ORIGINAL RESEARCH

Expression of P53 in Oral Squamous Cell Carcinoma – an IHC Study; Deep Insight into P53

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Abstract

Background: Oral squamous cell carcinoma (OSCC) entails quite noteworthy morbidity and mortality rates instead of immense amount of research and advances. Mutation in the TP53 gene is the most common genetic change found in oral squamous cell carcinoma (OSCC). Once activated, the p53 protein can induce the growth arrest, as well as cell death. Mutation and subsequent inactivation of a tumor suppressor gene causes 'loss of function'. This can damage its deoxyribonucleic acid (DNA) binding properties and transcription factor function, thus, inhibiting its normal function in cell cycle control and in cell proliferation.

Aim: A comparative study of P53 expression in oral squamous cell carcinoma using immunohistochemical techniques.

Materials and Methods: To study the relationship between histological grades of oral squamous cell carcinoma and P53 protein expression and also to compare along with normal mucosa, a total of 40 cases of histologically diagnosed OSCC were considered for the study group and 10 paraffin embedded normal mucosal tissue blocks were collected. The analysis of p53 positive cells was performed on IHC stained sections. Only staining of nucleus of epithelial cells was observed. Nuclei with clear brown colour regardless of staining intensity were considered as p53 positive. Counting of cells was done with graticule eyepiece. All these cases were reviewed and classified according to modified Anneroths histological malignancy grading system.

Results: When p53 expression was compared between the two groups i.e control and study groups. The expression of p53 was nil in normal oral mucosal tissue (control), while study group i.e OSCC expressed about 47.49% with Std.Dev of ±35.83%. And it was found to be a statistically significant ($p<0.001$).

Conclusion: The present study concludes a positive correlation between the histopathological grades of OSCC and immunohistochemistry (p53 expression). Expression of p53 above the basal layer is an early event in oral carcinogenesis and an indicator of a developing carcinoma preceding morphological tissue alterations. However, since immunohistochemistry cannot always detect changes in p53 expression in premalignant/malignant lesions, it is strongly recommended that p53 immunohistochemistry be used in conjugation with routine histology to increase the sensitivity of detection of cases that eventually progress to malignancy.

Keywords: OSCC, IHC, TP53

Introduction

Cancer is older than humanity S.U.Williston, in early 1920's, found a bone tumor on skeleton of dinosaur in Wyoming[1]. Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However this term tends to used interchangeably with oral squamous cell carcinoma (OSCC), which represents most frequent of all oral neoplasms[2]. The quantitative study of P53 suggested their aberrant expression in oral cancer. P53 over expression was also detected in 15-19% of oral-premalignant lesions, including lesions with mild dysplasia in head and neck cancer patients[3].

P53 protein was first described by Levine, Crawford, and Lane in 1979. TP53 (tumor protein 53) is a tumor suppressor gene (TSG) which is located on the short arm (p) of chromosome 17. TSGs encode proteins that typically transduce the negative growth regulatory signals. These are involved in cell cycle arrest and apoptosis. Mutation and subsequent inactivation of TSGs cause damage in their deoxyribonucleic acid (DNA) binding properties and transcription factor function,

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thus, inhibiting their normal function in cell cycle control and cell proliferation.

The gene is known as TP53; while the name of protein, encoded by this gene, is P53. The P53 is so termed because the molecular weight of protein is 53kDa. The protein has site of action in the nucleus and involved in the regulation of DNA replication. P53 has a very short half life (around 20 min) and therefore it is hard to detect in normal tissue. However, the protein can remain longer in the tissue for certain reasons, such as, a mutation defect in the degradation pathway, or by binding to other proteins[4].

Understanding the pathogenesis of cancer may well lead to improved diagnostic tests, preventive treatment and management. The routine diagnostic methods includes histologic and cytologic methods. The advanced diagnostic methods include immunohistochemistry, molecular diagnosis, flow cytometry and tumor markers. Histopathology is the gold standard in diagnosis though it is time consuming. Among all these new techniques, Immunohistochemistry has become a powerful tool in the armamentarium of pathologists, as it provides insight into tumor histopathogenesis and has contributed to more accurate determination of patients prognosis[5].

Materials and Methods

The control group comprise of 10 paraffin embedded blocks of normal oral mucosal tissue, collected from archives of Department of Oral Pathology and Microbiology, Mahe Institute of Dental Sciences during the study period of 2011-2019 were evaluated. As per the criteria by modified anneroth histological malignancy grading system of varying grades. Total of 40 cases of histologically diagnosed OSCC were considered for the study group. The study group was selected based on the following criteria

Inclusion Criteria

Stored tissue blocks of histopathologically confirmed cases of OSCC were included for the study.

Exclusion Criteria

Tissue blocks of patients without adequate clinical details and sufficient tissue for analysis.

Tissue which exhibited processing artefacts.

Methodology

Paraffin embedded tissue blocks were sectioned using a soft tissue microtome. Two sections each of 4 micron meters thickness were cut and subsequently stained with haemotoxylin and eosin staining and p53 immunohistochemical stain. The analysis of p53 positive cells was performed on IHC stained sections. Only staining of nucleus of epithelial cells was observed. Nuclei with clear brown colour regardless of staining intensity were regarded as p53 positive. The slide was scanned from one end to other and those areas which had no labelling at all were excluded. Counting of cells was done with graticule eyepiece. All these cases were reviewed and classified according to modified Anneroths histological malignancy grading system. Each morphological parameters in classification system was graded on the basis of 1-4 points. The final score for each case was defined as total sum of points attributed to the parameters evaluated.

MODIFIED ANNEROTH'S HISTOLOGICAL MALIGNANCY GRADING SYSTEM

Morphological parameter	1	2	3	4
Histological grading of malignancy of tumor cell population				
Degree of keratinization	Highly keratinized (50%of cells)	Moderately keratinized (20-50% cells)	Minimally keratinized (2-5%cells)	No keratinization
Nuclear polymorphism	Little nuclear polymorphism (75%mature cells)	Moderately abundant nuclear polymorphis-m (50-75% mature cells)	Abundant nuclear polymorphism (25-50%mature cells)	Extreme nuclear polymorphism (0-25% mature cells)
Number of mitosis/ HPF	0-1	2-3	4-5	5
Histological grading of malignancy of tumor – host population				
Pattern of invasion	Pushing well delineated infiltrating borders	Infiltrating, solid cords, bands and/or strands	Small groups or cords of infiltrating cells	Marked and widespread cellular dissociation in small groups of cells
Lymphoplasm-acytic infiltration	Marked	Moderate	Slight	None

The pattern of expression of p53:

When a distinct brown staining was confined to nuclei, p53 expression was considered positive.

In general, in well differentiated cases, p53 staining was seen in peripheral cells, where as in poorly differentiated carcinoma staining was observed consistently.

Counting procedure:

Haematoxylin and eosin stained tissue sections were first examined for Anneroth grading for histopathological verification and confirmation of the histopathological grade of oral squamous cell carcinoma using Trinocular Research Microscope Olympus Bx-53 Japan with Digital camera.

The immunohistochemically stained sections were first analysed for the expression of p53 under 4x, 10x, 40x. The selected fields were used for the quantitative assessment of p53 positive cells. Only staining of nucleus of epithelial cells was observed; nucleus with clear brown color regardless of staining intensity were regarded as p53 positive. At least 500 tumor cells were observed and counted in 5 different histological fields under a magnification of 40x using Computer and Image analysis software. An eye piece grid was used to prevent the overlapping of fields. Sections were viewed by 2 observer in order to avoid intraobserver bias.

Method of Determining the p53 Expression:

The cases were given scores as 0, 1, 2 and 3 depending upon percent of tumor cells exhibiting positive nuclear staining for mutant p53 protein. The scores given for p53 expression are as follows:

- Score 0: < 25% p53 positive cells
- Score 1: 25-50% p53 positive cells
- Score 2: 50-75% p53 positive cells
- Score 3: >75% p53 positive cells

The P53 indices were calculated as the percentage of positively stained cells among the total cells counted. The results were tabulated and subjected to statistical analysis.

Level of significance: "p" is level of significance

P > 0.05	Not significant
P < 0.05	Significant
P < 0.01	Highly significant
P < 0.001	Very highly significant

Results

The present study was conducted in the department of Oral Pathology and Microbiology, Mahe Institute of Dental Sciences and Hospital, with an objective to carry out a quantitative analysis of p53 expression in oral squamous cell carcinoma patients and to study the relationship between histological grades of oral squamous cell carcinoma and P53 protein expression and also to compare along with normal mucosa. For this purpose a total of 40 blocks of biopsy specimens and 10 paraffin embedded normal mucosal tissue blocks were collected. Two sec-

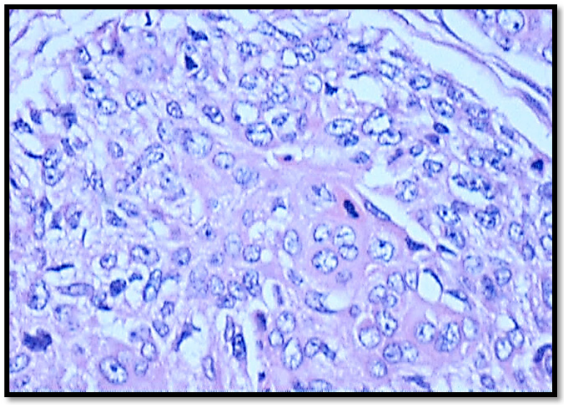


Fig. 1: H and E stained section of oral squamous cell carcinoma (40X)

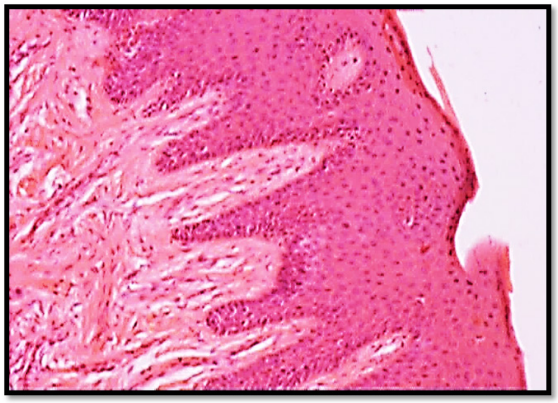


Fig. 2: H and E stained section showing normal oral mucosa (conrol)-10X

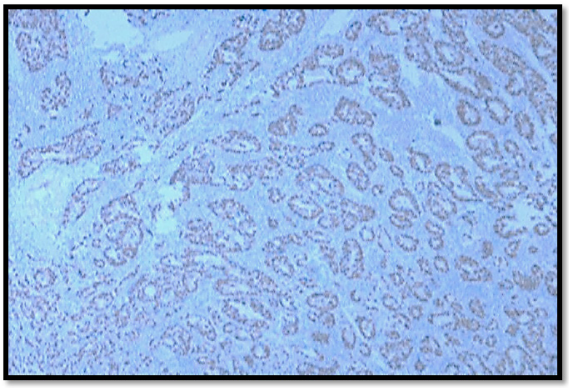


Fig. 3: Immunoreactive lymphnode showing p53 positive control

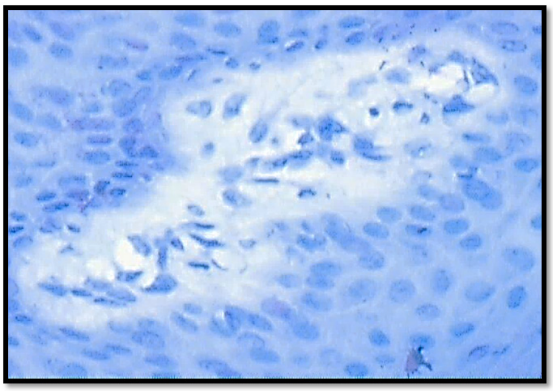


Fig. 4: p53 stained section showing negative result in Oral squamous cell carcinoma

tions each of 4µ thickness were cut and subsequently stained with haematoxylin and eosin and p53 immunohistochemical stain. Histopathological slides were viewed under 40 X objective using the "Trinocular Research Microscope Olympus Bx-53 Japan with Digital camera" and morphometric analysis was carried out using image analysis software "ProgRescapture® Pro 2.8.8".

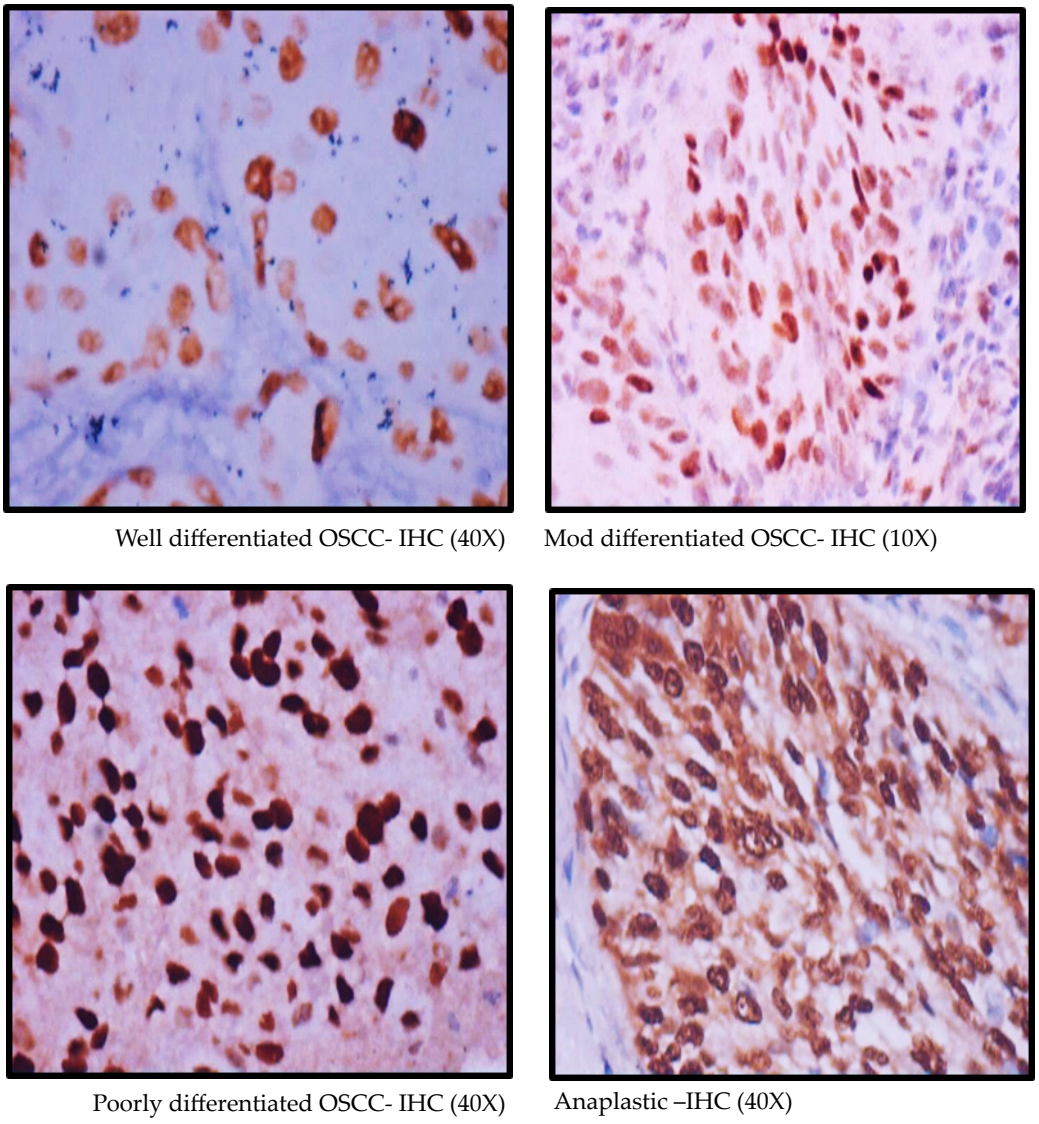
DISTRIBUTION OF SUBJECTS IN RELATION TO GENDER AND AGE

i. Comparison of Gender and OSCC (Table 1, Graph 1)

In present study a total of 40 subjects were included, out of 40, 18 were females comprising about 45% while 22 were males comprising about 55%. 10 were considered as control. Out of 10, 3 were females comprising about 30% and 7 were males comprising about 70% respectively. Chi square test is used to check the association between the gender and groups. We observed that there was no statistically significant association between the groups.

ii. Comparison of Mean Age and Histological grades of OSCC (Table 2)

Out of a total of 40 specimen, 10 each were categorised under the different grades of oral squamous cell carcinoma (well differentiated, moderately differentiated, poorly differentiated and anaplastic) according to Modified Anneroth's histological malignancy grading system. Among the subjects studied, mean age of well differentiated oral squamous cell carcinoma was 47.8 years with age ranging from 32-63 years. In Moderate squamous cell carcinoma mean age was 71.7 with minimum expression of 60 years and maximum expression of 85 years, while in poorly differentiated carcinoma mean age was 47.2 with 24 and 63 years revealing the minimum and maximum expression. And in anaplastic variant 38.3 was the mean age with 24 and 60 years was the minimum and maximum expression in age groups. Statistically, no significant difference was observed among age groups and histological grades of OSCC.



Well differentiated OSCC- IHC (40X) Mod differentiated OSCC- IHC (10X)

Poorly differentiated OSCC- IHC (40X) Anaplastic –IHC (40X)

Fig 5: p53 expression in different grades of OSCC showing minimal expression in well differentiated OSCC and maximum expression in anaplastic OSCC.

Gender Wise Comparison of Histological grades of OSCC (Table 3)

When we compared gender with histological grades of OSCC we observed that Grade 1 OSCC having equal distribution of both genders i.e 50% each, Grade II OSCC observed more in males than female's i.e 60% in males and 40% in females. While Grade III incidents were noted more in females i.e around 60% and 40% in males when compared. In case Grade IV, male predilection (70%) was observed when compared to female (30%).

Age wise Comparison between control and study groups (Table 4, Graph 2)

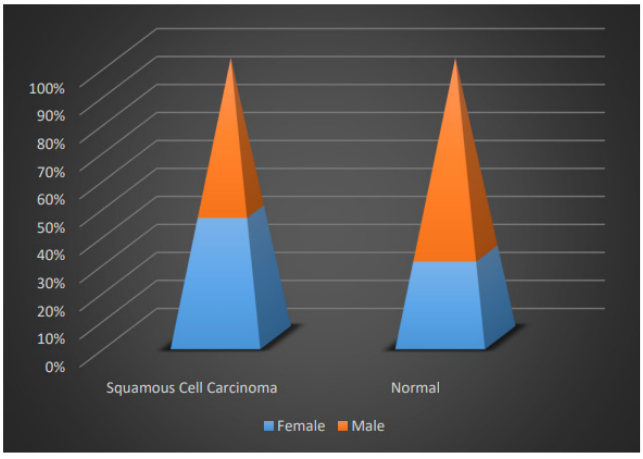
When compared mean age between control and study group, we observed mean age of control group was lower than study group. Mean age and Std. Deviation of control and study group was 44.7±11.01 and 51.25 ± 17.54 respectively. Independent t test is used to compare the age between the groups. We observed that there was no statistically significant difference between the two groups.

COMPARISON OF EXPRESSION BETWEEN THE GROUPS (Table 5, Graph 3)

When p53 expression was compared between normal and various grades of Oral Squamous cell carcinoma, we observed that, there was no expression of p53 in normal oral mucosa. While squamous cell carcinoma expressed the staining characteristic of p53. Expression of p53 was observed minimum in well differentiated oral squamous cell carcinoma (15.7000 ±24.50510) and maximum in anaplasic type (81.0080 ± 15.76424). While 32.4040 ± 28.44063 and 60.8715 ± 32.89721 was observed

Table 1: Comparison of Gender and OSCC

Groups	Female	Male	P-value
Squamous Cell Carcinoma	18	22	0.404
	45.0%	55.0%	
Normal	3	7	
	30.0%	70.0%	
Total	21	29	
	42.0%	58.0%	



Graph 1: Comparison of Gender and OSCC

Table 2: Comparison of Mean Age and Histological grades of OSCC

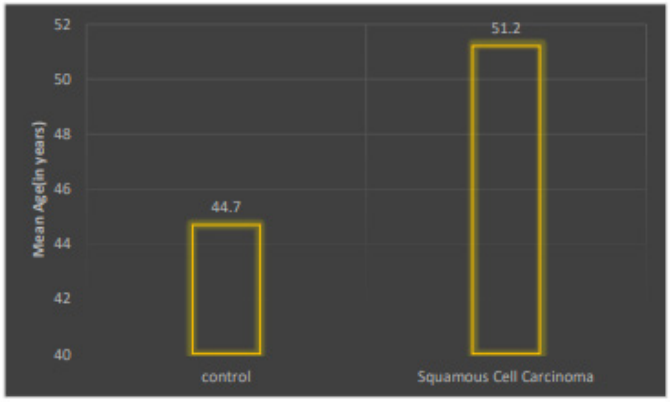
Group	No	Mean (Age)	Range (Age (years))	
			Minimum	Maximum
Well differentiated squamous cell carcinoma	10	47.8	32	63
Moderately differentiated squamous cell carcinoma	10	71.7	60	85
Poorly differentiated squamous cell carcinoma	10	47.2	24	63
Anaplastic	10	38.3	24	60

Table 3: Gender Wise Comparison of Histological grades of OSCC

s.no	Gender	Grade I		Grade II		Grade III		Grade IV	
		NO	%	NO	%	NO	%	NO	%
1	Male	5	50	6	60	4	40	7	70
2	Female	5	50	4	40	6	60	3	30

Table 4: Age wise Comparison between control and study groups

Parameter	Group	N	Mean	Std. Deviation	p-value
Age	Squamous cell carcinoma	40	51.250	17.5423	0.268
	control	10	44.700	11.0156	



Graph 2: Age wise Comparison between control and study groups

in Moderately and Poorly differentiated Oral squamous cell carcinoma respectively. An increasing trend in the mean p53 expression was observed with progressing histological grade of Oral squamous cell carcinoma. Statistically, a significant difference was observed among the groups (p< 0.001).

COMPARISON OF EXPRESSION BETWEEN THE 2 GROUPS (Table 6, Graph 4)

When p53 expression was compared between the two groups i.e control and study groups. The expression of p53 was nil in normal oral mucosal tissue (control), while study group i.e OSCC expressed about 47.49% with Std.Dev of ±35.83%. Independent 't' test was used to compare the expression between the two groups and it was found to be a statistically significant (p<0.001).

Post Hoc Tests for multiple comparison (Table 7)

Post Hoc Test done to compare the one group with the other. We observed that statistically significant expression between well differentiated OSCC with poorly and anaplastic differentiated carcinoma with p< 0.001. While comparing the other grade such as control group and moderately differentiated OSCC, values were not statistically significant.

When comparing Anaplastic with other groups we observed that the values were statistically significant between well differentiated, moderately differentiated and control p < 0.001. While in poorly differentiated carcinoma values showed no statistical significance.

Moderately differentiated carcinoma were compared with other groups we noted statistically significant results with anaplastic group only p< 0.001. While all others such as well differentiated, poorly differentiated and the control showed no statistical significance.

When comparing control with all grades of OSCC, anaplastic and poorly differentiated showed statistically significant results p<0.001. While other two groups such as well differentiated and moderately differentiated showed no statistical significance.

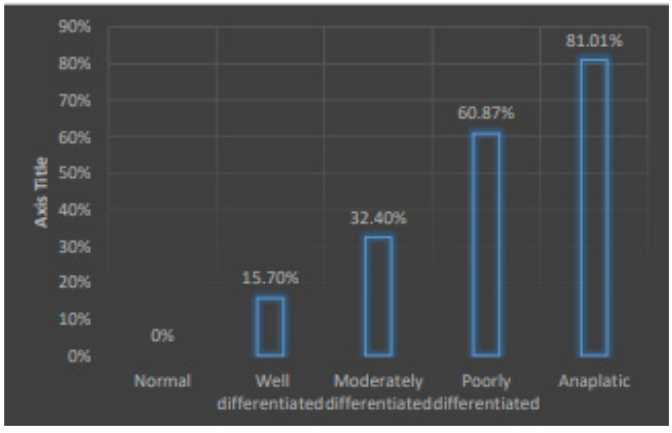
Poorly differentiated carcinoma showed statistically significant with well differentiated and control groups p<0.001. While values of anaplastic and moderately differentiated OSCC showed no statistical significance.

Discussion

Oral malignancy is complex and multi-factorial cancer. It is suspected that in India widespread malnutrition together with high risk behaviour like betel chewing may contribute to the high incidence of OSCC6. One of the most important genes in the regulation of apoptosis is p537. One important function of wild-type (wt) P53 is that it can cause cells to either undergo apoptosis or cell cycle arrest, following stress or damage to

Table 5: Comparison of Expression between the Groups

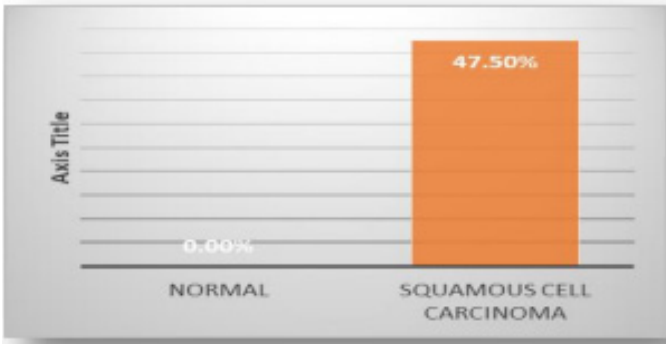
Grades	Mean	Std. Deviation	p-value
Normal oral mucosa	0%	0%	
Well differentiated	15.7000%	24.50510%	<0.001
Moderately differentiated	32.4040%	28.44063%	
Poorly differentiated	60.8715%	32.89721%	
Anaplastic	81.0080%	15.76424%	



Graph 3: Comparison of expression between the groups

Table 6: Comparison of expression between the 2 groups

S. NO	Groups	No	Mean	Std. Deviation	p-value
1	Control	10	0.0000%	0.0000%	
2	Squamous cell carcinoma	40	47.4959%	35.83011%	<0.001



Graph 4: Comparison of Expression between the 2 groups

DNA. Many other genes are likely to be activated or deactivated. Furthermore, mutations in p53 can not only inactivate proapoptotic functions, but can induce some other tumor promoting functions[8].

Our study was conducted to analyse the pattern of P53 expression in patients with oral squamous cell carcinoma and to study the relationship between histological grades of oral squamous cell carcinoma and P53 protein expression and with that of a normal oral mucosa.

The control group consisted of 10 samples of normal oral mucosa and 40 cases under study groups. Out of 40 each 10 cases are categorised under different grades of oral squamous cell carcinoma (Well differentiated, moderately differentiated, poorly differentiated and anaplastic) according to Modified Anneroths histological malignancy grading system.

Table 7: Post Hoc Tests for multiple comparison

I(G)	J(G)	Mean difference(I-J)	Std.error	P-value
Well differentiated	Control	15.70000%	10.46921%	0.141
	Moderately differentiated	-16.70400%	10.46921%	0.118
	Poorly differentiated	-45.17150%	10.46921%	<0.001
	Anaplastic	-65.30800%	10.46921%	<0.001
Anaplastic	Control	81.00800%	10.46921%	<0.001
	Well differentiated	65.30800%	10.46921%	<0.001
	Moderately differentiated	48.60400%	10.46921%	<0.001
	Poorly differentiated	20.13650%	10.46921%	0.061
Moderately differentiated	Control	32.40400%	10.46921%	0.003
	Well differentiated	16.70400%	10.46921%	0.118
	Poorly differentiated	-28.46750%	10.46921%	0.009
	Anaplastic	-48.60400%	10.46921%	<0.001
Control	Well differentiated	-15.70000%	10.46921%	0.141
	Moderately differentiated	-32.40400%	10.46921%	0.003
	Poorly differentiated	-60.87150%	10.46921%	<0.001
	Anaplastic	-81.00800%	10.46921%	<0.001
Poorly differentiated	Control	60.87150%	10.46921%	<0.001
	Well differentiated	45.17150%	10.46921%	<0.001
	Moderately differentiated	28.46750%	10.46921%	0.009
	Anaplastic	-20.13650%	10.46921%	0.061

In our study out of 40 samples, 28 (70%) samples showed positive p53 expression and remaining 12 (30%) samples showed negative p53 expression. Our results were similar to those of Ruchi Dhuria et al (70% positivity)[9]. Other studies which have assessed p53 expression in histologically proven case of OSCC have shown variable results ranging from (57.7% positivity) by (Gonzalez-Moles M A et al.)[10] to (96% positivity) by (Khor Goot Heah et al.)[11]. This ambiguity in results of p53 in HNSCC probably attributed to different methodology, variation in fixation procedures, interpretive errors in IHC technique, applied antibodies, the arbitrary cut- off points used to define p53 expression, false positive and false negative results, difference in ethnicity and involved risk factors[9].

This increase in the TP53 expression could be due to the increased transactivation or decreased destruction of the protein in the cytoplasm. Ionizing radiation, chemical carcinogens, and mutagens can cause DNA damage, which may activate or modify TP53 gene. Normally, TP53 activates MDM2 by binding to it and causing nuclear transcription. Tumor suppressor protein called p14ARF (the human homolog of mouse p19ARF [Alternative Reading Frame]) can bind to the MDM2 protein and form a complex with MDM2/TP53, causing its retention in the nucleus.[12] On the contrary, Dundy G et al. (2016) found that there was no statistical difference in p53 expression with histological grades of OSCC[13]. N Pooja et al (2010) and Ruchi Dhuria et al. (2020) also found no increase in the p53 positive expression with progress in the grades of OSCC[14].

All cases of normal oral mucosa (control) assessed in the present study were p53 (0.00%) negative, While study group i.e. OSCC expressed about 47.49% with Std.Dev of ±35.83%. And this was found to be a statistically significant (p<0.001). It might be due to the fact that in normal cells, p53 protein has a very short half-life and cannot be detected immunohistochemically. In contrast, the mutant forms are more stable and thus have an extended half-life and can be detected using immunohistochemical techniques[15]. This was consistent with the studies of N Pooja et al (2010) and Dundy G et al. (2016)[13] and with most other reports in the literature, regardless of differences in antibodies, immunohistochemical procedures, and types of specimens (frozen or paraffin- embedded sections) used[16]. On the contrary, the normal oral mucosa was found to be p53 positive by C Thode et al. (2010)[17] and GR Ogden et al. (1997)[18]. One of the explanation for this was p53 regulates cell cycle proliferation, and so all tissues may be expected to exhibit some amount of cell proliferation. Hence some p53 positive cells are expected in all cases including normal tissue[5].

Overexpression of p53 in normal oral mucosa which is not associated with tumor but has been exposed to tobacco or alcohol has also been reported .This may indicate probably the epithelium is at increased risk of malignant transformation. The cellular response to these carcinogens may consist of accumulation or stabilization of wild- type p53 protein to prevent cells from entering G1 in order to allow DNA repair, or to avoid the addition of oncogenic mutation[19].

A study conducted by Piffko j et al. (1995) described that tissue adjacent to malignant epithelium which might represent

an early event in oral carcinogenesis. This might also indicate a normally working p53 system activated in genetically – stressed cells which could either be restored or pushed towards apoptosis or enter the vicious circle of malignant transformation[20].

In a pairwise comparison of OSCC there was no significant difference obtained between the grades of OSCC. When values of p53 expression was compared between the various grades of OSCC i.e. well differentiated, moderately differentiated, poorly differentiated and anaplastic, we observed that statistically significant expression between well differentiated OSCC with poorly differentiated and anaplastic carcinoma (45.17150%) and (-65.30800%) With p value of <0.001. While in other grades such as control group and moderately differentiated OSCC when compared, the values obtained showed no statistical significance. On the contrary, Ghaghoria S et al. (2015)[21] found that in pairwise comparison, a significant difference was obtained between normal mucosa against WDSCC, MDSCC, and PDSCC (p<0.001).

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.Thus detection of high levels of p53 protein by IHC signifies poor prognosis[22].

Immunohistochemistry (IHC) is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions. It is useful as an auxillary diagnostic procedure in histopathology. The introduction of prognostic and predictive markers in IHC has made a tremendous impact on patient's treatment and management. These methods are particularly useful to a pathologist in ambiguous areas of neoplastic process enhancing the accuracy to large extent[23]. Mutations of TP53 gene is one of the most common event in human carcinogenesis. As mutated protein is not easily digestible, therefore it accumulates inside the cancer cell leading to immunohistochemical overexpression. P53 overexpression in OSCC is considered a marker of poor prognosis[24].

Conversely, absence of p53 expression does not necessarily indicate there is no mutation; it could be that mutation of p53 exists but did not result in a stable form, or alternatively p53 expression is degraded after forming complex with viral proteins and hence appears absent[25].

So immunohistochemistry cannot always detect changes in p53 expression in premalignant/malignant lesions, but it is strongly recommended that p53 Immunohistochemistry be used in conjunction with routine histology to increase the sensitivity of detection of cases that eventually progress to malignancy.

Conclusion

The present study concludes a positive correlation between the histopathological grades of OSCC and immunohistochemistry (p53expression). It implies that these tumor protein provide a supportive clue to the clinician and histopathologist to understand the biological behaviour of tumor. This help to bridge the gap that arises due to inter- observer variability while diagnosing different grades of OSCC. Further investigations on larger group of patients are still required to elucidate the clinical and prognostic potential of p53 in HNSCC as small sample size limits the conclusiveness of the current study. P53 expression in HNSCC may provide clinicians more accurate information to evaluate tumor aggressiveness and survival rates for better future management of the disease.

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