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Inactivation of the p53 Gene by Either Mutation or HPV Infection is Extremely Frequent in Human Oral Squamous Cell Carcinoma Cell Lines

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The state of p53 tumour suppressor and the frequency of high-risk human papillomavirus (HPV) infections were studied in nine human oral cancer cell lines. Three cancer cell lines (SCC-4, Tu-177 and FaDu) had similar amounts of p53 transcripts to normal cells, but contained significantly higher levels of p53 protein than the normal control cells. Sequencing highly conserved open reading frames of the p53 gene of these cancer cells showed point mutations in the SCC-4 and Tu-177 cell lines, a base transition from CCC to TCC occurred at codon 151; and in the line FaDu, a mutation of CGG to CTG occurred at codon 248. The HEp-2 and 1483 cancer lines contained significantly lower levels of p53 protein compared to the normal counterpart. Sequencing of p53 cDNA for HEp-2 and 1483 lines showed no mutations, but northern analysis revealed that these cell lines expressed HPV-18 E6/E7 messages. Four cell lines (SCC-9, SCC-15, SCC-25, and Tu-139) expressed negligible amounts of p53 transcripts compared to the normal counterpart and undetectable levels of p53 protein. These cell lines contained mutations in the highly conserved open reading frames of the p53 gene as follows: the SCC-9 had a deletion of 32 base pairs between codons 274 and 285; the line SCC-15 had an insertion of five base pairs between codons 224 and 225; the line SCC-25 had a deletion of two base pairs in codon 209; and the Tu-139 line had a deletion of 46 base pairs between codons 171 and 186. These data indicate that aberrant expression of p53 and altered levels of p53 protein are extremely frequent in oral cancer cell lines, and that these changes may result from either mutations or infection with high-risk HPV.

Keywords: oral cancer cells, p53, tumour suppressor gene, human papillomavirus

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INTRODUCTION

WILD-TYPE p53, a nuclear phosphoprotein, is a negative regulator of cell proliferation and functions as a tumour suppressor gene [1]. Wild-type p53 protein inhibits the co-transforming activity of *ras* with either mutant p53, c-*myc* or E1A proteins in rodent cells [2]. Also, alterations of the p53 gene through either rearrangement, deletion, or point mutation result in either no expression of wild-type p53 or overexpression of mutant p53 protein that, in turn, act as oncogene products. Such products are often found in epithelial cells that have escaped from senescence, i.e. human squamous cell carcinoma cells [3–7]. Mutations in p53 may result not only in loss of the suppressor activity associated with the gene product, but may also induce expression of mutant p53 protein that may be intrinsically oncogenic. Transfection of primary cells with plasmids expressing mutant p53 yields immortalised cells that are tumorigenic in concert with activated *ras* [8, 9]. Further, mutant p53 expression vectors enhance the tumorigenicity of weak tumorigenic cell lines and increase the metastatic potential of cancer cells [10]. Mutations of the p53 gene are present in most cancer subtypes, i.e. Friend virus-induced mouse leukaemic cells [11], non-small cell lung carcinomas [12], colorectal carcinomas [13], hepatocellular carcinomas [14], cervical cancer cells [7], bladder cancers [15], oesophageal cancers [16], and oral cancer cell lines [17]. The protein expressed from the mutant p53 gene has been implicated in growth deregulation and malignant progression of these cancers [1].

Human papillomavirus (HPV) infection is closely associated with the development of female genital epithelial cancers: over 90% of cervical cancer biopsy specimens contain HPV DNA [18]. Of more than 60 HPV types, HPV-16 and HPV-18 are

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most frequently associated with malignant genital lesions [19]. Similarly, HPV infection is also closely linked to benign and malignant oral lesions [20]. Recent studies show that up to 30–40% of oral cancer biopsy specimens contain the viral DNA [21]. Inasmuch as the oral mucosal epithelium resembles the female genital tract and is continuously challenged by innumerable environmental influences, close association between HPV infection and oral malignancies is not surprising. HPV's role in carcinogenesis derives from its transforming capacity. Transformations of normal human exocervical, cervical and oral epithelial cells—the major *in vivo* target cells for HPV infection—were established with cloned HPV-16 and -18 DNA [22–24]. These transformed cells are immortalised, contain integrated viral DNA expressing various HPV messages including E6/E7 mRNAs. Though the mechanism of HPV-induced cell malignancy remains unknown, expression of the viral E6 and E7 proteins may be closely associated with the tumorigenesis. In fact, high-risk HPV E6 and E7 proteins bind and inactivate the functions of wild-type p53 and Rb proteins, respectively.

In the present study, we investigated the status of the p53 gene (transcription, translation, and mutations) in nine human oral cancer cell lines, along with the expression of viral E6/E7 genes. Three oral cancer cell lines (SCC-4, Tu-177, and FaDu) expressed significantly higher levels of p53 protein than the normal counterpart, but these cells did not contain HPV-16 or -18 DNA. Sequencing of p53 cDNA showed that the p53 coding genes in these cell lines containing higher levels of p53 protein have point mutations. Two cell lines (HEp-2 and 1483) having lower levels of p53 protein than normal cells expressed HPV-18 E6/E7 genes. The p53 cDNA of these HPV-positive cancer cell lines did not show any mutations. Four cancer cell lines (SCC-9, SCC-15, SCC-25, and Tu-139) transcribed extremely low levels of p53 mRNA compared to the normal counterpart and contain undetectable amount of p53 protein. Sequence analysis of the p53 cDNA of these cell lines showed base deletions or insertion. These results indicate that aberrant expression of p53 is frequently found in oral cancer cells and that such aberrations may result from mutations and infection with high-risk HPV.

MATERIALS AND METHODS

Cancer cell lines, HPV-immortalised cells and preparation of normal human oral keratinocyte cultures

Nine human oral cancer cell lines and two HPV-immortalised oral keratinocyte cell lines were used in the present study. The cancer cell lines SCC-4, SCC-9, SCC-15, SCC-25, HEp-2 and FaDu were purchased from the American Type Culture Collection (ATCC; Rockville, Maryland). The Tu-139, Tu-177 and 1483 cancer cell lines were obtained from Drs G.L. Clayman and E. Shillitoe (University of Texas, Houston, Texas). The HPV-immortalised cell lines include the HOK-16B and HOK-18A cell lines that were immortalised by transfection with HPV-16 and HPV-18, respectively [24, 25]. All oral cancer cell lines except the HEp-2 and FaDu lines were cultured in Dulbecco's modified minimum essential medium (DMEM)/Ham's F12 (GIBCO/BRL, Bethesda, Maryland) supplemented with 10% fetal bovine serum (FBS) and 0.4 μg/ml hydrocortisone, while the HEp-2 and FaDu lines were grown in DMEM supplemented with 10% FBS. Normal human oral keratinocytes (NHOK) were prepared as described previously [24].

Northern analysis

To determine the transcription of p53, HPV-16 E6/E7, HPV-18 E6/E7 and β-actin genes, cytoplasmic poly(A^+)RNA was extracted from cells using standard procedures. Probes used for northern analysis included: *Bam*HI-restricted 1.2 kilobase pair (kbp) HPV-16 DNA fragment containing intact E6/E7 genes [26]; *Eco*R1-*Bam*H1-digested 2.4 kbp fragment of HPV-18 DNA representing major early HPV-18 message including E6/E7 genes [25]; p53 cDNA (from Dr E. Harlow; Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts); and human β-actin gene (from Dr L. Kedes; Stanford University, Palo Alto, California). All were labelled with [^{32}P]dCTP (ICN Radiochemicals, Irvine, California) by multiprime labelling (Amersham Corp., Arlington Heights, Illinois). Specific radioactivity of labelled probes was always higher than 5×10^8 cpm/μg of DNA.

Five micrograms of poly(A^+)RNAs were denatured and run on a 1.2% formaldehyde agarose gel with marker RNAs (9.5, 7.5, 4.4, 2.4, 1.4 and 0.24 kb RNA ladder, GIBCO/BRL). The RNAs were transferred to nylon filters (Amersham Corp.) and crosslinked with ultraviolet light for 5 min. The filter was hybridised to ^{32}P-labelled probe at 42°C for 24 h in 50% formamide/10% dextran sulphate/5 × SSPE (0.15 mol/l NaCl, 0.01 mol/l Na_2HPO_4, and 0.001 mol/l EDTA)/5 × Denhardt's solution/denatured salmon sperm DNA (20 μg/ml). Filters were washed twice in 5 × SSPE for 15 min at 42°C, then in 1 × SSPE/0.1% sodium dodecyl sulphate (SDS) for 30 min at 42°C, and finally in 0.1 × SSPE/0.1% SDS for 30 min at room temperature. Filters were then autoradiographed on SB-5 X-ray film (Eastman Kodak, Rochester, New York) for 12 h at −70°C. After exposure, the probe was stripped off the filter for rehybridisation to the next radiolabelled probe.

Western analysis

Cells grown in Petri dishes were lysed on ice for 30 min in lysis buffer (10 mmol/l Na_2HPO_4 pH 7.2, 9 mg/ml NaCl, 1% Triton X-100, 5 mg/ml sodium deoxycholate, 1 mg/ml SDS, 2 mg/ml sodium azide and 40 μg/ml sodium fluoride). The cell lysate was centrifuged at 14000 rpm for 20 min, and the supernatant containing 1 mg/ml of protein was denatured by boiling for 2 min in a sample buffer (62.5 mmol/l Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 1% β-mercaptoethanol and 0.001% bromophenol blue). An aliquot of supernatant containing 100 μg of protein was electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto an immobilon-P membrane (Millipore Corp., Bedford, Massachusetts). After incubating in blocking buffer (0.2% I-block, phosphate buffered saline, and 0.05% Tween 20) for 3 h at room temperature, the membrane was exposed to mouse antihuman monoclonal antibody for p53 (Ab-2 [PAb1801]; Oncogene Sciences, Manhasset, New York) at room temperature for 1 h. After washing with blocking buffer, the membrane was treated with antimouse IgG-alkaline phosphatase conjugate (Tropix Inc., Bedford, Massachusetts), and again washed with blocking buffer and assay buffer (0.1 mol/l diethanolamine and 1.0 mmol/l $Mgcl_2$). The membrane was then incubated in Nitroblock reagent, washed again with assay buffer, and incubated in chemiluminescent substrate solution using the Western-Light^R kit (Tropix Inc., Bedford, Massachusetts). The membrane was exposed to SB-5 X-ray film for 4 min at room temperature.

Immunohistochemistry

The avidin–biotin–horseradish peroxidase complex (ABC) procedure was used to stain p53 protein using the DAKO LSAB® Kit (DAKO Corp., Carpinteria, California). Cancer cell monolayers were fixed in acetone for 3 min and treated with 3% hydrogen peroxide for blocking the intrinsic peroxidase activity. After an initial blockage with non-immune goat serum, the cells were exposed to mouse antihuman monoclonal antibody for p53 (Ab-2 [PAb1801]; Oncogene Sciences). The cells were then treated with biotinylated antimouse IgG and peroxidase-conjugated streptavidin followed by an incubation in peroxidase substrate solution (3-amino-9-ethylcarbazole) and counterstaining with Mayer's haematoxylin.

Determination of HPV DNA using polymerase chain reaction (PCR)

The specific primers for HPV-6/11, -16 and -18 DNA were constructed at the UCLA Jonsson Cancer Center to amplify specific DNA sequences of HPV-6/11 (157 bp), HPV-16 (98 bp), and HPV-18 (80 bp), all from unique areas in the E6/E7 gene [27]. These primers from E6/E7 open reading frames (ORFs) are chosen because (1) the region E6/E7 remains intact during the integration of HPV DNA sequences into the human genome and (2) these ORFs are responsible for cellular transformation [28]. The primers of the HPV-6/11 are: 5'GTGTTTTGCAGGAATGCACTGACCA3' (sense primer) and 5'CAGCATAATTAAAGTGTCTATATTG3' (antisense primer). HPV-16 primers are 5'ACCGAAACCGGTTAGTATAAAAGC3' (sense primer) and 5'ATAACTGTGGTAACTTTCTGGGTC3' (antisense primer). HPV-18 primers are 5'CGGTCGGGACCGAAAACGGTG3' (sense primer) and 5'CGTGTTGGATCCTCAAAGCGCGCC3' (antisense primer). The PCR were carried out in a DNA thermal cycler using 25 µl of DNA extract, 200 µmol/l each of dATP, dCTP, dGTP and dTTP, 1.0 µmol/l of each primer and 2.5 units of *Taq* DNA polymerase in a final volume of 100 µl. After an initial denaturation for 2 min at 94°C, a typical cycle profile was 2 min at 55°C (annealing) and 1 min at 72°C (extension), followed by 1 min at 94°C to denature the DNA for the next cycle. A total of 30 cycles were run with a final extension step at 72°C for 7 min. Positive controls consisting of purified HPV DNA of various types were included in each run as well as negative controls of DNA from cell lines known to be HPV negative, and primers run with no DNA whatsoever.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis, cloning and DNA sequencing

The synthesis and amplification of p53 cDNAs were carried out using an RNA PCR kit (Perkin-Elmer Cetus, Irvine, California). In a total volume of 20 µl, 100 ng of poly(A$^+$)RNA was dissolved in a solution containing 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.01% gelatin, 1 mmol/l each of the four deoxyribonucleoside triphosphates, 2.5 µmol/l of oligo(dT) primer, 1 unit of RNAase inhibitor, and 2.5 units reverse transcriptase. Reverse transcription mixture was incubated at 42°C for 15 min, at 99°C for 5 min and at 5°C for 5 min.

The resulting cDNA products were then amplified by addition of 80 µl of PCR mixture containing 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 2.5 units of recombinant *Taq* DNA polymerase and 0.5 µmol/l of p53 primers. The sense primer for p53 cDNA amplification extends from nucleotide (nt) 296 to nt 315 and the antisense primer extends from nt 996 to 1015 of the cDNA (sense primer, 5'-CCCAGAAAACCTACCAGGGC-3'; antisense primer, 5'-CGAAGCGCTCACGCCCACGG-3'). Each amplification cycle consisted of 1 min of denaturation at 94°C, followed by 2 min of annealing (60°C) and 3 min of extension (72°C). A total of 35 cycles were run with a final extension step at 72°C for 7 min. Amplified cDNAs were ligated to pCRII vector using TA™ Cloning Kit (Invitrogen, San Diego, California) under the conditions recommended by the manufacturer.

The nucleotide sequences of the cloned p53 cDNA were determined by the primer extension method. Plasmid DNAs (3–5 µg) were denatured in 0.2 mol/l NaOH and 0.2 mmol/l EDTA at 37°C for 30 min. The denatured DNA was neutralised with 0.1 volume of 3 mol/l sodium acetate (pH 5.2), precipitated with three volumes of ethanol, and centrifuged. The DNA pellet was redissolved in 7 µl of distilled water, 2 µl of Sequenase (T7 DNA polymerase, US Biochemical Corp., Cleveland, Ohio) reaction buffer and 1 µl of sequencing primer (20 pmol) followed by incubation at 65°C for 2 min for annealing. To the annealed mixture, 2 µl of Sequenase (1.625 units), 1 µl of 0.1 mol/l dithiothreitol, 2 µl of labelling mix (1.5 µmol/l dGTP, 1.5 µmol/l dCTP and 1.5 µmol/l dTTP), and 0.5 µl of [^{35}S]α-dATP (10 mCi/ml, Amersham Corp., Arlington Heights, Illinois) were added. After incubation at room temperature for 5 min, 3.5 µl of the mixtures were added to each of four different tubes, containing 2.5 µl of the appropriate termination mixtures. After a 5-min incubation at 37°C, the reaction was stopped with 95% formamide dye. Samples were heated to 75°C for 2 min, and 1.8 µl of each sample were loaded onto a 7% acrylamide urea sequencing gel. Samples were run at a constant voltage for 3–7 h. The gel was fixed in acetic acid and methanol, dried, and exposed to XAR-5 X-ray film for 2 days (Eastman Kodak) at −80°C.

RESULTS

Expression of p53

Figure 1 shows that a 2.7-kb mRNA is the major transcript of the p53 gene in NHOK, in the HPV-immortalised cell lines, and in the cancer cell lines SCC-4, Tu-177, HEp-2, FaDu and 1483. Enhanced p53 transcription from the immortalised HOK-16B and HOK-18A was similar to previous reports [24, 25]. The SCC-4, Tu-177 and HEp-2 cell lines transcribed notably higher amounts of p53 mRNAs than did NHOK. The amount of p53 mRNA expressed from the cell line FaDu was similar to that from NHOK. The cancer cell lines SCC-9, SCC-15, SCC-25, Tu-139 and 1483 contained significantly lower levels of p53 transcripts compared to NHOK.

The SCC-4, Tu-177, and FaDu cancer cell lines contained approximately 35–40 times higher levels of p53 protein compared to NHOK (Fig. 2 and Table 1) when determined by western analysis. The cell lines HOK-16B, HOK-18A and HEp-2 had notably lower levels of the tumour suppressor gene product, and their levels were approximately one third of that found in NHOK. The p53 protein was not detected from cell lines SCC-9, SCC-15, SCC-25, Tu-139 and 1483 by western analysis, though p53 transcripts were detected from these cell lines (Figs 1, 2 and Table 1). The results of immunohisto-

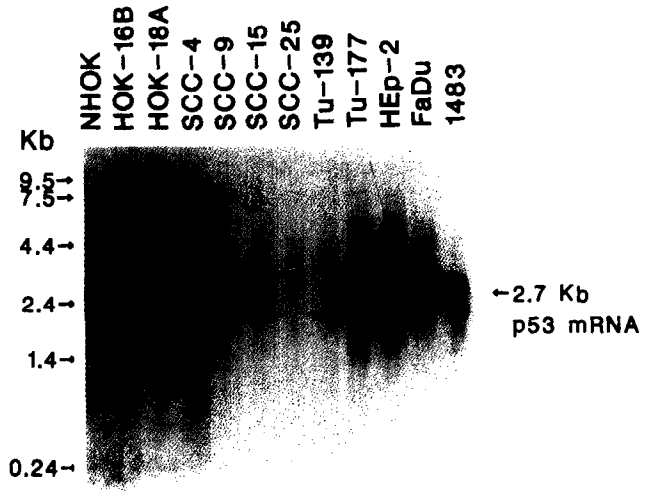

Fig. 1. Autoradiogram of the northern blot hybridisation of poly(A$^+$)RNA from NHOK, HOK-16B, HOK-18A and cancer cell lines indicated above to , ^{32}P-labelled human p53 cDNA. After the filter was exposed to X-ray film, the probe was stripped from the filter for subsequent hybridisations for detecting the presence of HPV-16, HPV-18 transcripts and β-actin (lower panel).

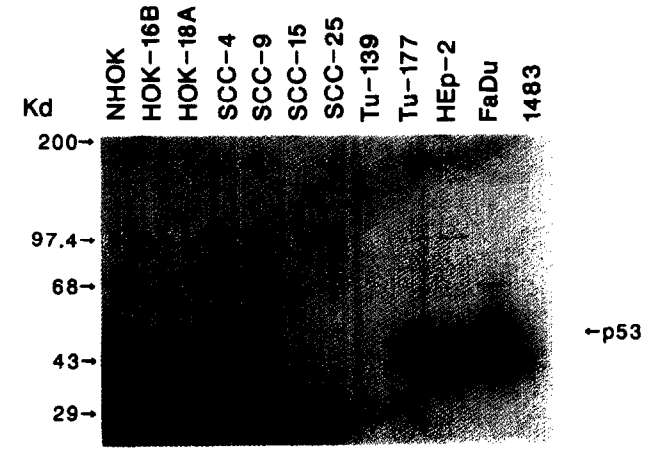

Fig. 2. Western analysis of electrophoretically separated p53 protein from NHOK, HOK-16B, HOK-18A and cancer cell lines indicated above. The bands whose sizes are smaller than p53 protein may be the metabolites of p53.

chemical staining of the p53 protein were similar to those of western analysis: cell lines SCC-4, Tu-177 and FaDu showed notably higher immunostaining compared to NHOK. The HOK-16B, HOK-18A, and cancer cell lines SCC-9, SCC-15, SCC-25, Tu-139, HEp-2 and 1483 revealed negligible intensity of staining, and did not show a significant difference from that of NHOK (Fig. 3).

Mutational analysis of p53 cDNA

Most point mutations in the p53 gene extend the half-life of p53 protein, resulting in a higher level of p53 protein in cells [27]. Thus, PCR amplification of the cDNA spanning the conserved p53 region (codons 117–309) was carried out, and nucleotide sequences were determined to detect, if any, mutations of this gene in oral cancer cell lines. Three cancer cell lines, SCC-4, Tu-177 and FaDu, containing notably higher amounts of p53 protein (compared with NHOK) had point mutations. This resulted in amino acid substitutions at codons 151 and 248, respectively: the SCC-4 and Tu-177 cell lines showed a mutation of CCC to TCC (C→T transition) at codon 151 resulting in an amino acid change from arginine to histidine (Figs 4 and 5); and the FaDu cell line had a mutation of CGG to CTG (G→T transition) at codon 248 resulting in the substitution of arginine with leucine (Fig. 6). These mutations were verified from at least three different clones. No p53 mutations were found in the amplified region of p53 cDNA originating from either NHOK, HOK-16B, HOK-18A, HEp-2 or 1483 cell lines. The SCC-9, SCC-15, SCC-25, and Tu-139 cell lines also presented mutations in the highly conserved ORF of p53 gene (Fig. 7). The SCC-9 cell line had a deletion of 32 bp from codon 274 to 285. The SCC-15 cell line showed an insertion of five bp (ATCTG) between codons 224 and 225. The cell lines SCC-25 showed deletion of the last two bp (AG) of codon 209. Finally, the Tu-139 cell line showed a deletion of 46 base pairs from the last nucleotide of codon 171 to codon 186 (Fig. 7).

Presence and expression of HPV-16 and HPV-18 DNA

As expected, the HOK-16B and HOK-18A cell lines expressed HPV-16 and HPV-18 E6/E7 genes, respectively. None of the cancer cell lines expressed HPV-16 E6/E7 transcripts (Fig. 8). Both HEp-2 and 1483 cell lines expressed HPV-18 genes: abundant poly(A$^+$)RNAs of 1.6 kb and 3.5 kb from the HEp-2 cell line, and rather small amounts of poly(A$^+$)RNAs with sizes of 1.6 kb, 4.6 kb, 7.0 kb and 9.7 kb in 1483 cell line were detected (Fig. 9). To confirm the absence of HPV-6, 11, 16 and 18 DNA in NHOK, SCC-4, SCC-9, SCC-15, SCC-25, Tu-139, Tu-177 and FaDu cell lines, PCR was performed using primers specific for HPV-6, 11, 16 and 18 DNA. None of the cell lines were positive.

DISCUSSION

The molecular mechanisms underlying the development of oral cancer remain largely unknown. Several studies indicate the overexpression and/or amplification of c-*erb*B-1/epidermal growth factor receptors (EGFR), c-*myc*, c-*bcl* and c-Ha-*ras* are closely associated with the development of oral cancer in humans [28–30]. Furthermore, amplification and overexpression of c-*erb*B-1/EGFR and c-Ha-*ras*1 gene can be induced by application of the chemical carcinogen, 7,12-dimethylbenz(*a*)anthracene (DMBA), in hamster cheek pouch epithelium [31], again indicating the close linkage between the activation of cellular proto-oncogenes and oral carcinogenesis. Inactivation of tumour suppressors through gene mutations and high-risk HPV infections are also linked to oral carcinogenesis. For example, high levels of p53 protein resulting from point mutation of the p53 gene are frequently found in head and neck cancer tissue [32, 33]. High-risk HPV DNA is also found in a high percentage of human oral cancer specimens [20]. Moreover, the loss of tumour suppressor activity is consistently observed from cancer cells derived from experimentally induced animal oral tumours [34]. Therefore, it appears that the inactivation of tumour suppressor genes, along with the dominant activation of proto-oncogenes is involved in oral carcinogenesis.

Northern and western analyses indicate that aberrant

Table 1. *Status of* p53 *tumour suppressor and HPV infection in human oral cancer cell lines*

Cell lines	p53 mRNA	p53 protein	p53 mutation	HPV-16/18 E6/E7 expression
NHOK	Yes	1.00	No	No
HOK-16B	Yes	0.30	No	Yes (HPV-16)
HOK-18A	Yes	0.32	No	Yes (HPV-18)
SCC-4	Yes	35.3	Yes (point mutation)	No
SCC-9	Yes (low)	0.00	Yes (deletion)	No
SCC-15	Yes (low)	0.00	Yes (insertion)	No
SCC-25	Yes (low)	0.00	Yes (deletion)	No
Tu-139	Yes (low)	0.00	Yes (deletion)	No
Tu-177	Yes	40.2	Yes (point mutation)	No
HEp-2	Yes	0.35	No	Yes (HPV-18)
FaDu	Yes	38.1	Yes (point mutation)	No
1483	Yes	0.32	No	Yes (HPV-18)

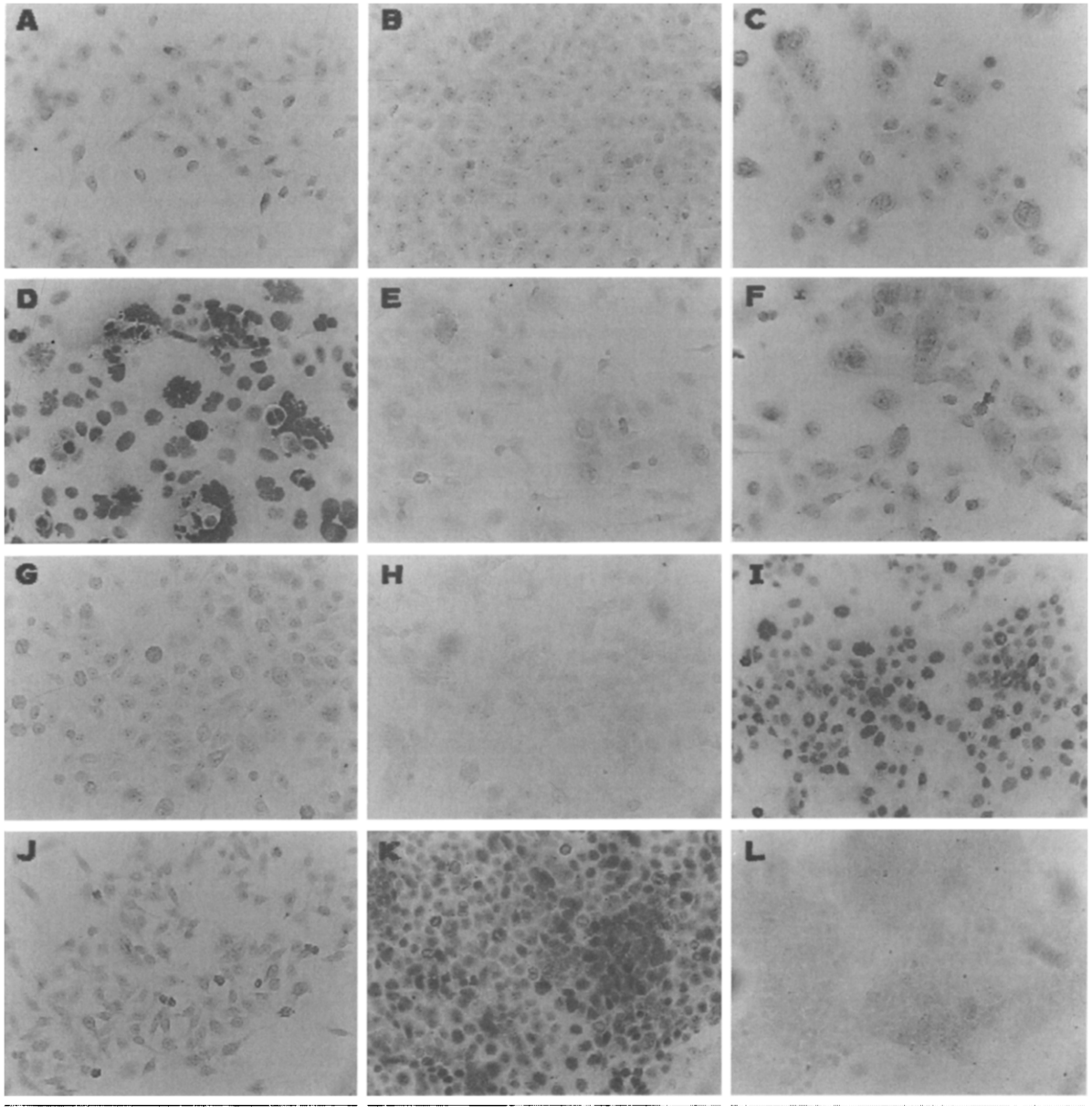

Fig. 3. Immunoperoxidase staining of NHOK (A), HOK-16B (B), HOK-18A (C), SCC-4 (D), SCC-9 (E), SCC-15 (F), SCC-25 (G), Tu-139 (H), Tu-177 (I), HEp-2 (J), FaDu (K) and 1483 (L) cell lines with p53 monoclonal antibody.

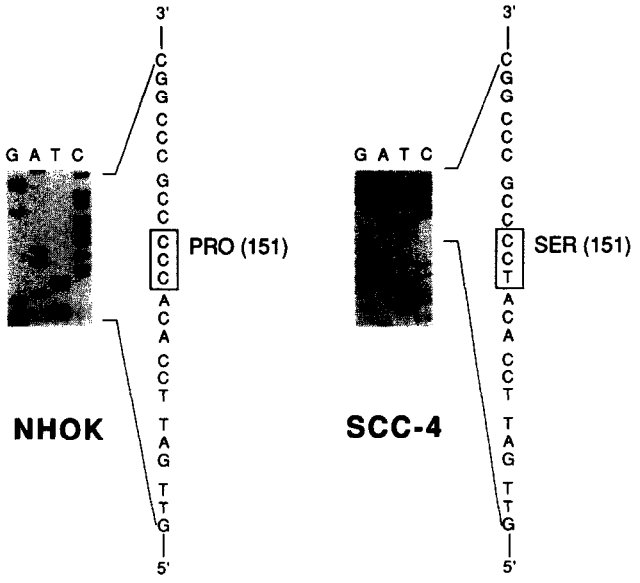

Fig. 4. Nucleotide sequence analysis of p53 cDNA of the cell line SCC-4. The wild-type sequence in NHOK in the same region is shown alongside. A point mutation CCC→TCC at codon 151 resulting in amino acid change of proline to serine.

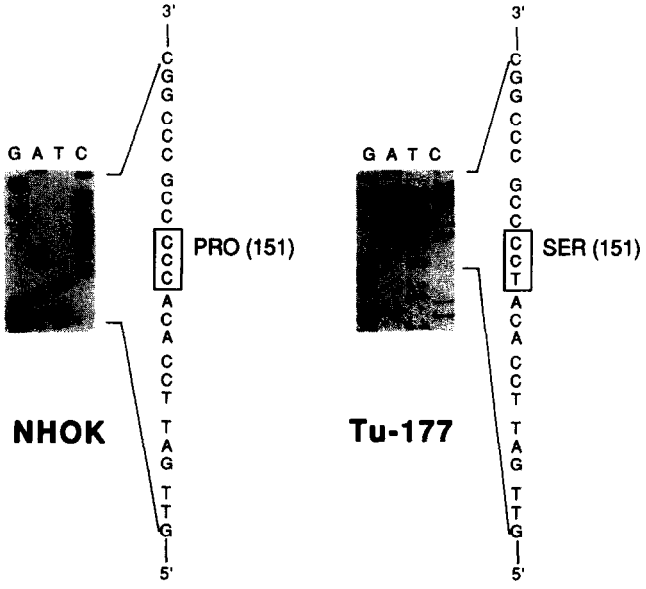

Fig. 5. Nucleotide sequence analysis of p53 cDNA of the cell line Tu-177. The wild-type sequence in NHOK in the same region is shown alongside. A base transition from CCC to TCC at codon 151 resulting in amino acid change of proline to serine.

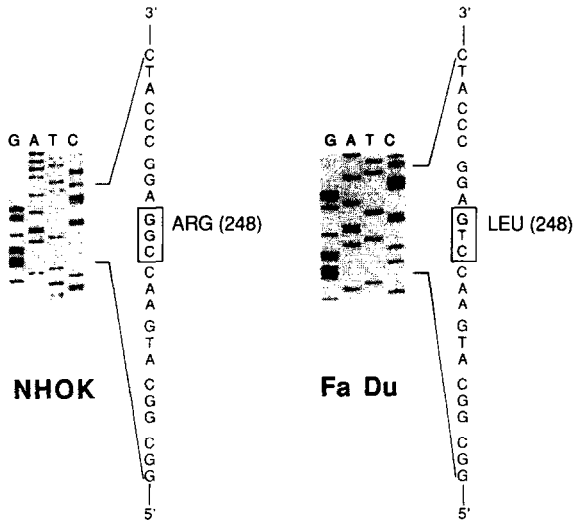

Fig. 6. Nucleotide sequence analysis of p53 cDNA of the cell line FaDu. The wild-type sequence in NHOK in the same region is shown alongside. A base transition from CGG to CTG at codon 248 resulting in amino acid change of arginine to leucine.

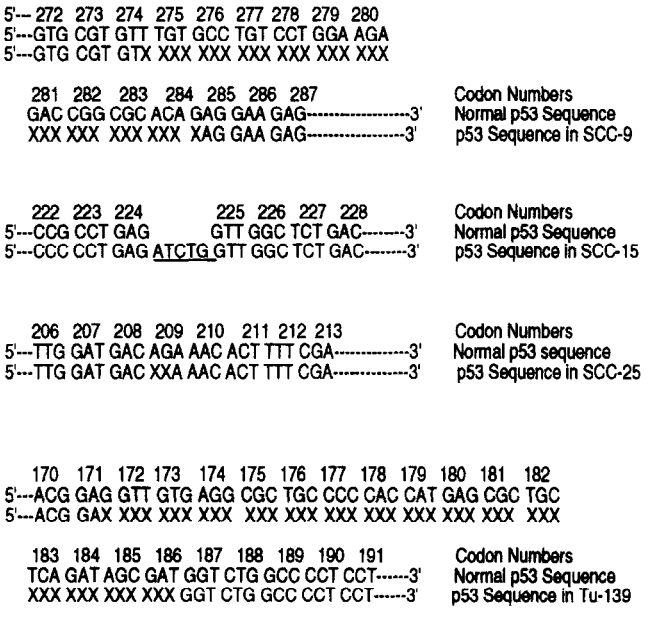

Fig. 7. Genomic mutations in the p53 gene in the SCC-9, SCC-15, SCC-25, and Tu-138 cell lines. In the SCC-9 cell line, deletion of 32 bp from codon 274 to 285; the SCC-15 cell line, insertion of five bp between codons 224 and 225; in the SCC-25 line, deletion of 2 bp in codon 209; and in the Tu-139 line, deletion of 46 bp from codon 171 to codon 186. X (in SCC-9, SCC-25 and Tu-138 lines) indicates the deleted nucleotide. Underlined sequence of nucleotides (in SCC-15 line) are inserted sequences.

transcription and altered levels of p53 protein, respectively, are frequent in oral cancer cell lines: three cell lines show notably higher levels of mutant p53 protein, two cell lines show a lower level of wild-type p53 protein compared with the normal counterpart, and four cell lines do not contain a detectable amount of p53 protein. Inasmuch as point mutations in the p53 gene result in the expression of mutant p53 protein with a longer half-life than the wild-type [27], increased levels of p53 protein in the SCC-4, Tu-177 and FaDu cell lines are assumed to be due to point mutations in the p53 gene in these HPV-negative cells. The mutations occurred at codons 151 and 248, and mapped to the conserved region where most mutations have been detected in many human cancers [12]. The results of immunohistochemical analysis of p53 in these three cell lines were similar to western analysis. Therefore, the immunostaining of p53 may be as useful as western analysis for the determination of the enhanced level of mutant p53 in cells.

Low levels of p53 protein in the HEp-2 and 1483 cell lines harbouring HPV-18 DNA may be associated with the ex-

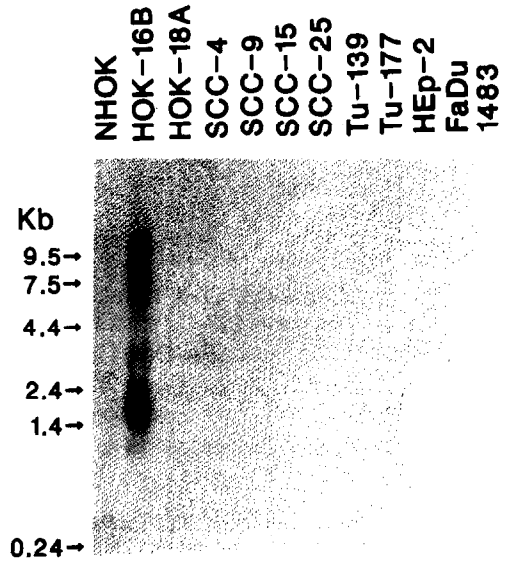

Fig. 8. Autoradiogram of the northern blot hybridisation of poly(A$^+$)RNA from NHOK, HPV-immortalised cells and cancer cell lines indicated above to ^{32}P-labelled 1.2 kbp fragment of HPV-16 DNA.

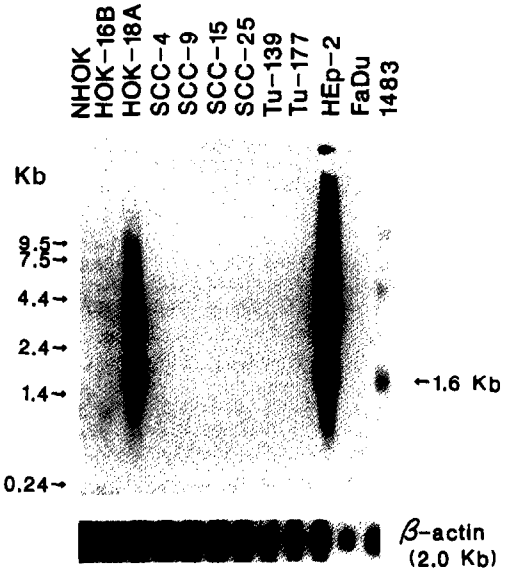

Fig. 9. Autoradiogram of the northern blot hybridisation of poly(A$^+$)RNA from NHOK, HPV-immortalised cells and cancer cell lines indicated above to ^{32}P-labelled 2.4 kbp fragment of HPV-18 DNA.

pression of viral E6 transcripts. Since the viral E6 protein makes a complex with wild-type p53 protein [35] and promotes the ubiquitin-dependent degradation of the latter *in vitro* [36] and *in vivo* [37], the low levels of p53 protein in the HEp-2 and 1483 cell lines might result from the enhanced degradation of the protein by the association of the viral E6 protein. These results confirm previous studies demonstrating lower levels of wild type p53 protein in human cervical cancer cell lines habouring high-risk HPV DNA [7] and in human oral keratinocytes transformed with cloned HPV DNA, as compared with the normal control cells [38]. However, the intensity of immunohistochemical staining of p53 protein in NHOK, HOK-16B, HOK-18A, HEp-2 and 1483 were similar to each other, indicating that the immunohistochemical staining may not be sensitive enough to determine the different levels of wild-type p53 protein in normal cells and cells expressing "high risk" HPV E6/E7 genes.

We also observed four oral cancer cell lines that transcribed very low levels of p53 transcripts but contained no p53 protein. Inasmuch as these cell lines neither contain "high risk" HPV DNA nor express the viral E6/E7 genes, we cannot explain the reason why these cells have such low amounts of intracellular p53 protein. However, low expression of p53 transcripts must, in part, be responsible for the absence of p53 protein in these cells. Though the mode of low expression of p53 transcripts in these cancer cell lines remains unknown, mutations such as insertions and deletions of the p53 gene in four cell lines (SCC-9, SCC-15, SCC-25, and Tu-139) may be linked to it. Again, the intensity of immunohistochemical staining of p53 protein in these cell lines was similar to that of the normal counterpart. These data indicate that immunohistochemical analysis may be useful for screening the increased level of p53 protein with point mutations in cells, but other analysis such as western blotting and sequencing of the gene may be necessary for rather accurate determinations of p53 protein levels and the nature of mutations.

These data provide evidence that an aberrant expression of the p53 gene inactivates p53 tumour suppressor activity. Such inactivation can be found in oral cancer cells, and thus, may be closely linked to oral carcinogenesis. Our present data coincide with other studies that have demonstrated frequent inactivation of p53 function by gene mutation or high-risk HPV infection in head and neck cancer cell lines and/or cancer tissue [41–47]. This study demonstrates that the inactivation of p53 gene is extremely frequent in human oral cancer cells, and the inactivation is induced by either mutations or high-risk HPV infections. The role of p53 inactivation in oral carcinogenesis remains unknown and should be further investigated.

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