ORIGINAL ARTICLE

IMMUNOHISTOCHEMICAL EXPRESSION OF CYCLIND1 IN CONVENTIONAL SQUAMOUS CELL CARCINOMAOF ORAL CAVITY

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Background: Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity, the incidence of which has significantly increased in the last 10 years. In Pakistan, it is reported to be the most common cancer among men and the second most common in women. Cyclin D1 is a protein involved in cell cycle regulation from G1 phase to S phase. Down regulation of this molecule causes inhibition of cell cycle transition and may lead to carcinogenesis. We studied the expression of Cyclin D1 in biopsies of oral squamous cell carcinoma to evaluate the staining patterns in various grades and sites of the oral cavity. Cyclin D1 was expressed in 53.8% cases of OSCC and showed a significant association with tumour differentiation, with higher intensity staining seen in poorly differentiated cases of OSCC. Thus, Cyclin D1 can be regarded as a marker of malignant potential of OSCC and can help identify cases with poorer outcome.

Keywords: Oral squamous cell carcinoma; Cyclin D1; Tumour differentiation

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INTRODUCTION

Squamous cell carcinoma accounts for more than 90% of all head and neck tumours.[1] The incidence of oral squamous cell carcinoma (OSCC) has drastically increased in past 10 years. Oral cavity tumours automatically refer to oral squamous cell carcinoma.[2]

Around 145,000 deaths have occurred around the world because of oral squamous cell carcinoma, according to 2012 statistical analysis. The highest incidence is reported in South-east Asian region, i.e., 6.4 per 100,000. In American region, the incidence is 4.1 per 100,000.[3] In India, it is the most common tumour in men and 5th most common cancer among females.[4] Due to lack of cancer registry, the exact incidence of OSCC in Pakistan is not known, however, it is the most common cancer in men and second most common in women.[5,6] According to a cancer survey which was done in Karachi in the year 2006, oral squamous cell carcinoma was the leading cancer of head and neck cancers. Unfortunately, most of the cases reach hospitals at a very advanced stage.[7]

Lips, hard palate, buccal mucosa, tongue, trigone, floor of mouth are the most common sites for oral squamous cell carcinoma.[8] Tobacco and betel nut chewing are the two most important risk factors for OSCC.[9] Drug addiction and alcohol are the other risk factors for oral squamous cell carcinoma.[10]

Oral squamous cell carcinoma is classified into 3 categories according to WHO guidelines: well, differentiated, moderately differentiated and poorly differentiated.[5] This classification is based on Broder's system of grading which is developed in the year 1920.[11] It has classified the OSCC on the basis of mitotic activity, pleomorphism, degree of differentiation and percentage of keratinization.[12]

Cyclin D1(a 45 KDa protein) is part of the molecular system which regulates the cell cycle in G1 to S transition phase.[13] Dysregulation of this pathway is the main cause of oral and other carcinogenesis. Studies have shown Cyclin D1 to be associated with poor prognosis and outcomes.[14] In addition, the genes involved in cell cycle control are the targets for oncogenic abnormalities and Cyclin D1 might prove to be a target for purpose of treatment.[15] For example, according to the clinical trial done by Shanghai Jiao Tongat University School of Medicine, Cyclin D1 levels may be used to plan TPF induction chemotherapy for OSCC patients at clinical N2 stage.

The rationale of our study was to determine the expression of Cyclin D1 in oral mucosal biopsies as a diagnostic aid for evaluation of OSCC in our target population. Most of the biopsies have scanty material, and this study might prove beneficial in early detection of malignancy and prompt treatment despite limited tissue in biopsies, so to avoid the hassle of re-biopsy. In addition, the patients showing Cyclin D1 expression may also be potential candidates for individualized treatment against this protein.

Objectives of the study were to determine

the frequency of the immuno-histochemical expression of Cyclin D1 in OSCC and to analyze the correlation of Cyclin D1 expression with clinic-pathological parameters such as differentiation and site of the tumour as well as age and gender of the patient

MATERIAL AND METHODS

This cross-sectional study was conducted in the Department of Pathology, Shaheed Zulfiqar Ali Bhutto Medical University (SZABMU), Pakistan Institute of Medical Sciences (PIMS), Islamabad, Pakistan. All oral biopsies and resection specimens of adult patients were collected from January to October 2020. After fixation of the specimen in 10% formalin, gross examination was done according to American Joint Committee on Cancer (AJCC) protocols, followed by sectioning, embedding in paraffin blocks, cutting, slide preparation and staining of the tissue with Haematoxylin and Eosin (H&E). The slides were examined under light microscope by consultant residents and pathologists and histopathological diagnoses were recorded. Fifty-two cases diagnosed as Primary Oral squamous cell carcinoma were taken for study. Four-micron sections of these blocks were prepared. Immunohistochemistry was applied for Cyclin D1. For Cyclin D1 immunostain, EP12 monoclonal mouse anti-human anti-body clone, oncoprotein by Leica, Germany was used. IHC was then examined under light microscope for analysis of expression by the tumour cells.

Presence or absence of nuclear brown coloured staining was recorded and percentage of positive cells in each slide was calculated from representative fields. Scoring of Cyclin D1 was done as shown in table 1.[16] For statistical analysis, *p*-value less than 0.05 was considered significant.

Table-1: Scoring of Cyclin D1 expression

Labelling index (LI)	Intensity (I)	Expression score (LIxI)	Cyclin D1 interpretation	
≤10% positive cells- 0	Nil- 0	0	Negative	
11–30% positive cells- 1	Faint- 1	1–12	Positive	***p*-value** 0.02
31–50% positive cells- 2	Moderate- 2			
>50% positive cells- 3	Intense- 3			

RESULTS

Out of the 52 patients, 29 (55.8%) were male and 23 (44.2%) females. The age of the patients ranged between 39 years to 83 years, having a mean age of 58.28±10.7 years. Of the age groups, the maximum number of patients, i.e., 20 (38.5%) were found in age group 51 to 60 years (Table-2).

The distribution of cases of OSCC according to site is shown in table-3. The commonest site was found to be tongue, followed by buccal mucosa.

Of the 52 cases, 13 were well differentiated keratinizing, 22 were moderately differentiated keratinizing, 15 were poorly differentiated keratinizing and 2 were non-keratinizing SCC (Table-4).

Cyclin D1 expression was seen in 28 (53.8%) of 52 cases of OSCC. Of the 23 female patients, 13 were positive for Cyclin D1 while 10 were negative. Of the 29 male patients, 15 were positive for cyclinD1 while 14 were negative. The correlation of Cyclin D1 expression with gender of the patient was insignificant (p=0.7) (Table-5).

Similarly, the correlation of Cyclin D1 with age group of the patient and site of OSCC were also insignificant (p=0.44 and p=0.68 respectively).

The correlation of CyclinD1 with differentiation of the tumour was significant (p=0.00). Of the 13 well differentiated cases, 12 (92%) were cyclin D1 negative while only 1 (8%) was positive. Of the 22 moderately differentiated cases, 12 (54%) were negative for cyclinD1 and 10 (46%) were positive. Of the 15 poorly differentiated cases, all, i.e., 100% were positive for CyclinD1. Of the 2 non-keratinizing cases, both, i.e., 100% were positive for CyclinD1 (Figure-1). Hence, there was a higher association of Cyclin D1 with poorly differentiated and non-keratinizing OSCC.

Out of the 13 well differentiated cases, 10 showed mild staining with Cyclin D1, 1 showed moderate and 2 showed intense staining pattern. Of the 22 moderately differentiated cases, 12 showed mild staining, 7 showed moderate and 3 showed intense staining. Of the 15 poorly differentiated cases, 10 showed moderate staining and 5 showed intense staining. Of the 2 non-keratinizing cases, 1 case showed moderate staining and 1 showed intense staining.

The distribution of expression score of Cyclin D1 obtained by multiplying the labelling index with intensity score according to grade of OSCC is shown in table-6. The expression score increased with loss of differentiation and the *p*-value came out to be 0.00.

Table-2: Distribution of cases according to age groups*

Age group	Frequency	Percent
1	1	1.9
2	14	26.9
3	20	38.5
4	9	17.3
5	7	13.5
6	1	1.9
Total	**52**	**100.0**

*Group 1: 31–40 years, group 2: 41–50 years, group 3: 51–60 years, group 4: 61–70 years, group 5: 71–80 years, group 6: 81–90 years

Table-3: Distribution of OSCC according to site

Site	Frequency
Alveolar ridge	7
Buccal mucosa	13
Lower lip	6
Submandibular region	5
Tongue	14
Upper lip	7
Total	**52**

Table-4: Frequency distribution according to grade of OSCC

Tumour differentiation	Frequency	Percent
Well differentiated keratinizing	13	25.0
Moderately differentiated keratinizing	22	42.3
Poorly differentiated keratinizing	15	28.8
Non-keratinizing	2	3.8
Total	**52**	**100.0**

Table-5: Correlation of Cyclin D1 expression with gender of the patient

Gender	Cyclin D1 expression		Total	
	negative	positive		
Female	10	13	23	***p*-value 0.7**
Male	14	15	29	
Total	24	28	52	

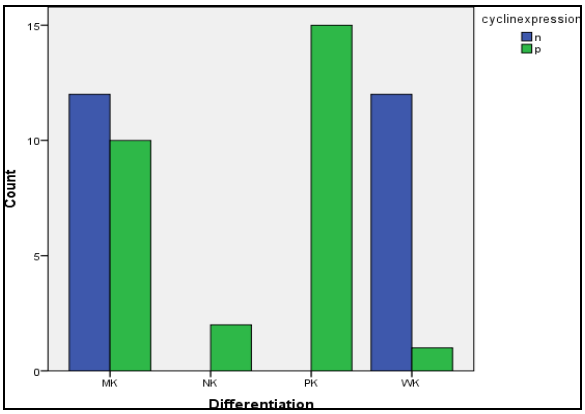
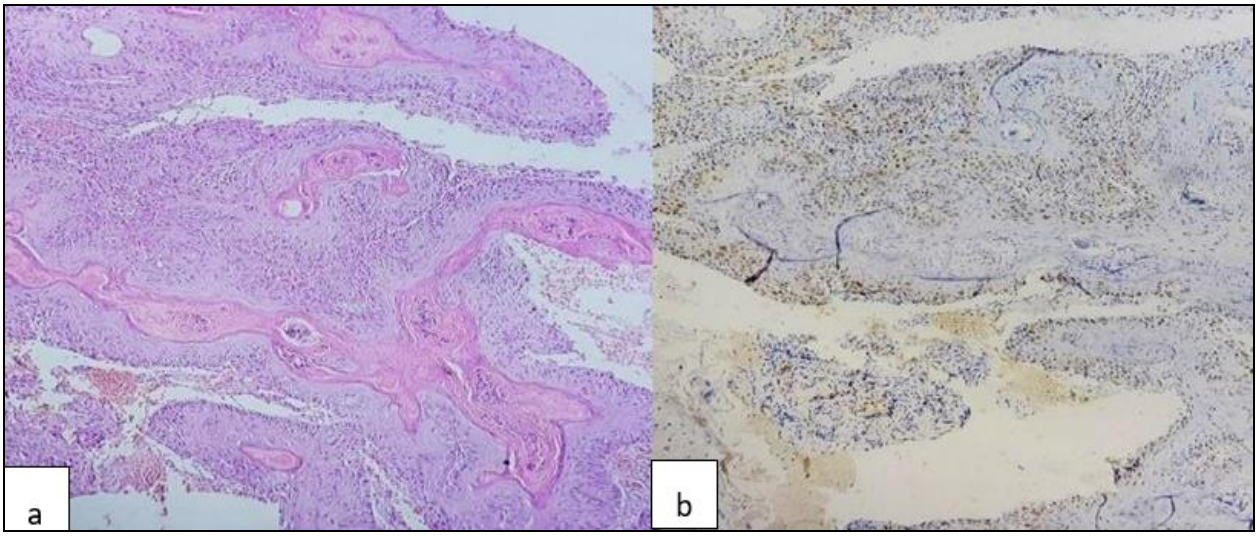


Figure-1: Bar chart showing Cyclin D1 expression in OSCC*

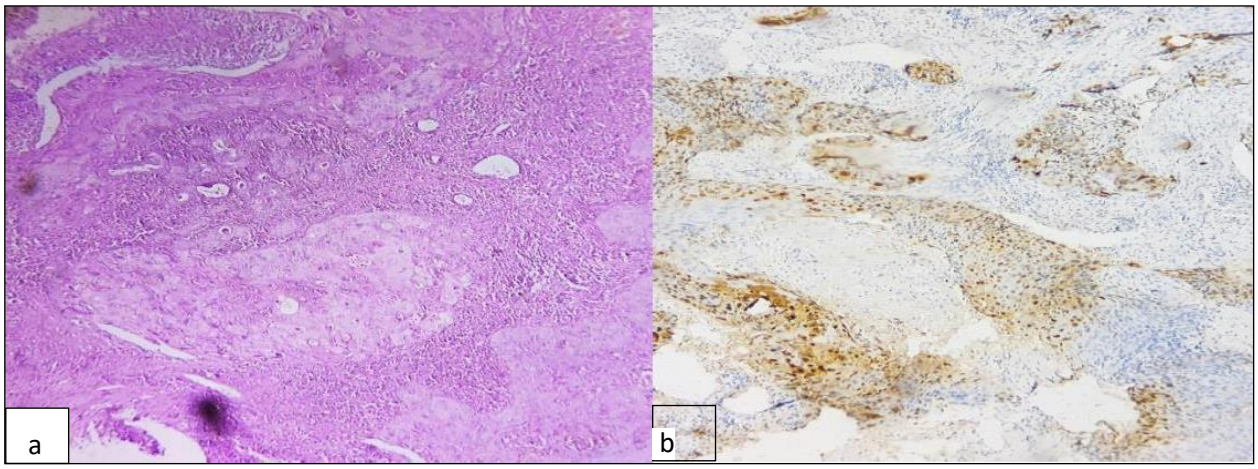
*WK: well differentiated keratinizing, MK: moderately differentiated keratinizing, PK:poorly differentiated keratinizing, NK: non-keratinizing

Table-6: Distribution of Cyclin D1 expression score according to degree ofdifferentiation

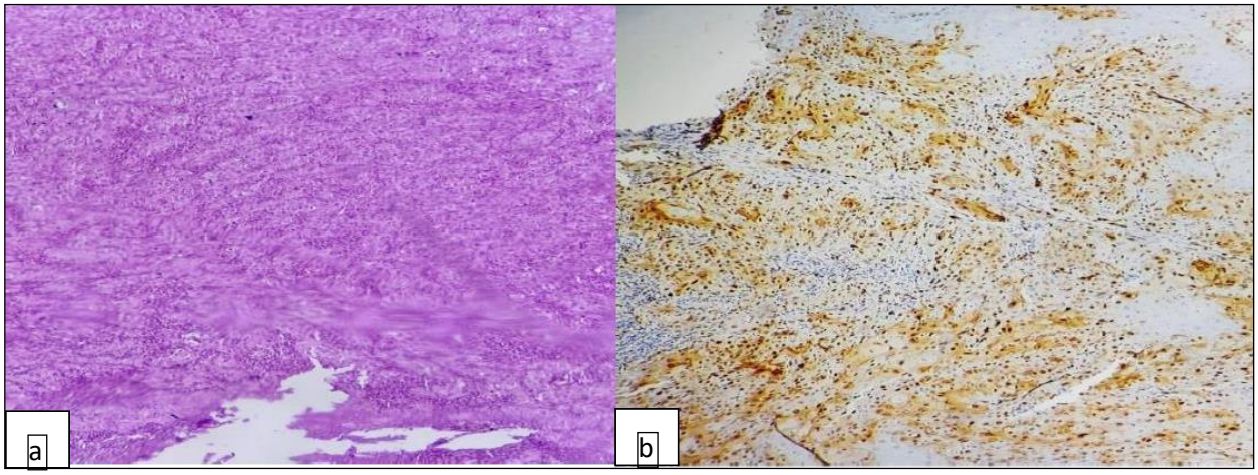
Differentiation of tumour	Cyclin D1score						Total	
	0	**1**	**2**	**3**	**4**	**6**		
Well-differentiated keratinizing	12	1	0	0	0	0	13	***p*-value 0.00**
Moderately-differentiated keratinizing	12	4	2	2	1	1	22	
Poorly- differentiated keratinizing	0	0	6	3	3	3	15	
Non- keratinizing	0	0	1	0	0	1	2	



Photomicrograph 1: Well differentiated keratinizing OSCC; a) H&E at 100Xmagnification, b) Cyclin D1 Immunostaining score 1 at 100X magnification



Photomicrograph 2: Moderately differentiated keratinizing OSCC; a) H&E at 100Xmagnification, b) Cyclin D1 Immunostaining score 3 at 100X magnification



Photomicrograph 3: Non-keratinizing OSCC; a) H&E at 100X magnification, b)Cyclin D1 Immunostaining score 6 at 100X magnification

DISCUSSION

Of the 52 cases, 55.8% cases were male and 44.2% were female with male to female ratio of 1.26:1, comparable to literature showing a general trend of OSCC in male gender e.g., Nazar *et al.*[17] Shenoi *et al.* showed an even higher male to female ratio, i.e., 4.2:1[18]. The lesser difference in proportion between male and female patients may be explained due to a rising trend of oral squamous cell carcinoma in female patients of Asia-pacific region as described in a multinational analysis by Satgunaseelan *et al.*[19]

The mean age of our patients came out to be 58.28 years, which is comparable to other studies. Silva *et al*. from Brazil reported a mean age of 63.3 years in 2017.[20] From India, Shenoi *et al*. presented a mean age of 49.73 years in 2012.[18] In 2020, Nazar *et al*. from AFIP Rawalpindi, Pakistan reported a mean age of 53.4 years.[17] The largest number of cases was found to be of age group 51–60 years, which was similar to the age group showed by Shenoi *et al.*[18] The results shown by our study are more relatable to those shown by Nazar *et al*. and Shenoi *et al*. because of the similar demographics of the Indo-Pak region. Silva et al. showed a higher mean age, which may have been contributed by several factors such as difference in demography, ethnicity and access to health-care facilities.

Grading of the OSCC lead to highest frequency of cases being found as moderately differentiated, similar to Ramos-Garcia *et al.*[21] In contrast, Patel *et al*. in their study found a higher frequency of well differentiated OSCC.[13] The difference may be due to an undeniable, however little, proportion of subjectivity of the observers and the difference in grading systems that may have been used. The commonest site encountered in our study was tongue, followed by buccal mucosa and alveolar ridge. This is similar to Nazar *et al*. from Pakistan and Vicente *et al*. from Spain.[14,17] In contrast, Shenoi

et al. from India showed the commonest site to be alveolar ridge.[18] The variance in pattern of presentation may be due to genetic differences and also due to difference in addiction patterns of the two regions, e.g., a higher trend of betel nut chewing in the Indian part of subcontinent.

Cyclin D1 expression was seen in 53.8% of OSCC in our study. This result is comparable to Choudhary *et al*. who showed a positivity of Cyclin D1 in 68% of OSCC.[16] Although the mentioned study used similar labelling index for Cyclin D1, a higher rate of expression in their study may be due to difference in the antibody used and possible differences or skill in the staining techniques. Saawarn *et al*. who showed a positivity of CyclinD1 in 45% of OSCC have used a different scoring system for Cyclin D1.[22]

The expression of Cyclin D1 showed no correlation with gender and age group of the patient in our study. Vicente *et al*. also did not show any correlation with gender of the patient[14] and Lam *et al*. did not show any correlation with age[23]. In contrast, Choudhary *et al*. showed a higher Cyclin D1 expression in younger age group.[16] Likewise, the expression of Cyclin D1 was also not related with site of the tumour, similar to Saawarn *et al*.[22] Cyclin D1 expression was significantly associated with loss of differentiation of the tumour, i.e., poorly differentiated and non-keratinizing tumours. This result is similar to the ones shown by Choudhary *et al*., Vicente *et al* and Ramos-Gracia *et al*. who have shown Cyclin D1 to be a poor prognostic marker.[14,16,21] In contrast, Saawarn *et al*. showed that Cyclin D1 is associated with better differentiated tumours[22], while Vicente *et al*. and Nazar *et al*. showed that Cyclin D1 expression was not related to histological differentiation of OSCC.[14,17]

The staining intensity of Cyclin D1 increased with loss of differentiation. It was manifest in our study as the final labelling score of Cyclin D1 which was obtained after multiplying the staining intensity with labelling index. The higher score of Cyclin D1 was significantly correlated with loss of degree of differentiation/ higher grade of OSCC (Table-6). This was similar to the result shown by Ramos-Gracia *et al*.[21] In contrast, Saawarn *et al*. showed that differentiation of OSCC and Cyclin D1 were not statistically correlated. The difference in result may be in part due to the use of a different scoring criteria for Cyclin D1 by Saawarn *et al*.[22]

CONCLUSION

Cyclin D1 expression was seen in 53.8% of OSCC and was significantly associated with loss of tumor differentiation and intensity of Cyclin D1 increased with loss of differentiation.

Limitation:

Our study did not include the follow up of the patients, due to which the exact prognostic significance of Cyclin D1 expression could not be determined.

Recommendation:

Further studies with larger pool are required to establish a concrete evidence of Cyclin D1 as a prognostic marker. Also, the correlation of Cyclin D1 overexpression with gene amplification might prove to be a better analysis. In addition, such studies might also be helpful for segregation of patients for target therapy.

Conflict of interest:

None

AUTHORS' CONTRIBUTION

MR: Conceptualization of the study design, data collection, write-up, proof reading. HA, SS: Design, data collection, write-up, proof reading. AA: Data entry, analysis. SK, JF: Data interpretatin, entry.

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