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RESEARCH ARTICLE

p53 IMMUNOEXPRESSION IN ORAL SQUAMOUS CELL CARCINOMAS FROM DIFFERENT ANATOMICAL SITES: A COMPARATIVE STUDY

Yogish. P[1], Girish, H.C[2], Asha[3], Ravikumar Pujari[4], Vidya Rani[5], Kavitha[6] and Umapathy[7]

[1]Assisstant Professor, Department of Oral Pathology & Microbiology, Sharavathi Dental College and Hospital, Shimoga, Karnataka, India
[2]Head of the Department, Department of Oral Pathology & Microbiology, Rajarajeswari Dental College and Hospital, Bangalore, Karnataka, India
[3]Post graduate student, Department of Oral Medicine & Radiology, Coorg Institute of Dental Sciences, Virajpet, Karnataka, India
[4]Reader, Department of Oral Pathology & Microbiology, Rajarajeswari Dental College and Hospital,Bangalore, Karnataka, India
[5]Post graduate student, Department of Oral Pathology & Microbiology, Rajarajeswari Dental College and Hospital, Bangalore, Karnataka, India
[6]Post graduate student, Department of Paediatric Dentistry, V.S. Dental College and hospital, Bangalore, Karnataka, India
[7]Reader, Department of Paediatric Dentistry, Rajarajeswari Dental College and Hospital, Bangalore, Karnataka, India

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ABSTRACT

Background and Objectives: Squamous cell carcinoma is the most common intraoral malignancy. Despite intense research in the area of squamous cell carcinomas of the head and neck (SCCHN), its 5-year survival rate remains low. p53 alterations have been implicated in oral carcinogenesis process. Oral squamous cell carcinoma (OSCC) lesions mostly occur at the tongue, followed by floor of the mouth, alveolar ridge/ gingiva, buccal mucosa, labial mucosa and hard palate. This study was undertaken to analyze the p53 immunoexpression in OSCC at different sites.

Methods: This study was conducted on samples collected from patients with oral squamous cell carcinoma who visited Rajarajeswari Dental College & Hospital, Mysore Road, Bangalore. Tissue sections were subjected to Immunohistochemistry using p53 marker and cells were counted in three different histological fields. The obtained data was subjected to Anova test.

Results: On performing Anova test between different sites of oral cavity to know the immunoexpression of p53 we got a p value of 0.270 which was not statistically significant.

Interpretation & Conclusion: Further investigations are required to elucidate the specific mechanisms involving tongue cancer as p53 did not appear to have influence in the higher frequency and more aggressiveness of the disease in this site particularly.

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INTRODUCTION

Squamous cell carcinoma is the most common intraoral malignancy. It mainly affects male patients with ages varying between 40-80 years, although recent studies have reported the occurrence of this malignancy in younger patients (Claudia, 2006). Oral cancers represent 4% of all cancers in the West, whereas, in developing countries including India, it accounts for up to 45% of all cancers. Globally, oral cancer ranks as the sixth most common cancer (Tandle, 2001) Oral squamous cell carcinoma (OSCC) lesions mostly occur at the tongue, followed by floor of the mouth, alveolar ridge/ gingiva, buccal mucosa, labial mucosa and hard palate (Claudia, 2006).

**Corresponding author: Yogish, P.,*
Department of Oral Pathology & Microbiology, Sharavathi Dental College and Hospital, Shimoga, Karnataka, India

Mutations in the *TP53* gene is the most common genetic change found in human tumours; in SCCHN, 40–50% of the tumours studied have shown a mutation in this gene (Nylander, 2000). In normal cells, wild type p53 protein has a short half life and is usually undetectable immunohistochemically. Alterations to the p53 gene are usually due to mutations, particularly missense point mutations. Inactivation of wild type p53 by binding with viral or cellular proteins can cause both elimination of the suppressor function and stabilization of the protein (Kerdpon, 1997)

MATERIALS AND METHODS

Source and collection of data

This study was conducted on samples from patients with oral squamous cell carcinoma who had visited Rajarajeswari Dental

College & Hospital, Mysore Road, Bangalore and Rajarajeswari Medical College and Hospital, Bangalore. The sample size for this research work comprised of 30 histologically proven cases of oral squamous cell carcinoma i.e. 5 cases in every site- buccal mucosa, palate, lip, gingivobuccal sulcus, tongue, floor of the mouth. Paraffin embedded specimens of oral squamous cell carcinoma was also selected from the tissue archives of the Department of Oral Pathology and Microbiology, Rajarajeswari Dental College & Hospital.

Inclusion Criteria

All cases of untreated squamous cell carcinoma which are confirmed with biopsy affecting the oral cavity.

Exclusion Criteria

- Benign epithelial growths
- Epithelial hyperplasias
- Recurrence after the primary lesion has been treated.

Preparation of the slides

The wax blocks of histologically diagnosed cases of oral squamous cell carcinoma of different sites were selected. Then 4µm of tissue section was made using a semiautomatic microtome. The sections were placed on poly L silane coated slides. The slides were preserved in a slide holding box until they are stained for p53.

Principle

The method used for detection of the expression of the p53 protein was Immunohistochemistry. The reaction in IHC usually takes place generally between two protein macromolecules: an antigen, which may be a glycoprotein, lipoprotein or just a protein and the antibody, which is a glycoprotein.

Procedure

Immunohistochemical Staining

p53 protein was detected by using immunohistochemical method. Tissue sections were made from formalin fixed, paraffin embedded specimens of 30 OSCC patients included in the study. From each specimen, two 4 µm section were cut and one placed on Poly L Silane coated slide and another on albumin coated slide. The albumin coated slide were stained with H and E, while the Poly L Silane coated slide was subjected to IHC procedure, which were incubated overnight at 56 C, deparaffinised on slide warmer for 10 mins, deparafinised in xylene (3 times, 5 mins each), rehydrated in ethanol 100%, 95%, 70% and finally in distilled water. Antigen retrieval was performed by incubating slides in a jar containing 10 mM Sodium citrate buffer, pH 6.0 in preheated pressure cooker at 115^0c for 20 minutes, then cooled to room temperature. Slides were then rinsed twice in Tris buffer saline (TBS 0.05M, ph 7.6), 5 mins each time. The slides were then transferred into a humidified chamber and incubated in 3% Hydrogen peroxide for 20 mins to block the endogenous peroxidase activities. Sections were then incubated with a highly effective universal protein blocking reagent (Caesin and proprietary additives in PBS with 15mM sodium azide, Levamisole). Sections were incubated with primary antibody against p53 (mouse monoclonal anti-p53, clone DO-7, diluted 1:50, BIOGENEX) for 60-75 minutes at room temperature followed by wash with TBS for 5 minutes where excess was wiped off using a blotting paper. The sections were incubated with biotin-labeled secondary antibody for 30 minutes at room temperature. The secondary antibody Streptavidin conjugated with horse radish peroxidase, HRP was applied to cover the tissue samples and was incubated for 30 minutes at room temperature. The sections were stained for 20 min in 3-diamino- benzidine tetrahydrochloride (DAB) and counterstained in harris hematoxylin for 3-5 mins. Slides were sequentially dipped in 70%, 95%, 100% ethanol and finally in xylene. These were coated with DPX and covered with coverslips.

Quality control for IHC

One positive and one negative control were run with each batch of immunostained sections. Positive controls for p53 protein consisted of breast cancer tissue sections with known positivity. All reactions were performed with negative controls using TBS instead of primary antibody.

Interpretation of the p53 staining

The slides stained for p53 were observed under light microscope with a magnification of 400X. The tissue samples were thoroughly examined and total number of 450 cells (150 cells per field) was counted. Then, among the 150 cells, the number of cells which had taken up the stain was counted. A brown precipitate seen within the nucleus confirmed the presence of p53 protein. The p53 positive samples were then evaluated semiquantitatively on a 4- point scale based on the percentage of cells showing p53 staining.

The protein expression was scored as follows:

- +++ when > 50% (>251) of cells staining positive.
- ++when 25% - 50% (126- 250) of cells staining positive.
- + when 5% - 25% (26- 125) of cells staining positive.
- −when < 5% (< 25) of cells staining positive were considered.

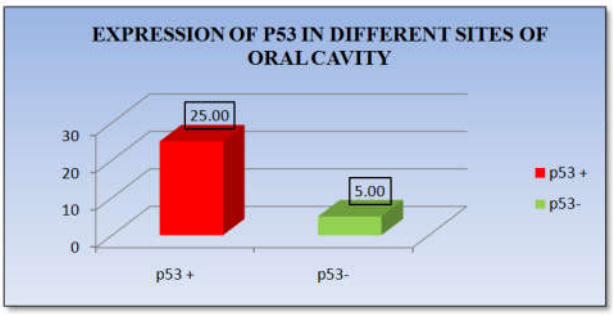
Results and Observations

This study was undertaken to compare the p53 expression in different sites of oral cavity. The study also comprised of comparison between grading of the tumor and intensity of staining. The study comprised of different sites i.e. buccal mucosa, floor of the mouth, gingiva, lip, palate and tongue. 5 samples were taken from each site and control group included tissue sample of breast carcinoma. The data obtained was subjected to ANOVA test, the statistical software SPSS 20 was used for the analysis of the data.

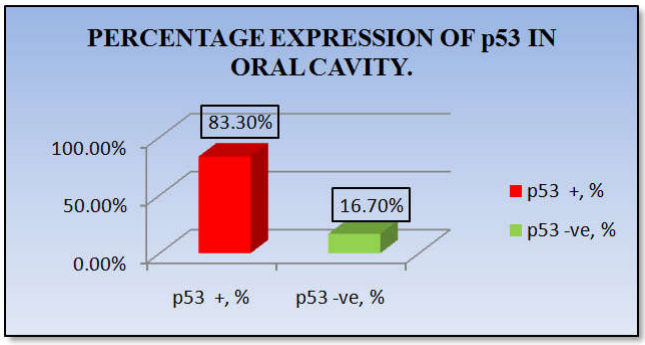
Statistical Analysis

Comparison of expression of p53 in different sites

Level of Significance: $p = < 0.05$
Statistical test used: **ANOVA**, analysis of variance test.



Graph 1. Expression of p53 IN OSCC i.e Positive and Negative Cases



Graph 2. Percentage of Expression of p53 in OSCC

Immunohistochemical analysis of p53 was performed on the OSCC lesions of 30 patients. The OSCC samples were grouped according to their site and p53 expression was determined. Graph 1 and 2 shows that p53 immunoexpression positivity was seen in 25 cases (83.3%) and negative was seen in 5 cases

Table 2 shows that standard deviation of each group is too high, and unequal variance of the groups. The ANOVA test shows that there is no statistical significant difference among the averages of the group.

Table 1. p53 Expression in different sites of oral cavity

Site	--ve negative	+ mild	++ moderate	+++ severe
Buccal mucosa	1	4	0	0
Palate	0	3	1	1
LIP	2	2	1	0
Gingiva	1	2	2	0
Tongue	1	3	1	0
Floor of Mouth	0	4	1	0

ANOVA: Analysis of Variance

Table 2. Table for Descriptive Statistics

		N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum	Between- Component Variance
					Lower Bound	Upper Bound			
buccal mucosa		5	140.40	50.816	77.30	203.50	53	184	
Floor- mouth		5	288.60	130.268	126.85	450.35	124	486	
Gingiva		5	332.20	253.632	17.27	647.13	23	682	
Lip		5	237.40	261.756	-87.61	562.41	23	648	
Palate		5	453.80	301.238	79.76	827.84	177	908	
Tongue		5	198.20	151.815	9.70	386.70	56	402	
Total		30	275.10	217.274	193.97	356.23	23	908	
Model	Fixed Effects			210.630	195.73	354.47			
	Random Effects				159.32	390.88			3298.085

Table 3. Anova test between groups and within groups

ANOVA					
Total					
	Sum of Squares	Df	Mean Square	F	Significance
Between Groups	304276.700	5	60855.340	1.372	0.270
Within Groups	1064758.000	24	44364.917		
Total	1369034.700	29			

Table 1 shows that negative p53 immunoexpression were seen in lip, gingiva and tongue cases. Samples of tongue and floor of the mouth shows 1 case of moderate and nil cases of severe p53 expression whereas palate shows 1 severe p53 expression.

Descriptive Statistics

Calculates the number of cases, mean, standard deviation, standard error of the mean, minimum, maximum, and 95% confidence intervals for each dependent variable for each group.

The Table 3 shows that p value as 0.270, which is greater than 0.05, hence we accept the null hypothesis and conclude that the groups p53 expressions are not different.

DISCUSSION

The study of the molecular pathogenesis of oral squamous cell carcinoma can help in the search for molecular markers that might predict the clinical behavior of the tumor, which is not

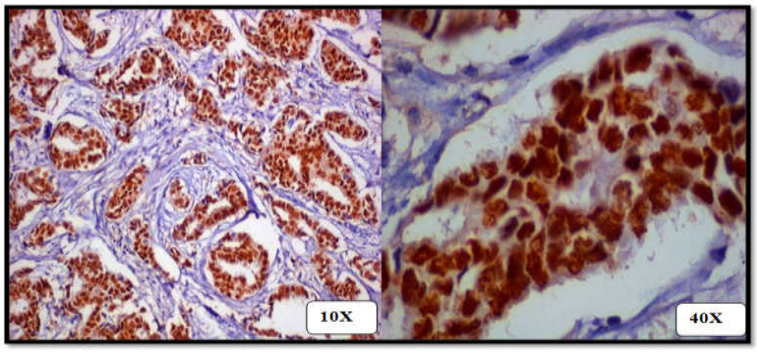


Figure 1. Photomicrograph: Immunohistochemical staining of p53 in breast carcinoma (control) as seen under Bright field Microscopy

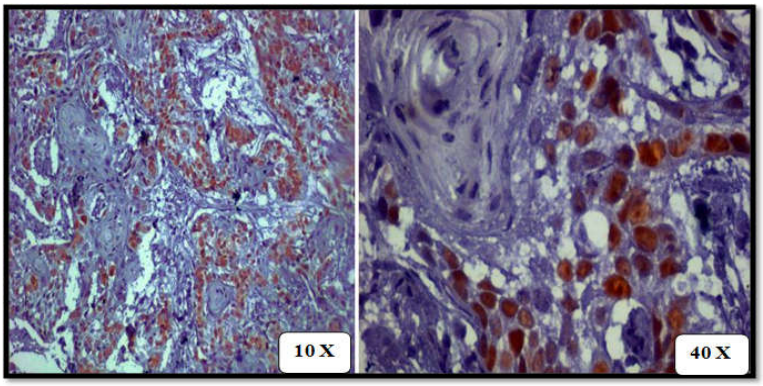


Figure 2. Photomicrograph: Immunohistochemical staining of p53 in OSCC as seen under Bright field Microscopy

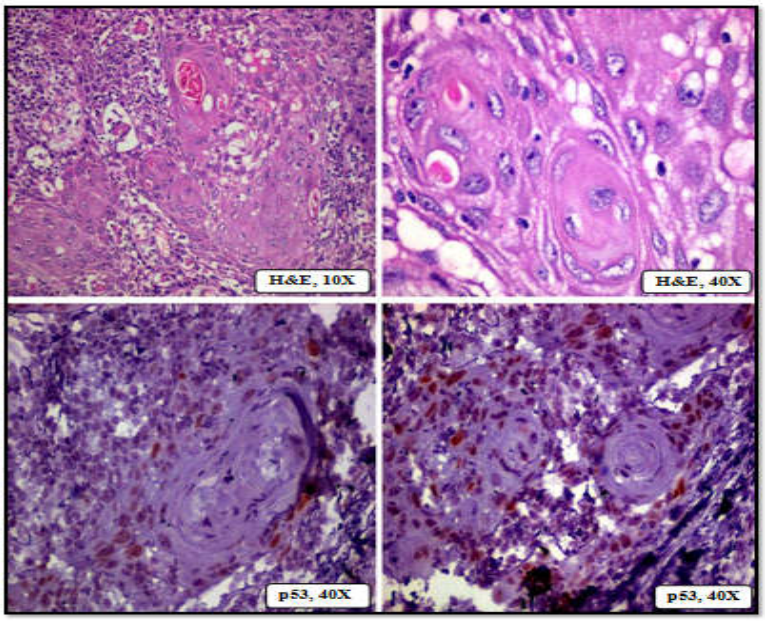


Figure 3. Photomicrograph: H and E staining and Immunohistochemical staining of p53 in OSCC as seen under Bright field Microscopy

strictly related to TNM staging or histological grade (Rich ., 1999). p53, a well- known tumor suppressor gene that is believed to serve as a gatekeeper against carcinogenesis. Under normal circumstances, the function of p53 protein is to prevent the propagation of genetically damaged cells. Cells with loss of p53 function are speculated to undergo malignant transformation. To date, mutation of the p53 gene is one of the most common events in human cancers, including oral squamous cell carcinoma. The mutation produces accumulation of p53 protein, which can be detected by molecular biological methods and immunohistochemical methods in several human malignant lesions (Rich, 1999). p53 behaves as a multifunctional transcription factor involved in: control of cell cycles, programmed cell death, senescence, differentiation and development, transcription, DNA replication, DNA repair and maintenance of genomic stability. In view of these activities, p53 has been rightfully called a "GUARDIAN OF THE GENOME". Inactivation of this gene leads to the inability of a cell with DNA damage to induce cell cycle arrest to allow time for DNA repair or the induction of apoptosis. Wild-type p53

may be inactivated by complex formation with mutant p53, viral or aberrant host-binding proteins. The mutated p53 gene product is more stable than the wild-type and can be demonstrated immunohistochemically in tissue sections (Lane, 1992).

The present study was carried out in oral squamous cell carcinoma to determine p53 expression in different sites of oral cavity. In our study 25 out of 30 cases, 83.3% were positive for p53 in oral squamous cell carcinoma and only 5 cases, 16.70% were negative. Our results were in accordance with that of Kaur (1994) who reported 24/32 cases i.e 75% positivity, Kerdpon (1997) who reported 45/48 cases, 94% of p53 positive expression. The cut off value of 25% appeared to be a good predictor for TP53 mutations (Kerdpon, 1997). The aim of our study was not to analyze samples at gene level, thus the detection of mutant p53 cannot be confirmed although immunohistochemistry technique has assured its importance by the concordance level found in some recent studies between p53 protein expression mean-indexes and the presence of TP53 gene alterations. It is noteworthy that these studies have analyzed not only hotspots regions (exons 5-9), but the entire coding region of TP53 gene. Nevertheless, the study of Cruz . has established a cut-off value of 25% of p53 immunostained tumoral cells as indicative of TP53 mutations. False-positive results may have occurred leading to our 83.3% p53-positive cases, such as p53 up regulation in an attempt to promote DNA repair or apoptosis. Similarly, p53 stabilization and accumulation could be the result of p53 binding to cellular or viral proteins.

Type and dilution of primary antibody as well as pre-treatment use are also factors of influence in immunohistochemistry results (Claudia ., 2006). On the other hand, p53-negative cases, apart from technique failures, may result from biallelic deletion of TP53 gene, very low levels of mutant or wild-type p53, a nonsense mutation or a truncated p53 protein in its N-terminal portion, which would not be recognized by DO-1 antibody. Another possible explanation would be an accumulation of Mdm2 protein caused by gene amplification, promoting p53 degradation and non-detection by Immunohistochemistry (Claudia ., 2006). In the present study we did not find any correlation between p53 immunoexpression with respect to primary anatomical site in 30 samples of oral squamous cell carcinoma. The rich lymphatic network of the tongue and floor of the mouth seems to favor prognosis values lower than 20%. Moreover, experimental studies have shown that tongue tissue displays a higher amount of some enzymes responsible for the metabolic activation of chemical carcinogens than other oral tissues. Nevertheless, the floor of the mouth and lateral and ventral tongue surfaces are characterized by a higher permeability due to their thinner, nonkeratinized mucosa, providing less protection against carcinogens (Tandle ., 2001). These features may give a possible explanation for the high frequency and aggressiveness of tongue tumors. Perineural invasion, tumoral stage (T) and nodal metastasis have been considered as the most important parameters to predict tongue SCC recurrence (Ünal ., 1999). In our study we aimed to compare p53 protein immunostaining in tongue and floor of the mouth SCC cases with the remaining oral cavity sites. However, no statistically significant results were found. Even when tongue cases were excluded from the analysis, the remaining cases did not show correlation with p53. Our results were in accordance with that of a study done by Sauter , Brennen , 1994 (Kerdpon, 1997). Equally, we should consider that the diagnosis of these lesions is established, most of the times, lately, and sometimes both sites are affected simultaneously. This creates difficulty in determining the primary site of occurrence. Hence, it is possible that the assembly of these two sites, compared with the remaining oral cavity regions, could have presented different results with respect to p53 immunostaining (Claudia, 2006).

Conclusion

This lack of association between p53 immunostaining in tongue SCC and the remaining oral cavity sites supports that other molecular and clinicopathological factors may have some relationship with the prevalence of the disease at the tongue. Probably, mechanisms other than p53 alterations may be of relevance for oral carcinogenesis, thus, other investigations considering different variables deserve to be continued until the elucidation of the specific mechanisms involving tongue carcinogenesis process.

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