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p53 GENE MUTATIONS IN ORAL CARCINOMAS FROM INDIA

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In this study, we analyzed 53 oral squamous-cell carcinomas among Indians for the presence of alterations in the tumor-suppressor gene *p53* by PCR-SSCP and sequencing methods. Our results showed that 21% (11/53) of oral carcinomas analyzed carried mutations within the exons 5-8 of the *p53* gene. We have identified 11 single-base pair substitutions consisting of 10 mis-sense mutations and one at the splice acceptor site, and one deletion mutation involving 4 consecutive bases. The majority of the base substitutions were transitions (5 TA to CG and 5 GC to AT), while only one transversion (TA to GC) was observed. Probable hot-spots for the mutation induction were identified at codons 149 and 274, which have not been observed before in head-and-neck cancers. The mutational spectrum might have originated from base alkylations at guanine and thymine residues, caused by some alkylating agents. The present results are thus consistent with the involvement of tobacco-related nitrosoamines in the etiology of oral squamous-cell carcinoma.

Oral squamous-cell carcinoma is relatively uncommon in most parts of the world. However, in India, it occupies the top rank among male cancers and ranks within the first 5 among female cancers (Gupta, 1993). Oral cancers of Indian origin thus represent a unique system for studying the role of carcinogens, because they are often linked to the tobacco-chewing habit.

The tumor-suppressor gene *p53* is the most frequently mutated gene in human cancers, where loss of one allele is often coupled with a point mutation in the other (Caron de Fromentel and Soussi, 1992; Levine *et al.*, 1994). Studies on *p53* gene mutations in oral cancer from Japan, China, USA, Switzerland and Papua New Guinea indicate that the *p53* gene is mutated in 17 to 63% of the tumors and most of these mutations occur in highly conserved regions [exons 5–9 of the gene (Sakai and Tsuchida, 1992; Sakai *et al.*, 1992; Chang *et al.*, 1992; Somers *et al.*, 1992; Brachman *et al.*, 1992; Boyle *et al.*, 1993; Chung *et al.*, 1993; Zariwala *et al.*, 1993; Thomas *et al.*, 1994). There are only 2 reports on the mutational profile of tobacco-chewing-related oral cancers. Ranasinghe *et al.* (1993) reported no mutation in Sri Lankans when 5 neoplasms overexpressing *p53* gene were analyzed, while Thomas *et al.* (1994) observed mutations in 17% of 30 tumors from tobacco chewers.

The present study was conducted to determine the mutational profile in the tumor-suppressor gene *p53* in tobacco-related oral tumors of Indians. To screen the *p53* mutations, the DNAs were amplified by PCR and the amplified products were subjected to single-strand conformation polymorphism (SSCP) analysis, then the SSCP-positive samples were sequenced for identification of the mutations. The results showed that 21% of SCCs of the oral cavity had mutations in the *p53* gene.

MATERIAL AND METHODS

Patients and tumor samples

Samples of 53 surgically resected oral squamous-cell carcinomas arising from the cheek (27), tongue (8), alveolus (8), angle of mouth (5), lips (3), floor of the mouth (1) and palate (1) were collected from patients at the Government Rajaji Hospital, Madurai, India. The tumors were put in cold PBS and immediately transported on ice to the laboratory. They were either snap-frozen in liquid nitrogen and stored at −70°C or immediately processed for DNA isolation. None of the patients had received chemotherapy or radiotherapy prior to tumor resection.

DNA extraction

High-molecular-weight DNA was prepared from tumor tissues by phenol/chloroform extraction (Maniatis *et al.*, 1989).

PCR and sequencing primers

Four sets of primers were used to amplify exons 5–8 of the *p53* gene. The same sets of primers were used for DNA sequencing. The sequences of these primers are:

E5S	TGTTCACTTGTGCCCTGCT
E5A	CAGCCCTGTCGTCTCTCCAG
E6S	GCCTCTGATTCCTCACTGAT
E6A	TTAACCCCTCCTCCAGAGA
E7S	ACTGGCCTCATCTTGGGCCT
E7A	TGTGCAGGGTGGCAAGTGGC
E8S	TAAATGGGACAGGTAGGACC
E8A	TCCACCGCTTCTTGTCCTGC

These primers were selected from the previous study (Sakai and Tsuchida, 1992) and obtained by custom ordering from Oligos Etc. (Wilsonville, OR).

Polymerase chain reaction and single-strand conformation polymorphism (SSCP) analysis

Exons 5 to 8 of the *p53* gene were PCR-amplified from tumor DNAs and mutations were detected by SSCP using a 6% non-denaturing polyacrylamide gel containing 5% glycerol as described previously (Sakai and Tsuchida, 1992). Cell-line DNAs from HOC605, NA, Ca922 and ZA (Sakai and Tsuchida, 1992) were used as positive controls.

Direct sequencing

Abnormal bands detected by SSCP were excised from the dried gel, placed in 50 μl of distilled water and incubated at 80°C for 30 min. An aliquot (5 μl of the supernatant) was then used for PCR. The double-stranded PCR products were purified from agarose gel by electrophoretically transferring the DNA band to Whatman DE-81 paper, then eluting with 1.5 M NaCl. The DNA sequence of the PCR product was determined by using a PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster

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City, CA). The gel electrophoresis, data collection and analysis were performed on an Applied Biosystems Model 373 A automated sequencer.

RESULTS

PCR/SSCP analysis

Fifty-three squamous-cell carcinomas of the oral cavity were examined for *p53* gene mutations. Exons 5–8 of the *p53* gene were amplified in the presence of alpha-^{32}P dCTP by PCR and analyzed by SSCP. DNA fragments showing electrophoretic mobility shifts in comparison to those of normal cell DNAs were scored as positive for mutation. Human placental and peripheral lymphocyte DNAs were used as normal controls and HOC605, NA, Ca922 and ZA DNAs as positive controls of mutated fragments of exons 5, 6, 7 and 8, respectively, and included in SSCP analyses (Sakai and Tsuchida, 1992). In comparison with normal cell DNAs, 11 tumors showed a mobility shift (Figs. 1 and 2*a*). Some PCR-amplified fragments of tumor DNAs showed mobilities similar to those of wild-type controls with varying intensities. The amplified DNAs showing only mutant bands were used directly for sequencing. Wherever additional bands corresponding to normal cell DNAs were also present, the mutant fragment was excised and the DNA from the band was reamplified and subsequently used for sequencing. A representative sequence analysis is shown in Figure 2*b*.

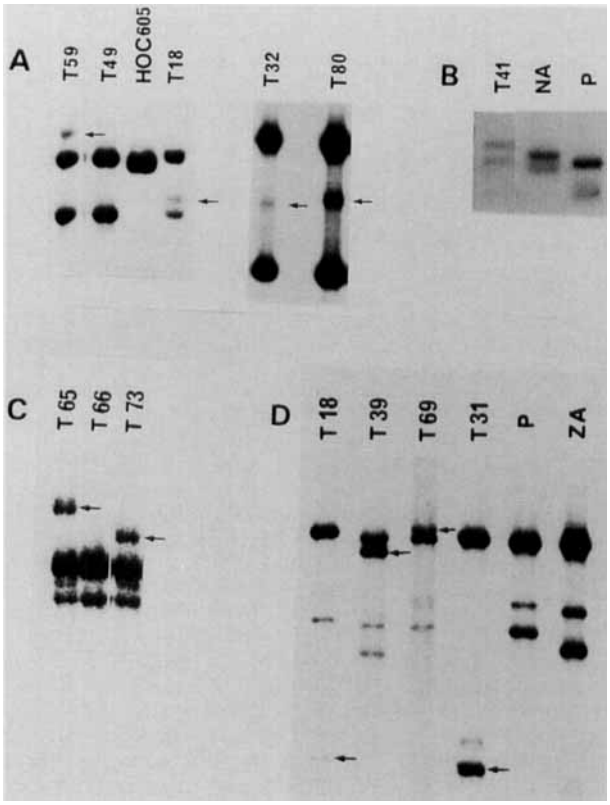

FIGURE 1 – PCR-SSCP analysis of tumor DNAs. *(a)* Exon 5; *(b)* Exon 6; *(c)* Exon 7; *(d)* Exon 8. DNAs from tumors are indicated by T followed by tumor number. P, placental DNA. HOC605, NA and ZA are DNAs from cell lines having mutated *p53* genes of 126 Tyr > Stop, 220 Tyr > His, and 279 Gly > Glu, respectively. Two major unmarked bands shown in *a* and *c*, and bands corresponding to placental DNA shown in *d*, are those from the wild-type sequence in tumor DNAs.

Determination of mutated sequences

Direct sequencing of SSCP-positive samples revealed several base-substitution mutations and a deletion mutation (Table I). Five tumor samples had mutations in exon 5: these mutations consisted of 3 different base substitutions in codon 143 (GTG to ATG), codon 149 (TCC to CCC) and codon 181 (CGC to CAC). Only one tumor had a mutation in exon 6 in which a TAT to GAT conversion was observed in codon 205. Two different mutations in exon 7, one in codon 237 (ATG to ATA) and the other in the splice acceptor site (AG/GT to AG/AT) were observed in 2 different tumors (Table I). Four samples had mutations in exon 8: in 2 cases GTT was converted to GCT in codon 274, in 1 case CGT was changed to TGT in codon 273, and in 1 case a 4-bp deletion, involving the last 2 bases of codon 273 and first 2 bases of codon 274, was seen.

Although the frequency of *p53* mutations in all oral tumors, taken together, was low (21%), it is to be noted that when a site-wise comparison was made, tumors from the angle of mouth and alveolus showed a higher frequency (40–50%) of mutations (Table II).

The sequence alterations summarized in Table I show that the mutations and the deletion were found between codons 143 and 274, corresponding to exons 5 through 8 of the *p53* gene. One tongue tumor (T18) showed a double mutation: one in codon 149 and the other in codon 274. Three tumors—from tongue (T18), cheek (T22), and alveolus (T80)—had mutations in the same position of exon 5, codon 149 (TCC to CCC). Similarly, a mutation in exon 8, codon 274 (GTT to GCT) was common to a tumor of tongue (T18) and a tumor of the angle of mouth (T31).

DISCUSSION

Mutations in the *p53* gene are the most frequently observed genetic changes in human cancers (Caron de Fromentel and Soussi, 1992; Levine *et al.*, 1994). Our results showed that 21% (11/53) of oral carcinomas analyzed carried mutations within exons 5–8 of the *p53* gene. It is to be noted that we could have missed a few mutations since we sequenced only the SSCP-positive DNAs. We identified 12 mutations in 11 carcinomas, and most (11/12) of the mutations were single-base substitutions of transitions at either AT or GC pairs (Table I). This type of mutagenesis is reminiscent of the activity of alkylating agents such as ethyl nitrosourea (Bronstein *et al.*, 1991), which can produce O^6-alkylguanine and O^4-4alkylthymine. Further, 9/11 transitions were G and T on the coding strand where repair was reported to be slower than on the template strand (Bohr, 1991). Overall, the spectrum of the *p53* gene is consistent with the involvement of tobacco-related nitrosoamines in the etiology of oral squamous-cell carcinoma (Hoffman and Hecht, 1985). In fact, most (8/11) of the cases with identified mutations were from patients with a long-term (more than 5 years) habit of tobacco chewing (Table I). Since high frequencies of transition mutations were also noted in studies of oral squamous-cell carcinomas in Japan (Hirano *et al.*, 1995), other factors such as tobacco smoking and alcohol consumption could have caused similarities in the mutational spectrum. It has been reported that there were no correlation of alcohol drinking with the *p53* mutation frequency in cancers of the upper aero-digestive tract (Franceschi *et al.*, 1995). The mutations observed by us may not be due to the carcinogenic hydrocarbons of tobacco smoke, since these chemicals were shown to be implicated in transversions and, in our analysis, all mutations except one were transitions.

Relatively low frequencies of *p53* gene mutation were observed in developing countries, where 79% of total oral cancers in the world occur, suggesting the involvement of other genes in the genesis of oral cancer in addition to the *p53* mutation. Even in our cases derived mostly from the tobacco-

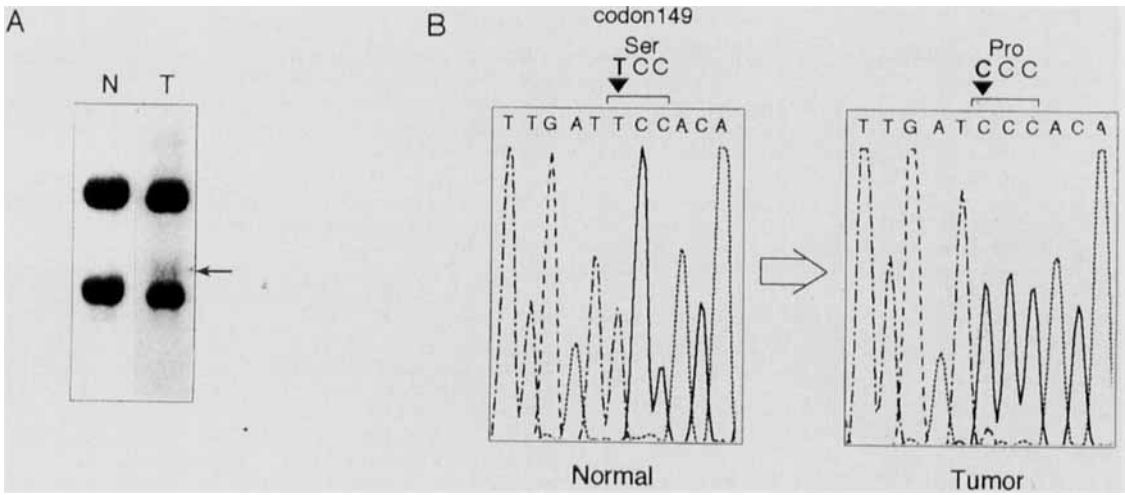

FIGURE 2 – Sequence analysis of the T-22 Exon 5 DNA. *(a)* PCR-SSCP analysis showing mobility shift in tumor DNA. The fragment indicated by the arrow was subjected to sequencing. N indicates DNA from normal tissue: T, DNA from tumor. *(b)* ABI electropherograms of normal and tumor DNA sequences. Mis-sense mutation at codon 149 is shown. This T → C transition resulted in a ser-pro alteration.

TABLE I – *p53* MUTATIONS IN ORAL CANCERS OF INDIANS

Tumor number	Age/sex	Primary site	Chewing history	TNM stage[3]	Mutated codon	Amino- acid change
Exon 5						
T-18	45/F	Tongue	No	2.1.0	149 TCC - CCC	ser-pro
T-22	—[2]	Cheek	Yes	3.1.0	149 TCC - CCC	ser-pro
T-32	50/M	Cheek	Yes	—	181 CGC - CAC	arg-his
T-59	55/F	Cheek	Yes	4.1.0	143 GTG - ATG	val-met
T-80	50/F	Alveolus	Yes	4.1.0	149 TCC - CCC	ser-pro
Exon 6						
T-41[1]	60/M	Alveolus	Yes	4.1.0	205 TAT - GAT	tyr-asp
Exon 7						
T-65	47/M	Angle of mouth	No	4.0.0	AG/GT E7 S.A	—
T-73[1]	45/F	Alveolus	Yes	4.1.0	237 ATG - ATA	met-ile
Exon 8						
T-18	45/F	Tongue	No	2.1.0	274 GTT - GCT	val-ala
T-31	50/M	Angle of mouth	Yes	4.3.0	274 GTT - GCT	val-ala
T-39	60/F	Alveolus	Yes	4.3.0	4 base pair deletion	—
T-69	45/F	Cheek	Yes	—	273 CGT - TGT	arg-cys

[1]Mutations identical to those in T-41 and T-73 were reported by others, while the other mutations have not hitherto been reported in head-and-neck cancers (Cariello *et al.*, 1994).–[2]Data not available or not applicable.–[3]TNM, tumor-nodes-metastasis.

TABLE II – ASSOCIATION OF *p53* MUTATIONS WITH TUMOR SITES

Tumor site	Total number of tumors analyzed	Number of tumors with mutations
Cheek	27	4 (15%)
Tongue	8	1 (13%)
Alveolus	8	4 (50%)
Angle of mouth	5	2 (40%)
Lips	3	—
Floor of mouth	1	—
Palate	1	—

and betel-quid-chewing population of India, the observed incidence of *p53* mutation was 21%, which is relatively low. Thus, tobacco-related nitrosamines, which could explain the mutational profile observed in the *p53* gene, may also cause mutations in other cancer-related genes such as *ras*, since it has been shown that *ras* mutations in oral cancers were low in Japan (Sakai *et al.*, 1992), Europe (Rumsby *et al.*, 1990), and the USA (Somers *et al.*, 1990) while they were relatively high in India (Saranath *et al.*, 1991). Our preliminary results with the same samples also showed a relatively high incidence (32%) of *ras* mutation. It should also be noted that, when tumor samples were dissected, no previous therapeutic intervention with either radiation or chemicals had occurred in these cases, in contrast to those examined in developed countries.

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