

ALTERATIONS OF P53 (GENE MUTATION AND PROTEIN EXPRESSION) IN ORAL EPITHELIAL DYSPLASIA

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ABSTRACT

Oral Squamous Cell Carcinoma (OSCC) is usually preceded by precancerous lesions. These lesions appear white or red clinically (Leukoplakia and Erythroplakia respectively) and show dysplastic epithelial changes on histopathological examination (Abbas et al, 2007). If p53 alterations (gene mutations and protein expression) in premalignant lesions are detected and treated in their early stages, it might help in prevention of progression to cancer (Patton et al, 2008).

Aim was to determine the frequency of p53 gene mutation and protein expression in oral epithelial dysplastic lesions. This was a descriptive study carried out at the Armed Forces Institute of Pathology (AFIP), Rawalpindi and was of one year duration from 8th May 2010- 5th May 2011. Thirty cases of oral epithelial dysplasia (OED) were retrieved from the record files. Some fresh / frozen sections were also included. Gene p53 mutation was detected in these cases by 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 protein p53 expression was recorded.

Mutations of the p53 gene were detected in 20% of the dysplastic lesions. Immunohistochemical staining of marker p53 was positive in 60% of the cases. Gene p53 mutation and protein expression was not coexistent.

Key Words: Oral Epithelial Dysplasia (OED), Tumour suppressor gene p53, Mutation, Polymerase chain reaction (PCR), Gel Electrophoresis.

INTRODUCTION

Leukoplakia and erythroplakia are the two most important precursor lesions of the oral cavity that show dysplasia on histopathological examination of biopsy specimens.¹ If not treated, they are at a higher risk of developing into invasive oral squamous cell

carcinoma (OSCC), especially the dysplastic lesions of the tongue and floor of the mouth.² About 20% of the non-homogenous leukoplakic lesions progress to invasive OSCC.³

Alcohol and tobacco are independent risk factors but there is an added effect when alcohol is consumed along with tobacco in precancer and cancers.⁴ High risk HPVs (types 16&18) have been found in 15-42% of oral leukoplakias.⁵

Leukoplakia is the most common oral precancerous lesion accounting for 85% of such lesions. The age of the patients is approximately 5 years less than those affected by OSCC, and is more common in males older

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than 40 years of age. There are various phases of clinical appearances from mild to severe, which correlate with the histopathological changes. Follow-up investigations have demonstrated that carcinomatous transformation usually occur 2 to 4 years after the onset of white plaque, but it may occur within months or after decades.⁶

Oral epithelial dysplasia (OED), as defined by Pindborg, is a lesion in which architectural disturbance of the epithelium is accompanied by cytological atypia. Clinically leukoplakic lesions show a range of histopathological features under microscope, which may be non-dysplastic epithelial hyperplasia and/or hyperkeratosis, or eventually leading to epithelial dysplasia.⁷

The tumour suppressor gene p53 has 11 exons in total.⁸ Mutation of p53 gene is the most common genetic alterations found in precancer as well as cancer.¹ Most of the mutations are located in Exons 5-8 of the p53 gene. These are mostly missense mutations which lead to amino acid substitutions in the wild type protein. Mutated gene encodes for a mutant protein whose cellular stability is increased.⁹ About 50% cases of HNSCC show point mutations of p53.¹⁰

In Southeast Asia, more than 90% of malignancies in the oral cavity arise from preexisting potentially malignant disorders.¹¹ If premalignant lesions are detected in their early stages and the patients get benefit from early treatment, then it is assumed that the lesion may not progress to cancer.¹²

In Pakistan, emphasis should be directed towards the early detection and diagnosis of oral dysplasia so that its transformation to oral cancer may be prevented. There is paucity of significant data on p53 gene mutations in oral epithelial dysplastic lesions. The study was thus planned to evaluate the malignant potential of OED by determining the frequency of p53 gene mutation and protein expression in OED and OSCC. This will eventually help the pathologists and oral and maxillofacial surgeons in better treatment planning of oral dysplastic lesions.

METHODOLOGY

This was a descriptive study carried out at the Histopathology Department and Molecular Biology Laboratory, Armed Forces Institute of Pathology

(AFIP), Rawalpindi and was of one year duration from 8th May 2010- 5th May 2011. A total of 30 cases of oral dysplasia were selected by consecutive sampling. Of the 30 cases, 23 formalin-fixed and paraffin-embedded tissue samples were retrieved from the archives of the Armed Forces Institute of Pathology, Rawalpindi between January 2007 and December 2010. While 7 fresh/frozen sections were taken from the patients (with informed written consent) reporting to the Oral Surgery Department of the Armed Forces Institute of Dentistry (AFID) from June 2010 to December 2010.

The data on age, gender and site of involvement was extracted from the clinical records. Histopathological features of the selected samples were reviewed from freshly prepared Hematoxylin & Eosin sections. Diagnosis of oral dysplasia (regardless of previous diagnosis) was made according to the criteria given by the WHO, and the new diagnosis was confirmed by two consultant histopathologists.

Extraction of DNA: DNA from Fresh/Frozen tissue was extracted by the Phenol Chloroform Method. Two to three chunks of tumour tissue about 1-2 mm in size were taken in an eppendorf tube. Approximately 700 microlitres of Cell lysis solution (Sigma) and 30µl of 'Proteinase K' (Sigma) were added and placed in the incubator at 37°C overnight.

The next day, 250 µl each of buffered phenol (Sigma) and chloroform (Sigma) was added to the lysate. The protocol was followed till the DNA precipitated as a white pellet and washed with 500 µl absolute ethanol. About 150 µl of DNA hydration solution (Sigma) was added and was incubated at 56°C for fifteen minutes to dissolve the DNA pellet. The DNA was stored at 4°C.

PureLink™ Genomic DNA Mini Kit (Catalog no. K1820-02 ordered from Invitrogen, USA) was used to extract DNA from the formalin-fixed and paraffin-embedded tissue specimens, according to the manufacturer's manual.

PCR-SSCP Analysis: 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, 1992)¹³ and obtained by custom ordering from e-Oligos, GeneLink (Table 1).

The PCR was done in 23 μ l reaction mixture containing 2.3 μ l of 10 x PCR Buffer, MgCl₂ 25 mM, dNTPs 200 mM (Fermentos, USA), 1 μ l of primer (10 pmol each), 0.5U/ μ l Taq polymerase (Fermentos, USA) and template DNA (about 100ng). Thirty cycles of the PCR reaction were run in an automated thermocycler(Cetus); 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 was done for 7 min at 72°C.

DNA of all samples was amplified by using Forward & Reverse Primers of Exon 5, Exon 6, Exon 7, and Exon 8 & 9 consecutively.

Denaturation of the amplified DNA: Five microlitres of amplicons of each sample were mixed with 5 μ l of 0.1% SDS and 5 μ l of 10mM EDTA (Merck-Germany). Bromophenol dye (15 μ l) was added to this mixture. These samples were then denatured by heating at 99°C for 5 minutes, and immediately chilled on ice. A volume of 4 μ l was applied to 6% Polyacrylamide Gel. Electrophoresis was performed at 100V for 20 hours at room temperature. Gel was stained with silver nitrate and counter stained with sodium hydroxide and formalin (100ml: 2 ml) for 20 minutes. Gel was 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 167bp fragment.

All mutations were confirmed by a complete repeat of the experimental procedure using the same samples. No bands were detected in PCR-SSCP analysis when DNA was not included in the amplification reactions. Colon carcinoma was taken as positive control and Human DNA (free of p53 mutation) was used as negative control.

Immunohistochemistry: Sections of 5 μ m thicknesses were cut from formalin-fixed and paraffin-embedded (FFPE) tissue. Antigen retrieval was done in citrate using pressure cooker. Primary antibody p53, a mouse monoclonal antibody recognizing mutant p53 (NovoCastra, 15Mm Sodium Azide, NCL-p53-BP) was applied to the sections at a dilution of 1:20. This was followed by incubation with biotinylated secondary antibody and Streptavidin-Biotin complex for 10 min-

utes each. Visualisation was with 3'-3 diaminobenzidine chromogen. The sections were then stained with Hematoxylin. Phosphate buffered saline (Ph: 7.4) was used for rinsing in between the reaction steps. Colon carcinoma was taken as positive control. For negative control, all the steps of IHC were followed except that the primary antibody p53 was omitted. Nuclear staining of more than 10% cells was taken positive for p53.

The percentage was evaluated by counting the 100 tumour cells in the area of best staining and were 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

+++, staining of greater than 66% of keratinocytes.¹⁴

The staining intensity was graded as follows; 1+ for definite but light stain; 2+ for darker stain and 3+ for most intense stain.¹⁵

Data Analysis: The data was analyzed using the SPSS version 17.0. Frequency of occurrence of study variables i.e. p53 gene mutation and protein expression was calculated along with 95% confidence intervals. Spearman's rank correlation analysis was used to determine any correlation between the variables.

RESULTS

The age of the patients ranged between 32-80 years with a mean age of 54.23 years (SD = 11.76). Among patients affected by oral dysplasia, 15 (50%) were males and 15 (50%) were females with male to female ratio of 1:1. Majority of dysplastic lesions were found on the tongue (40%) followed by buccal mucosa (30%), labial mucosa (13.3 %), floor of the mouth (10%) and buccal vestibule (6.7%). Twelve (40%) dysplastic lesions were of mild grade, whereas 8 (26.7%) were of moderate grade and 10 (33.3%) were of severe dysplasia.

Gene p53 mutation was present in 6 (20%; CI: 7.7% - 38.6%) cases while in 24 (80%; CI: 62.3% - 90.9%) cases p53 gene mutation was absent. Polyacrylamide gel showing mutations in Exon 5 of the p53 gene is shown in Figure 1. About 13.3% of the p53 gene mutations were detected in severe dysplasia while 6.7% were

TABLE 1: PRIMERS

Amplified Fragment	Primers		
Genomic DNA	Designation	Sequence	
EX5	EX5U	5' TTCCTCTTCCTGCAGTACTC3'	
	EX5D	5' GCAAATTCCCTCCACTCGG3'	
EX6	EX6U	5' ACCATGAGCGCTGCTCAGAT3'	
	EX6D	5' AGTTGCAAACCAGACCTCAG3'	
EX7	EX7U	5' GTGTTGCCTCCTAGGTGGC3'	
	EX7D	5' CAAGTGGCTCCTGACCTGGA3'	
EX8, 9	EX8U	5' CCTATCCTGAGTAGTGTAA3'	
	EX9D	5' CCAAGACTTAGTACCTGAAG3'	

TABLE 2: CORRELATION BETWEEN PROTEIN P53 EXPRESSION AND HISTOPATHOLOGICAL GRADES

Grades	p53 expression				p-value
	-	+	++	+++	
Oral Dysplasia					
Mild	7(23.3%)	5(16.7%)	-	-	r = 0.461, p = 0.01
Moderate	2(6.7%)	2(6.7%)	4(13.3%)	-	
Severe	3(10%)	1(3.3%)	4(13.3%)	2(6.7%)	

TABLE 3: CORRELATION BETWEEN P53 GENE MUTATION AND PROTEIN EXPRESSION

p53 Gene Mutation	p53 expression				p-value
	-	+	++	+++	
Oral Dysplasia					
Absent	9(30%)	6(20%)	7(23.3%)	2(6.7%)	r = - 0.162, p = 0.391
Present	3(10%)	2(6.7%)	1(3.3%)	0(0%)	

found in moderate dysplasia. A significant correlation was seen between p53 mutation and histopathological grades of oral epithelial dysplastic lesions ($r=0.429$, $P=0.018$)

Protein p53 immunoexpression was seen in 60% (42.3% - 75.4%) of the cases (Figure 2). In 9 (30%) cases marked staining intensity (3+) was seen with p53 marker followed by 5 (16.7%) of moderately stained (2+) and 4 (13.3%) showed mild staining intensity (1+). Very strong positive and highly significant correlation was observed between p53 staining and staining intensity in OED ($r = 0.972$, $p < 0.001$).

Positive and strong correlation was observed between p53 protein expression and histopathological grades in OED (Table 2).

In this study, 50% (3 of 6) of OED that contained p53 gene mutations did not show any p53 expression while 50% (3 of 6) of OED that contained p53 gene mutations showed positive p53 expression. Gene p53 mutation and protein expression was not present simultaneously (Table 3).

DISCUSSION

Several researches worldwide have proved that p53 tumour suppressor gene and its protein are altered very early in the process of carcinogenesis.¹⁶ Oral epithelial dysplasia has shown varying range of malignant transformation worldwide, after follow-up period of several months or years.¹⁷

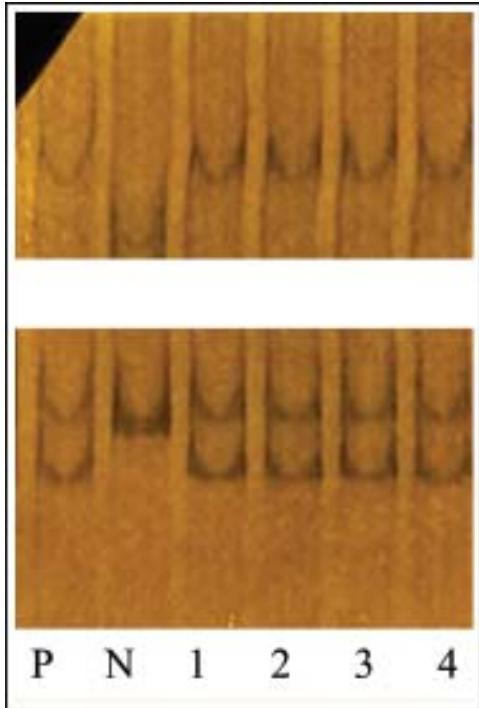


Fig 1: Polyacrylamide gel showing positive cases for mutations in Exon 5 of the p53 gene.

P: Positive control

N: Negative control

1- 4: Positive samples of dysplasia

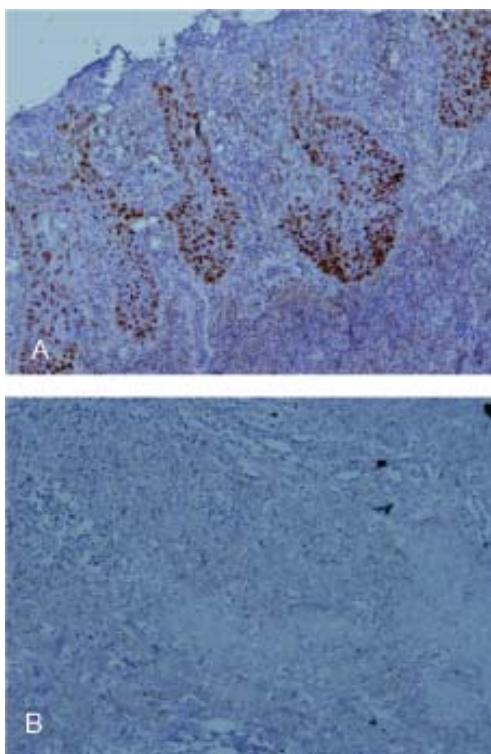


Fig 2: A. Photomicrograph of (++) immunohistochemical p53 expression in dysplastic lesion of the buccal mucosa (x100). B. Negative control(x100).

The prognostic significance of p53 protein expression in oral dysplastic lesions has been studied worldwide, but few studies have focused on p53 gene mutation in OED. The present study was thus conducted to detect p53 alterations (gene mutation and protein expression) in dysplastic lesions of the oral cavity.

In this study, 20% of the dysplastic lesions showed mutations mostly in Exon 5 of the gene, while over expression of p53 protein was present in 60% of the dysplastic lesions. Similar results were found in a North Indian study conducted by Ralhan et al in which gene mutation was detected in 17% of the precancerous lesions, predominantly in Exon 5 of the p53 gene.¹⁸

In this study, protein expression and gene mutation were not coexistent; suggesting that mutation of the gene may be one of the mechanisms responsible for protein stabilization. A study conducted by Rowley et al had showed 77% of dysplastic lesions positive for p53 protein expression, while mutations of the gene were detected in 44% of OED.¹⁵

Alteration of p53 is a strong indicator of malignant transformation potential of oral dysplastic lesions.¹⁹ The dysplastic lesion having mutations detected are candidates for aggressive treatment and closer follow-up.

There is paucity of significant data of p53 gene mutation in Pakistani population. As this study is a baseline study, a further study may be planned by performing p53 gene sequencing analysis to determine amino acid change in a large sample size.

Genetic studies applied to the detection of mutations in precancerous lesions have opened new windows in our understanding of the detrimental effects exerted by the carcinogens on the mucosa of the oral cavity. Several studies worldwide have revealed that the presence of mutation is a definite indicator of progression to malignancy. A simple and cost effective PCR-SSCP analysis can be used to find out whether mutation is present or absent in the precancerous tissue. If mutation detected, the dysplastic lesion can be treated and cured in early stages and progression to cancer can be reduced. The early detection of mutations in dysplastic lesions can save mutilating ablative surgeries and may improve survival rate of patients.

CONCLUSION

The detection of mutations in the p53 gene may be considered an important marker suggesting the possible progression of the oral dysplastic lesions to oral squamous cell carcinoma, and it can have a major role in gene therapy in future.

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