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Presence of Human Papillomavirus Sequences in Tumour-derived Human Oral Keratinocytes Expressing Mutant p53

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A series of eight oral epithelial cell lines derived from untreated human oral squamous cell carcinomas, which had arisen in patients with different tobacco histories, were examined for the presence of human papillomavirus (HPV) DNA, expression of stable p53 protein and *p53* point mutation. Polymerase chain reaction (PCR)-based screening, but not Southern blot analysis, showed HPV-16 early region sequences to be present at low copy number (< 1 copy per cell) in two cell lines at early passage (3–5) *in vitro* (H400, T45), implying that only subpopulations of cells harboured viral DNA. HPV sequences were undetectable in cells at later passage (12–15), suggesting that viral sequences had been lost during growth *in vitro*, or that negative selection of HPV-containing cells had occurred. High levels of p53 were detected in the two HPV-positive cell lines and in three others (H103, H314, H357) by Western blotting, suggesting expression of mutant (stable) p53 molecules. A sixth cell line (H157) expressed a truncated p53. Sequence analysis of exons 2–11 of the *p53* gene revealed missense mutations in six cell lines, one of which (H413) did not result in high levels of protein, and nonsense mutations in the remaining two cell lines (H157, H376). The results suggest that *p53* mutation is a frequent genetic event in oral cancer. In addition, the expression of mutant p53 in oral cancer cells does not preclude a papillomaviral aetiology for these tumours. Analysis of p53 expression alone may result in underestimation of the frequency of *p53* mutations in human cancers. In contrast to other studies, we demonstrate that positive staining of p53 in oral cancer does not necessarily reflect a tobacco aetiology.

Keywords: *p53*, gene mutation, oral cancer, chemical carcinogens, tumour suppressor, tumour progression, viral oncogenesis, human papillomavirus

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INTRODUCTION

ALTERATIONS OF *p53* are manifest by allelic loss, mutation and by modulation of expression. In many inherited and sporadic cancers, gene mutations are frequently found within four highly conserved regions of *p53* which include exons 5–9 [1]. The consequences of such mutations include an increase in protein stability, which manifests as high steady state levels of p53 and contributes to the immunocytochemical detection of mutant p53 molecules. In addition to loss of normal p53 function in malignancy, dominant negative mutant p53 species have the ability to immortalise primary cells and act in concert with viral or cellular oncogenes to effect cell transformation [2].

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There is good evidence to show that p53 expression is modulated by a variety of other mechanisms. These include stabilisation by SV40 T antigen and adenovirus E1b [3, 4] or cellular proteins [5], or increased degradation by human papillomavirus (HPV) E6 via the ubiquitin protease system [6]. Indeed, the role of viruses in the aetiology of human cancers is becoming increasingly evident, with specific HPV types being implicated in the pathogenesis of a wide range of human cancers [7]. Although viral DNA persists and continued expression of papillomavirus oncoproteins E6 and E7 is found in premalignant cervical lesions and in carcinomas with or without viral integration into the host DNA [8], the situation appears to be different with regard to oral epithelium [9]. Papillomaviral integration is not a common event in oral cancer [10, 11] and expression of viral RNA or protein has not been reported. In addition, an inverse correlation between the expression of HPV E6 and mutant p53 in cervical carcinomas has been suggested, with the majority of HPV-positive cervical tumours expressing normal p53 [12, 13], although more recent evidence suggests that there may be exceptions to this attractive hypothesis as HPV-related primary cancers of the cervix [14, 15] and epidermis [16], and cervical carcinoma

metastases [14] have been shown to express mutant p53. These may represent dominant-negative mutants which are resistant to E6-mediated degradation and confer an additional growth advantage on cancer cells [14]. By contrast, most oral carcinomas studied to date harbour mutant p53 [17–19] and no reports have documented a relationship between HPV and p53 in oral malignancies. In view of the conflicting reports of HPV and mutant p53 in cervical carcinomas and the paucity of information as regards oral malignancies, it is at present unclear what relationship exists between HPV and p53 in tumour development at both of these sites.

It has been suggested that *p53* mutations reflect the effects of specific aetiological agents. For example, tobacco-related cancers have been associated with G–T transversions in *p53* and have been documented in non-small cell lung, oesophageal and head and neck carcinomas [20–23]. Perhaps more strikingly, aflatoxin from *Aspergillus flavus* and hepatitis B virus are suspected primary agents in the development of liver cancers which are associated with a high rate of G–T transversions at a specific hotspot of *p53* [24]. Oral cancer has a strong association with tobacco and alcohol [25], but limited data exist regarding the nature of *p53* mutations in oral squamous cell carcinoma [17–19, 23, 26] and their relationship to tobacco usage.

This study demonstrates the presence of human papillomavirus DNA and *p53* mutations in cell lines derived from eight untreated intraoral squamous cell carcinomas. For the first time we have shown *p53* mutations in oral cancer cell lines containing HPV sequences at low copy number in early passage cells. Expression of stable p53 was examined in the context of p53 mRNA levels, specific *p53* mutations and tobacco exposure of patients. The data indicate that *p53* mutation is a frequent genetic alteration in oral squamous cell carcinoma, but that increased protein stability is not the invariable result of point mutation. Cells expressing high steady state levels of mutant p53 did not necessarily show increased p53 mRNA. In addition, *p53* mutations were detected in cells derived from patients with no positive tobacco history.

MATERIALS AND METHODS

Cell lines

Keratinocyte cultures derived from oral squamous cell carcinomas have been described previously [27, 28]. Cell lines, including HT29 colon carcinoma cells as positive controls, were cultured in the absence of 3T3 fibroblast support in DMEM supplemented with 5% fetal calf serum, 0.075% $NaHCO_3$, 0.6 μg/ml L-glutamine and 0.5 μg/ml hydrocortisone at 37°C in an atmosphere of 95% air/5% CO_2.

Western blot analysis of p53

Oral keratinocyte cell lines and control cell lines were cultured to 80% confluence, trypsinised, 1×10^6 cells were pelleted and lysed in 1 × SDS gel buffer at 100°C for 10 min and proteins resolved in denaturing 10% polyacrylamide gels. Samples were transferred onto Immobilon membranes (Millipore, Bedford, Massachusetts, U.S.A.), blocked in 4% (w/v) dried milk in TBST (50 mM Tris HCl pH 7.6, 0.15 M NaCl, 0.05% Tween 20) for 1 h at ambient temperature and incubated overnight with anti-p53 antibody (Pab 1801, which recognises wild-type and mutant p53 molecules [29]) diluted 1:1000 in TBST/milk. Membranes were washed twice in TBST for 10 min, incubated in a 1:500 dilution of secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, Sigma, St Louis, Missouri, U.S.A.) for 1 h, washed twice in TBST and protein detected by chemiluminescence and autoradiography.

Northern blot analysis

Total cellular RNA was prepared according to standard protocols and 20 μg aliquots were subjected to northern blot analysis [30]. Blots were autoradiographed at −70°C for up to 10 days.

Preparation of DNA

DNA was prepared from cell lines according to standard protocols [30], resuspended in 10 mM Tris HCl, 1 mM EDTA (TE) pH 8 and quantified spectrophotometrically.

Oligonucleotides

Oligonucleotides for use as PCR primers or hybridisation probes were obtained from Keystone Laboratories Inc. (Menlo Park, California, U.S.A.), and are listed in Table 1.

Polymerase chain reaction

PCR was performed as described previously [11]. Thirty-five cycles of amplification were carried out (*p53*: 95°C–55°C–72°C, 1 min each; HPV-16, 18: 95°C–50°C–72°C, 1 min each). PCR products were resolved in 6% polyacrylamide. Bands corresponding to *p53* DNA fragments were excised from gels and the DNA recovered as described previously [11]. HPV PCR gels were Southern blotted and hybridised to ^{32}P-end-labelled internal oligonucleotide probes as described previously [11].

Cloning of PCR products

p53 amplification products, purified as described, were ligated into the plasmid vector pT7Blue-T (Novagen, Madison, Wisconsin, U.S.A.) and recombinant clones selected on the basis of *lacZ* inactivation. Recombinants were pooled and plasmid DNA prepared by alkaline lysis (Magic Minipreps, Promega, Madison, Wisconsin, U.S.A.).

DNA sequence analysis

Cloned PCR products were sequenced using a commercially-available kit (Sequenase v2.0, United States Biochemicals, Cambridge, U.K.). The sequencing primers were T7 (5′-AATACGACTCACTATAG-3′), M13-40 (5′-GTTTTCCCAGTCACGAC-3′), or sense or antisense PCR oligonucleotides (Table 1). Sequencing reactions were resolved in 5% polyacrylamide, dried and autoradiographed.

RESULTS

Presence of papillomavirus sequences

DNA prepared from oral keratinocyte lines was screened by PCR and Southern blot hybridisation for the presence of HPV-16 and HPV-18 sequences. Figure 1a shows a Southern blot of PCR products hybridised with an oligonucleotide probe specific for HPV-16 sequences internal to the target

Table 1. Sequence of oligonucleotides used in the PCR

P2S	5′-GACGCGCAGTCAGATCCTAG-3′	592 bp
P4A	5′-CTCAGGGCAACTGACCG-3′	
P5S	5′-TGTTCACTTGTGCCCTGACT-3′	269 bp
P5A	5′-CAGCCCTGTCGTCTCTCCAG-3′	
P6S	5′-GCCTCTGATTCCTCACTGAT-3′	181 bp
P6A	5′-TTAACCCCTCCTCCCAGAGA-3′	
P7S	5′-CTCCTAGGTTGGCTCTGA-3′	132 bp
P7A	5′-CAAGTGGCTCCTGACCTGGA-3′	
P8S	5′-CCTATCCTGAGTAGTGGTAA-3′	151 bp
P8A	5′-CTGGTGTTGTTGGGCTGTGC-3′	
P9S	5′-ACTAAGCGAGGTAAGCAAGC-3′	210 bp
P9A	5′-CTGGAAACTTTCCACTTGAT-3′	
P10S	5′-CTCTGTTGCTGCAGATC-3′	136 bp
P10A	5′-GCTGAGGTCACTCACCT-3′	
P11S	5′-TTCTGTCTCCTACAGCCACC-3′	117 bp
P11A	5′-GGAACAAGAAGTGGAGAATG-3′	
16E6S	5′-TTAATTAGGTGTATTAACTG-3′	166 bp
16E6A	5′-TGCATGATTACAGCTGGGTT-3′	
18E6S	5′-ATCTGTGCACGGAACTGAAC-3′	100 bp
18E6A	5′-AATGCAAATTCAAATACCTC-3′	
16I	5′-GACAAAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGA-3′	
18I	5′-CTGCAAGACATAGAAATAACCTGTGTATATTGCAAGACAG-3′	

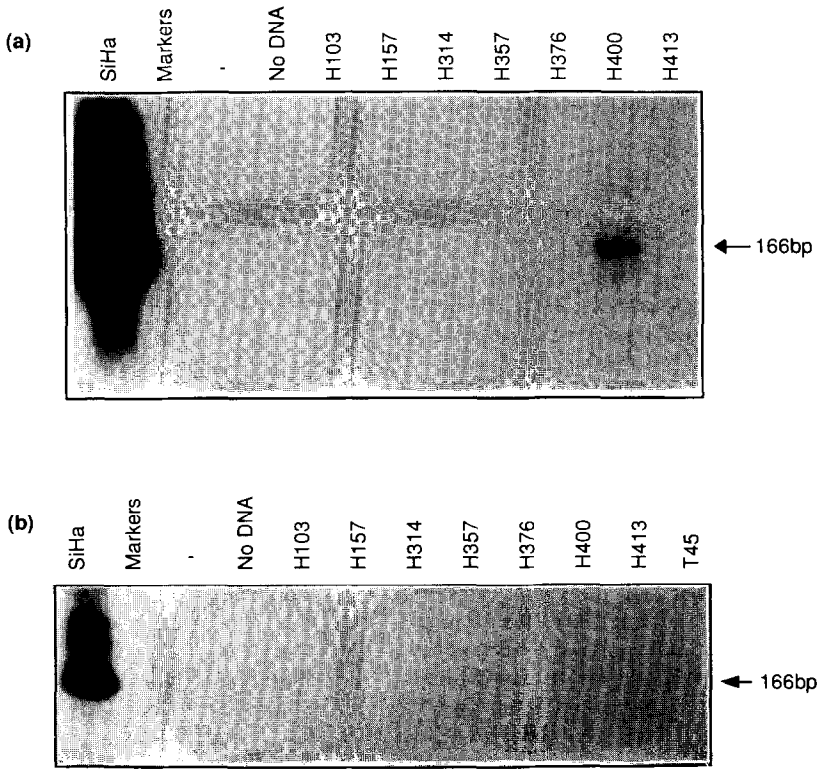

Fig. 1. Southern blot of PCR products amplified from 1 μg of genomic DNA from oral SCC cell lines at passage 3–5 (a) or passage 12–15 (b), and from control SiHa DNA (1 HPV genome per cell), hybridised to an internal oligonucleotide probe.

sequence and demonstrates HPV-16 sequences in H400 cells at passage 6. Early passage T45 cells (passage 3) have been documented to contain HPV-16 early region DNA in a previous study [11]. Conventional Southern blot hybridisation of genomic DNA failed to detect papillomavirus sequences, suggesting that viral DNA was present at low copy number (less than one HPV genome per cell); the data indicates that only subpopulations of cells were HPV-16-positive. PCR and Southern blot analysis of DNA from later passage cells (passages 12–15) did not reveal the presence of HPV-16 sequences in H400 or T45 (Fig. 1b), suggesting either selection against HPV-containing cells *in vitro* or a "hit-and-run" action for viral DNA in these tumours. None of the cell lines were found to harbour HPV-18 DNA (data not shown). Southern hybridisation of the products of PCR amplification using these primers allows routine detection of one viral genome in a background of 1×10^5 uninfected cells from *in vitro* reconstruction experiments (W.A. Yeudall, unpublished observations).

Expression of p53 in squamous cell carcinoma-derived oral keratinocytes

Oral keratinocyte lines, together with HT29 cells as a positive control, were examined for p53 expression by Western blot analysis of whole cell lysates. The results are shown in Fig. 2 and Table 2. H314 and H357 cells were found to express high steady state levels of p53 comparable with those observed in HT29 colon carcinoma cells; in HT29 and H314 a doublet was noted. Three other cell lines also expressed stable 53 kD p53 molecules, namely T45, H103 and H400 (higher to lower expression levels), and a sixth cell line, H157, expressed a p53 species of approximately 43 kD. No protein was detected in the remaining two cell lines, H376 and H413, by this method.

To confirm that the high levels of p53 detected were not due to high levels of p53 transcription, northern blot analysis was performed on 20 μg aliquots of total RNA using a radiolabelled p53 cDNA probe. Three cell lines (H157, H376, H413) were found to express low levels of p53 mRNA; the remaining five cell lines, which expressed high levels of stable p53 protein, overexpressed p53 mRNA (H400 = T45 > H103 > H314 > H357, Fig. 3). Equal loading was confirmed by the intensity of the 28S and 18S ribosomal bands after staining with ethidium bromide (not shown).

Nucleotide sequence analysis of p53

To further characterise *p53* abnormalities in tumour-derived oral keratinocytes, nucleotide sequence analysis of the entire coding region of the gene was carried out following *in vitro* amplification and molecular cloning. Single point mutations were found in six of eight oral cell lines (Table 2), and in H314 two distinct point mutations were evident. Six cell lines (H103, H314, H357, H400, H413 and T45) harboured missense mutations, while H157 and H376 contained nonsense mutations which would be predicted to result in premature termination of translation, thereby explaining the presence of a 43 kD protein in H157 (Fig. 2); no detectable protein was observed in H376. In three cell lines (H357, H413, T45) point mutations were present in exon 4; in both H357 and T45 codon 110 was altered, while codon 68 was mutated in H413. A further three cell lines (H157, H376, H400) harboured point mutations in exon 8; one cell line (H103) showed mutation of exon 7 of *p53*. In H314, two point mutations were detected: at codon 176 near to a mutational hotspot in exon 5 and in exon 11 in the oligomerisation domain. The presence of a normal allele was only detected in one cell line (H413). The authenticity of the mutations was confirmed by sequencing cDNA clones obtained by reverse transcription of p53 mRNA followed by PCR. In addition, mutations were further confirmed in DNA isolated from cells which had been cultured for a limited time (up to passage 6). The results obtained were consistent with those documented previously, suggesting that it was unlikely that mutations were the result of extended growth *in vitro*.

Tobacco history and p53 *mutation*

Mutations characteristic of tobacco usage (G–T, G–A) comprised 70% of the total *p53* mutations detected. There was no clear correlation between the nature of the *p53* mutations and the use of tobacco by the patients from which the cell lines were derived (Table 3). For example, H103 and H314 were non-smokers but had G–T mutations in *p53*. By contrast, H413 was a moderate smoker and showed an A–G substitution.

DISCUSSION

Studies of *p53* mutations have been carried out in a wide spectrum of neoplasms [1]. Previous reports of *p53* mutations in cancer of the head and neck have included tumours of the larynx [19, 31], hypopharynx [19, 23], oesophagus [22] and epidermis [19]. By contrast, the present study examined *p53* mutations in a unique series of cell lines derived from untreated intraoral squamous cell carcinomas. We believe it is important to distinguish between different tumours of the head and neck, in view of their contrasting aetiologies and disparate cellular origins [32]. In addition, therapy of oral cancer commonly involves radiotherapy [18, 26] and, in many cases, treatment regimes have not been documented [17]. In the present study, *p53* mutations were found to be frequent genetic alterations in intraoral cancer as all eight cell lines

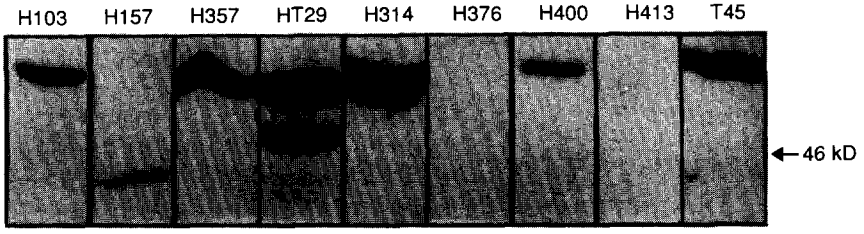

Fig. 2. Western blot analysis of p53 expression in oral SCC cells and HT29 control. Lysates were prepared from 1×10^6 cells, as described in the text.

Table 2. p53 expression and mutation in SCC-derived oral keratinocytes

Cell line	p53 expression: Western blot	p53 expression: Immunostaining	Mutated codon (exon)	Nucleotide sequence change	Predicted amino acid change	Presence of normal alelle	HPV status
H103	++	++	244 (7)	GGC–TGC	Gly–Cys	N	—
H157	+	+	306 (8)	CGA–TGA	Arg–Stop	N	—
H314	+++	++	176 (5)	TGC–TTC	Cys–Phe	N	—
			373 (11)	AAG–GAG	Lys–Glu	N	—
H357	+++	++	110 (4)	CGT–TGT	Arg–Cys	N	—
H376	−	+/−	266 (8)	GGA–TGA	Gly–Stop	N	—
H400	+	++	283 (8)	CGC–GGC	Arg–Gly	N	HPV-16
H413	−	+	68 (4)	GAG–GGG	Glu–Gly	Y	—
T45	++	++	110 (4)	CGT–CTT	Arg–Leu	N	HPV-16

contained point mutations within the coding region of the gene. The findings confirm previous observations in head and neck cancers [17–19, 23, 26]. The consistently high frequency of *p53* mutations in this and in the above studies implies its importance in the pathogenesis of these tumours.

High-risk HPV types, such as HPV-16 and HPV-18, are closely associated with the development of several human cancers, in particular anogenital malignancies [7]. Recent reports suggest that loss of p53 function through point mutation in papillomavirus-negative tumours or E6-mediated degradation in HPV-positive lesions may also be important for tumour development [12, 13, 33]. Mutant p53 is also able to substitute for HPV-16 E6 in *in vitro* immortalisation assays with E7 [34]. Although loss of normal p53 function via E6-mediated degradation may be important for tumour establishment, a potential "gain-of-function" *p53* mutation is likely to confer an additional growth advantage on tumour cells. The presence of HPV sequences and mutant p53, therefore, are not necessarily mutually exclusive and it is not surprising that two cell lines in this study which expressed highly stable mutant p53 proteins (H400, T45) also harboured HPV-16 sequences. Indeed, recent studies have shown that cells transformed by HPV-16 E7 and *RAS* become further transformed by co-expression of mutant p53 [35]. It is currently unknown whether a mechanism similar to bovine papillomavirus type 4-mediated "hit-and-run" transformation [36] occurs in human oral epithelial cells. A significant difference between viral carcinogenesis in both human oral and bovine alimentary carcinomas and human cervical neoplasms is the presence of papillomavirus integration in the latter tumour types, and it may be that loss of viral sequences may occur more readily in oral cancer cells [10, 11]. Alternatively, the dual presence of HPV sequences and mutant p53 in the keratinocyte cultures of the present study could represent polyclonal tumours which have arisen through a field change *in vivo*, particularly as the cells in this study have not been cloned. Immunocytochemical data indicated that mutant p53 was expressed in all T45 and H400 cells (unpublished observations). The possibility exists, therefore, of HPV-positive and negative subpopulations or, alternatively, HPV sequences are lost as a consequence of tumour progression, with maintenance of the transformed phenotype being controlled by expression of cellular genes. Thus far, we have been unable to isolate any HPV-positive clonal cell populations.

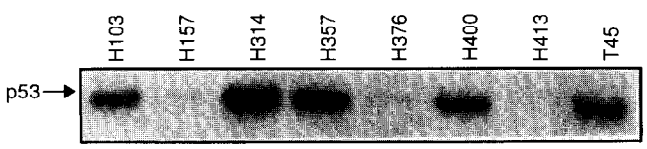

Fig. 3. Northern blot analysis showing p53 mRNA levels in oral SCC cell lines. Exposure was for 10 days at −70°C.

The results of this study highlight several points of importance in the analysis of p53 alterations in human cancers. Some previous studies of both oral and non-oral cancers have carried out sequence analysis limited to specific regions of the *p53* gene which have previously been shown to contain a high proportion of point mutations [1], in particular exons 5–9. While these may represent particular mutation "hotspots", such a narrow examination must lead to a proportion of false negative results. In this study, 40% of mutations were found outwith exons 5–9 and, interestingly, exon 4 was a common site (three of eight cell lines); in two cell lines, Arg^{110} mutations were detected. Furthermore, examination of the entire coding region in the present study demonstrated more than one mutation in one cell line. To our knowledge, this has not been reported previously in head and neck cancers, although double mutations have been reported in leukaemias [37] and in lung carcinomas [38], and may reflect a high dose of carcinogen.

Alterations within the coding region of *p53*, such as deletions, nonsense and or frameshift mutations, may lead to loss of wild-type p53 function. Alternatively, gain of function mutations may result where both wild-type activity is lost and the mutant p53 acts as a dominant transforming oncoprotein [39]. In this study, putative loss of function mutations were restricted to two cell lines (H157 exon 8; H376 exon 7); the remaining six cell lines contained point mutations which resulted in non-conservative amino acid substitutions and in five of which a high level of p53 protein was detected. No frameshift mutations or in-frame deletions or insertions were found in this study. These findings contrast with the results of previous studies of oral cancer where predominantly deletions and nonsense mutations were noted [18, 19, 23], although missense mutations were more common in tumours outwith the oral cavity (larynx [31]; larynx, hypopharynx, vulva [23]; epidermis [19]).

The site of mutation has important implications for the

Table 3. Tobacco history and p53 mutations related to clinical characteristics of patients

Cell line	Age (years)	Sex	Site	Grade*	Tobacco usage	p53 mutation
H103	32	M	T	I	Non-smoker	G-T
H157	84	M	BM	II	5 cigarettes/day	G-A
H314	82	M	FOM	II	Non-smoker	G-T A-G
H357	74	M	T	I	Smoker (aged 17–20 years only)	G-A
H376	40	F	FOM	III	Tobacco chewer	G-T
H400	55	F	AP	II	Pan chewer (no tobacco) 2 cigarettes/day	C-G
H413	53	F	BM	II	10–20 cigarettes/day	A-G
T45	NK	M	T	NK	NK	G-T

T, tongue; BM, buccal mucosa; FOM, floor of mouth; AP, alveolar process; NK, not known. *Untreated primary oral carcinomas are classified clinically according to their site of origin (S), tumour size (T), lymph node (N), metastatic involvement (M) and tumour pathology (P). Each parameter is weighted numerically and the maximum index of severity is used to express the STNPM clinical stage. The 5-year survival rates are 51.5% (grade I), 25.3% (grade II), 21.5% (grade III), 8.3% (grade IV) (Henk & Langdon, 1985).

stability of expressed protein. In particular, mutation at codon 110 (exon 4; H357, T45) in this study resulted in high levels of p53 as determined by Western blotting, but a third cell line which contained a mutation at an alternative site within exon 4 (codon 68; H413) did not express a stabilised p53. It is possible that this heterozygous change may represent an amino acid polymorphism; we have been unable to confirm or refute this due to a lack of normal tissue, but we have not noted this nucleotide alteration in other samples sequenced in this laboratory, or in reports from other workers. However, non-stabilising missense mutations have been described previously [39]. Three cell lines (H314, H103, H400) which had mutations at codon 176 (exon 5), codon 244 (exon 7) and codon 283 (exon 8), respectively, also expressed stable p53. These mutations fall within three of the four "hotspots" reported in human colorectal cancers [40], and the codon 176 mutation has been detected previously in oral cancer [23]. The presence of a second missense mutation in H314 at codon 373 (exon 11) was an unexpected finding and, to our knowledge, represents a novel mutation. This is located within the basic C-terminal region of the protein and results in substitution of an acidic residue (glutamate) for the wild-type lysine, which may affect the ability of p53 to oligomerise.

The failure to detect p53 protein in H376 by Western blotting may be the result of the absence of C-terminal oligomerisation sequences because this cell line contained a nonsense mutation at codon 266. A truncated p53 protein, however, was observed in H157 and the apparent lack of protein in H376 is more probably due to either lowered transcription as p53 mRNA was virtually undetectable by northern blot analysis. It may be, therefore, that additional factors contribute to the levels of p53 present in cancer cells, and mutation of *p53* promoter sequences or decreased/absent trans-acting transcriptional enhancers may be influential in this context. Alternatively, the difference in protein levels between these two cell lines could be explained by stabilisation of p53 in H157 via complex formation with other cellular or viral proteins. Thus, the lack of detectable protein does not exclude the presence of a mutation, a fact which is of importance when interpreting immunochemical analyses in the absence of nucleotide sequence data, as many loss of function mutations might not be evident using these techniques. Indeed, if frameshift and nonsense mutations are as frequent as some data suggest [19], then previous immunocytochemical studies of p53 in head and neck cancer [26, 41–43] may have generously underestimated the frequency of *p53* mutations present, with obvious implications if the presence of detectable mutant p53 were to be used as a prognostic indicator.

Previous studies have documented specific nucleotide changes in *p53* in relation to agents important in the aetiopathogenesis of certain human tumour types, including hepatocellular carcinoma (G–T [44]), epidermal squamous cell carcinoma (C–T [45]) and tobacco-related cancers such as lung (G–T [20, 46]) and head and neck (G–T [23]). The nature of *p53* mutations detected in this study supports the concept of tobacco-related nucleotide changes in intraoral squamous cell carcinomas, with G–T transversions being present in three cell lines and G–A transversions in two cell lines, and thereby confirms previous reports of *p53* mutations and tobacco usage [19, 23, 24]. It is not possible to compare point mutations found in the present day study with the results of Sakai *et al.* [47] because these authors sequenced only a few *p53* mutants in some 30 oral carcinomas. Such comparison might further emphasize the importance of tobacco-related carcinogens in the aetiology of oral cancer.

Several studies have demonstrated the synergistic transforming ability of p53 mutants with cellular and viral oncogenes, including *HRAS*, *MYC* and HPV-16 E7 [48, 49]. Interestingly, in H357 a similar G–A nucleotide change was found at codon 13 of *HRAS* and thus one carcinogen may have targeted two separate growth-related genes in this patient. It is cautionary to note, however, that mutation of *RAS* genes is rare both in the cell lines of the present study [50] and in oral cancers of the western world [50, 51]; the status of other cellular oncogenes in these cell lines is currently under investigation.

In summary, this study demonstrates frequent *p53* mutations in cell lines derived from untreated human oral squamous carcinomas. Mutations detected in tobacco-related

cancers were not uncommon. In addition, we propose that the expression of mutant p53 species does not necessarily exclude a viral aetiology for such tumours.

1. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991, **351**, 453–456.
2. Shaulsky G, Goldfinger N, Rotter V. Alterations in tumor development *in vivo* mediated by expression of wild-type or mutant p53 proteins. *Cancer Res* 1991, **51**, 5232–5237.
3. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. *Nature* 1979, **278**, 261–263.
4. Sarnow P, Ho YS, Williams J, Levine AJ. The adenovirus E1b-55Kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54K cellular protein. *Cell* 1982, **28**, 387–394.
5. Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 1992, **358**, 80–83.
6. Scheffner M, Munger K, Byrne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA* 1991, **88**, 5523–5527.
7. Hausen H zur. Papillomaviruses in human cancers. *Mol Carcinogen* 1988, **1**, 147–150.
8. Yee C, Krishnan-Hewlett I, Baker CC, Schlegel R, Howley PM. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am J Pathol* 1985, **119**, 361–366.
9. Yeudall, WA. Human papillomaviruses and oral neoplasia. *Oral Oncol, Eur J Cancer* 1992, **28B**, 61–66.
10. Maitland NJ, Cox MF, Lynas C, Prime SS, Meanwell CA, Scully C. Detection of human papillomavirus DNA in biopsies of human oral tissue. *Br J Cancer* 1987, **56**, 245–250.
11. Yeudall WA, Campo MS. Human papillomavirus DNA in biopsies of oral tissues. *J Gen Virol* 1991, **72**, 173–176.
12. Crook T, Wrede D, Vousden KH. p53 point mutation in HPV negative human cervical carcinoma cell lines. *Oncogene* 1991, **6**, 873–875.
13. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1991, **63**, 1129–1136.
14. Crook T, Vousden KH. Properties of p53 mutations detected in primary and secondary cervical cancers suggest mechanisms of metastasis and involvement of environmental carcinogens. *EMBO J* 1992, **11**, 3935–3940.
15. Borresen AL, Helland A, Nesland J, Holm R, Trope C, Kaern J. Papillomaviruses, p53 and cervical cancer. *Lancet* 1992, **339**, 350–351.
16. McGregor JM, Levison DA, MacDonald DM, Yu CC. Papillomaviruses, p53 and cervical cancer. *Lancet* 1992, **339**, 351.
17. Sakai E, Tsuchida N. Most human squamous cell carcinomas in the oral cavity contain mutated p53 tumor-suppressor genes. *Oncogene* 1992, **7**, 927–933.
18. Brachman DG, Graves D, Voks E, *et al.* Occurrence of p53 gene deletions and human papilloma virus infection in human head and neck cancer. *Cancer Res* 1992, **52**, 4832–4836.
19. Burns JE, Baird MC, Clark LJ, *et al.* Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines. *Br J Cancer* 1992, **67**, 1274–1284.
20. Iggo R, Gatter K, Bartek J, Lane D, Harris AL. Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* 1990, **335**, 675–679.
21. Hollstein MC, Metcalfe RA, Welsh JA, Montesano R, Harris CC. Frequent mutation of the p53 gene in human esophageal cancer. *Proc Natl Acad Sci USA* 1990, **87**, 9958–9961.
22. Bennett WP, Hollstein MC, Metcalf RA, *et al.* p53 mutation and protein accumulation during multistage human esophageal carcinogenesis. *Cancer Res* 1992, **52**, 6092–6097.
23. Somers KD, Merrick MA, Lopez ME, Incognito LS, Schechter GL, Casey G. Frequent p53 mutations in head and neck cancer. *Cancer Res* 1992, **52**, 5997–6000.
24. Hollstein MC, Sidransky D, Vogelstein B, Harris CC. p53 mutation in human cancers. *Science* 1991, **253**, 49–53.
25. CRC Factsheet 14. *Oral Cancer* 1991, 1–5.
26. Gusterson BA, Anbazhagan R, Warren W, *et al.* Expression of p53 in premalignant and malignant squamous epithelium. *Oncogene* 1991, **6**, 1785–1789.
27. Prime SS, Nixon SV, Crane IJ, *et al.* The behaviour of human oral squamous cell carcinoma in cell culture. *J Pathol* 1990, **160**, 259–269.
28. Parkinson EK, Yeudall WA. Epidermis. In Masters J, ed. *Human Cancer in Primary Culture: A Handbook.* Boston, Kluwer Academic, 1991, 187–197.
29. Banks L, Matlashewski G, Crawford LV. Isolation of human-p53-specific monoclonal antibodies and their use in the study of p53 expression. *Eur J Biochem* 1986, **259**, 529–534.
30. Sambrook J, Fritsch E, Maniatis T. *Molecular Cloning: A Laboratory Manual,* second edition. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989.
31. Maestro R, Dolcetti R, Gasparotto D, *et al.* High frequency of p53 gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. *Oncogene* 1991, **7**, 1159–1166.
32. Boyle P, Zheng T, MacFarlane GJ, *et al.* Recent advances in the etiology and epidemiology of head and neck cancer. *Curr Opin Oncol* 1990, **2**, 539–545.
33. Crook T, Wrede D, Tidy JA, Mason WP, Evans DJ, Vousden KH. Clonal p53 mutation in primary cervical cancer: association with human-papillomavirus-negative tumours. *Lancet* 1992, **339**, 1070–1073.
34. Sedman SA, Hubbert NL, Vass WC, Lowy DR, Schiller JT. Mutant p53 can substitute for human papillomavirus type 16 E6 in immortalization of human keratinocytes but does not have E6-associated trans-activation or transforming activity. *J Virol* 1992, **66**, 4201–4208.
35. Peacock JW, Benchimol S. Mutation of the endogenous p53 gene in cells transformed by HPV-16 E7 and EJ c-ras confers a growth advantage involving an autocrine mechanism. *EMBO J* 1994, **13**, 1084–1092.
36. Smith KT, Campo MS. "Hit and run" transformation of mouse C127 cells by bovine papillomavirus type 4: the viral DNA is required for the initiation but not for maintenance of the transformed phenotype. *Virology* 1988, **164**, 39–45.
37. Slingerland JM, Minden MD, Benchimol S. Mutation of the p53 gene in human acute myelogenous leukemia. *Blood* 1991, 77, 1500–1507.
38. Mitsudomi T, Steinberg SM, Nau MM, *et al.* p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. *Oncogene* 1992, 7, 171–180.
39. Halevy O, Michaelovitz D, Oren M. Different tumor-derived p53 mutants exhibit distinct biological activities. *Science* 1990, **250**, 113–116.
40. Baker SJ, Preisinger AC, Jessup JM, *et al.* p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res* 1990, **50**, 7717–7722.
41. Field JK, Spandidos DA, Malliri A, Gosney JR, Yiagnansis M, Stell PM. Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of the head and neck. *Br J Cancer* 1991, **64**, 573–577.
42. Langdon JD, Partridge M. Expression of the tumour suppressor gene p53 in oral cancer. *Br J Oral Maxillofac Surg* 1992, **30**, 214–220.
43. Matthews JB, Scully C, Jovanovic A, Waal I van der, Yeudall WA, Prime SS. Relationship of tobacco/alcohol use to p53 expression in patients with lingual squamous cell carcinomas. *Oral Oncol, Eur J Cancer* 1993, **29B**, 285–289.
44. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wand NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* 1991, **350**, 427–428.
45. Brash DE, Rudolph JA, Simon JA, *et al.* UV induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci USA* 1991, **83**, 10124–10125.
46. Chiba I, Takahashi T, Nau MM, *et al.* Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. Lung Cancer Study Group. *Oncogene* 1990, **5**, 1603–1610.
47. Sakai E, Rikimaru K, Ueda M, *et al.* The p53 tumor suppressor

gene and ras oncogene mutations in oral squamous cell carcinoma. *Int J Cancer* 1992, **52**, 867–872.
48. Peacock JW, Matlashewski GJ, Benchimol S. Synergism between pairs of immortalizing genes in transformation assays of rat embryo fibroblasts. *Oncogene* 1990, **5**, 1769–1774.
49. Taylor WR, Egan SE, Mowat M, Greenberg AH, Wright JA. Evidence for synergistic interactions between *ras*, *myc* and a mutant form of p53 in cellular transformation and tumor dissemination. *Oncogene* 1992, **7**, 1383–1390.
50. Yeudall WA, Torrance LK, Elsegood KA, Speight P, Scully C, Prime SS. Ras gene point mutation is a rare event in premalignant tissues and malignant cells and tissues from oral mucosal lesions. *Oral Oncol, Eur J Cancer* 1993, **29B**, 63–67.
51. Chang SE, Bhatia P, Johnson NW, *et al.* Ras mutations in United Kingdom examples of oral malignancies are rare. *Int J Cancer* 1991, **48**, 409–412.

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