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p53 protein expression in malignant, pre-malignant and non-malignant lesions of the lip

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Abstract

***Aim*—To elucidate the role of the p53 tumour suppressor gene in the pathogenesis of lip cancer.**

***Methods*—Expression of p53 was evaluated immunocytochemically in a retrospective study of formalin fixed, paraffin wax embedded tissue. Five cases each of four types of lip lesions were studied; these comprised squamous cell carcinoma (SCC), solar keratosis (SK), chronic hyperplastic candidosis (CHC), and lichen planus (LP). Five cases each of normal lip mucosa, SCC, and SK from sun exposed facial skin as well as LP, CHC, and SCC from buccal mucosa were also analysed. Immunolocalisation of p53 was scored semiquantitatively. The degree of apoptosis was also assessed in selected lesions by determining cell nuclear fragmentation.**

***Results*—All SCCs from lip lesions were immunopositive for p53. All cases of SK and two of five CHC lip lesions were also p53 positive. Normal lip mucosa samples were p53 negative. Sun exposed skin lesions of SCC and SK were all positive for p53, but only three of five cases of SCC from the buccal mucosa had detectable levels of p53. p53 expression was not detected in CHC and LP lesions of the buccal mucosa.**

***Conclusions*—The aberrant expression of p53 is likely to occur early in the pathogenesis of lip cancer and may be related to exposure to the sun. The immunopositive p53 cells identified in the benign LP lesions do not necessarily correlate with commitment of cells within the lesion to programmed cell death. In light of the prior reports which indicate that p53 positive cells may progress to form malignant tumours, it is suggested that patients with p53 positive but otherwise benign lesions should be followed more closely.**

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Keywords: lip lesions, p53, benign, pre-malignant, malignant.

Altered expression of the p53 tumour suppressor gene is the most common genetic mutation found in human cancer.[1 2] Aberrant expression of this gene often results in the build-up of the p53 protein product within affected cells, enabling it to be detected immunocytochemically. However, doubts about whether p53 overexpression is actually indicative of a p53 mutational event has called into question the clinical significance of p53 immunolocalisation in pathological specimens.[3] More recent elucidation of the alternative cellular caveats that can lead to the accumulation of cellular p53 in the absence of p53 mutations,[4] however, provides reassurance of the value of determining aberrant p53 expression in tissue sections and has reaffirmed the clinical interest in localising p53 immunocytochemically.[5]

The purpose of this study was to measure p53 immunoreactivity in non-malignant, pre-malignant and malignant lesions of the lip. It has been shown in previous investigations that p53 overexpression occurs in 88% of 26 lip cancers; this has clearly identified a significant role for p53 in this form of malignant disease.[6] In ascertaining p53 immunoreactivity in benign as well as pre-malignant lesions, this study sought to establish whether changes in p53 occur as early or late stage events in the pathogenesis of lip cancer and whether changes in p53 expression occur in non-malignant diseases of the lip. Furthermore, potential correlations between p53 immunoreactivity and a commitment to programmed cell death have been considered, for a selection of lip lesions, by assaying for DNA fragmentation in the tumour cell nuclei.

Methods

A retrospective study was performed on four different types of lip lesion: squamous cell carcinoma (SCC), solar keratosis (SK), chronic hyperplastic candidosis (CHC), and lichen planus (LP). For each type of lesion, five different cases were selected to give a representative sample. Similarly, five cases each of SCC and SK from sun exposed facial skin, and lesions of SCC, LP and CHC from buccal mucosa were obtained at random from the archives.

The histological criteria adopted for case selection were as recommended by the World Health Organisation (WHO).[7 8] In addition, for oral (including lip) LP and CHC, the criteria were those suggested by Cawson *et al*.[9] For both skin and oral mucosa, well differentiated (grade 1) cases of SCC were selected.

All tissues had been fixed in buffered formal saline (pH 7.2) and embedded in paraffin wax. Samples of normal lip mucosa, taken from sun

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Table 1 Patient details, site and type of lesion, and grade of p53 expression

Case number	Sex	Age (years)	Site	Lesion	p53 grade
1	M	69	Lip	SCC	4
2	M	61	Lip	SCC	4
3	M	55	Lip	SCC	3
4	M	54	Lip	SCC	2
5	F	0	Lip	Normal mucosa	1
6	F	18	Lip	Normal mucosa	1
7	M	37	Lip	Normal mucosa	1
8	M	38	Lip	Normal mucosa	1
9	F	57	Lip	Normal mucosa	1
10	F	35	Lip	Normal mucosa	1
11	M	68	Lip	CHC	1
12	M	79	Lip	CHC	2
13	F	41	Lip	CHC	2
14	M	80	Lip	CHC	1
15	F	37	Lip	CHC	1
16	F	67	Lip	LP	2
17	M	37	Lip	LP	2
18	M	?	Lip	LP	2
19	F	67	Lip	LP	2
20	F	43	Lip	LP	2
21	F	76	Lip	SK	2
22	F	67	Lip	SK	3
23	F	64	Lip	SK	3
24	M	66	Lip	SK	2
25	M	58	Lip	SK	2
26	M	81	Skin	SCC	4
27	M	79	Skin	SCC	2
28	M	84	Skin	SCC	2
29	M	79	Skin	SCC	2
30	M	84	Skin	SCC	3
31	M	76	Skin	SK	4
32	M	72	Skin	SK	3
33	M	58	Skin	SK	3
34	M	74	Skin	SK	2
35	M	70	Skin	SK	2
36	F	67	Buccal mucosa	SCC	3
37	F	69	Buccal mucosa	SCC	3
38	M	50	Buccal mucosa	SCC	2
39	M	66	Buccal mucosa	SCC	1
40	F	63	Buccal mucosa	SCC	1
41	M	64	Buccal mucosa	LP	1
42	F	39	Buccal mucosa	LP	1
43	F	29	Buccal mucosa	LP	1
44	F	67	Buccal mucosa	LP	1
45	F	29	Buccal mucosa	LP	1
46	M	43	Buccal mucosa	CHC	1
47	M	68	Buccal mucosa	CHC	1
48	F	31	Buccal mucosa	CHC	1
49	F	56	Buccal mucosa	CHC	1
50	F	?	Buccal mucosa	CHC	1

Table 2 Lip lesions by type and grade

Grade	SCC	SK	CHC	LP	Normal mucosa
1	0	0	3	0	5
2	1	3	2	5	0
3	1	2	0	0	0
4	3	0	0	0	0

Table 3 Lesions from sun exposed skin tissue by type and grade

Grade	SCC	SK
1	0	0
2	3	2
3	1	2
4	1	1

exposed tissue overlying mucous extravasation cysts, were also obtained.

Breast tumour tissue which had previously been identified as p53 immunopositive was used as a positive control.

IMMUNOCYTOCHEMISTRY

Sections, 5 µm thick, were cut from representative paraffin wax blocks of the aforementioned lesions and from the known immunonegative and immunopositive controls. Control tissue was run for each immunocytochemical experiment.

Table 4 Lesions from buccal mucosa by type and grade

Grade	SCC	CHC	LP
1	2	5	5
2	1	0	0
3	2	0	0
4	0	0	0

Samples were subjected to an indirect immunoperoxidase technique to localise p53 as follows: sections were mounted on poly-L-lysine coated slides and allowed to dry in air overnight at room temperature as the use of heat at this stage is not recommended.[10] Sections were dewaxed in xylene, ethanol and 70% ethanol. Endogenous peroxidase activity was blocked by submerging sections in a 5:1 (v/v) solution of methanol:10 mM phosphate buffered saline (PBS), pH 7.6, containing 0.3% hydrogen peroxide for 30 minutes. Sections were then washed in PBS for 10 minutes during which the PBS solution was changed three times. This washing procedure was carried out between each new application of reagents.

Where CM-1 (Novocastra Laboratories, Newcastle, UK) was used as primary antiserum, non-specific binding was blocked by first incubating the sections with normal goat serum (Vectastain ABC kit, Vector Laboratories, Peterborough, UK) diluted in PBS for 30 minutes. Excess serum was blotted away and primary antiserum, diluted 1 in 1500 in PBS containing 0.01% Tween 20, was applied. The sharpest intensity and most consistent results were obtained by overnight incubation at 4°C. Sections were then incubated with the goat anti-rabbit secondary antibody followed by avidin peroxidase, according to the manufacturer's instructions.

In addition, a pre-diluted mouse monoclonal antibody directed against p53 (IOpath, Immunotech International, Birmingham, UK) was used in place of the CM-1 primary antiserum. In this procedure, sections were first pretreated in a microwave oven in 10 mM citrate buffer, pH 6.0. After cooling for five minutes, the microwave treatment was repeated and sections allowed to cool to room temperature. They were then rinsed in PBS and incubated with the mouse monoclonal antibody directed against p53 at room temperature for one hour. This was followed by incubation with biotinylated horse anti-mouse secondary antiserum and avidin peroxidase according to the manufacturer's instructions (Vector Laboratories).

For both procedures, after washing the slides in PBS, reaction products were developed by immersion of the sections (for an appropriate length of time to allow the development of a colour reaction product, but keeping background staining to a minimum) in equal volumes of 0.1% diaminobenzidine tetrahydrochloride (DAB), made in 0.1 M Tris buffer, pH 7.2, and 0.02% hydrogen peroxide (made in distilled water from a 30% H_2O_2 stock

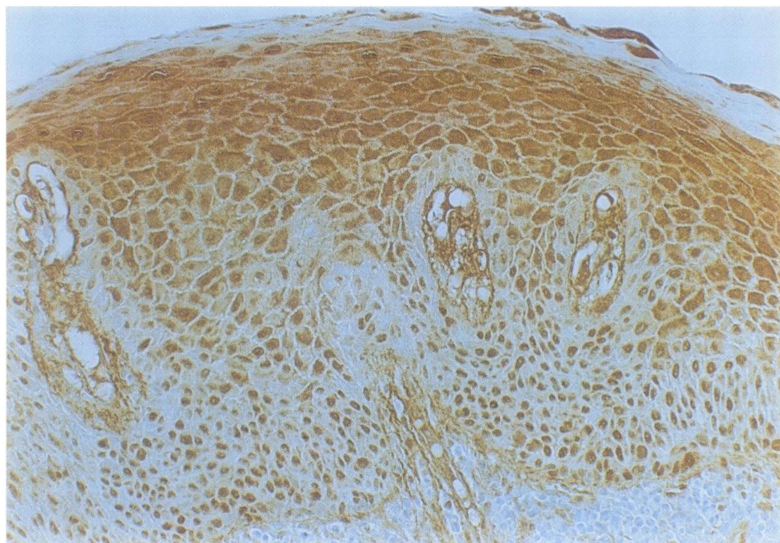

Figure 1 Chronic hyperplastic candidosis of the lip showing p53 localisation in the basal and parabasal layers of the epithelium. Non-specific cytoplasmic staining is also seen and is possibly due to antiserum cross reactivity with keratins. CM-1 antiserum (Novocastra) and Harris's haematoxylin counterstain (×200).

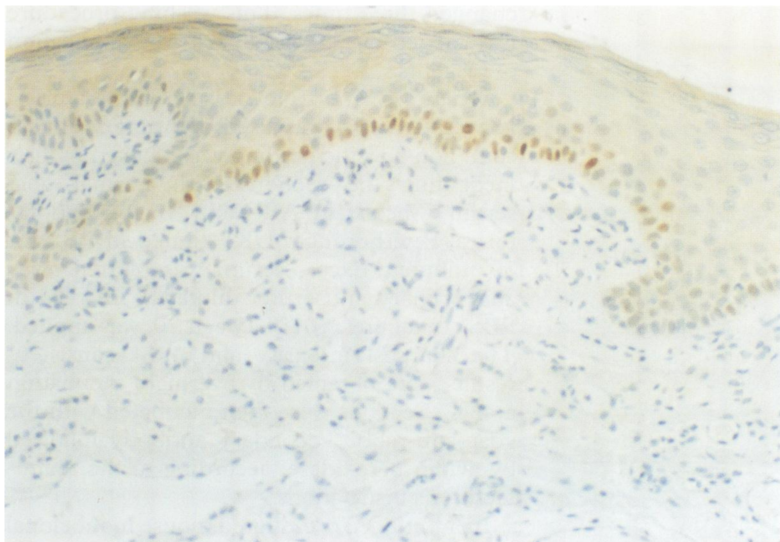

Figure 2 p53 immunopositivity in the epithelial basal cells in the lichen planus of the lip identified using a monoclonal antibody directed against p53 (IOpath). Harris's haematoxylin counterstain (×200).

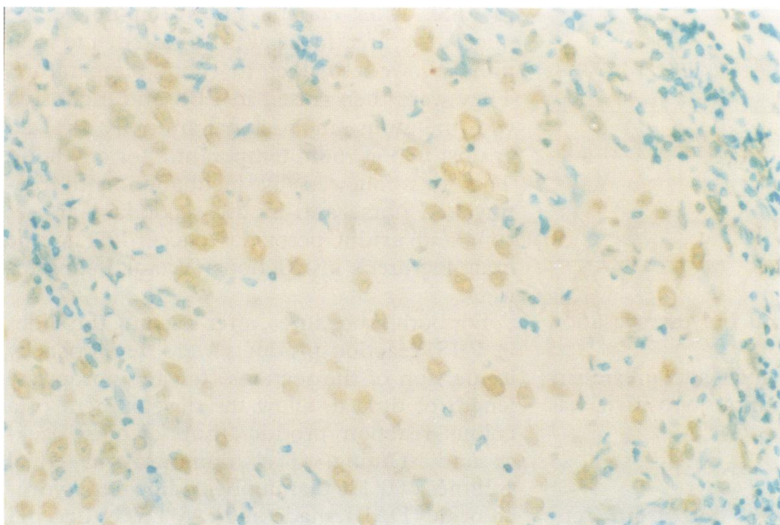

Figure 3 Nuclear fragmentation in a squamous cell carcinoma of the lip detected by 3' end nucleotide labelling (ApopTag). Methyl green counterstain (×400).

solution). Slides were then immersed in running tap water for five minutes, lightly counterstained in Harris's haematoxylin, dehydrated, cleared, and mounted.

For each immunocytochemical run the following were used: (1) two negative control sections, one with the primary antibody, the other with PBS alone; and (2) two sections of a p53 positive breast tumour, treated as described above.

SEMIQUANTITATIVE ANALYSIS

Interpretation of staining was based on a semiquantitative grading system, as described previously.[10 11] The proportion of cells with immunoreactive p53 were scored as follows: grade 1 = no staining; grade 2 = < 10% of tumours cells p53 positive; grade 3 = 10–70% tumour cells p53 positive; grade 4 = > 70% tumour cells p53 positive. Sections were examined in a double blind manner to reduce bias and ensure consistency of examination.

LABELLING OF APOPTOTIC CELLS

To determine cellular commitment to apoptosis in tissue samples, the method of terminal deoxynucleotidyl transferase (TDTase) catalysis was used, by way of an apoptosis detection kit (ApopTag, Oncor Inc., Gaithersburg, USA).

Sections were incubated in the presence of digoxigenin labelled d-UTP and d-ATP, and TDTase. The 3' ends of DNA are recognised by TDTase which catalytically replaces short sequences from the exposed 3' end by up to 11 nucleotides. This ensures that all fragmented DNA, which is present abundantly in cells undergoing apoptosis, is labelled with digoxigenin. After being washed in PBS, sections were incubated with immunoperoxidase labelled anti-digoxygenin antibody, followed by labelling with DAB as described earlier.

Results

IMMUNOLOCALISATION OF P53

The p53 related immunohistochemical results are summarised in table 1. Tables 2, 3, and 4 provide a breakdown of lesions and grading by site.

The expression of immunoreactive p53 phosphoprotein was analysed in 50 independent cases; 30 of these exhibited positive staining to some degree. The reaction product, which denoted the binding of CM-1 antiserum (or monoclonal antibody directed against p53), was localised to cell nuclei. Generally, p53 positive nuclei were stained homogeneously, but with varying intensity. A more granular pattern was observed occasionally in some lesions. However, there was no discernible relation between staining intensity or pattern of nuclear staining and the type of lesion.

The CM-1 antiserum showed a degree of cross reactivity with the keratins. This made it difficult to assess the rating of some lesions, particularly for well differentiated SCCs which produce keratin. In contrast, staining was localised to cell nuclei in poorly differentiated SCCs. Problems with CM-1 were circum-

vented, however, by the additional use of a p53 monoclonal antibody which has negligible cross reactivity with the cytokeratins.

In this investigation all the cases of SCC from sun exposed lip and skin were positive for p53, as were three lesions of SCC from buccal mucosa. Three SCCs from the lip and one from the skin scored grade 4 and all were widely invasive lesions. It was noted that SCCs which were widely invasive represented the best examples of distinct, intense nuclear p53 overexpression, particularly in the cells found in more deeply invasive malignant epithelium. Focal areas of basal and parabasal p53 immunolocalisation in adjacent normal or even hyperplastic epithelium was also a consistent finding in SCC sections.

Solar keratosis is regarded as a potential pre-malignant lesion and tables 2 and 3 highlight the high degree of p53 overexpression exhibited in all SK lip and skin lesions examined. One SK lesion from sun exposed skin gave an unusual result in that p53 expression was absent from the epidermis but was present in dermal fibroblasts. In addition, some, but not all, of the SK specimens (from both lip and skin) also provided examples of a distinct cut-off point between tissue expressing p53 and normal p53 negative adjacent tissue and some showed p53 positive staining in adjacent, apparently normal epithelium.

Lesions from patients with CHC of buccal mucosa did not express p53; however, two of the five CHC lesions from lip tissue did (fig 1). Within these two lesions p53 staining was focal and some areas were given an overall score of grade 3, whereas in other areas of the same lesion p53 expression was absent.

Cases of LP were included in this study as known benign lesions with minimal potential for malignant change. All of the LP lesions of the buccal mucosa were p53 negative, however, all five LP lesions of the lip showed some degree of p53 immunopositivty localised to the basal layer. Staining was very weak in three cases, and only identifiable in some basal cells in two of these (fig 2).

All samples of normal lip mucosa were p53 negative.

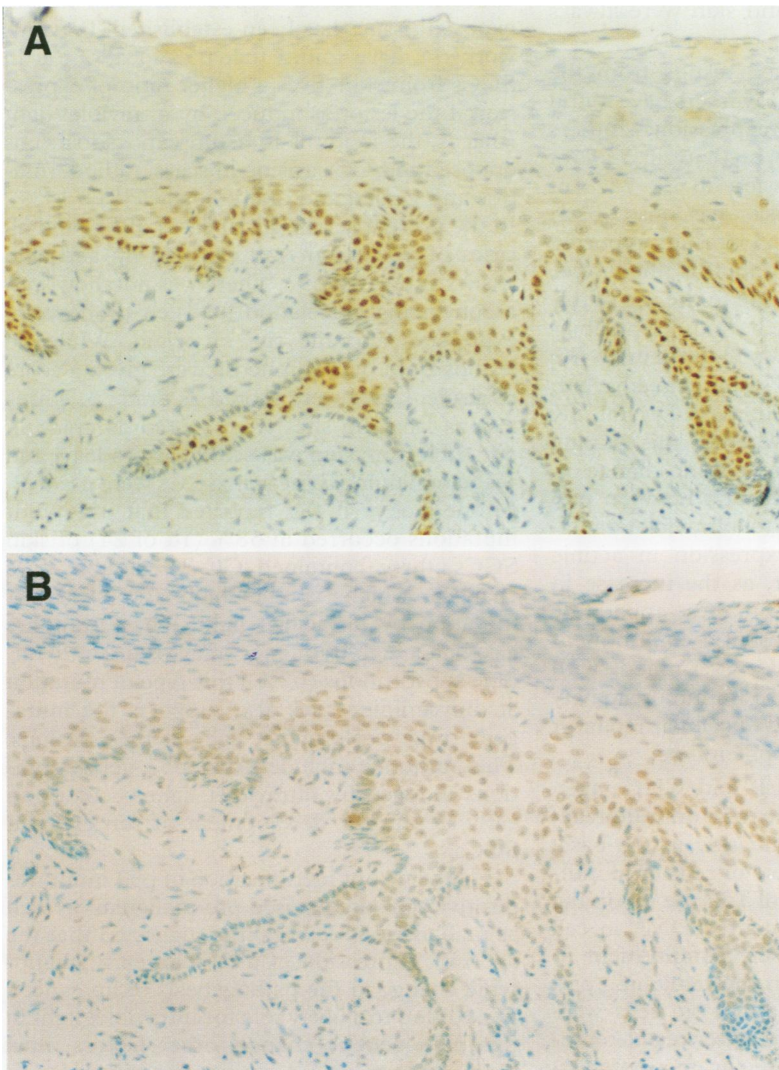

Figure 4 (A) p53 immunopositivity in an area of hyperplasia in solar keratosis of the lip identified using a monoclonal antibody directed against p53 (IOpath). Harris's haematoxylin counterstain (×200). (B) Serial section to that depicted in (A) showing the distribution of apoptotic cells by 3' end nucleotide labelling (ApopTag). Methyl green counterstain (×200).

DNA FRAGMENTATION STUDIES

In order to distinguish whether p53 immunopositivity signified a commitment to programmed cell death, a selection of p53 immunopositive lesions was analysed for nuclear DNA fragmentation, a marker of cellular apoptosis. These included SCC, SK and LP lesions of the lip.

Considerable variability was seen both between and within the SCC lesions sampled. In some tumours, no nuclear DNA fragmentation could be detected. However, in some up to 50% of cells were undergoing apoptosis in what seemed to be a random fashion, whereas in some other SCC lesions, nuclear fragmentation was restricted to the periphery of the tumour mass (fig 3).

Three of the SK lesions of the lip showed a similar proportion of cells with nuclear DNA cleavage to their p53 staining; this was localised to the basal layer. In one of the three lesions, however, p53 was localised to an area of hyperplasia (fig 4A), but the apoptotic cells were also seen in a similar distribution (fig 4B).

Of the five LP lesions of the lip, four showed nuclear fragmentation which had a more diffuse localisation in all layers of the epithelium (fig 5).

Discussion

To our knowledge, this study provides the first analysis of p53 protein expression in benign and pre-malignant lip lesions. In addition, we have measured p53 immunoreactivity in a series of lip SCCs; for comparison, skin and buccal mucosa biopsy specimens provided examples of sun exposed and non-sun exposed tissue, respectively.

Results obtained from the intra-oral malignancies are similar to those of other workers and show that p53 localisation is variable both in terms of expression and the degree of nuclear intensity.[12-17] To date, it seems that there is no oral site in normal or lesional tissue that is specific for p53 positivity. In one study[17] a correlation between p53 expression and

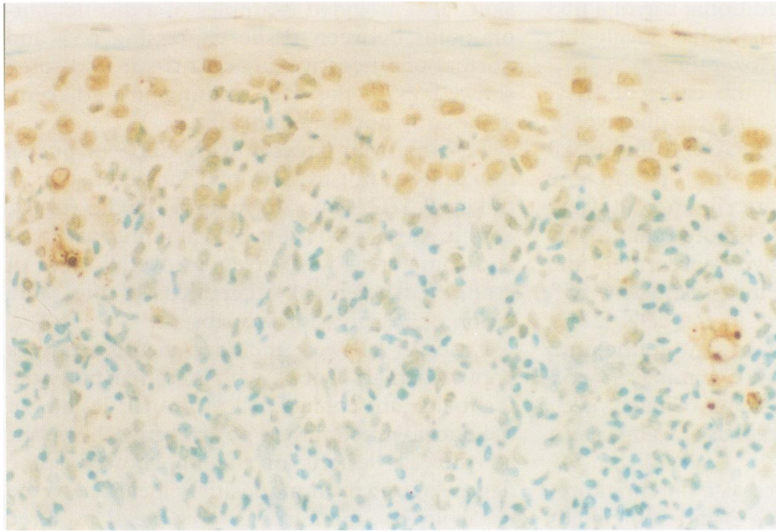

Figure 5 Lichen planus of the lip showing nuclear fragmentation throughout many of the epithelial layers by 3' end nucleotide labelling (ApopTag). Methyl green counterstain (×200)

TNM staging was reported with p53 highest in T1. The same workers also noted an increase in p53 expression with increasing tumour invasion. Our findings also support a correlation between p53 and degree of invasion as p53 grade 4 SCCs from lip and skin were always highly invasive lesions.

We have obtained mixed results from the analysis of SK. While some workers have found no evidence of p53 overexpression,[18] others have shown p53 immunoreactivity in 74% of SK lesions.[19] p53 expression in 27–74% of cases of actinic keratosis has also been noted.[20 21] The range of p53 results reported may reflect true variants of the SK phenotype. Alternatively, this may be due to the use of different antibodies that may have differing abilities to recognise mutant p53 proteins.[22] We also observed positive p53 staining in adjacent, apparently normal epithelium in several SK samples. It is not clear whether this finding represents potential early neoplastic change or, given the well documented link between p53 expression and exposure to ultraviolet radiation,[23] predictable p53 expression in actinic damaged tissue. However, as the increase in p53 protein concentrations in these apparently normal cells did not represent a commitment to cellular apoptosis, as determined with ApopTag, we believe the p53 immunoreactivity can only represent a period of cellular repair or an aberrant change in cell phenotype.

In two cases each of LP and CHC taken from sun exposed lip tissue, p53 immunoreactivity was located predominantly in the basal layer, either in small clusters of cells or in individual cells. In one case of LP, the ApopTag procedure identified fragmented DNA in cell nuclei, indicating their likely commitment to programmed cell death. In the remaining p53 positive lesion, however, apoptotic cells were not identified. Whether the latter result represents a period of cellular repair or a potential for progression to a more insidious lesion remains to be elucidated.

So far, clinical studies have shown that p53 protein expression correlates with both neoplasia and lesions which can become malignant at a later date. For example, one longitudinal study of a patient's dorsal lingual hyperkeratosis found no p53 overexpression in 1986, yet in 1987 and 1989 positive results for p53 were obtained when clearly there was no histopathological evidence of dysplasia. Examination of a subsequent biopsy specimen in 1991 resulted in a diagnosis of carcinoma in situ.[24] Furthermore, from a recent prospective study, there is now good evidence of the clinical value of p53 immunohistochemical data.[25] In the latter study, Dowell *et al* determined p53 expression in 1333 non-gynaecological cytological specimens without prior knowledge of the diagnosis. Of 108 cases of p53 overexpression, 86 proved to be malignant lesions. Of 43 cytopathologically suspicious lesions, seven were found to exhibit p53 expression; all seven cases were eventually diagnosed as malignant. It might be deduced from these studies, therefore, that the presence of immunoreactive p53 in lip lesions is indicative of a potential for malignant change. Hence, the presence of p53 immunoreactivity in apparently benign LP lesions of the lip might be a useful indicator that such lesions should be viewed with greater concern. A method of p53 activation in sun exposed tissues has been established. Previous workers[26] have found that p53 localisation in SCCs from skin gives a higher ratio of expression if the lesion is induced by ultraviolet light (that is, the lesion is in a sun exposed site) as opposed to SCCs arising in scars, radiodermatitis and genital and oral lesions (54% *v* 19%). In the present series, all 10 cases of SK and all cases of SCC from sun exposed lip and skin tissues expressed p53. There was not, however, a convincing correlation between the level of expression and the site of origin. While no lesions from buccal mucosa were given a score of grade 4, two were scored as grade 3. Three lesions from lip sites were scored as grade 4, one each from skin was scored as grades 3 and 4; the remaining three skin SCCs were grade 2. In one study[27] it was reported that p53 point mutations occurred in 58% (14 of 24) of skin SCCs, three contained CC-TT double base changes which is the hallmark of DNA mutation by ultraviolet light. These tumours also had a high frequency of cytosine to thymidine substitutions (62%); this type of mutation at dipyrimidine sites also supports the mutagenic role of ultraviolet light in these lesions. The authors concluded that p53 gene mutations induced by ultraviolet light occur as an early step in the development of sun exposed SCCs.

Following our identification of p53 immunopositivity in sun exposed pre-malignant as well as malignant lip lesions it is suggested that the aberrant expression of p53 is an early phenotypic change in the pathogenesis of lip cancer. In addition to a likely role for ultraviolet light in this pathogenetic change, other factors must also be taken into account. For example, an association between heavy drinking and smoking with raised p53 expression has been established.[28] Tobacco is a known carcinogenic agent and has been implicated in the pathogen-

esis of lip cancer.[29] Viruses may also play a role in this rise in p53 expression.[30]

In conclusion, we have localised p53 immunocytochemically in malignant, pre-malignant and non-malignant lesions of the lip. From our results, it is suggested that aberrant p53 expression is likely to be an early event in the pathogenesis of lip cancer. Furthermore, we have shown that the immunopositive p53 cells identified in the benign LP lesions do not necessarily correlate with a commitment of cells within the lesion to programmed cell death. In light of the prior reports which indicate that p53 positive cells may progress to form malignant tumours,[24 25] we suggest that patients with p53 positive but otherwise benign lesions should be followed more closely.

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