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RESEARCH PAPER

Histological significance of p53 gene expression in squamous cell carcinoma of the buccal mucosa

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Abstract
Introduction Oral Squamous Cell Carcinoma (SCC) results from genetic mutations which activate the oncogenes and inactivate the tumor suppressor gene namely TP53. Despite the use of multimodality treatments the prognosis of oral SCC has not changed significantly.
Purpose To evaluate 1) if there is any correlation between the two prognostic indicators i.e. p53 over expression and histological grade of the tumor 2) if any of the parameters of histological grading correlate significantly with p53 over expression. This information would help in understanding the exact role of TP53 gene mutation in cellular progression of oral SCC.
Method Study was conducted on 90 resected specimens of Stage IV SCC of buccal mucosa. Slides from these specimens were evaluated for histological grading by Anneroth's method and p53 over expression by Immunohistochemistry.
Results Statistically significant co-relation was seen between the total histological grade and p53 over expression. Also 4 individual histological parameters which indicated high cellular turnover were also significantly associated with p53 over expression.
Conclusion TP53 mutation histologically signifies an early event in cellular progression of oral SCC.

Keywords Oral squamous cell carcinoma · Anneroth's grade · Histology · p53 · Immunohistochemistry

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Introduction

Squamous Cell Carcinoma (SCC) accounts for 90% of all oral malignancies. Globally it represents for 5% and 2% of all cancers in males and females respectively [1]. This disease is now accepted to be caused by genetic damage to the mucosal cells in form of accumulated mutations causing the cells to proliferate rapidly in an uncontrolled manner and also to metastasize to regional lymph nodes. A variety of causative factors are associated with this disease, significant amongst them are tobacco, alcohol and human papilloma virus [2,3]. Although a lot of research is being carried out and new therapeutic regimens are being introduced in management of oral cancer, the five year prognosis of the disease has not changed significantly [4,5].

It is now widely acknowledged that, the genetic changes leading to oral cancer result in inactivation of the tumor suppressor gene namely TP53 and activation of a multitude of proto-oncogenes [5]. Mutations and alterations in the tumor suppressor pathways of TP53 gene have been implicated in almost all human cancers [6, 7]. This gene and its altered pathway have also been extensively studied in pre-malignant oral lesions and oral cancer. p53 protein also has been extensively investigated as a tumor marker for prognostic indicator. A strong correlation has been shown between p53 expression and poor outcome in patients with oral SCC [4,6–10].

The other accepted reliable prognostic indicator in oral SCC is the histological grade of the tumor. Although many grading systems are available, Anneroth's grading (modified in 1987) system shows a good statistical correlation between its histological grading and the disease prognosis [11–14].

Aims and objectives

The aim of this study was to evaluate

1. If there is any correlation between these two prognostic indicators i.e. p53 over expression and histological grade of the tumor
2. If any of the parameters of histological grading correlate significantly with p53 over expression.

This information would help in understanding the exact role of TP53 gene mutation in cellular progression of oral

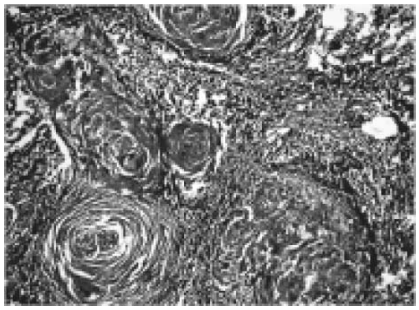


Fig. 1 Squamous cell carcinoma showing keratin pearls and lympho plasmocytic infilterate (x100 H and E)

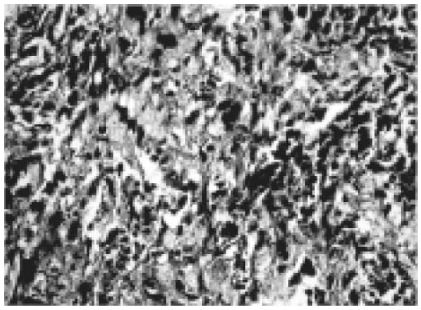


Fig. 2 H and E(x400) staining showing nuclear polymorphism and mitotic figures

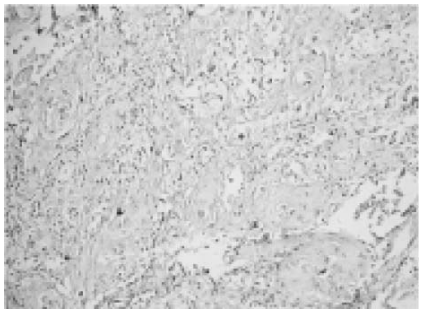


Fig. 3 p53 grade 1(x100)

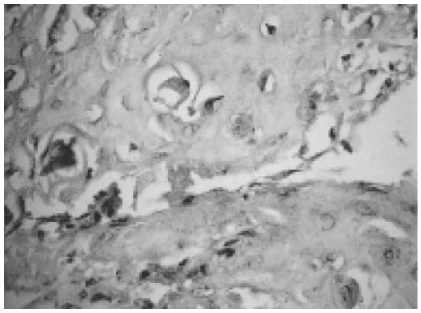


Fig. 4 p53 grade 2 (x400)

SCC. It would also help in improving the understanding the histological significance of TP53 mutation.

Materials and method

The study was jointly done at the Karnataka Cancer Therapy and Research Institute (KCTRI) Hubli, India and Maratha Mandal's N.G.H. Institute of Dental Sciences, Belgaum, India. 90 patients with clinically stage IV (TNM staging) disease of the buccal mucosa who had undergone therapy (resection and other modalities subsequently) at KCTRI were randomly included in this study. This was done to standardize the patient sample. p53 expression has been shown to have variations with the site and stage of the disease [6]. Paraffin blocks (of the tissue) of the resected specimens of these patients were retrieved from the pathology archives and fresh sections of 4 microns were prepared using a semi-automated microtome. Three slides were prepared from each block. One slide was subjected to Hematoxylin & Eosin (H&E) staining and two slides were used for p53 immuno-staining. The best p53 stained slide was used for the study. The H&E stained slides were reviewed by a senior oral pathologist for Anneroth's grading and the p53 slide was reviewed by a senior general pathologist. All the slides were reviewed by only one person respectively to avoid inter-personal subjective bias. Both the reviewers worked independently and were blinded to results of the other reviewer.

Anneroth's grading

The H & E stained slides were graded as per the grading system given by Anneroth [12,13]. Morphological parameters of the malignant cells were graded on a 1–4 scale (Table 1). The sum total of the grades of all the parameters was recorded as the total histological grade of the tumor.

Immunohistochemistry

The tissues on the slides were deparaffinized and rehydrated by using gradients of xylene and alcohol by using standard protocol. The antigen retrieval was carried out by immersing the slides in a solution of 0.25% Trypsin with 0.1% Calcium hydroxide. After rinsing the slides in Tris buffer the staining was carried out by using anti-p53 protein and super sensitive polymer – HRP Detection system (BioGenex, USA). Briefly the slides were treated with peroxide block and power block for 10 mins each followed by anti p53 primary antibody for a period of 2 hours. The slides were rinsed thoroughly in Tris buffer and treated with super enhancer reagent for 20 mins followed by poly-HRP reagent for 30 minutes. Finally the sections were treated with DAB chromogen substrate for 10 mins. The slides were again rinsed with Tris buffer and counter stained with Mayer's Hematoxylin for 10 mins. Slides were rinsed with water and mounted in a permanent mounting medium. The p53 antibody used was monoclonal mouse anti-p53 protein which can detect both wild and mutant p53. Its efficacy was first established by using normal skin as negative control and breast cancer cells as positive control as recommended by the manufacturers.

p53 grading

As mentioned previously the p53 grading for all the slides was done by a single senior pathologist who was blinded to the results of the histological grading to avoid any kind of bias. Five different areas were evaluated and the immune-expression limit was set at 25% i.e. the TP53 gene mutation was considered positive if 25% or >25% of the tumor cells showed nuclear staining for p53 protein (average of 5 fields). The p53 positive slides were further graded as 1, 2, 3 and 4 according to the number of cells that showed positive nuclear staining [15].

1. 25% +ve
2. 25–50% +ve
3. 50–75% +ve
4. 75–100% +ve

The most densely stained area was considered for this grading.

Results

Out of 90 patients 57 were male and 33 were females with an age range of 37 years to 83 years. From the records a definite history of regular tobacco association was present in 73 patients (81.1%). As mentioned all the patients had biopsy proven SCC and the slides were done from the blocks of the resected specimens. All the patients had malignancy of the buccal mucosa only and they were clinically Stage IV (TNM staging)

Anneroth's grading: (Fig. 1 and 2)

The total Anneroth's grade for all the patients varied from 6–18 with a mean of 11.416 (Std dev. 2.199 and SE 0.233) Table 2. The range of the individual grades of the 6 parameters of the Anneroth's grading system, are summarized in Graph 1.

Table 1 Anneroth's histological grading

Morphological parameters	1	2	3	4
Degree of Keratinitization (DK)	Highly keratinized >50% cells	Moderately keratinized (20–50% cells)	Minimally keratinized (5–20% cells)	No keratinization (0–5% Cells)
Nuclear Polymorphism (NP)	Minimal polymorphism (>75% cells mature)	Moderate polymorphism (50–75% mature cells)	Abundant polymorphism (20–500% mature cells)	Extreme polymorphism (<20 % mature cells)
Number of Mitoses (NM)	0–1	2–3	4–6	>6
Pattern of Invasion (PI)	Pushing well delineated infiltrating border	Infiltrating solid cords bands or cords	Small groups or cords of infiltrating cells	Marked and widespread cellular dissociation in small groups of cells or single cells
Stage of Invasion (SI)	Carcinoma in situ	Distinct invasion mainly in lamina propria	Invasion deep to lamina propria into adjacent structures	Extensive deep invasion replacing normal stromal tissues
Lympho-Plasmocytic Infiltration (LPI)	Marked	Moderate	Slight	None

Table 2 Summary statistics of individual histological grading and their total

Variables	Mean	Std.Dev.	SE	Minimum	Maximum	Range
DK	1.775	0.735	0.078	1	4	3
NP	1.742	0.731	0.078	1	4	3
NM	1.472	0.605	0.064	1	3	2
PI	1.865	0.643	0.068	1	4	3
SI	2.764	0.477	0.051	1	3	2
LPI	1.798	0.481	0.051	1	3	2
Total	11.416	2.199	0.233	6	18	12

Table 3 Summary statistics of p53

Variables	Mean	Std.Dev.	SE	Minimum	Maximum	Range
p53	1.202	0.919	0.097	0	3	3

Table 4 Comparison of patients with positive and negative p53 with respect to different histological grading and its total by Mann-Whitney U-test

Variable	Summary	Pos	Neg	U-value	Z-value	P-value
DK	Means	1.9559	1.1905	290.50	-4.0922	0.0000*
	Std.Dev.	0.7214	0.4024			
NP	Means	1.8676	1.3333	421.50	-2.8264	0.0047**
	Std.Dev.	0.7311	0.5774			
NM	Means	1.5735	1.1429	428.00	-2.7636	0.0057**
	Std.Dev.	0.6063	0.4781			
PI	Means	2.0000	1.4286	373.00	-3.2950	0.0010**
	Std.Dev.	0.5985	0.5976			
SI	Means	2.8235	2.5714	590.00	-1.1982	0.2309
	Std.Dev.	0.3841	0.6761			
LPI	Means	1.8235	1.7143	645.00	-0.6667	0.5049
	Std.Dev.	0.4869	0.4629			
Total	Means	12.0588	9.3333	191.50	-5.0488	0.0000*
	Std.Dev.	1.9308	1.6833			

*p<0.001, **p<0.01

Table 5 Spearman's rank correlation between p53 and histological grading and its total

Histological grades	Spearman's r-value	p53 t-value	p-level
DK	0.5728	6.5183	0.0000*
NP	0.4248	4.3764	0.0000*
NM	0.4240	4.3664	0.0000*
PI	0.4319	4.4667	0.0000*
SI	0.1258	1.1825	0.2402
LPI	0.0560	0.5236	0.6019
Total	0.6262	7.4906	0.0000*

*p<0.001

Table 6 Comparison of patients with categories of p53 (0, 1–2, 2–3) with respect to different histological grading and its total by Kruskal Wallis ANOVA test

Variable	Summary	0 p53	1–2 p53	2–3 p53	H-value	P-value
DK	Means	1.1905	1.8983	2.3333	21.5770	0.0000*
	Std.Dev.	0.4024	0.6616	1.0000		
NP	Means	1.3333	1.8475	2.0000	10.2264	0.0060**
	Std.Dev.	0.5774	0.7614	0.5000		
NM	Means	1.1429	1.5593	1.6667	10.7070	0.0047**
	Std.Dev.	0.4781	0.6234	0.5000		
PI	Means	1.4286	1.9831	2.1111	15.0979	0.0005*
	Std.Dev.	0.5976	0.5411	0.9280		
SI	Means	2.5714	2.8475	2.6667	4.2627	0.1187
	Std.Dev.	0.6761	0.3626	0.5000		
LPI	Means	1.7143	1.8475	1.6667	1.7925	0.4081
	Std.Dev.	0.4629	0.4847	0.5000		
Total	Means	9.3333	12.0000	12.4444	26.6544	0.0000*
	Std.Dev.	1.6833	1.9740	1.6667		

*p<0.001, **p<0.01

p53 grading: (Fig. 3 and 4)

Out of the 90 specimens 69 patient samples tested positive for p53 while 21 were negative. The p53 grading varied between 0–3 with a mean value of 1.2 (Std deviation 0.919 & SE .097) Graph 2 and Table 3.

Comparison between Anneroth's grading and p53 scores

This was done using Mann Whitney U test. This analysis showed that there was a highly significant correlation statistically between the total Anneroth's score and p53 positivity (p<0.001). Also amongst the individual parameters the degree of keratinization scores showed a highly statistically significant correlation with p53 expression (p<0.001) whereas nuclear polymorphism number of mitoses and pattern of invasion scores were having a significant correlation with p53 expression statistically (p<0.01). No correlation was seen between p53 expression and stage of invasion and lympho-plasmocytic infiltration (Table 4).

This was also further confirmed using the Spearman's rank correlation test (p<0.001) (Table 5)

Comparison between the amount of p53 expression and the Anneroth's grades

Highly statistically significant correlation was seen between the increased total Anneroth's score and increased amount of p53 grading (p<0.001). Similar relationship was seen between high individual scores of degree of keratinization and pattern of invasion and increased p53 expression. High scores in the parameters nuclear polymorphism and number of mitosis had a statistically significant correlation with increased amount of p53 expression (p<0.01). However as mentioned above no correlation was seen between the scores of stage of invasion and lympho-plasmocytic infiltration and the p53 grading. This comparison was done using Kruskal Wallis ANOVA test (Table 6).

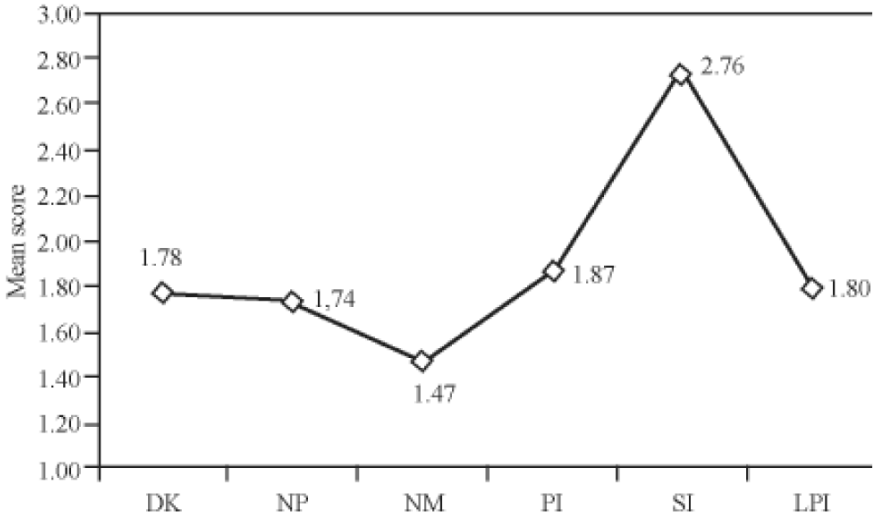
Discussion

Histological grade of the SCC of the oral cavity and p53 gene over-expression have individually been investigated for their significance as determinants of prognosis. However very few studies have been found, where a direct comparison between the two has been done [13,16,17]. We feel that a direct comparison of these two parameters will not only provide an insight into the role of p53, in cellular progression of oral SCC but also help in understanding the role of TP53 mutation in bringing about histological changes.

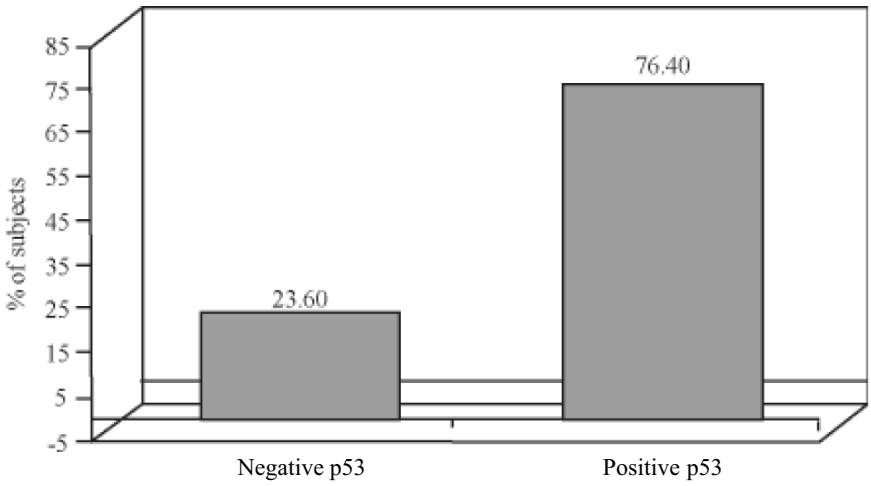
TP53 gene ('Guardian of the Genome') is usually in a standby mode and its activation occurs in response to a variety of exogenous and endogenous cellular stresses. Activation of the gene normally results in formation of the p53 protein which has 393 amino acids and this protein ultimately causes either cell cycle arrest or Apoptosis. As it is a very biologically active molecule, in normal cells its activity is strictly regulated. The protein is very rapidly degraded and cannot be detected by Immunohistochemistry (IHC) [4,6,7,15]. Upon mutation of the gene in cancer cells its growth suppressor activity gets reduced. The protein gets stabilized and its degradation is slowed down. Such cells (Tumor) which have p53 mutations begin to accumulate the protein and these are detected by IHC. It has also been postulated mutations not only lead to loss of growth suppressor functions but the accumulation of the proteins can have a gain of function effect on the cells thus making them more resistant to anti-cancer drugs and radiotherapy [6,7,15,18]. Thus detection of high levels of p53 protein by IHC signifies poor prognosis [19].

Different studies have shown different percentage of incidence of p53 protein detection. In our study we found p53 positivity in 76.4% of the patients. This is similar to the percentages found by other different studies from the region [9,16]. Although other studies have found a lower percentage of detection our figures can be attributed to the high incidence of use of tobacco. Many studies have shown that use of tobacco increases the frequency of p53 mutations [6,7,9,15]. In our series 73 out of 90 patients (81.1%) of the patients had a strong history of tobacco use. Absence of p53 mutations in 23.6% of our patients who had stage IV malignancy can be attributed to other mechanisms compromising p53 function like HPV virus inactivation of the gene (we were not able to do viral protein detection), MDM2 inactivation of the gene etc [6].

Clinical factors that determine the prognosis of oral SCC are the volume of the tumor, the stage of the tumor, lymph node metastasis and the histological grade of the tumor. Amongst these the histological grading of the tumor as given by Anneroth has shown good statistical co- relation with the prognosis of the disease because it considers both, cellular changes in the epithelium and the relationship between the tumor and the underlying connective tissue. As this grading system has 6 parameters that assess both tumor cell population and tumor host relationship we adopted this system in our study to reflect the biological activity of the tumor [11,12,13]. In our study the total malignancy score varied from 6–18 with a mean of 11.4. The individual parameter which showed highest mean grade was stage of invasion at 2.76. This probably co-relates with the advanced stage of the disease (Stage IV).



Graph 1 Mean of individual histological gradings of study subjects



Graph 2 Percentage distribution of study subjects according to positive and negative p53

Comparing p53 expression and histological grading our study showed a significant correlation between increased p53 expression and total malignancy score. Also higher scores of p53 directly corresponded to individual parameters of degree of Keratinization, nuclear polymorphism number of mitoses and pattern of invasion. There was no correlation with parameters stage of infiltration and Lympho-plasmocytic infiltration.

These results compare with other similar studies [13,16,17]. However there are some differences in the results of our study and some of the above mentioned studies [13,17]. In the study by De Araujo VC et al. they reported no correlation between pattern of invasion and p53 over expression. However our study and the study by Kurokowa H et al. shows that there is increased p53 expression at invasive tumor front signifying that only rapidly proliferating biologically active cells have the capacity to infiltrate. Whether the p53 mutations by themselves give the cells metastatic potential has to be further investigated.

Panjwani S and Sadiq S in their study [16] noticed that with increasing malignancy score between 13–20 there was a reduction in the p53 expression. We found no such co-relation. This may be attributed

to the fact that our total malignancy scores did not exceed 18 and the mean was 11.4.

Conclusion

Findings of our study support the postulation that TP53 mutations in cancers caused by exogenous carcinogens are an early event [7]. Histologically, p53 over expression is seen in rapidly proliferating cells which have a high degree of keratinization, nuclear polymorphism, increased number of mitoses and an aggressive pattern of invasion. Delayed events like actual invasion and the host inflammatory response to the tumor may not be affected by TP53 mutation. Also there is a statistically significant co-relation between Anneroth's grading and p53 over expression both signifying poor prognosis for oral SCC.

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