Original Article

Association of Cyclin D expression with malignant transformation of oral mucosa in tobacco users

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Objectives: To assess the expression of Cyclin D in transition of normal oral mucosa to oral squamous cell carcinoma (OSCC) and to find the possible association of immunostaining in various clinicopathological and histopathological distribution of OSCC.

Methodology: In this cross sectional analytical study, 120 diagnosed paraffin embedded blocks were included comprising of 60 samples of normal oral mucosa (Group 1) and 60 cases of various grades of OSCC (Group 2). All tissue samples belonged to tobacco chewers. Patients' record files were studied for age, gender, tobacco habits and various clinicopathological details. Immunohistochemistry was performed using Cyclin D monoclonal antibodies on all samples. Staining with Cyclin D was done to find its possible association with various clinicopathological and histopathological variables. Association of Cyclin D expression was also observed in transition of normal mucosa to OSCC.

Results: In Group 1, 75% were negative for Cyclin D. In Group 2, 100% were positive for Cyclin D. We found significant association for Cyclin D staining in transition of normal oral mucosa to OSCC in tobacco users. Nonsignificant association was found in Cyclin D positivity in various grades and stages of OSCC.

Conclusion: The overall Cyclin D expression was shown not to be increased with increasing grades and stages of OSCC in tobacco users. Positive association was seen in Cyclin D immunopositivity with transition of healthy mucosa to OSCC among tobacco users. (Rawal Med J 202;45:342-346).

Keywords: Cyclin D, normal oral mucosa, oral squamous cell carcinoma, immunohisto-chemistry.

INTRODUCTION

In Southeast Asian countries, Oral squamous cell carcinoma (OSCC) is most common type of head and neck cancers (90%).[1] Tobacco is one of the most important risk factor for OSCC, which results in epigenetic changes which interfere with activation and degradation of carcinogens as a result of disturbances in DNA repair by Cyclin D mutations.[2] Southeast Asian countries including Pakistan, tobacco is available in open market and supported on the cultural basis.[3]

Oral carcinogenesis is a multistage molecular process involving disruption in cell cycle like activation of oncogenes and deactivation of tumor suppressor genes.[4] Along with clinical examination, numerous immunohistochemical methods have used to highlight the ongoing disturbances in biochemical of cell cycle causing OSCC.[5,6] One of the most critical event in transformation of normal oral mucosa into malignant mucosa is error in G1/S phase of cell cycle which is hosted by biochemical Cyclin D. Cyclin D is encoded by proto-oncogene CCDN1 (B-cell leukemia/lymphoma 1) located on chromosome 11q13.[7] Cyclin D is known to be a human oncogene.

Overexpression of cyclin D is marker of poor prognosis in variety of cancers such as head and neck cancer, pancreatic cancer, lung cancer, breast cancer and cutaneous cancers.[8] Currently, Cyclin D enhancement is considered to be an independent factor in OSCC progression and one of the important treatment regimens include anti cyclin D inhibitors. Therefore, it is important to understand the role of Cyclin D in tobacco related OSCC.[9] Our study provides evidence of tobacco mediated activation of Cyclin D pathway in normal oral mucosa and OSCC.

METHODOLOGY

This cross sectional observational study was done in Al Tibri Medical College and Hospital (ATMCH) and Dow University of Health Sciences (DUHS) from May 2017 to November 2018 after approval from ethical review committee. A total of 120 diagnosed paraffin embedded blocks were collected from histopathology departments of ATMCH and DUHS after institutional permission to use these blocks for research purpose only. Being an observational study it did not required informed consent from subjects.

Medical records of patients were studied for demographic, clinicopathological and histopathological variables. The blocks were divided into two groups; group 1 and group 2. Group 1 comprised of blocks of normal oral mucosa (n=60) and Group 2 included paraffin blocks of various grades of OSCC (n=60). Group 1 samples were retrieved from patients undergoing surgical extractions of third molars, frenectomies and gingivectomies. Group 2 samples were subclassified into a, b and c according to the histopathological grading of OSCC; 'a' for well differentiated squamous cell carcinoma (WDSCC), 'b' for moderately differentiated squamous cell carcinoma (MDSCC) and 'c' for poorly differentiated squamous cell carcinoma (PDSCC) according to WHO Broder's classification of OSCC.[10] It was ensured that all blocks belonged to tobacco chewers using any type of tobacco for more than 5 years. Both male and females were included in our study regardless of age. Any material which belonged from patients with tobacco habit, metastatic cancer and undergoing chemotherapy or radiotherapy was excluded from the study.

Two sections of 5 microns were cut by conventional microtome from each of the paraffin embedded blocks. One section was used for H&E staining and second for immunohistochemical staining with anti Cyclin D (clone P2D11F11, RTU-CYCLIN-GM, dilution 1:100 Dako cytomotion), which was done by following Manual's instructions protocol. The validity of immunohistochemistry (IHC) staining was done by using B cell lymphoma as positive control for Cyclin D. Negative controls were taken by omitting primary antibody.

Qualitative assessment of IHC stained sections of normal mucosa and OSCC was done by subjective perception of experienced pathologists. Immunohistochemical staining assessment was done by randomly selecting 5 high power fields (x40) and was classified under two labels. Negative label was given for no color change or light heterogenous nuclear stain in <10%. similarly homogenous nuclear discoloration ranging from light yellow to dark brown in >10% area was considered as positive. Grading of immunostaining was done on the basis of intensity (No staining as 0 grade, mild staining as 1+, moderate staining as 2+ and strong staining as 3+).[11]

Statistical Analysis: Data analysis was performed by SPSS version 20. Associations between categorical variables were analyzed using chi-square tests. $p \leq 0.05$ was considered as significant.

RESULTS

There were more males (78%) in both the groups (Table 1). We noted that 89% cases in both the groups were regular tobacco users with 70% >40 years of age (Table 2). Overall, the prominent histopathological grade among group 2 were PDSCC making 38% of group II and most prominent clinical stage was I with 58% (Table 3).

Table 1. Association of demographic features of study groups using Chi square test.

Variable	No. of cases (n=120)	Normal mucosa (n=60)	Oral squamous cell carcinoma (n=60)	X^2 value	P value
Gender					
Male	93(78%)	33 (35%)	60 (100%)	34.83	0.001*
Female	27(22%)	27 (45%)	0		
Age					
<40Years	30 (25%)	30 (50%)	0	40.00	0.001*
>40years	90 (75%)	30 (50%)	60 (100%)		
Tobacco habits					
Regular	107 (89%)	50 (83%)	57 (95%)	4.22	0.04*
Occasional	13 (11%)	10 (17%)	3 (5%)		

P value significant at ≤0.05. Confidence interval 95%. Group I M:F ratio 1.2:1. Group II M:F ratio 1:0. Group I Mean±SD for age 48±17 years. Group II Mean±SD for age 65±5 years

Table 2. Association of Cyclin D expression in relation to type of mucosa and tobacco habit.

OSCC features	No. of cases (n=60)	Staining expression			X^2 value	P value
		1+	2+	3+		
Site of lesion Buccal mucosa Alveolus	 52 (87%) 8 (13%)	 10 (19%) 3 (37%)	 9 (17%) 5 (63%)	 33 (63%) 0	12.2	0.002*
Grading WDSCC(2a) MDSCC(2b) PDSCC(2c)	 18 (30%) 19 (32%) 23 (38%)	 2 (11%) 3 (15%) 7 (30%)	 5 (28%) 6 (32%) 3 (13%)	 11 (61%) 10 (53%) 13 (57%)	3.9	0.40
Staging I II III	 35 (58%) 20 (33%) 5 (8%)	 10 (28%) 5 (25%) 1 (20%)	 10 (28%) 7 (35%) 1 (20%)	 15 (43%) 8 (40%) 3 (60%)	0.88	0.92

P value significant at ≤0.05. OSCC=Oral squamous cell carcinoma

WDSCC=Well differentiated squamous cell carcinoma

MDSCC=Moderately differentiated squamous cell carcinoma

PDSCC=Poorly differentiated squamous cell carcinoma

1+=Mild IHC staining. 2+=Moderate IHC staining.

3+=Strong IHC staining

Table 3. Association of Cyclin D expression in relation to clinicopathological and histopathological distribution of OSCC.

Type of mucosa	Staining expression				X^2 value	P value
	0	1+	2+	3+		
Normal oral mucosa (n=60)	45 (75%)	15(25%)	0	0	93.33	0.001*
Oral squamous cell carcinoma (n=60)	0	12(20%)	14(23%)	34(57%)		
Tobacco habit						
Regular (n=107)	35 (33%)	24(22%)	14(13%)	34(32%)	11.87	0.008*
Occasional (n=13)	10 (77%)	3 (23%)	0	0		

P value significant at ≤ 0.05

Buccal area or cheeks were the most commonest site for OSCC lesions making 87% and showed significant association for Cyclin D immunohistochemistry ($p<0.05$). Strength of association of staining expression of Cyclin D in malignant transformation of oral mucosa and frequency of tobacco habit was also significant (Table 2). However, non significant associations were seen in between Cyclin D intensity and increasing tumor grades and increasing clinical stage of tumor (Table 3).

DISCUSSION

Oral squamous cell carcinoma primarily effects male as compared to females.[12] In our study, we had 100% of OSCC cases who were males. Similar results were reported by Akhtar et al in 2016 which showed OSCC as a real health burden of old age group in Pakistani males.[2] It is believed that OSCC is an age related carcinoma.[3] In the present study, OSCC was found in older males with mean age 65±5 years. Khammissa et al found that OSCC was higher in middle aged males i.e. 58±13.[13] These studies were done in patients using tobacco along with alcohol, which might be the causative factor for the early appearance of OSCC in these patients.

In our study, most commonly observed anatomical site for OSCC was buccal mucosa i.e. 87% which is similar to study by Sailan et al.[14] The current study revealed most encountered OSCC was PDSCC i.e. 38%. This was considerably lower than the findings shown by Pires et al showing WDSCC more prevalent than other two grades.[15] Our study showed 75% cases of normal oral mucosa with negative scores for Cyclin D however, 25% samples showed mild staining. It has been previously shown by Bogozi et al that Cyclin D was totally negatively expressed in normal oral mucosa.[16]

In contrast to our results, study by Basnaker et al showed that 80% of normal oral mucosal samples with moderate staining.[17] Similarly, another study by Ramakrishna et al showed 100% of normal oral mucosa mildly stained with cyclin D.[18] These differences might be due to inclusion of dysplastic mucosa in their studies. Present study showed 100% positive immune staining of cyclin D with loss of differentiation in OSCC but failed to show significant association (p=0.04). These results were in similarity with study by Choudary et al which showed non significant association (p=0.07) between cyclin D staining intensity and increase in loss of differentiation in OSCC.[19] However, this was in disagreement with a previous study by Huang et al showing significant association of cyclin D with loss increasing grades of OSCC (p-0.02).[20]

Our study revealed increasing staining expression of Cyclin D was not dependent upon decrease in differentiation of OSCC which was in similarity with a previous study by Khan et al showing moderate expression was seen in 86% cases of various grades of OSCC.[21] Our study revealed that

Cyclin D reactivity was not significantly associated with TNM staging and histopathological grading of OSCC which was in concordance with previous studies which showed no significant association of Cyclin D with tumor grade and tumor stage.[22] In contrast Zhao et al observed a significant association of Cyclin D1 expression with tumor stage as well as tumor grade.[23] Although Cyclin D was not significantly associated with tumor grading and staging, we noticed major transitions that occur in normal oral mucosa which leads to oral cancer by tobacco usage ($p<0.05$) which was also explained by Ramakrishna et al[18] and Mishra et al.[24]

There were many exciting things which were yet to explore but there was very less data documented regarding cyclin D reactivity in malignant transition of healthy oral mucosa in tobacco users. Therefore, we were unable to elaborate our discussion. Hence, it is expected to continue such studies with cohort designs and larger sample size.

CONCLUSION

Present study found that the alteration of cyclin D1 protein was strongly positive for malignant transition of oral mucosa due to tobacco but was not associated with the process of tumor differentiation and clinical staging.

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Conception and design: Sarwat Batool
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