplified cDNA by random priming using the Megaprime Labelling Kit (Amersham Int, Aylesbury, U.K.) as per manufacturer's instructions. cDNA probe was prepared from the normal cDNA library (normal cDNA probes) and from the oral SCC library (cancer cDNA probe).

*Single probes.* Suspensions of individually picked bacteriophage plaques were employed for the preparation of individual radiolabelled cDNA probes for cDNA library screening and northern dot blot analysis. An aliquot (1 µl) of each selected freshly resuspended agar plug was taken without further purification and amplified directly using PCR as described above except that an extension time of 3 min was employed as the solution was homogeneous for one insert. Amplified DNA was separated by agarose gel electrophoresis and bands corresponding to the amplified DNA products were purified using a Mermaid Purification Kit (Stratech Scientific Ltd., Luton, U.K.) as per manufacturer's instructions.

*Differential plaque screening*

Plaque lifts of the oral SCC cDNA library were carried out using Hybond N+ nylon membranes (Amersham) as described by the manufacturers and differentially screened with radiolabelled normal and oral SCC cDNA probes (see below) at high density (approximately $3 \times 10^3$ pfu/8 cm plate). Plaques that gave differential signals on the resultant autoradiographs were eluted into 100 µl SM buffer. Because of the close proximity of individual pfus a low density differential screen (approximately 300 pfu/8 cm plate) of the picked plaques using the same PCR generated normal and oral SCC probes was performed so that individual differentially-expressed plaques of virus could be obtained. Radiolabelled probes were also prepared from these individual cDNA inserts and used to screen aliquots of the unamplified libraries to determine the percentage of plaques containing the insert. This percentage should reflect the relative abundance of the insert mRNA in the population used for library construction [17].

*Prehybridisation, hybridisation, washing and autoradiography*

For all plaque differential screens, northern and Southern blots, prehybridisation and hybridisation were carried out in bags in a Hybaid hybridisation oven in the presence of 50% (v/v) formamide at 42°C (for northern blots) and without formamide at 65°C (for Southern blots) as per manufacturer's instructions (Amersham). Membranes were put up for autoradiography with intensifying screens at 20°C. Where duplicate membranes were autoradiographed the exposure time was identical. Exposure times varied from 24 h (differential plaque screens) to 1 week (northern blots). Autoradiographs were developed using a Durr Med 250 automatic developer.

Densitometry of autoradiographs was carried out using a Bromma Ultrascan Lazer Densitometer. The areas underlying the densitometer curves were compared using the Student's *t*-test.

*Sequencing reactions*

Sequencing reactions were carried out using the Sequenase version 2.0 sequencing kit following manufacturer's instruc-

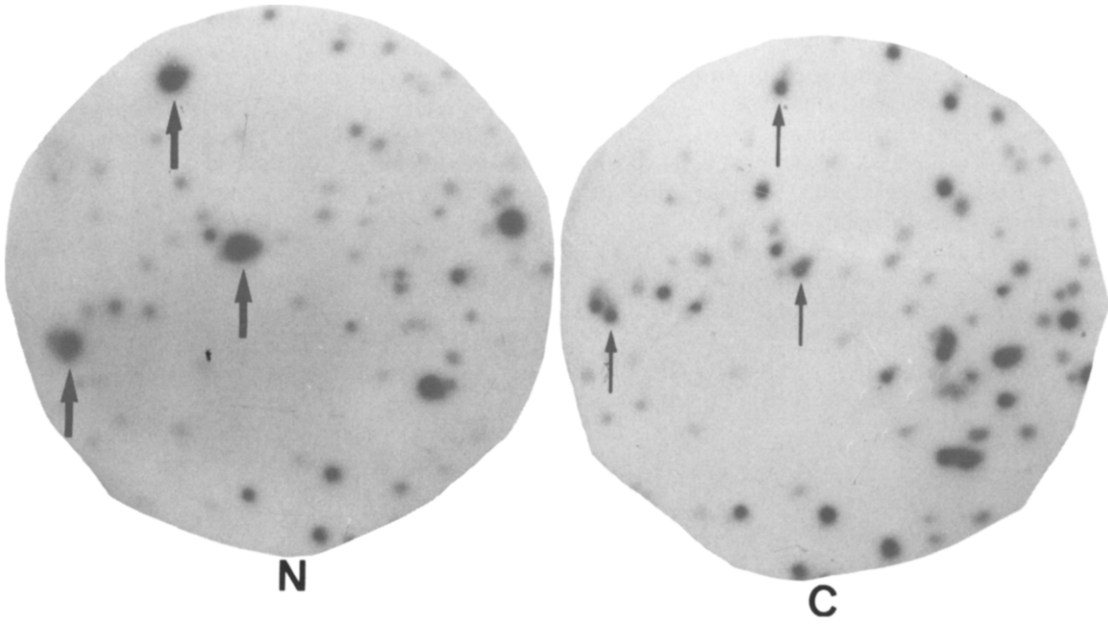


**Fig. 2. Low density differential screening of picked plaques. An example of duplicate membranes of a low density plating of plaque X is shown. These were screened with normal (N) and cancer (C) library cDNA probes. Putatively differentially expressed plaques are indicated with arrows.**

tions (U.S. Biochemical Corporation, distributed by Cambridge Bioscience, Cambridge, U.K.). The reaction products were electrophoresed on denaturing polyacrylamide electrophoresis gels using the Sequi-Gen Sequencing System. (BioRad Laboratories Ltd, Watford, U.K.) as per manufacturer's instructions.

## RESULTS

*Differential screening of normal and oral SCC libraries*

A high density differential screening of the cancer cDNA library was performed and duplicate membranes of a high density plating of the oral SCC cDNA library were probed with normal and cancer cDNA probes. Plaques were identified whose expression was increased in the cancer population and also those whose expression was decreased. A total of 20 plaques were selected for further characterisation.

A low density differential screening of picked plaques was carried out to obtain a population consisting of a single clone. An example of duplicate membranes of a low density plating of a selected plaque (plaque X) from the high density screen is shown in Fig. 2. This was differentially screened with normal and cancer cDNA probes solutions as before. Putatively differentially expressed plaques are indicated with arrows.

It appeared the insert of plaque X was under-expressed by 50% in the oral SCC cDNA library compared to that observed with the normal cDNA library used for the differential screening (Fig. 3). It was present in a total of 0.5% of the total population of plaques in the normal cDNA library. Due to its apparent under-expression in oral SCC compared with the normal tissue it was selected for further investigation.

A comparison of the deduced protein sequence data of the cDNA insert of plaque X with those in the OWL protein data base (Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, U.K.) revealed that this cDNA insert was homologous to part of the amino acid sequence of coil 1B of human cytokeratin 14.

*Northern dot blots*

A dot blot analysis of total RNA extracted from a range of normal oral SCC and "white patch" tissue with the cytokeratin 14 cDNA probe was performed (Fig. 4, Table 3). The intensities of the resultant autoradiographic signals were assessed by lazer densitometry compared to a chick β-actin control screen of the stripped membrane. All RNA samples expressed cytokeratin 14 (Fig. 5). However, it was apparent that mRNA corresponding to cytokeratin 14 was significantly under-expressed in oral SCC samples ($n=9$) compared with normal tissue ($n=21$), using the Student's $t$-test ($P<0.01$). There was a trend for increased expression of cytokeratin 14 in those "white-patch" lesions exhibiting various degrees of atypia ($n=8$), as described in Table 2 compared with the malignant lesions ($P<0.05$). This may suggest that the observed decrease in its expression in oral cancer is a late event.

## DISCUSSION

The approach adopted in this study was based on the concept that differential gene expression accompanies malignant conversion and progression [8]. In order to identify these differentially expressed genes the technique of plaque differential hybridisation was employed [9]. We identified a cDNA insert using this technique that coded for cytokeratin 14. This appeared to be under-expressed in oral SCC library examined, as well as in a range of oral SCCs, compared with normal oral tissue.

Human cytokeratins belong to a group of structural proteins known as intermediate filaments and are characteristic of epithelial cells [18]. Together with the actin-based microfilaments and tubulin-containing microtubules, the inter-

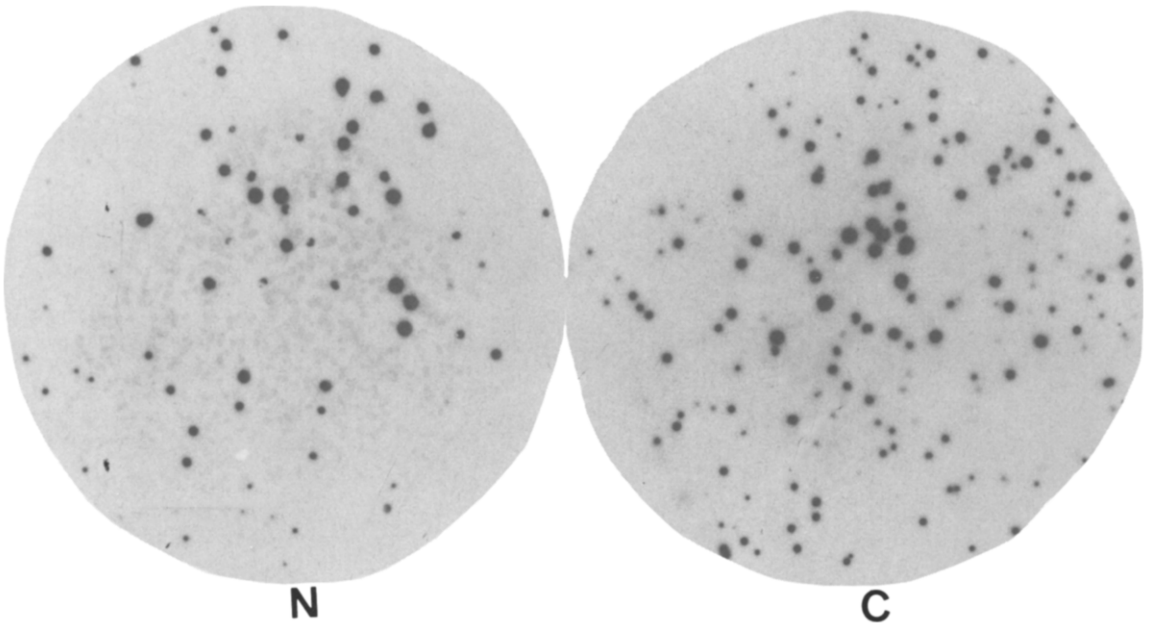


**Fig. 3. Normal and cancer cDNA libraries probed with plaque X (cytokeratin 14) cDNA insert. Plaque lifts were taken of original normal (N) and cancer (C) libraries at equal pfu densities. These lifts were then screened with radiolabelled cytokeratin 14 cDNA probe to confirm the differential expression of this moiety.**

*Table 3. Key of tissue sample position on dot blot*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| A | N | N | N | 5 | 4 | 18 | 25 | 34 | 35 |
| B | N | N | N | 6 | 1 | 19 | 26 | 28 | |
| C | N | N | N | 7 | 12 | 20 | | 29 | |
| D | N | N | N | 8 | 13 | 21 | | 30 | |
| E | N | N | N | 9 | 14 | 22 | | 31 | |
| F | N | N | | 2 | 15 | 23 | | 32 | |
| G | N | N | | 10 | 16 | 3 | | 33 | |
| H | N | N | | 11 | 17 | 24 | | 27 | |

N, Normal. Numbers indicate sample number (see Table 2).

mediate filaments make up the three fibrous systems of the cytoskeleton [19].

Cytokeratin expression patterns are site-specific [18] and indicative of epithelial differentiation patterns. These patterns can vary in pathological states, including malignancy [20]. Their use as diagnostic markers in these cases relies on the identification of consistent changes in the expression pattern and with this in mind a number of workers have attempted to assess the variations in expression patterns of these proteins in normal and abnormal oral mucosa, including oral premalignant and malignant lesions [21–24].

These studies have used biochemical and immunohistochemical techniques [25] which can prove problematical [22]. Recent investigations in similar fields at the level of RNA *in situ* hybridisation have been undertaken which overcome some of these difficulties [26].

It is important, however, to ascertain whether the observed variations in the expression of this moiety are related to the disease process or are merely normal site-specific variations. Attempts have been made to deduce a base-line of cytokeratin 14 protein expression for various normal sites and it appears to be decreased in areas of low cornification [21]. Arguments which would relate our observed expression of cytokeratin 14 to the site of a particular lesion can be answered, at least in part, as the premalignant material (see Table 2) presented in areas of low cornification and yet this moiety was over-expressed compared to malignant tissue arising from areas of well cornified mucosa.

In addition, it is also important to ensure that variations in the expression pattern of cytokeratin 14 are not due to quantitative variations in cell numbers in the heterogeneous cell populations of the tissue biopsies under examination. We, therefore, attempted to screen a wide range of tissue sample RNA. These were considered to have comparable proportions of cell types and would, therefore, help to eliminate erroneous results due to cell population differences and select truly differentially expressed moieties. Preliminary *in situ* hybridisation studies using PCR-generated cDNA probes also tended to confirm our observations (data not shown).

Human cytokeratin 14 co-exists as a heterodimer with a smaller basic intermediate filament cytokeratin 5 and is found in the basal cell layer of stratified squamous epithelium [22]. Recent studies have highlighted its structural importance in the integrity of the epidermis [27, 28]. Very little is known about its role in oral carcinogenesis. It is not inconceivable that its basal distribution and its apparent decrease in expression in oral SCC may be a factor in the genesis of invasive SCC. It is interesting that the levels of expression of cyctokeratin 14, as well as other cytokeratins, actually decrease on treatment with retinoic acid (RA) [29]. RA and its derivatives have been proposed as topical and systemic medications for the treatment of oral cancer and precancer, amongst other micronutrients [30]. This finding would appear to conflict with our speculations, but may merely reflect the predominant anti-tumour functions of retinoids in modulating epithelial terminal differentiation and stimulating tissue Langherans cells [31, 32].

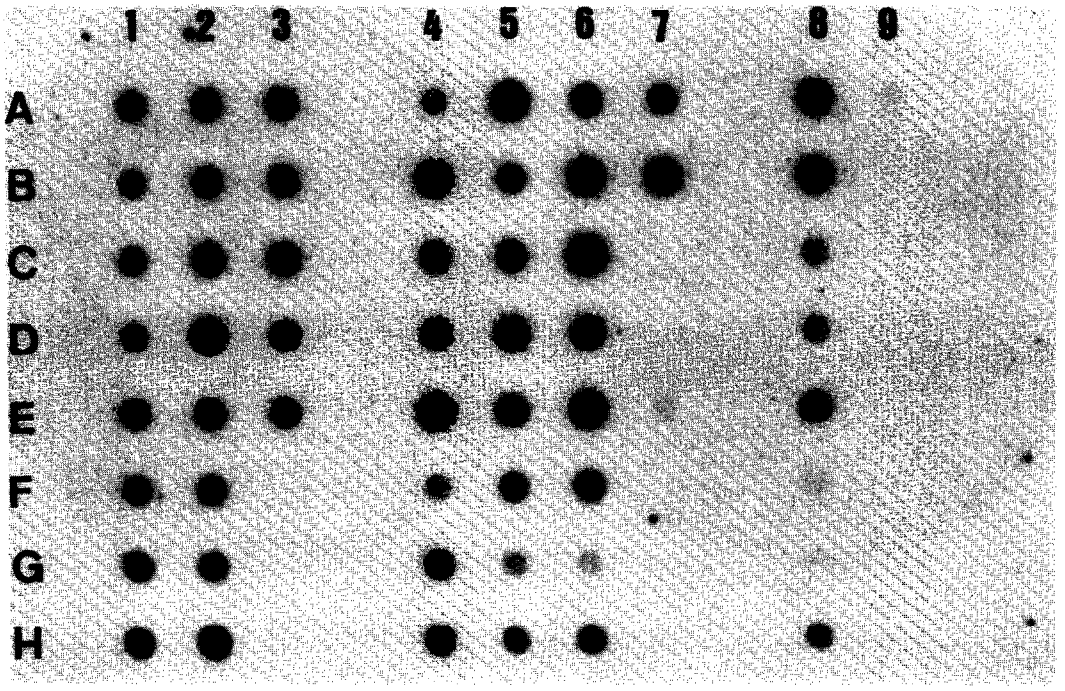


**Fig. 4. Analysis of cytokeratin 14 expression in total cellular RNA extracted from normal, premalignant and malignant oral tissue. A dot blot analysis of total RNA extracted from a range of normal, white patch (including premalignant) and oral SCC with cytokeratin 14 was performed as described in Materials and Methods. See Table 2 for description of lesions. β-actin screening of the stripped membrane was carried out as a control (data not shown).**

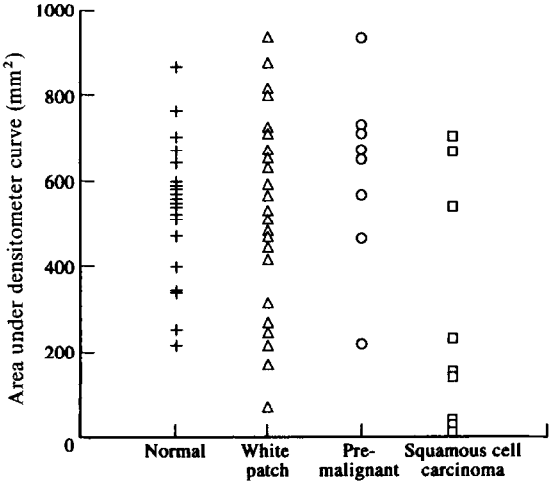


**Fig. 5**

The major problem for both library construction and probe preparation was the paucity of tissue available for RNA extraction and, consequently, the limited supply of mRNA from biopsy samples. It was reasoned that attempts to purify mRNA from small quantities of total cellular RNA, e.g. by oligo(dT)-cellulose chromatography and ethanol precipitation, would lead to further loss of already limited mRNA through the inefficiency of the purification techniques and also potentially exposure of the purified mRNA to ribonuclease contamination. A method was, therefore, developed to prepare representative cDNA libraries from small quantities of total RNA [13]. In addition, we had to develop a procedure to prepare DNA probe solutions, that were representative of the mRNA populations that were used for library construction. Probes were, therefore, constructed directly from the libraries themselves based on work carried out by others [16, 33, 34].

These results confirm the usefulness of the assay system for the detection and characterisation of putative differentially expressed clones that may have the potential to monitor malignant transformation and progression. The further development of this approach could produce nucleic acid probes that may (i) allow the earlier detection of potentially malignant changes at a molecular level in biopsy tissue, (ii) introduce a complementary method to assist in the histological assessment of oral premalignant lesions and (iii) assist in the surgical management of these lesions by allowing surgical resection margins to be probed for molecular abnormalities. It is felt that such an approach would have extremely important prognostic implications in the management of oral precancer and cancer. The other differentially expressed moieties identified in this study are currently under investigation [35].

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