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IMMUNOHISTOCHEMICAL EXPRESSION OF P53 AND CYCLIN D1 IN SQUAMOUS CELL CARCINOMA AND EPITHELIAL HYPERPLASIA OF ORAL MUCOSA.

Oral Pathology

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ABSTRACT

Oral cancer is among the top three types of cancers in India. Most invasive oral carcinomas are preceded by a pre-invasive stage, that may last for many years. . Understanding the molecular mechanisms involved in the initiation and progression to malignancy of oral cancer will help to improve its prognosis and in the elaboration of new forms of treatment. The most common genetic alterations, include gene amplification and overexpression of oncogenes such as myc, erbB-2, Epidermal Growth Factor Receptor (EGFR), Cyclin D1 and mutations, deletions and hyper-methylation leading to p16 and p53 tumor suppressor gene inactivation. There still remains the problem of differentiating epitheliomatous hyperplasia from squamous cell carcinoma (SCC) especially on biopsies.

AIMS:

1. To study and evaluate the expression of p53 and Cyclin D1 in oral SCC and epithelial hyperplasia.
2. To correlate the expression of p53 and Cyclin D1 in different grades of oral SCC.

SETTINGS AND DESIGN: Cross-sectional study conducted over a period of 18 months.

METHODS AND MATERIAL: 30 cases (n=30) of oral squamous cell carcinoma & 30 cases (n=30) of epithelial hyperplasia diagnosed on H & E were included in this study. All these cases of oral SCC were further classified into different histological grades according to BRODERS' HISTOLOGICAL GRADE.

STATISTICAL ANALYSIS: The data was collected and the association between different groups was analysed by using Chi square test and quantitative variables were compared using Independent T test/Mann-Whitney Test (when the data sets were not normally distributed) between the two groups and ANOVA/Kruskal Wallis test between three groups. A p value of ≤ 0.05 was considered statistically significant.

RESULTS: There was a significant increase in the expression of p53 in oral SCC compared to epithelial hyperplasia. The results were statistically significant (p value <0.0001). Similarly , Cyclin D1 also showed an increased expression in cases of SCC in contrast to epithelial hyperplasia (p value <0.0001). The expression of p53 significantly increased with increase in grade of the tumor. The results being statistically significant, p value=0.004. Similarly, poorly and moderately differentiated SCC also showed an increased expression of Cyclin D1 compared to well differentiated SCC (p value=0.03, statistically significant).

CONCLUSION: Thus the author concludes that the expression of both p53 and Cyclin D1 is increased in oral SCC compared to epithelial hyperplasia of oral mucosa and these markers can be used to differentiate between these two lesion especially on biopsy cases.

KEYWORDS

Squamous cell carcinoma, p53, Cyclin D1, Epithelial hyperplasia

INTRODUCTION:

Oral cancer constitutes the sixth most common cancer worldwide. [1] Oral cancer is among the top three types of cancers in India.[2] In India, where tobacco chewing is used with betel nuts and reverse smoking is practiced, there is a striking incidence of oral cancer. The commonly affected sites are tongue, inferior lips and floor of mouth. [3] The genetic alterations observed in head and neck cancer are mainly due to oncogene activation and tumor suppressor gene inactivation, leading to de-regulation of cell proliferation and death. These genetic alterations, include gene amplification and overexpression of oncogenes such as myc, erbB-2, Epidermal Growth Factor Receptor (EGFR), Cyclin D1 and mutations, deletions and hyper-methylation leading to p16 and p53 tumor suppressor gene inactivation. [4] The tumor suppressor gene p53 , is called as 'Guardian of the Genome', having a role in maintaining genomic stability, cell cycle progression, cellular differentiation, DNA repair and apoptosis. [5] Activated p53 drives transcription of CDKNIA (p21) which prevents Rb phosphorylation, thereby causing G1-S block in the cell cycle. [6] Hence mutations in p53 abrogates its trans-activating activity leading to uncontrolled cell growth. [7] Dysregulated expression or over expression of Cyclin D1 leads to shortening of G1 phase, increased cellular proliferation and reduced dependency on growth factors leading to disturbance in the normal cell cycle control and tumour formation. [8,9]

Most invasive oral carcinomas are preceded by a pre invasive stage, that may last for many years. Tumor progression in epithelia has been classified as normal, hyperplastic (non-dysplastic), dysplastic carcinoma in situ and invasive carcinoma. [10] Studies have suggested that approximately half of all the oral squamous cell carcinomas (SCC) contain p53 mutations, and about one third contain mutations in Cyclin D1. [11] Therefore, understanding the molecular mechanisms involved in the initiation and progression to malignancy of oral cancer will help to improve its prognosis and in the elaboration of new forms of treatment. .There still remains the problem of differentiating epithelial hyperplasia from squamous cell carcinoma especially on biopsies. The study is thus being conducted to find whether the expression of these markers can help in differentiating well differentiated SCC from epithelial hyperplasia that often causes a diagnostic problem on small biopsy specimens. This study will also help us to know whether the expression of p53 and Cyclin D1 in squamous cell carcinoma can be correlated with the grade of the tumour.

MATERIAL & METHODS:

This was a cross-sectional study conducted over a period of 18 months. 30 cases (n=30) of oral SCC & 30 cases (n=30) of epithelial hyperplasia diagnosed on H & E were included in this study. All these cases of oral SCC were further classified into different histological grades according to BRODERS' HISTOLOGICAL GRADE. [12]

Well differentiated (Grade I) - <25% undifferentiated cells
Moderately differentiated (Grade II) - <50% undifferentiated cells
Poorly differentiated (Grade III) - <75% undifferentiated cells

All oral biopsies suspected of SCC or epithelial hyperplasia were fixed in 10% formalin and representative tissue were grossed and processed as routine. Routine 4 to 5 µm sections were cut and sections were stained with haematoxylin and eosin. Microscopic diagnosis along with grade of the tumour was given.

Sections taken on poly-L-lysine coated slides were used to perform immunohistochemistry for p53 and Cyclin D1. p53 immunohistochemistry was done using p53 antibody (Rabbit Polyclonal Antibody). Cyclin D1 immunohistochemistry was done using Cyclin D1 antibody (Rabbit Polyclonal Antibody).

Interpretation:
>5% of cells with positive nuclear staining was taken as positive for p53.
>5% of cells with both positive nuclear and cytoplasmic staining was taken as positive for Cyclin D1.

Statistical Analysis:
The data was collected and the association between different groups was analysed by using Chi square test and quantitative variables were compared using Independent T test/Mann-Whitney Test (when the data sets were not normally distributed) between the two groups and ANOVA/Kruskal Wallis test between three groups. A p value of ≤0.05 was considered statistically significant.

RESULTS:
The study population of epithelial hyperplasia ranged between 21-67 years, whereas the study population for oral SCC ranged between 30-80 years. The mean age for epithelial hyperplasia was 47.6 years and the mean age for oral SCC was 56.33 years (p value=0.02). Both the study populations showed a male predominance. Epithelial hyperplasia showed a male: female ratio of 2.3:1, similarly oral SCC showed male: female ratio of 2:1. These results however, showed a p value=0.781, hence were not statistically significant.

It was observed epithelial hyperplasia occurred most commonly in the floor of the mouth (33.33%) followed by lateral border of the tongue (26.67%). For SCC the most common site was gingiva-buccal sulcus (30%) followed by lateral border of lip (20%) and lateral border of lip (20%). These results showed a p value=0.531, hence were not statistically significant.

p53:
The study showed that the expression of p53 was significantly higher in patients with oral SCC, showing nuclear positivity in 27 out of 30 cases (90%) compared to epithelial hyperplasia which showed nuclear positivity in only 03 out of the 30 (10%) cases studied. This comparison was statistically significant with a p value of <0.0001. (Table 1)

Table 1: Comparison Between Expression Of p53 In Epithelial Hyperplasia And Oral SCC.

		EPITHELIAL HYPERPLASIA	SCC	Total	P value
p53	NEGATIVE	27 (90.00%)	03 (10.00%)	30 (50.00%)	<0.0001
	POSITIVE	03 (10.00%)	27 (90.00%)	30 (50.00%)	
Total		30 (100.00%)	30 (100.00%)	60 (100.00%)	

It was observed that the nuclear positivity of p53 was seen throughout the epithelial islands of tumor cells in oral SCC, whereas the positivity in epithelial hyperplasia was confined mostly to basal layer and partially the supra-basal layer of the epithelium. (Fig 1)

CYCLIN D1:
The study showed that the expression of Cyclin D1 was significantly higher in patients with oral SCC, showing nuclear as well as cytoplasmic positivity in 26 out of 30 cases (86.67%) compared to epithelial hyperplasia which showed nuclear and cytoplasmic positivity in only 03 out of the 30 (10%) cases studied. This comparison was statistically significant with a p value of <0.0001. (Table 2)

It was observed that the nuclear and cytoplasmic positivity of Cyclin D1 was seen throughout the epithelial islands of tumor cells in oral SCC whereas the positivity in epithelial hyperplasia was confined mostly to basal layer and partially the supra-basal layer of the epithelium. (Fig 2)

Table 2: Comparison Between Expression Of Cyclin D1 In Epithelial Hyperplasia And Oral SCC.

		EPITHELIAL HYPERPLASIA	SCC	Total	P value
CYCLIN D1	NEGATIVE	27 (90.00%)	04 (13.33%)	31(51.67%)	<0.0001
	POSITIVE	03 (10.00%)	26 (86.67%)	29 (48.33%)	
Total		30 (100.00%)	30 (100.00%)	60(100%)	

Histological grading of SCC:
The 30 cases of oral SCC that were studied, were further sub categorized into different histological grades based on BRODERS' classification, into well differentiated, moderately differentiated and poorly differentiated SCC. Out of the 30 cases, 07 cases (23%) were well differentiated, 17 cases (56.67%) were moderately differentiated and 06 cases (20%) were poorly differentiated oral SCC.

Immunohistochemical Expression Of Markers In Histological Grades Of Oral SCC:
p53:
It was seen that the immunohistochemical expression of p53 was found to be positive in 04 out 07 (57.14%) cases of well differentiated oral SCC. All the 17 cases (100%) of moderately differentiated and all the 06 (100%) cases of poorly differentiated oral SCC included in the study showed a positivity for p53 These results showed a p value of 0.004 and hence were statistically significant. (Table 3) It was also seen that the nuclear positivity of p53 was present diffusely throughout the epithelial islands of the tumor cells in all the grades of oral SCC. (Fig 3)

Table 3: p53 Expression In Different Histological Grades Of SCC.

p53	WELL diff. SCC	MODERATE diff. SCC	POOR diff SCC	Total	P value
NEGATIVE	03 (42.86%)	00 (00%)	00 (00%)	03 (10%)	0.004
POSITIVE	04 (57.14%)	17 (100%)	06 (100%)	27 (90%)	
Total	07 (100%)	17 (100%)	06 (100%)	30 (100%)	

CYCLIN D1:
It was seen that the immunohistochemical expression of Cyclin D1 was found to be positive in 04 out of 07 (57.14%) cases of well differentiated SCC. 16 out of 17 (94.12%) cases of moderately differentiated and all the 06 (100%) cases of poorly differentiated SCC included in the study, showed a positivity for Cyclin D1. These results showed a p value of 0.03 and hence were statistically (Table 4) It was also seen that the nuclear and cytoplasmic positivity of Cyclin D1 was present diffusely throughout the epithelial islands of the tumor cells in all the grades of SCC. (Fig 4)

Table 4: Cyclin D1 Expression In Different Histological Grades Of SCC.

	WELL diff. SCC	MODERATE diff. SCC	POOR diff. SCC	Total	P value
NEGATIVE	03 (42.86%)	01 (05.88%)	00 (0%)	04 (13.33%)	0.030
POSITIVE	04 (57.14%)	16 (94.12%)	06 (100%)	26 (86.67%)	
Total	07 (100%)	17 (100.00%)	06 (100%)	30 (100%)	

DISCUSSION:
Our study showed the age distribution of oral SCC ranging between 30-80 years showing a peak in the 7th decade (30%) followed by 6th decade (26.67%). According to US National Cancer Institute SEER program the mean age of diagnosis of oral cancer was 65 years.[13] However, Sankaranarayan et al. [14] found that the peak-age frequency of occurrence (the fifth decade of life) in India was at least a decade earlier than that described in the western literature. Similarly Gupta et al. [15] observed an increase in the incidence of oral cancer in the younger (less than 50 years) age group.. Hence, the incidence of SCC in our study was slightly higher in the older population showing a peak in the 7th decade compared to other Indian studies showing increased

incidence in a more younger population. The age distribution of epithelial hyperplasia in our study was found to be ranging between 21-67 years, showing a peak in the 6th decade (50%) followed by 4th decade (20%). The mean age for epithelial hyperplasia was 47.6 years compared to SCC with the mean age of 56.33 years. Hence, it was seen in our study that the mean age for epithelial hyperplasia was almost a decade lower compared to SCC.

Our study showed a male predominance in both the study populations. Epithelial hyperplasia showed a male: female ratio of 2.3:1, similarly oral SCC showed male: female ratio of 2:1. These findings were in concordance with the findings of Salain V et al. [16]whose study showed a male: female ratio of 2.6:1 In our study as well, there is significant bias in the incidence of oral cancer amongst males, due to increased prevalence of smoking and alcohol consumption in males compared to the females.

It was seen in our study that epithelial hyperplasia occurred most commonly in the floor of the mouth (33.33%) followed by lateral border of the tongue (26.67%). For SCC the most common site was the gingivo-buccal sulcus (30%) followed by lateral border of tongue (20%) and lateral border of lip (20%). Although the border of tongue is considered the most common site for oral SCC in America and Europe, the buccal mucosa is the most common site for oral SCC in south eastern Asia, due to habit of areca nut and tobacco-chewing. [17,18] A study in western UP, by Sharma et al. [19]reported that the most common site of oral SCC was buccal mucosa, followed by the retro molar area, floor of mouth, lateral border of tongue, labial mucosa, and palate.. Our study, in accordance to most of the Indian studies, showed the most common site of oral SCC to be the gingivo-buccal sulcus. This can be explained on the basis of increased prevalence of the habit of tucking the betel nut in the mouth while chewing by the Indian population.

The expression of p53 in oral SCC was found to be showing nuclear positivity in 27 out of 30 cases (90%) and positivity in epithelial hyperplasia was found in only 03 out of the 30 (10%) cases studied.. Uma Swaminathan et al. [11] in their study found that p53 was positive in 30% and 65% cases of normal mucosa and oral SCC respectively. The staining in their study was confined to the basal layer of the epithelium in the normal mucosa, whereas it was found to be in the basal and supra-basal layers of the epithelium in oral SCC but two samples showed positivity throughout the epithelial island. In our study, it was observed that the nuclear positivity of p53 was seen throughout the epithelial islands of tumor cells in oral SCC whereas the positivity in epithelial hyperplasia was confined mostly to basal layer and partially the supra-basal layer of the epithelium. L.P.Dragomir et al. [20] in their study found 82.3% positivity for p53 in oral SCC. Hence concluding from our study, the expression of p53 was significantly more in oral SCC (90%) compared to epithelial hyperplasia (10%) and also that the staining became more diffuse with the development of malignancy. Thereby, concluding from present study that the expression of p53 increases with the progression towards SCC. The positivity in 10% cases of epithelial hyperplasia can be explained as a possible premalignant condition, however further studies are needed to conclude this result.

The expression of Cyclin D1 was seen as nuclear and cytoplasmic positivity in 26 out of 30 cases (86.67%) in oral SCC and in only 03 out of the 30 (10%) cases of epithelial hyperplasia. Uma Swaminathan et al. [11], in their study found that Cyclin D1 was positive in 40% and 95% cases of normal mucosa and oral SCC respectively. However, Saawarn et al. [21], showed Cyclin D1 positivity in 45% cases of oral SCC. The study by Patel S.B. et al. [22], found 70.0% cases of leukoplakia and 76.7% cases of oral SCC to be positive for Cyclin D1. In our study it was observed that the nuclear and cytoplasmic positivity of Cyclin D1 was seen throughout the epithelial islands of tumor cells in oral squamous cell whereas the positivity in epithelial hyperplasia was confined mostly to basal layer and partially the supra-basal layer of the epithelium. Hence, the expression of Cyclin D1 was significantly higher in oral SCC (86.67%) compared to epithelial hyperplasia (10%) in our study and also that the staining became more diffuse with the development of malignancy. The over expression of Cyclin D1 suggests as expected, that there is increased proliferation in oral SCC as compared to epithelial hyperplasia. The basal and partial supra-basal positivity for Cyclin D1 in epithelial hyperplasia can be attributed to the high proliferating activity of the basal layer of the epithelium, as Cyclin D1 is a positive regulator of the transition from G1 phase to S phase in cell cycle progression.

Out of the 30 cases of oral SCC that were studied, 07 cases (23.33%) were well differentiated, 17 cases (56.67%) were moderately differentiated and 06 cases (20%) were poorly differentiated according to the BRODERS' classification.[12] It was seen that the immunohistochemical expression of p53 was found to be positive in 04 out 07 (57.14%) cases of well differentiated SCC. All the 17 cases (100%) of moderately differentiated and all the 06 (100%) cases of poorly differentiated SCC included in the study showed a positivity for p53 showing a gradual increase in staining from well to poorly differentiated SCC. These results were in contrast to the study conducted by Li.Y et al. [23] who showed no expression of p53 in well diffentiated SCC, 55% positivity in moderately and 100% positivity in poorly differentiated oral SCC. It was also observed in our study that nuclear positivity of p53 was seen diffusely throughout the epithelial islands of the tumor cells in all the grades of SCC. Based on these findings it can be postulated that the expression of p53 increases with the grade of the tumor, though moderate and well differentiated oral SCC showed diffuse positivity for p53 in all the cases that we studied, well differentiated oral SCC showed positivity in only 57.14% of the cases.

The immunohistochemical expression of Cyclin D1 was found to be positive in 04 out of 07 (57.14%) cases of well differentiated SCC, 16 out of 17 (94.12%) cases of moderately differentiated and all the 06 (100%) cases of poorly differentiated SCC included in the study showed a positivity for Cyclin D1. Goto et al. [24]and Patel S.B. et a.l. [22], in their studies indicated that Cyclin D1 expression increases and is correlated with poor histological grade in accordance to findings in our study. It was also observed in our study that the nuclear and cytoplasmic positivity of Cyclin D1 was present diffusely throughout the epithelial islands of the tumor cells in all the grades of SCC.

Hence based on our study, p53 and Cyclin D1 are found to be expressed significantly more in oral SCC, compared to epithelial hyperplasia, thereby their expression can be implicated in tumor genesis. Also, these markers can be used to differentiate between these two lesion especially on biopsies. However, due to our small sample size and conflicting results in literature, regarding the expression of Cyclin D1 in different histological grades of SCC, studies with a larger study population is required to reach to more conclusive results.

CONCLUSION:

From the study it can be concluded that the expression of IHC markers p53 and Cyclin D1 is significantly higher in oral SCC as compared to epithelial hyperplasia. The expression of both p53 and Cyclin D1 is more diffuse, spread throughout the epithelial islands in oral SCC, whereas in epithelial hyperplasia, in case of positivity their expression is limited to the basal and partial supra-basal layers of the epithelium. Hence, owing to the diffuse positivity of p53 and Cyclin D1 mostly throughout the lesion in oral squamous cell carcinomas, they can also be used as potential markers to differentiate between these two lesions in case of confusion in diagnosis especially on small biopsies. However, caution should always be rendered as a small percentage of malignant oral SCC especially well differentiated tumors can also show negativity for these markers.

We also correlated the expression of p53 and Cyclin D1 with the different histological grades of oral SCC (well differentiated, moderately differentiated and poorly differentiated). Based on the results that were found, it can be concluded that the frequency of expression of both p53 and Cyclin D1 increases with the grade of the tumor, thereby signifying poor prognosis with increasing expression of these markers.

Hence based on our study, p53 and Cyclin D1 are found to be expressed significantly more in oral SCC, thereby their expression can also be implicated in tumor genesis. However, due to our small sample size and conflicting results in literature, regarding the expression of Cyclin D1 in different histological grades of SCC, studies with a larger study population is required to reach more conclusive results.

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