

# Frequency of p53 Gene Mutation and Protein Expression in Oral Squamous Cell Carcinoma

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## ABSTRACT

**Objective:** To determine the frequency of p53 gene mutation and protein expression in Oral Squamous Cell Carcinoma (OSCC) and to establish correlation between the two.

**Study Design:** Analytical study.

**Place and Duration of Study:** Histopathology Department and Molecular Biology Laboratory, Armed Forces Institute of Pathology (AFIP), Rawalpindi, from May 2010 to May 2011.

**Methodology:** Thirty diagnosed cases of OSCC were selected by consecutive sampling. Seventeen were retrieved from the record files of the AFIP, and 13 fresh/frozen sections were selected from patients reporting to the Oral Surgery Department, Armed Forces Institute of Dentistry (AFID). Gene p53 mutation was analyzed in all the cases using PCR-SSCP analysis. DNA was extracted from the formalin-fixed and paraffin-embedded tissue sections and fresh/frozen sections. DNA thus extracted was amplified by polymerase chain reaction. The amplified products were denatured and finally analyzed by gel electrophoresis. Gene mutation was detected as electrophoretic mobility shift. The immunohistochemical marker p53 was applied to the same 30 cases and overexpression of protein p53 was recorded.

**Results:** Immunohistochemical expression of marker p53 was positive in 67% [95% Confidence Interval (CI) 48.7 - 80.9] of the cases. Mutations of the p53 gene were detected in 23% (95% CI 11.5 - 41.2) of the OSCC. No statistically significant correlation was found between p53 gene mutation and protein p53 expression ( $r_s = -0.057$ ,  $p = 0.765$ ).

**Conclusion:** A substantial number of patients have p53 gene mutation (23%) and protein p53 expression (67%) in oral squamous cell carcinoma (OSCC).

**Key Words:** Gene p53 mutation. PCR-SSCP analysis. Gel electrophoresis. Oral squamous cell. Immunohistochemical expression.

## INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is the third most common cancer in developing countries, usually arising from the precancerous lesions.<sup>1</sup> Lip and oral cavity cancer was among the top three malignancies in all age groups and both gender combined at the Shaikat Khanum Memorial Cancer Hospital and Research Centre (SKMCH and RC) registry database in the year 2009.<sup>2</sup>

Tumour suppressor gene p53 (Tp53) is positioned on the short arm of chromosome 17 at 13.1 gene locus with a total of 11 Exons. Tp53 guards the genome stability and encodes a 393 amino acid phosphoprotein (protein p53) that regulates the cell growth cycle, repair the

damaged DNA and control cell proliferation through apoptosis of the damaged cells.<sup>3</sup>

Mutation or inactivation of the Tp53 leads to deregulation of the p53-dependant DNA repair pathway. It is incapable of repairing the damaged DNA or inducing apoptosis in cells.<sup>4</sup> Mutant p53 protein loses the function of tumour suppressor activity and the cells replicate with the damaged DNA transforming into malignancy.<sup>5</sup>

Mutation in the p53 gene is the most common genetic alteration found in oral premalignant lesions and squamous cell carcinoma *in situ* (OSCC).<sup>6</sup> These mutations are usually located in the Exons 5 - 8 of the p53 gene and are associated with poor prognosis in several human tumours including oral, lung, and breast, prostate and colorectal tumours. Mutated gene encodes for a mutant protein having an increased cellular stability that form the basis for detection by immunohistochemistry (IHC).<sup>7</sup>

The incidence of p53 mutations in head and neck SCC varies between 30 - 70%.<sup>8</sup> Tumours with mutations of the p53 gene grow faster and have a worse prognosis with an overall reduced survival rate.<sup>9</sup>

Histopathology and IHC is the gold standard in diagnosing oral cancer. Molecular marker p53 augments and rationalizes the diagnosis as mutations confirm the aggressive nature and poor prognosis of OSCC.

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There is a lack of data of p53 gene mutation regarding OSCC in Pakistani population. Considering the situation, this study was carried out as a baseline study to determine the frequency of p53 gene mutation and protein p53 expression in OSCC and to establish any correlation between them.

## METHODOLOGY

This analytical study was carried out at the Histopathology Department and Molecular Biology Laboratory, AFIP, Rawalpindi, Pakistan, from May 2010 to May 2011. The study protocol was reviewed and approved by the Ethical Committee at the AFIP and the study was carried out in accordance with the declaration of Helsinki of 1975 as reviewed in 1983. Incisional and excisional biopsies of diagnosed cases of OSCC were included in the study. Patients with incomplete information on the records, poorly processed tissue and samples with poor amplification of DNA were excluded.

Seventeen of 30 cases were FFPE tissue blocks retrieved from the archives of the AFIP, from January 2007 to December 2010. Thirteen fresh/frozen sections of OSCC in saline were taken from the patients of Oral Surgery Department, AFID from June 2010 to December 2010. Informed written consent was taken from all such patients. The data on age, gender and site of involvement was extracted from the clinical histories. The slides were independently reviewed by two observers without prior knowledge of the diagnosis.

Chelex Method was used to extract DNA from the fresh/frozen sections. PureLink Genomic DNA Mini Kit (Invitrogen, USA) was used to extract DNA from FFPE tissue samples according to manufacturer's manual. The purified DNA was stored at 4°C. Spectrophotometer was used to determine the quality and quantity of the isolated DNA. Colon carcinoma with known p53 mutations was taken as positive control. Human DNA (free of p53 mutation) was used as negative control.

Genomic DNA obtained from paraffin embedded /fresh/ frozen sections was amplified by the PCR to double-stranded DNA fragments. Four sets of primers were used to amplify Exons 5 - 9 of the p53 gene. These primers were selected from the previous study (Ichikawa *et al.*)<sup>10</sup> and obtained by custom ordering from e-Oligos, GeneLink. DNA of all 30 samples was amplified by using Forward and Reverse Primers of Exon 5, Exon 6, Exon 7, and Exon 8 and 9 consecutively.

The PCR was done in 23 µl reaction mixture containing 2.3 µl of 10 x PCR Buffer, MgCl<sub>2</sub> 25 mM, dNTPs 200 mM (Fermentos, USA), 1 µl of primer (10 pmol each), 0.5 U/µl Taq DNA polymerase (Fermentos, USA) and 2 µl template DNA (100 ng). Thirty cycles of the PCR reaction at denaturation for 5 minutes at 94°C, melting for 30 seconds at 94°C, annealing for 30 seconds at 60°C, extension for 1 minute at 72°C and final

elongation for 7 minutes at 72°C were run in a thermocycler (Cetus).

Five microlitres of amplicons (PCR amplified products) of each sample were mixed with 5 µl of 0.1% Sodium Dodecyl Sulphate (SDS) and 5 µl of 10 mM Ethylene-Diamine Tetra Acetic acid (EDTA, Merck-Germany). Fifteen microlitres of bromophenol dye (40% sucrose solution 1 mg bromophenol dye) was added to this mixture. These samples were then denatured by heating at 95°C for 5 minutes, and promptly chilled on ice.

A volume of 4 µl of the denatured products was applied to 6% Polyacrylamide gel. Electrophoresis was performed at 100 V for 20 hours at room temperature (26°C). Gel was stained with silver nitrate and dried on filter paper in a gel drier for 20 minutes. A single nucleotide change (gene mutation) was detected as electrophoretic mobility shift. The primer pair of Exon 5 of the p53 gene mutation amplified 167 bp fragment.

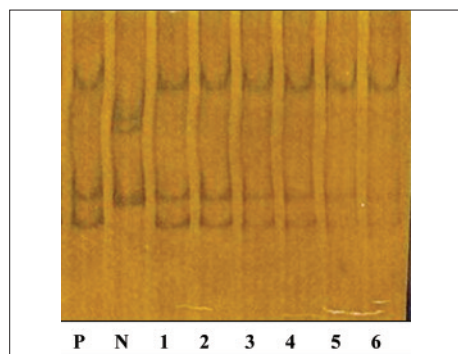
Immunohistochemical staining (expression of protein p53) was detected by Streptavidin Biotin technique. Antigen was retrieved in citrate using pressure cooker. A mouse monoclonal antibody recognizing mutant p53 (Novo Castra, 15 Mm Sodium Azide, NCL-p53-BP, concentrated, 1:20 dilution) was used. The percentage was evaluated by counting the stained cells per 100 tumour cells in the area of best staining and was then subdivided broadly into four groups: - = absence of staining or occasional keratinocytes staining; + = staining of 10 - 33% of keratinocytes; ++ = staining of 33 - 66% of keratinocytes and +++ = staining of greater than 66% of keratinocytes.<sup>11</sup>

The immunoreactivity was considered positive if greater than 10% of the tumour cells were stained.<sup>12</sup> The staining intensity was graded as follows; 1+ for definite but light stain; 2+ for darker stain and 3+ for most intense stain.<sup>13</sup>

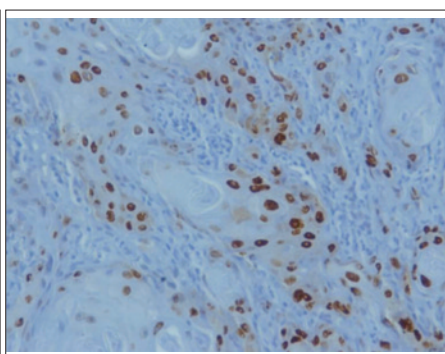
Statistical analysis of all the data was entered in Statistical Package for Social Sciences (SPSS) version 17. Frequency of p53 gene mutation and p53 protein expression was calculated along with 95% confidence intervals. Correlation between qualitative variables (p53 gene mutation and protein p53 expression) was assessed using Spearman's rank correlation analysis. P-value of < 0.05 was considered statistically significant.

## RESULTS

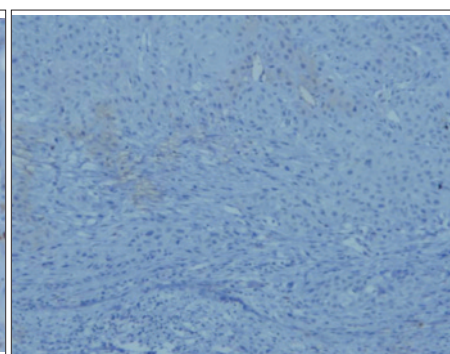
Demographic data including age, gender and site were obtained from the record files of AFIP and AFID. All the patients were Pakistanis and their ages were between 21 - 86 years. Nineteen (63.3%) were males and 11 (36.7%) were females with male to female ratio of 1.7:1. The most common site was buccal mucosa 13 (43.3%) followed by tongue 7 (23.3%), palate 4 (13.3%), alveolar mucosa 3 (10%), floor of the mouth 2 (6.7%)



**Figure 1:** Polyacrylamide gel showing positive cases for mutations in Exon 5 of the p53 gene. P = Positive control. N = Negative control. 1 - 6 = Positive samples of oral carcinoma.



**Figure 2A:** Photomicrograph of (+++) p53 expression in squamous cell carcinoma of the tongue (Original magnification x200).



**Figure 2B:** Negative control (x100).

**Table I:** Correlation between p53 expression and staining intensity.

Grades of staining intensity	p53 expression				p-value
	-	+	++	+++	
-	10 (33%)	0 (0%)	0 (0%)	0 (0%)	$r_s = 0.896$ $p < 0.001$
1+	0 (0%)	3 (10%)	1 (3.3%)	0 (0%)	
2+	0 (0%)	3 (10%)	4 (13.3%)	1 (3.3%)	
3+	0 (0%)	0 (0%)	5 (16.7%)	3 (10%)	

and labial mucosa 1 (3.3%). Seventeen (56.7%) squamous cell carcinomas were graded as well differentiated, 9 (30%) were graded as moderately differentiated and 4 (13.3%) were poorly differentiated OSCC.

Gene p53 mutation was detected in 7 (23.3%, 95% CI=11.5 - 41.2) of the cases (Figure 1). In OSCC, 4 (13.3%) of mutations were observed in moderately-differentiated SCC while 3 (10%) of the p53 gene mutations were found in well-differentiated SCC. No mutations were detected in poorly-differentiated SCC. No significant correlation was observed between p53 gene mutation and histopathological grades ( $r_s = -0.364$ ,  $p = 0.728$ ).

Protein p53 expression was seen in 20 (67%, 95% CI=48.7 - 80.9) of 30 cases of OSCC (Figure 2). No significant correlation was observed between p53 protein expression and histopathological grades ( $r_s = -0.166$ ,  $p = 0.381$ ). In OSCC, highly significant correlation was observed between p53 expression and staining intensity ( $r_s = 0.896$ ,  $p < 0.001$ ) shown in Table I. No significant correlation was observed between p53 gene mutation and protein p53 expression ( $r_s = -0.057$ ,  $p = 0.765$ ).

### DISCUSSION

Several researches worldwide have proved that p53 tumour suppressor gene and its encoded protein are altered in oncogenesis.<sup>14</sup> Single-Strand Conformation Polymorphism (SSCP) analysis is one of the simplest and inexpensive techniques to detect the presence of unknown gene mutations in DNA fragments.<sup>15</sup>

In the present study, p53 gene mutation was detected in 23% of the OSCC and protein p53 expression was seen in 67% of the cases. Similar results were found in a North Indian study by Ralhan *et al.* to determine the correlations between p53 gene mutations, protein accumulations and serum antibodies in potentially malignant oral lesions and oral cancer. All the subjects were either tobacco or betel-quid users. Gene mutation was detected in 23% of OSCC. Majority of these mutations were base substitution mutations predominantly located in Exon 5 of the p53 gene.<sup>16</sup> These results are comparable with the present study.

Higher frequency (76.8%) of protein p53 expression was reported in OSCC by Panjwani and Sadiq.<sup>17</sup> As the samples were collected from Karachi, betel-quid chewing might be responsible for higher frequencies. Kannan *et al.* conducted a study on 50 tissue samples to see p53 expression in normal mucosa, dysplastic and malignant lesions of the oral mucosa.<sup>18</sup> Over-expression of p53 was seen in 67% of OSCC similar to the present study. Habitual usage of tobacco, betel-quid, naswar or alcohol was not considered in the present study. It was broadened to include DNA extraction and gene analysis along with other variables.

The current study showed no significant correlation between mutational status and protein expression even though most of the cases (5 of 7) with p53 gene mutation showed positive staining for p53 protein. These findings are consistent with a previous study by Saunders *et al.*<sup>19</sup> on 42 patients of laryngeal carcinoma, and by Rowley *et al.* in OSCC.<sup>13</sup>

Immunoexpression and gene mutation of p53 was not simultaneously present in our samples of OSCC. Two of the 7 (28.5%) samples with p53 gene mutation did not express protein by IHC. This may be due to the presence of frameshift mutations that resulted in small quantities of the stabilized, truncated protein undetectable by IHC.<sup>20</sup> In 15 of 23 (65.2%) samples with undetected p53 mutation, positive immunoexpression was observed by IHC. This may be attributed to the fact

that protein p53 may be stabilized by binding either to viral oncoproteins (HPV 16, 18) or to cellular proteins (MDM2).<sup>15</sup>

A study conducted in UK by Rowley *et al.* analyzed p53 gene mutation and p53 protein expression in oral dysplasia and oral SCC. Positive p53 protein expression was seen in 77% of SCC.<sup>13</sup> Microwave antigen retrieval step was used in IHC that might have resulted in the increased sensitivity of protein p53 leading to higher frequencies compared to the present study. Mutations of the gene were detected in 55% of OSCC by PCR-SSCP and sequencing analysis. The present methods might have missed some mutations because sequencing was not performed due to limited institutional funding and mandate.

The present study is a baseline study conducted on local population in our own setup. An earlier study was carried out on Pakistani patients by Trivedy *et al.* but it was completed in UK using FFPE tissue blocks collected from Karachi, Pakistan.<sup>21</sup> Another study was conducted in Karachi by Rashid *et al.* to determine the prevalence of Tp53 germ line mutations in young Pakistani cancer patients. Oral cancer patients were not included in their study. One rare mutation c.499-500delCA in exon 5 was identified in the cancer patients.<sup>22</sup>

A recent study was conducted (after the present study) in Karachi to investigate the association of p53 gene mutation/polymorphism with OSCC patients consuming *guthka*, *naswar* and *manpuri*. Azhar *et al.* detected a novel 'AGT' to 'ACT' missense mutation in Exon 7 of the p53 gene using PCR-SSCP analysis.<sup>23</sup> Protein p53 expression by IHC was not performed in that study.

Oral and maxillofacial surgeons rely on histopathological assessment and IHC of surgical margins to ensure complete excision of OSCC. Molecular analysis of surgical margins can supplement the histopathological assessment and IHC and improve the likelihood of local recurrence of OSCC. The samples that did not express protein p53 on IHC and showed detection of mutations on molecular analysis is the one in which detection of recurrence at surgical margins is beneficial.

Small number of cases of poorly-differentiated OSCC could be retrieved from the archives of AFIP, and gene mutations were detected in none of them. Several FFPE-samples were excluded from the study as DNA could not be extracted. There might be a possibility of finding some mutations if the sample size was sufficiently large.

DNA was simply extracted from all the fresh/frozen sections in saline but was challenging regarding FFPE samples due to the presence of paraffin. About 30 - 80 microlitres of DNA were obtained from the FFPE-samples.

There is a need to determine the status of p53 gene mutation and p53 protein expression in OSCC of the

Pakistani population by conducting further studies. Since gene p53 plays a vital role in preventing injury to the genome, these genetic targets can be utilized for gene therapy in oral cancers using strategies such as suicide gene therapy or gene replacement therapy.<sup>24</sup> OSCC is the best candidate for trials of p53 gene therapy as they can be easily accessed by injection therapy.<sup>25</sup>

## CONCLUSION

A substantial number of patients have p53 gene mutation (23%) and protein p53 expression (67%) in OSCC. This will ultimately contribute to the future therapeutic improvements including gene therapy.

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