C-deletion in exon 4 codon 63 of *p53* gene as a molecular marker for oral squamous cell carcinoma: A preliminary study

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Abstract

Background: Exfoliated oral cancer cells in saliva samples from patients with oral squamous cell carcinoma (OSCC) can be used to determine the incidence and type of mutations of the *p53* tumor suppressor gene. The purpose of this study was to identify C-deletion mutation in exon 4 codon 63 of *p53* gene in the saliva of OSCC patients by polymerase chain reaction (PCR). **Materials and Methods:** Saliva samples of 20 newly histopathologically diagnosed OSCC patients and 5 healthy volunteers were subjected to isolation of the total genomic DNA and PCR amplification for C-deletion on exon 4 of *p53* gene. The resulting products were resolved by agarose gel electrophoresis, viewed and photographed on ultraviolet-transilluminator. **Results:** The relationship between the frequencies of genetic alterations was assessed by Chi-square test. Differences with values of $P < 0.05$ were statistically significant. **Conclusion:** The study concluded a 100% presence of C-deletion mutation in exon 4 codon 63 of *p53* in the saliva of OSCC patients. This study suggests that detection of mutation in exon 4 codon 63 of *p53* by PCR is a fast, reliable, accurate, and sensitive molecular method for OSCC diagnosis.

Keywords: Mutation, oral cancer, oral squamous cell carcinoma, *p53* C-deletion, polymerase chain reaction

Introduction

Oral cancer holds the eighth position in the cancer incidence ranking worldwide, with epidemiologic variations between different geographic regions and it is the third most common malignancy in the South Central Asia. It is the most frequent malignant tumor in the head and neck region and about 80–90% is squamous cell carcinoma (SCC). Pindborg *et al*., 1997 defined SCC as a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges.[1] Globally, about 50,000 new oral and pharyngeal cancers are diagnosed annually, and three quarters of these are from the developing world.[2] The etiology and risk factors of oral squamous cell carcinoma (OSCC) include primarily, tobacco-associated intraoral carcinogens. Since, carcinogenesis is a multistep process, in addition to genetic insult (mutation) by tobacco associated intraoral carcinogens, several additional factors, such as genetic susceptibility of individuals and external agents, such as alcohol, dietary factors and viruses like human papilloma virus (HPV) and Epstein–Barr virus, may play a synergistic role in oral carcinogenesis. Oral neoplasia has been associated with chewing of tobacco with betel quid in India and other Asian countries, whereas in the Western countries, cigarette smoking and heavy alcohol consumption are the main risk factors.[2] Clinically, it is important to note that the therapeutic modality currently offered to patients is based on traditional stage predicting indices (based mostly on the tumor-node-metastasis criteria) and on histologic grading. Unfortunately, these predictors are subjective and relatively unreliable, as often two tumors with identical staging and grading behave in totally different fashions, and although one responds to therapy, the other is lethal.[3]

Identifying mutations as very specific markers for the presence of tumor cells in a background of normal cells can

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Access this article online	
Quick Response Code:	Website: www.contempclindent.org
	DOI: 10.4103/0976-237X.166840

How to cite this article: Sukhija H, Krishnan R, Balachander N, Raghavendhar K, Ramadoss R, Sen S. C-deletion in exon 4 codon 63 of *p53* gene as a molecular marker for oral squamous cell carcinoma: A preliminary study. Contemp Clin Dent 2015;6:S227-34.

aid in evaluating the prognosis of SCC and in evaluating the risk of malignant transformation of potentially malignant lesions. Increasing interest has developed in using saliva to diagnose systemic diseases because of its simplicity in the collection. The collection of saliva is relatively safe, noninvasive, inexpensive to sample and may be collected repeatedly with minimal discomfort to the patient; thereby, rendering saliva as a very desirable diagnostic model. More importantly, saliva contains constituents that are frequently altered in the presence of systemic diseases. Since OSCC is bathed in readily accessible secretions, it is proposed that exfoliated cells from the tumor are possible to detect in saliva. The gene mutations in patients' primary tumor and the protein products would serve as markers for these cells.[4] Recent advances in polymerase chain reaction (PCR) have provided with unique possibilities for studying aberrations at the genetic level in practically no time.[5] Exfoliated oral cancer cells in saliva samples from patients with OSCC can be used to determine the incidence and type of mutations of the *p53* tumor suppressor gene. Earlier studies noted that most *p53* mutations occurred in the most highly conserved region of the gene that is, exons 5–8 (codons 126–306). Based on this preliminary data, most investigators confined their analyses to exons 5–8, few have investigated exons.[4] Partly because of this bias in searching, 95% of the reported mutations have been found in exons 5–8 and their intervening introns.[6] Liao *et al*., 2000, collected saliva from histologically confirmed OSCC subjects and controls from which DNA was extracted by using standard procedures. Exon 4 and intron 6 within the *p53* gene were amplified with PCR, followed by DNA sequence analysis, which revealed that the majority of the base substitutions were C-deletion. Probable hot spots for the mutation were identified at exon 4 codon 63, that had not been observed before in head and neck cancers.[4]

This study focuses on the evaluation of saliva as an additional tool for diagnosing OSCC. The objectives were to identify the presence of exon 4 in *p53* gene in the saliva of OSCC patients (experimental group) and apparently healthy individuals (control group) by PCR; and to identify C-deletion mutation in exon 4 codon 63 of *p53* gene in saliva of OSCC patients and apparently healthy individuals by PCR.

Materials and Methods

This study was carried out in the Department of Oral and Maxillofacial Pathology, Microbiology and Forensic Odontology, S.R.M. Dental College, Ramapuram, Chennai. The molecular laboratory procedures were performed in Eugeniks Genetic Laboratory and Research Institute, Nagpur.

Tris-HCl, Tris-buffer, sodium dodecyl sulfate, NaCl, KCl, dNTP, Taq DNA polymerase, ethidium bromide, agarose, and RNAase were obtained from Sigma Chemical Company, St. Louie, USA. The primers were synthesized and provided by Bengaluru Genei Company, India. All the others chemical used were of molecular and analytical grade.

The study was approved by Institutional Ethics Committee. Clinically, three patients presented with ulcers and 17 patients presented with intraoral growths. Saliva samples of 20 newly histopathologically diagnosed OSCC patients and 5 healthy volunteers were subjected to isolation of the total genomic DNA and PCR amplification for C-deletion on exon 4 of *p53* gene. The resulting products were resolved by agarose gel electrophoresis, viewed, and photographed on ultraviolet (UV)-transilluminator. Potentially malignant lesions and conditions were excluded while screening. Patients who presented with precancerous lesions or conditions, on confirmation through biopsy were not included in the study, so as to maintain the purity of samples. Inclusion criteria were stringently monitored, and patients with history or presentations of other medical ailments were not included in the study.

Sample collection

This study was conducted on 20 newly diagnosed OSCC patients. Saliva samples were collected from patients after taking subjects written informed consent.

Newly diagnosed and histopathologically proven cases of OSCC ($n = 20$) were considered, and information about their history, their clinical examination conducted, and histopathological findings were noted and their saliva samples were used for the study. Saliva samples from healthy subjects ($n = 5$) were used as a control. About 5 ml unstimulated saliva samples were collected from each of the OSCC patients and control subjects in 15 ml centrifuge tubes by instructing the subjects to directly spit into the tubes over about 30 min to a maximum, under supervision, and centrifuged. The supernatant was discarded, and RNA*later*® solution was micropipette into the residue in a ratio of 4:1 by volume for genomic stability while storage. This was stored at 4°C and was used for DNA isolation and PCR.

Genomic DNA isolation

For the isolation of the total genomic DNA, standard chloroform-phenol protocol was applied with slight modification. 1 ml saliva sample was placed in a 1.5 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany) and centrifuged to collect cell pellet. In the cells, 0.5 ml of lysis buffer (containing NaCl, ethylenediaminetetraacetic acid (EDTA), sodium lauriate sulfate and NP40), and 0.01% proteinase-K were added, mixed gently and incubated at 55°C for 45–60 minutes for complete lysis of cells. After incubation, 250 μl of chloroform and phenol were added, mixed gently and centrifuged at 10,000 rpm for 5 min. The supernatant was then transferred to a new microcentrifuge tube and repeat chloroform-phenol step. The DNA was precipitated from the supernatant with two volumes of iso-propenol (ice-cold). The DNA pellet was washed with 70% ethanol, dried, dissolved

in Tris-EDTA buffer (containing 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and stored at −20°C. The concentration of extracted DNA was adjusted to 1 µg/µl.

Polymerase chain reaction amplification for normal exon 4 of *p53* gene

PCR reaction was carried out in a 25 µl reaction volume containing 1 µg/µl of genomic DNA, 2.5 µl of 10X Taq polymerase buffer with 1.5 mM $MgCl_2$, 200 µM of each dNTPs (Q-Biogene, USA), 15 µg of each primer and 1 unit of Taq DNA polymerase (Q-Biogene, USA). The primer sequences were used for exon 4 of *p53* gene as upstream 5' (GATGCTGTCCGCGGACGATATT) 3' and downstream 5'(CGTGCAAGTCACAGACTTGGC) 3'. A negative control, without template DNA was included in each round of reactions. DNA amplification was performed in a Techne thermocycler, USA. PCR thermal was performed in 35 cycles. Each cycle consisted of 94°C denaturizing for 45 s, 57°C annealing for 45 s, and 72°C extension for 1-min. The thermal cycles were started with an initial denaturizing of 96°C for 5 min and a final 72°C extension for 10 min for polishing the ends (making smooth) of PCR products.

Construction of primer against C-deletion on exon 4 of *p53* gene

On the basis of sequence, the primers were constructed for C-deletion on codon 63 of exon 4 of *p*53 gene as 5'-GGTCCAGATGAAGTCCCAGAA (upstream) and 5'-CGTGCAAGTCACAGACTTGGC (Downstream). The length of primers were 21 nucleotides, GC content was kept to 40–60%. The annealing temperature of constructed primers was estimated as 58°C and the PCR amplification reaction was done as described above.

Agarose gel electrophoresis

Resulting PCR products were resolved (15 µl PCR product mixed with 2 µl gel loading dye) on 1.5% agarose gel using submarine gel electrophoresis for 1hour in 1X TBE buffer (Tris-HCl, boric acid, EDTA; pH 8.0). Subsequently, gels were stained with ethidium bromide (10 mg/l) and photographed on a UV transilluminator using a gel documentation system (AlphaDigiDoc-1201 documentation system, USA). A known DNA size marker was run with every gel (100 bp ladder from Bangalore Genei, India). Reproducibility of PCR was tested by performing duplicate reactions at different times, and only reproducible bands were scored.

Statistical analysis

The relationship between the frequency of genetic alterations and clinicopathological features was assessed by using the Chi-square test. Differences with values of $P < 0.05$ were found to be statistically significant. The statistical evaluation of the data was based on the WHO histopathological grading system.

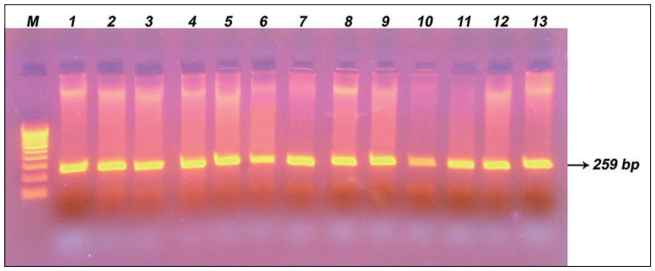

Figure 1: 259 bp polymerase chain reaction product of exon 4 of *p53* gene in lanes 1 to 13 (squamous cell carcinoma patients test samples)

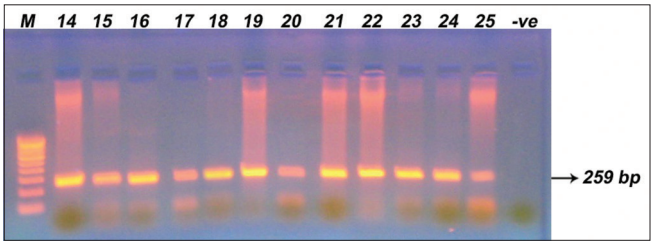

Figure 2: 259 bp polymerase chain reaction product of exon 4 of *p53* gene in lanes 14 to 20 (squamous cell carcinoma test samples), lanes 21 to 25 (apparently healthy control subjects) and negative control

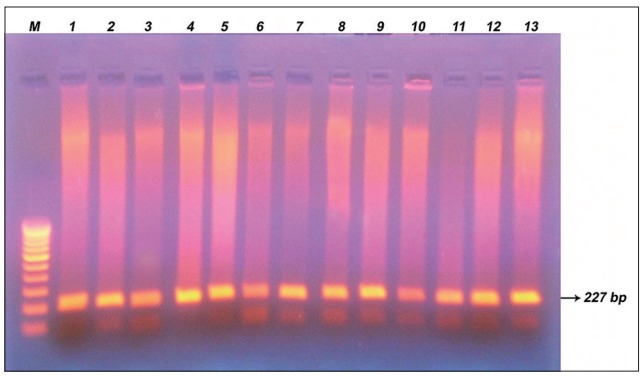

Figure 3: 227 bp polymerase chain reaction product of C-deletion in exon 4 codon 63 of *p53* gene in lanes 1 to 13 (squamous cell carcinoma patients test samples)

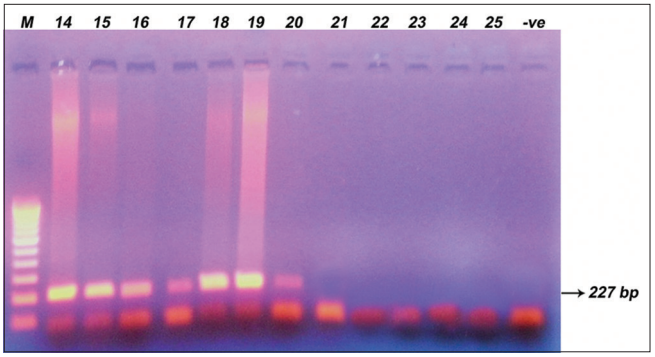

Figure 4: 227 bp polymerase chain reaction product of C-deletion in exon 4 codon 63 of *p53* gene in lanes 14 to 20 (squamous cell carcinoma patients test samples), lanes 21 to 25 (apparently healthy control subjects) and negative control

Results

Polymerase chain reaction analysis results

The genomic DNA was successfully extracted from the exfoliated cells in saliva. Furthermore, the genomic DNA was amplified through repeated cycles of denaturation, annealing, and extension of primers for exon 4 of *p53* gene and C-deletion in exon 4 codon 63 of *p53* gene. After PCR reaction, the product was separated on agarose gel by employing gel electrophoresis. The PCR results of all 25 patients are depicted in Figures 1-4.

Figures 1 and 2 show that a 259 bp product was generated from normal exon 4 of *p53* gene when compared with 100 bp DNA ladder (molecular weight marker), which was present in all patients' samples. By employing C-deletion in exon 4 codon 63 of *p53* gene primer, a 227 bp PCR product was generated [Figures 3 and 4] which was present in all the OSCC patients (samples 1–20) and absent in the normal individuals (samples 21–25). The systematic outcomes of the PCR products are presented in tabular columns [Tables 1-4] below each figure.

Evaluation of the data was based on the WHO histopathological grading system which showed that 17/20 patients (85%) had well-differentiated lesions in contrast to 2/20 (10%) who had moderately differentiated lesions and 1/20 (5%) with poorly differentiated lesion.

Table 5 shows that the mean age of OSCC patients was observed to be 52.35 ± 16.21 years with 16 (80%) being males and 4 (20%) females and that of normal subjects was 26.6 ± 1.67 years with 3 (60%) males and 2 (40%) females [according to Table 6]. Table 7 shows that the data got on distribution of OSCC patients according to their habits was highly significant with the highest group being that of tobacco chewers (52%) followed by bidi smokers, pan masala (tobacco and arecanut) chewers, cigarette smokers, arecanut chewers, betel leaf quid (with tobacco) chewers, in the decreasing order. None of the OSCC patients was found be devoid of habits. Table 8 shows that the PCR study of all the 25 samples (20 OSCC and 5 normal control) showed the 100% presence of 259 bp PCR product of exon 4 of *p53* gene. Table 9 show C-deletion in exon 4 codon 63 of *p53* gene in the OSCC samples that is, 20 samples and significantly absent in normal healthy control ($n = 5$).

Discussion

Oral cancer is an epithelial neoplasia generally beginning as a focal clonal overgrowth of altered stem cells near the basement membrane, expanding upward and laterally, replacing the normal epithelium. Oral carcinogenesis is associated with cumulative gene alterations. The development of OSCC has been hypothesized to be a process of "field cancerization" in that the premalignant lesions often proceed and occur in areas neighboring frank malignancy. It, thus, provides an ideal system to study and verify the theory of multistep tumorigenesis. Because the activation of oncogenes (promote cell growth) and inactivation of tumor suppressor genes (suppresses cell growth) are believed to be essential events in the process of neoplastic transformation, oral cancers and their frequently associated premalignant lesions should yield clues as to the characteristic mutational sequences involved.[7]

p53 is a tumor suppressor gene located on the short arm (p) of chromosome 17, as well as the protein encoded by this gene. Once activated, the *p53* protein can induce growth arrest, as well as cell death.[5] The human *p53* gene can be divided into

Table 1: 259 bp PCR product of exon 4 of *p53* gene

Lane number	Identity number	Result
M	100 bp DNA ladder	
1	C 24	Positive
2	C 25	Positive
3	C 26	Positive
4	C 27	Positive
5	C 28	Positive
6	C 29	Positive
7	C 30	Positive
8	C 31	Positive
9	C 32	Positive
10	C 33	Positive
11	C 34	Positive
12	C 35	Positive
13	C 36	Positive

PCR: Polymerase chain reaction

Table 2: 259 bp PCR product of exon 4 of *p53* gene

Lane number	Identity number	Result
M	100 bp DNA ladder	
14	C 2	Positive
15	C 6	Positive
16	C 8	Positive
17	C 12	Positive
18	C 16	Positive
19	C 17	Positive
20	C 18	Positive
21	N 1	Positive
22	N 2	Positive
23	N 4	Positive
24	N 5	Positive
25	N 6	Positive
26	Negative control	Negative

PCR: Polymerase chain reaction

Table 3: 227 bp PCR product of C-deletion in exon 4 codon 63 of *p53* gene

Lane number	Identity number	Result
M	100 bp DNA ladder	
1	C 24	Positive
2	C 25	Positive
3	C 26	Positive
4	C 27	Positive
5	C 28	Positive
6	C 29	Positive
7	C 30	Positive
8	C 31	Positive
9	C 32	Positive
10	C 33	Positive
11	C 34	Positive
12	C 35	Positive
13	C 36	Positive

PCR: Polymerase chain reaction

Table 4: 227 bp PCR product of C-deletion in exon 4 codon 63 of *p53* gene

Lane number	Identity number	Result
M	100 bp DNA ladder	
14	C 2	Positive
15	C 6	Positive
16	C 8	Positive
17	C 12	Positive
18	C 16	Positive
19	C 17	Positive
20	C 18	Positive
21	N 1	Negative
22	N 2	Negative
23	N 4	Negative
24	N 5	Negative
25	N 6	Negative
26	Negative control	Negative

PCR: Polymerase chain reaction

three distinct functional domains[8] that is, an acidic amino terminal transactivational domain (N), a central DNA binding domain and a basic carboxy terminal oligomerization domain. The transactivation domain is located at the N-terminus and mediates transcriptional activation. The central core region is responsible for sequence – specific DNA binding and is a frequent target for mutational inactivations in human cancer. An oligomerization domain localized to the C-terminus is involved in tetramerization.[8] *p53*, a tumor suppressor gene functions as the "guardian of the genome" and plays a pivotal role in "sensing" damaged DNA and in making critical decisions of whether a cell should undergo apoptosis.

Table 5: Age wise distribution of patients in cancer group

Age group (years)	Cancer group (%)
20-30	3 (15)
31-40	1 (5)
41-50	5 (25)
51-60	6 (30)
61-70	2 (10)
71-80	2 (10)
>81	1 (5)
Total	20 (100)
Mean age±SD	52.35±16.21

SD: Standard deviation

Table 6: Sex wise distribution of patients in cancer group

Sex	Cancer group (%)
Male	16 (80)
Female	4 (20)
Total	20 (100)

Table 7: Distribution of cancer patients according to habits

Habits	Cancer group (%)
Areca nut chewing	4 (16)
Betel leaf quid with tobacco	3 (12)
Bidi smoking	8 (32)
Cigarette smoking	5 (20)
Pan masala	6 (24)
Tobacco chewing	13 (52)
No habit	0 (0)

Table 8: PCR product of exon 4 of *p53* gene

PCR product of exon 4 of *p53* gene	Cancer group (%)	Normal group (%)	χ^2
Present	20 (100)	5 (100)	9.00 significant
Absent	0 (0)	0 (0)	
Total	20 (100)	5 (100)	

Significant value is calculated by using Chi-square test; Degrees of freedom=*n*–1=1; Chi-square tabulated value=3.84; Result: Chi-square calculated value is more that Chi-square tabulated value and hence the result is significant. PCR: Polymerase chain reaction

Table 9: C-detection in exon 4 codon 63 of *p53* gene

C-detection in codon 63 exon 4 of *p53* gene	Cancer group (%)	Normal group (%)	χ^2
Present	20 (100)	0 (0)	25.00 *P*<0.0001 HS
Absent	0 (0)	5 (100)	
Total	20 (100)	5 (100)	

P value is calculated by using Chi-square test. Result: HS at 1% level of significance. HS: Highly significant

This gene mediates cell cycle arrest, allowing for repair of damaged DNA or cell death by playing an important role in cell cycle control and the induction of apoptosis. Mutant *p53* loses these activities and thus permits the proliferation of cells which carry damaged DNA, eventually leading to their malignant transformation. More than 50% of human primary tumors have lost the wild-type *p53* suppressor gene and instead express accentuated levels of mutant *p53* protein.[9] *p53* protein has the ability to sense different kinds of stress to which cells are exposed. This protein has a very short half-life and, therefore, can be hard to detect in normal tissue. However, the protein can remain in the tissue longer for certain reasons, such as a mutation, a defect in the degradation pathway, or by binding to other proteins. In the currently accepted model of *p53* function, the inactive *p53* protein, once activated by phosphorylation at specific residues, non-specifically binds to DNA. The non-specific binding is caused by the C-terminus of the protein binding to, and accordingly blocking the central domain. This binding and blocking is, however, reversible by phosphorylation or acetylation within the C-terminus; and by reversing the blockade, binding of *p53* becomes specific and *p53* can thus act as a transcription factor.[5] It is clear that the activated and modified *p53* protein acts as a transcription factor through binding to specific sequences and hence, transactivating downstream target genes which in turn activates the expression of proteins that regulate the cell-cycle, either halting the cycle until damage can be repaired, or in more extreme cases, causing the cell death.[10]

Due to its high catabolic rate, it is not usually possible to demonstrate *p53* protein in normal tissues using immunohistochemical procedures, whereas mutated *p53* exhibits a much lower catabolic rate and accumulates in the cells. Status of *p53*, bcl-2 and bax, members of the *p53* dependent apoptotic pathway has previously been evaluated in oral cancers/oral lesions by immunohistochemistry. Overexpression of antiapoptotic members of the bcl-2 family namely bclxL and Mcl-1, in oral cancer cell lines, have also been demonstrated. Therefore, it has been conjectured that evasion of apoptosis via abnormal expression of bcl-2, bclxL, mcl-1, and *p53* may contribute to oral cancer pathogenesis. Activation of genes frequently involves a single point mutation at specific sites, usually referred to as "*hot spots,*" which results in expression of an abnormal protein. This abnormal protein harbors an amino acid substitution in areas important for protein synthesis.[7] The location and type of mutations in a specific sequence define a mutational spectrum. When all tumor *p53* mutations are grouped together, they identify several codons at which exceptionally high numbers of tumor mutations are clustered (hot spots). When mutations are examined separately by cancer type, clear differences in spectra emerge, both with respect to the position of the hot spots and with respect to the frequency of transitions and transversions.[11] A hallmark of *p53* is that alleles are generally altered during transformation, which usually represents a loss of heterozygosity. Mutations in the *p53* gene on human chromosome 17 at p13 (p: Short arm, 13: Band) (spanning 20 kb of DNA and yielding a 2.8 kb mRNA transcript from 11 exons) have the greatest effect when they occur in the conserved regions II–V in exon 4 (codons 129–146), exon 5 (codons 171–179), exon 7 (codons 234–260), and exon 8 (codons 270–287). Mutations in *p53* occur mainly as missense, resulting from base pair substitutions, but some are insertions and deletions and exert a dominant negative effect.[9] Analysis of base substitution mutations is of interest for two reasons,[11] namely that endogenous and exogenous mutagens generate specific kinds of base substitutions at certain preferred sites, the *p53* mutational spectrum in tumors may provide information about the origins of the mutations that give rise to human cancers, and that the positions of tumor mutations in the *p53* gene sequence define regions of the *p53* protein that are likely to be essential for its biological activities and for its interaction with other cellular and viral proteins.

Somers *et al*.[12] 1992 demonstrated that mutations in the tumor suppressor gene *p53* are common in head and neck cancers. Their inactivation, either by deletion or mutation, results in loss of function and unrestrained cellular growth. In their study, head and neck tumor were analyzed for mutations in the tumor suppressor gene *p53* by immunocytochemistry and complementary DNA sequencing. A striking finding was that G to T transversions were detected in the majority of head and neck SCC (HNSCC) cell lines and that codons 245 and 248 were postulated to represent mutational hot spots in head and neck cancer. It was worth noting that benzo (a) pyrene, a major carcinogen in tobacco smoke is known to induce G to T transversions. Thus, the data link tobacco smoke, a known risk factor in HNSCC to induce G to T transversions in *p53* and suggested that mutations might be the footprints of tobacco carcinogens.

Boyle *et al*.[13] 1993 analyzed colorectal cancer, in which the progression from adenoma to carcinoma was correlated with specific genetic changes. Reports have suggested that alterations of *p53* may occur even earlier in squamous epithelium during progression to cancer. As demonstrated by the genetic model of colorectal carcinoma, it was the accumulation and not necessarily the order of genetic missteps that determine the progression. Their findings clearly indicated that early lesions in squamous epithelium can have *p53* mutations and establish mutation of the *p53* gene in the general progression from non-invasive to invasive disease.

Boyle *et al*.[13] 1994, examined the possible value of *p53* in saliva as a marker for OSCC. They detected and identified tumor specific mutations in *p53* in preoperative salivary samples of individuals suffering from HNSCC and observed 71% positive results.[14] Munirajan *et al*. 1996, analyzed 53 OSCCs among Indians for the presence

of alterations in the tumor suppressor gene *p53* by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and sequencing methods. Their results showed that 21% (11/53) of oral carcinomas analyzed carried mutations within the exons 5–8 of the *p53* gene. Probable hot-spots for the mutation induction were identified at codons 149 and 274, which were observed for the first time in head and neck cancers. Chiba *et al*.[15] 1998, collected primary resected specimens from 23 oral SCCs, 7 leukoplakias and 2 oral submucous fibrosis from OSCC patients in Sri Lanka and used these for *p53* mutation analysis. Exons 5 through 8 of the *p53* gene were examined by PCR-SSCP and direct sequencing. Mutations in the *p53* gene were frequent (10/23) in OSCC specimens from Sri Lanka. Moreover, the mutations clustered significantly in exon 5 (7/10) of the *p53* gene, and small deletions and inclusions other than point mutations were observed. Saranath *et al*.[16] 1999, examined the inactivation of *p53* TSG with reference to point mutation, overexpression and degradation due to HPV 16/18 infection, in chewing tobacco-associated oral cancers and oral leukoplakias from India. The analysis of mutations was assessed by PCR with PCR-SSCP of exons 5–9 on DNA from 83 oral cancer cases, and the mutations confirmed by direct nucleotide sequencing of the PCR products. It was postulated that overexpression of *p53* protein in leukoplakias might serve as a valuable biomarker for identifying individuals at high risk of transformation to the malignant phenotype.

Many alternate sources of DNA have been used for genetic screening. In the study conducted by Liao *et al*.[4] 2000, saliva was chosen as the target sample. They selected 10 patients who had histologically confirmed OSCC lesions and 27 normal controls for the comparison of the results. From the saliva samples collected from the subjects, DNA was extracted by using standard procedures and stored at 4°C. Exon 4 and intron 6 within the *p53* gene were amplified with PCR, followed by DNA sequence analysis. DNA sequence analysis of PCR products revealed 5 out of 10 tumor saliva samples and 5 out of 27 healthy saliva samples contained *p53* exon 4 codon 63 mutations. The study revealed that the majority of the base substitutions were C-deletion. Probable hot-spots for the mutation were identified at exon 4 codon 63, which had not been observed before in head and neck cancers. Naggar *et al*.[17] 2001, performed microsatellite analysis at chromosomal regions frequently altered in head and neck squamous carcinoma on matched saliva and tumor samples from 37 patients who had OSCC. Epithelial cells in saliva from patients with head and neck squamous tumorigenesis provided suitable material for genetic analysis. Combined application of certain markers improved the detection of genetic alteration in these patients. The results indicated that clonal heterozygosity between saliva and matching tumor supports genetic instability of the mucosal field in some of these patients.

Conclusion

This study concluded that detection of mutation in exon 4 codon 63 of *p53* gene by PCR is a fast, reliable, accurate, and sensitive molecular method that may be employed as a potential molecular diagnostic marker for OSCC. As analyzed by PCR, presence of exon 4 in *p53* gene in saliva was positive for both 100% OSCC and 100% apparently healthy individuals respectively. The presence of C-deletion mutation in exon 4 codon 63 of *p53* gene in saliva was positive for 100% OSCC patients and negative for all the 5 apparently healthy individuals.

This study suggests that futuristic prospects for related research lie in aspects of oral cancer that need to be addressed by keeping in mind the following:

- A larger sample may be examined for C-deletion in exon 4 codon 63 of *p53* gene
- Correlation between histopathological grading of OSCC and *p53* mutation may be evaluated based on a larger sample
- Varied ethnic groups in the Indian population may be examined for C-deletion in exon 4 codon 63 of *p53* gene
- Potentially malignant lesions and conditions may be studied for C-deletion in exon 4 codon 63 of *p53* gene in comparison with OSCC for early diagnosis of OSCC
- Longitudinal prospective follow-up and investigations for *p53* mutation may be studied in order to evaluate the prognostic value of C-deletion in exon 4 codon 63 of *p53* gene.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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