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Expression of p53 Protein in oral Squamous Cell Carcinomas and its comparison with a well established Grading system for Histopathological Malignancy.

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Abstract
Objectives: *To evaluate the use of p53 as indicator of tumour prognosis in routine biopsies of oral squamous cell carcinomas and its comparison with a well established grading system for histopathological malignancy.*
Materials and methods: *A total of 20 patients with age ranging from 45-70, diagnosed as clinically malignant and 20 normal controls were selected for the study. One group of sections of the biopsy specimens was stained for routine H & E and the other group was used for immunohistochemical study The technique used in this kit is based on the labeled streptavidin-biotin method.*
Statistical analysis: *All the cases were reviewed and classified according to the modified histological malignancy grading system. The analysis of p53 positive cells was performed using Diracom 3, image processing software. The percentage of positive p53 nuclei was then calculated. The Pearson correlation co-efficient test was used to evaluate the relationship between the percentage of tumoral p53 Positive cells and the total score of malignancy and the relationship between the percentages of tumoral p53 positive cells with each of the parameters of the malignancy grading system.*
Results: *All the 20 cases had a clear positive reaction to p53. Nuclear p53 staining was found only in epithelial cells. Positivity of p53 staining shows a correlation with the individual histological grading criteria, related to the tumour cell population (degree of keratinisation, nuclear polymorphism, and number of mitosis. However there was no correlation between the p53 and the individual histological grading criteria expressing tumour host relationship as inflammatory infiltrate and pattern of invasion.*
Conclusion: *Altogether, these data indicated that p53 protein is related to tumour cells, but not involved with either the tumour architecture or the host response. Therefore, a significant association between p53 over expression and high grade of malignancy has been found.*
Keywords: *Oral Squamous cell carcinoma, p 53protein, Immunohistochemistry, Malignancy grading system.*

I. Introduction

Oral squamous cell carcinomas account for 5% of all malignancies among the world population and more than 30 - 40% of all malignancies in the Indian subcontinent. It is generally accepted that neoplasm arise from a series of genetic alterations and they lead to perturbation of cellular proliferation and differentiation. These genetic alterations include conversion of proto-oncogenes which are components of normal cells to oncogenes or inactivation of inhibitory genes, known as **tumour suppressor genes**[1], leading to altered cellular proliferation and differentiation. The **tumour suppressor genes** group includes the p53 gene. Mutations[2] in the p53 tumour suppressor gene are frequent in squamous cell carcinoma of the oral mucosa. Inactivation[3] 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. \According to most studies[4] p53 is not detected in normal oral mucosa, but it can be demonstrated with immunohistochemical techniques in oral mucosal squamous cell carcinomas and potentially malignant oral mucosal lesions[2]. Most reports have shown that there is an increase in the proportion of cases that show p53 abnormalities, as detected by immunohistochemistry, from hyperplasia to dysplasia to neoplasia. Mutations of p53[5,6] and over expression of p53[,7,8,9,10,11] protein have been found in approximately 40% of invasive head and neck squamous cell carcinoma and in more than 50% of oral malignant neoplasia. The purpose of this study is to assess the expression of p53 protein in oral squamous cell carcinomas and to compare it with a well established grading system for histopathological malignancy.

II. Materials And Methods

All the patients who attended the oral diagnostic department were screened for the purpose of the study. A total of 20 cases were selected for the study, diagnosed as clinically malignant apart from 20 normal controls. Besides these, paraffin embedded tissue blocks of malignant conditions were also retrieved from the archives of our department. Clinical data obtained from the patients records indicated that 14 were men, 6 were women with age ranging from 45-70. The predominant anatomic tumor site was the floor of the mouth - 9 cases, tongue – 6 cases, buccal mucosa- 3 cases and palate – 2 cases

The biopsies were obtained with patient's consent under local anesthesia. The specimens were immediately fixed in 10% neutral formalin, processed and embedded in paraffin after processing. Serial sections of 4 μm were obtained from both the archival material and also from the new cases. One group of sections was stained for routine haematoxylin and eosin examination in order to confirm the clinical diagnosis and also for comparison. The other group of sections was used for immunohistochemical study using monoclonal p53 antibody DO-7 (Dako lab).

Procedure for immunohistochemical staining:

1. **Armamentarium for Immunohistochemistry**

Step 1: The tissue specimen was covered with 3% hydrogen peroxide, incubated for 10-15 min. and gently washed with phosphated buffer saline.(Figure 1)
Step 2: Tissue section was heated for antigen retrieval.
Step 3: The tissue specimen was covered with 10% normal goat serum (Blocking solution) and incubated for 10 min and gently washed with phosphated buffer saline.
Step 4: The tissue specimen was covered with primary antibody, monoclonal anti p53, (DO-7, Dakopats, California, USA) incubated for one hour at room temperature and gently washed twice with phosphated buffer saline each time.
Step 5: The tissue specimen was then covered with yellow colored Biotinylated secondary link antibody, incubated for 30 minutes at room temperature and gently rinsed with phosphated buffer saline.
Step 6: The tissue specimen was treated with streptavidin conjugate, incubated for 30minutes at room temperature and gently rinsed with phosphated buffer saline twice.
Step 7: The substrate chromogen solution was added to the specimen, incubated for 10 minutes at room temperature and gently rinsed twice with phosphated buffer saline.
Step 8: The slides were then immersed in a bath of haematoxylin (Counter stain) for 2-5 min and washed under tap water for 5min. Kit used is based on the labeled streptavidin-biotin method. The reagents included in the kit are given in Figure 2.

2. **Lab kit and p53 Antibody and Immunohistochemistry**

Both positive control specimen and negative control specimen are included in each batch of staining to ensure quality control and also to ensure that all kit reagents are working properly and to check the specificity of the primary antibody.

Results: Positive p53 expression is seen as light brown, granular nuclear stain.

III. Results

All the cases were reviewed and classified[12, 13] according to the modified histological malignancy grading system (Tables 1&2) based on morphological criteria representing tumour cell population and additional criteria signifying and tumour / host relationship.

Table 1 Histological Grading Of Malignancy for Oral Squamous Cell Carcinoma

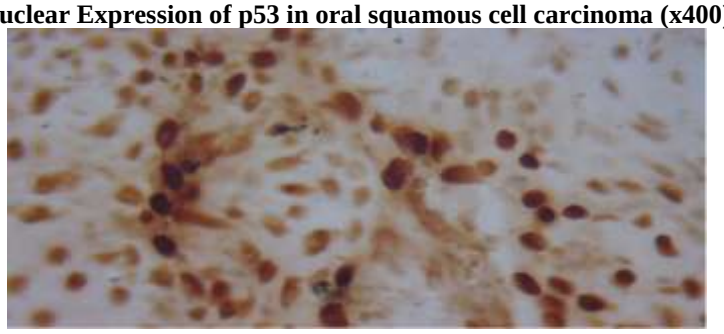
Morphological parameters	1	2	3	4
Degree of keratinisation	Highly keratinized (50% of cells)	Moderately keratinized (20% - 50% of cells)	Minimal keratinized (2% - 5% of cells)	No keratinisation (0-5% of cells)
Nuclear polymorphism	Little nuclear polymorphism (75% of mature cells)	Moderately abundant nuclear polymorphism (50-75% mature cells)	Abundant nuclear polymorphism (25-50% mature cells)	Extreme nuclear polymorphism (0-25% mature cells)
Number of mitoses / high power field	0-1	2-3	4-5	5

Table 2 Modified Histological Grading Of Malignancy and Tumour Host Relationship Points

Morphological parameters	1	2	3	4
Pattern of invasion	Pushing well delineated infiltrating borders	Infiltrating solid cords, bands and / or strands	Small groups or cords of infiltrating cells (n=15)	Marked and wide spread cellular dissociation in small groups of cells (n=15) and or in single cells
Stage of invasion	Carcinoma in situ and / or questionable invasion	Distinctive invasion but involving lamina propria only	Invasion below lamina propria adjacent to muscle, salivary tissue & periosteum	Extensive and deep invasion replacing most of the stromal tissue and infiltrating jawbone
Lympho-plasmocytic infiltration	Marked	Moderate	Slight	None

Each morphological parameter was graded on the basis of 1-4 points. The final score for each case was defined as the total sum of points attributed to the parameter evaluated. As incisional biopsies were used in this study, "Stage of invasion" was omitted and the numeral average score was used, i.e., the total sum of points divided by the number of parameters evaluated. The analysis of p53 positive cells was performed using Diracom 3 image processing software. For each case, a minimum of 1000 nuclei of tumoural cells from six microscopic fields at a 400x magnification, were examined. (Figure 3)

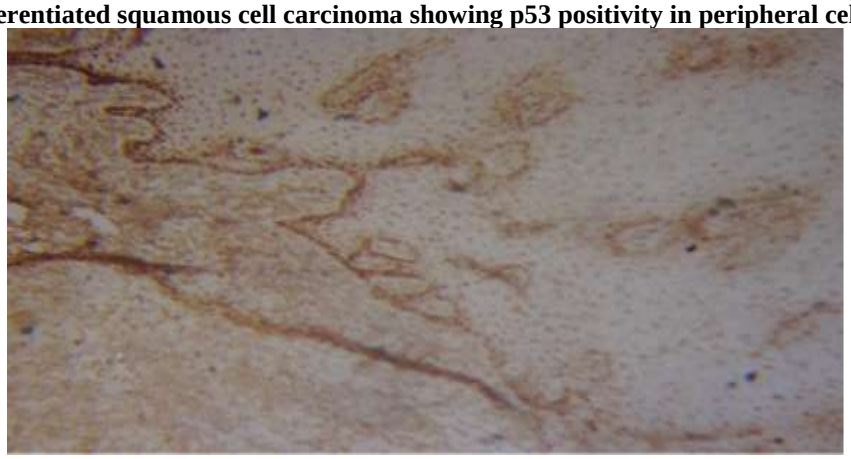
3. **Nuclear Expression of p53 in oral squamous cell carcinoma (x400)**



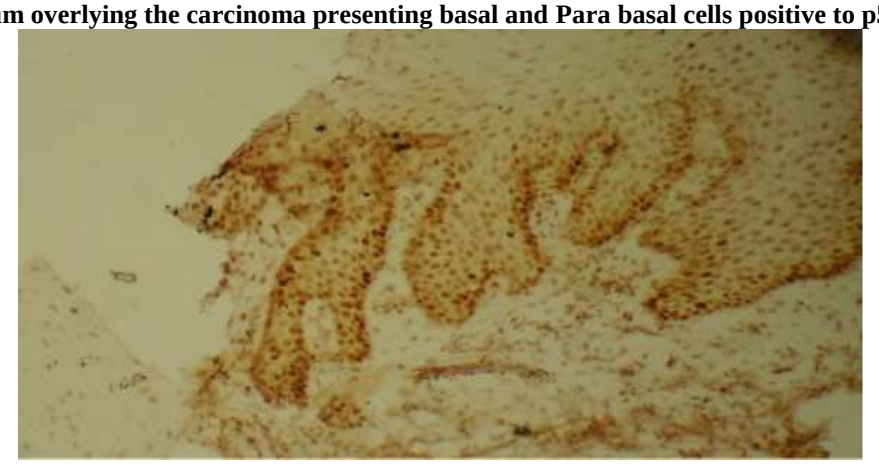
The percentage of positive p53 nuclei was then calculated. The Pearson correlation co-efficient test was used to evaluate the relationship between the percentage of tumoural p53 Positive cells and the total score of malignancy and the relationship between the percentages of tumoral p53 positive cells with each of the parameters of the malignancy grading system.

All the 20 cases had a clear positive reaction to p53. Nuclear p53 staining was found only in epithelial cells. In general, well differentiated squamous cell carcinoma with keratin formation, p53 was found only in the peripheral cells of neoplastic masses. (Figure 4 & 5)

4. **Well differentiated squamous cell carcinoma showing p53 positivity in peripheral cells (x100)**

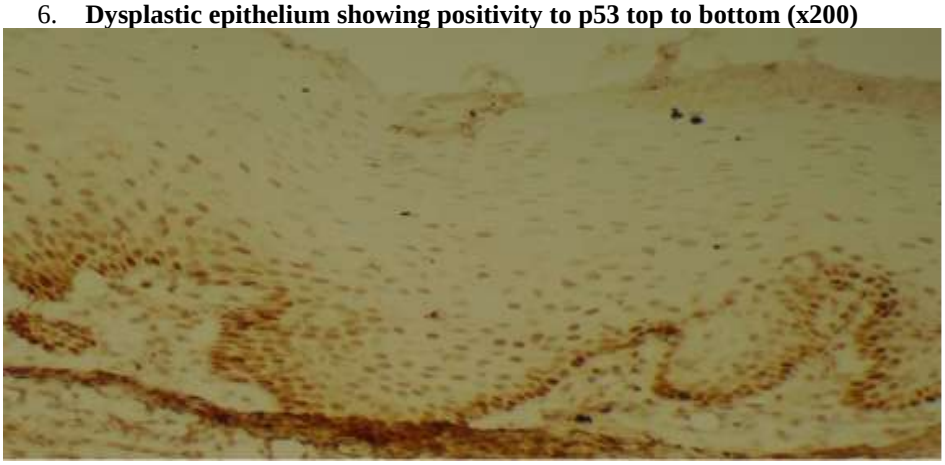


5. **Epithelium overlying the carcinoma presenting basal and Para basal cells positive to p53 (x100)**



However, in less differentiated cases, the pattern of staining was constantly observed. In severe dysplastic areas, staining was very intense. (Figure 6)

6. **Dysplastic epithelium showing positivity to p53 top to bottom (x200)**



The percentage of nuclear accumulation of p53 protein and the histological grade of malignancy are summarized in Table 3 which shows score of each parameter of the histological grading system.

Table 3 - Percentage of Tumoral P53 Positive Cells and Histological Scores of 20 Oral Squamous Cell Carcinomas Biopsy Tissues

Case	DK	NP	NM	P1	LP1	HS	% of p53 Positive cells
1.	1	2	2	4	3	2.4%	87
2.	1	3	1	2	3	2.0%	90
3.	2	3	1	3	2	2.2%	88
4.	1	2	4	1	3	2.2%	95
5.	3	1	1	2	3	2.0%	72
6.	1	2	1	2	2	1.6%	94
7.	2	2	1	2	2	1.8%	91
8.	2	4	2	2	3	2.6%	79
9.	2	2	1	1	2	1.6%	78
10.	1	2	1	1	3	1.6%	82
11.	1	2	4	1	2	2.0%	69
12.	1	2	2	2	2	1.8%	79
13.	2	3	2	2	3	2.4%	57
14.	2	3	2	2	3	2.6%	98
15.	1	2	2	3	2	2.0%	84
16.	1	3	3	2	3	2.4%	64
17.	1	3	2	3	3	2.4%	86
18.	1	3	1	2	3	2.0%	98
19.	1	4	1	4	3	2.6%	73
20.	1	4	2	1	3	2.2%	78

DK Degree of keratinisation NK Nuclear polymorphism
NM Number of Mitoses P1 Pattern of invasion
LP1 Lympho plasmocytic infiltration
HS Histological score using the modified grading system of Anneroth et al[12]

The correlation of percentage of nuclear accumulation of p53 protein was detected for degree of keratinization ($r = 0.449$ $x <= 0.05$), nuclear polymorphism ($r = 0.564$ $x <= 0.01$) and number of mitoses ($r = 0.649$ $x < 0.001$). There was no correlation between the expression of p53 and the pattern of invasion ($r = 0.035$ $x < 0.05$) Inflammatory infiltrate ($r = - 0.131$ $x < 0.05$) nuclear polymorphism ($r = - 0.107$ $x < 0.05$).

IV. Discussion

The p53 protein[3] was first discovered in 1970's as a 53kDa cellular protein complexed with the Simian SV40 virus. p53 is recognized as 'a pivotal regulatory protein involved in differentiation and development, DNA replication and transcription and senescence.' The p53 protein can regulate the cell in several ways. These are most notably cell-cycle arrest and apoptosis.

The p53 Signaling Pathway:

The tumour-suppressor protein p53 exhibits sequence-specific DNA-binding, directly interacts with various cellular and viral proteins[14], and induces cell cycle arrest in response to DNA damage. In response to signals generated by a variety of genotoxic stresses, e.g., UV irradiation or DNA damage, p53 is expressed and undergoes post-translational modification that results in its accumulation in the nucleus. The p53-dependent pathways help to maintain genomic stability by eliminating damaged cells, either by arresting them permanently or through apoptosis. For example, γ-irradiation activates p53 to turn on the transcription[15] of p21CIP1, that, in turn, binds to and inhibits cyclin-dependent kinases, causing hypophosphorylation of retinoblastoma (Rb), thus preventing the release of E2F and blocking the G1-S transition. Some of the cellular effects of p53 can be blocked by the deregulated expression of c-Myc, Bcl-2, or E2F. p53 activity is controlled through an autoregulatory loop involving Mdm2. The binding of Mdm2 to p53 targets p53 for degradation and inhibits p53-induced cell-cycle arrest and apoptosis.

Apoptosis and p53:

Cellular stresses such as genotoxic injury and oxidative stress[16, 17], viral proteins such as SV40 T antigen[18] and adenovirus E1a, and conditional expression of cellular factors such as myc and ras all induce p53

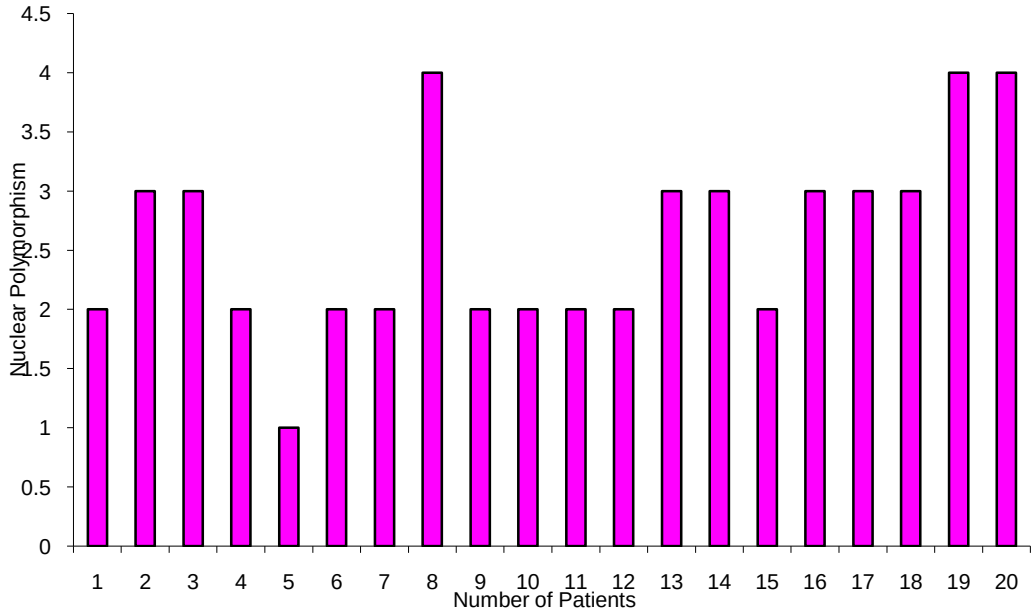
expression. p53 acting as a sequence-specific and DNA binding protein is known to activate p21WAF/CIP, Bax[19], and MDM2, which are linked to cell cycle arrest and apoptosis. p53 also increases levels of Fas/Apo1[20] or KILLER/DR5 in response to genotoxic stress in several cell types. Apoptosis evoked by DNA damage[21] may be triggered via a p53-dependent, but transcription-independent, pathway or by a p53-independent pathway.

Mechanisms of Tumour Formation from p53 Mutation[15] Patterns in Tumors:

p53 mutations are common in most types of cancer that include small but devastating amino acid replacements[22] as a result of single base changes along a continuous stretch of target DNA producing modifications characteristic of specific mutagens. Eighty percent of p53 mutations are missense mutations causing one amino acid to be substituted for another, usually altering protein conformation, and causing nuclear accumulation. Since mutant p53 proteins typically have a much longer half-life than the wild type protein[15], the diagnosis of tumors that harbor mutant p53 is feasible by immunohistochemical detection of its accumulation in the cell. Detection of these p53 gene alterations may allow very early detection of oral and oropharyngeal tumours[23]. These tests may also be used to better define surgical margins and to determine which tumors are most likely to respond to surgery or radiation therapy. Our results show a nuclear accumulation of p53 protein in almost all the cases of patients with oral squamous cell carcinoma, while the similar studies carried by V.C, DC Araujo et al., in 1997, show 62.5% for 100 cases since the number of cases were only 20, we could find positivity in all the cases. There is a large amount of research going on into new drugs for cancer therapy. Gene therapy, reactivating mutant p53 or finding a p53 analogue **are** the various ways used to restore p53 function.

This study has also demonstrated a correlation between the expression of p53 protein and the histological grade of malignancy. Positivity was found mainly in tumors of high histological grade of malignancy, showing that an over expression of p53 is an poor indicator of a poor prognosis of oral squamous cell carcinoma, Field et al.[5] have demonstrated a correlation between p53 over expression and a very poor prognosis for patients in end stage disease. M.C. Donald et al.[7] reported that p53 accumulation in early oral squamous cell carcinoma[23] may identify a sub group of patients with a tendency for more aggression behavior and another study associated the positivity of p53 protein with a high velocity of tumour growth and positive lymph nodes. Positivity of p53 staining also shows a correlation with the individual histological grading criteria, related to the tumour cell population (degree of keratinisation, nuclear polymorphism (Graph -1) and number of mitosis)

Graph -1 Nuclear Polymorphism



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Cell proliferation has been related to the presence of p53 in the cell nucleus and these individual criteria described above may also reflect the grade of proliferation. However there was no correlation between the p53 and the individual histological grading criteria expressing tumour host relationship as inflammatory infiltrate and pattern of invasion. Altogether, these data indicated that p53 protein is related to tumour cells, but it seems it is not involved with either the tumour architecture or the host response. These results contraindicate previous observations which reported a correlation between p53 over expression and the vascular / perineural

invasion, but no correlation with degree of differentiation, however, a significant association between p53 over expression and high grade of malignancy[24] has been found.

V. Summary And Conclusion

The present study is aimed with the relationship between the histological grading of malignancy and p53 protein expression in 20 biopsies of oral squamous cell carcinomas. The positively of p53 staining showed a correlation with the histological grade of malignancy, with the degree of keratinisation, nuclear polymorphism and number of mitosis. The results reveal that more number of men was affected than women. It may be due to constant use of tobacco, alcoholism among men. . This study could be a sibling for understanding more serious research project on oral squamous cell carcinoma due to its high incidence with the backing of immunohistochemical techniques, especially in the Indian scenario,

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