IMMUNOHISTOCHEMICAL EXPRESSION LEVELS OF CYCLIN E1 IN THREE HISTOLOGICAL GRADES OF ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Objectives: The objective of this study is to assess and compare the immunohistochemical expression levels of Cyclin E1 in three histological grades of oral squamous cell carcinoma.

Material and Methods: A descriptive cross-sectional study was designed. Sample size was 90 and categorised into three groups as i.e., well, moderate and poorly differentiated oral squamous cell carcinoma (OSCC) with 30 cases in each group. Specimens were collected from Histopathology Laboratories at Pakistan Institute of Medical Sciences (PIMS) and Foundation University Dental College and Hospital (FUDC&H) Islamabad, to evaluate the expression levels of Cyclin E1 (CCNE1) with the help of immunohistochemical (IHC) staining. Statistical analysis was performed using the statistical analysis software, SPSS version 25.

Results: In OSCC cases, average age of patients was 61.1±10.2 years. Over all age range of patients was 40-79 years (min – max) which was statistically significant (p=0.02). Male to female ratio was 1.1:1 (p=0.003). The most common site of development of OSCC was Buccal mucosa (p= < 0.001). According to immunoreactive scoring system (IRS) value of CCNE1 expression levels was low in 42.2% of cases and high in 57.7% of cases. The high final score of 6 and 9 was found in 80% cases of poorly differentiated OSCC cases and the results were found statistically significant (p<0.001)

Conclusion: It is concluded that increased expression levels of Cyclin E1 significantly correlated with lack of differentiation in OSCC.

Key Words: Cyclin E1, Immunohistochemistry, Oral squamous cell carcinoma.

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INTRODUCTION

The most common invasive epithelial carcinoma which affects the oral cavity is oral squamous cell carcinoma (OSCC) [1]. Globally, OSCC is one of the major public health problems [2]. According to the Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) 2020 report, OSCC is designated as the 16TH most commonly occurring cancer in the world and 8th most frequently occurring cancer in the United States [3]. GLOBOCAN 2020 report shows that OSCC accounted for 2% of all the cancers globally with 377,713 new cases in 2020 and rate of mortality is 1.8% with 177,757 new deaths in the year 2020 [4]. The areas with highest prevalence of oral cancer are Southeast Asia and several countries in southern and eastern Europe [5]. According to Global Cancer Observatory 2020 report in Pakistan, the incidence rate of OSCC is 10.9% with 16,959 new cases and mortality rate is 11.3% with 10,617 new deaths in both the genders. The incidence of OSCC in males is 12.9% with 11,395 new cases and in females it is 6.2% with 5564 new cases in the year 2020 [6].

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The main etiological factors are tobacco and alcohol, betel quid, poor oral hygiene, genetic and occupational factors [7]. The most commonly affected anatomical sites are buccal mucosa, lateral borders of tongue, palate, gingival region and buccal sulcus [8]. Men are more commonly affected by OSCC than women and most individuals are affected by this tumour in sixth to eight decades of their life. Average age of patients affected by OSCC is 60 years [9]. But recent researches have shown that OSCC is becoming common in individuals below the age of 45 years due to the habit of using smokeless and smoked tobacco at teen age or adolescence [10].

Although oral cavity is easily approachable for clinical examination, even then OSCC is routinely detected at advanced stages [11]. Most common causes are the initial incorrect diagnosis, unawareness of patients about the disease [12] and negligence by the primary health care physicians [13]. During the past three decades, despite the advancement in treatment options, morbidity and mortality rate because of OSCC have not been significantly improved [14]. There is five years survival rate for patients afflicted from OSCC and it varies from 40-60% [15]. WHO has also predicted a remarkable rise in worldwide occurrence of OSCC in the coming years [16].

OSCC occurs when sufficient gene alterations happen, which irreversibly alter the normal regulation of cell division and apoptosis [17]. E-type cyclins are main components of the cell cycle, which helps in normal cell proliferation and development and also assists in transition from G1 to S phase of cell cycle via activation of the enzyme such as cyclin dependent kinases [18]. Over expression of CCNE1 may lead to speedy transition from G1 to S phase which increases the duration of S phase, resulting in an increased instability of chromosome [19].

It is reported that CCNE1 has been overexpressed in many cancers including, breast cancer [20], stomach cancer [21], hepatocellular carcinomas [22] and ovarian cancer [23]. Over expressions of CCNE1 have also been seen in colon cancer, gall bladder cancer, prostate cancer, testicular cancer, urinary bladder cancer and also found in cancers of thyroid, lung, nasopharynx, bone, skin and the lymphatic system [24].

On the basis of previous researches on CCNE1, it is proved that this nuclear protein has oncogenic role in many tumour types and have a direct correlation with advance stage of carcinomas with lack of differentiation. Similarly, like other carcinomas, oral carcinogenesis occurs because of many genetic alterations, which basically involves proteins (that helps in normal cell cycle) such as cyclins and the cyclin-dependent kinases [25]. Recognition of these nuclear proteins which can be involved in these genetic changes can detect new biomarkers, that may act as prognostic markers for OSCC. CCNE1 plays a vital role in G1 to S phase of cell cycle. Therefore, it is possible it might play its role in oncogenesis of OSCC and can be used as a prognostic marker which can help in developing more accurate treatment plans for OSCC [26].

As no data is available on expression level of Cyclin E1 in OSCC, in population of Pakistan. So, in order to get benefit from targeted immunotherapy agents for CCNE1 we need to study CCNE1 in our population.

MATERIAL AND METHODS

This descriptive cross-sectional study was conducted at the department of Pathology, Peshawar Medical and Dental College, Peshawar. Sample size was measured by using G power software version (3.1.9.7), with an effective size of 0.3, alpha 0.05, power 80% and degree of freedom 2%. Sampling was done with non-probability convenient sampling technique. The research was conducted following the approval by the Institutional Review Board (IRB), Peshawar and IRB approval number is Prime/IRB/2020/237. Already diagnosed cases of OSCC (with 30 cases of each grade) well, moderate and poorly differentiated were included. Cases with poor formalin fixation where antigen retrieval issues may arise were excluded. Blocks of the patients receiving chemotherapies were also excluded. Cases with previous records were collected at Department of Oral Pathology, Pakistan Institute of Medical sciences (PIMS) and Foundation University Dental College and Hospital (FUDC&H), Islamabad. Laboratory procedures were done in Department of Pathology, Peshawar Medical and Dental College, Peshawar. Already Diagnosed formalin fixed, paraffin embedded OSCC tissue blocks were taken and sections were cut for both H&E staining and IHC procedure. Four to five microns (µm) thin paraffin embedded OSCC tissue sections were stained with Heamatoxylin and Eosin (H&E) by using standard protocols. Microscopic examination of H&E slides was done for the confirmation of clinical diagnosis of OSCC and selection of IHC staining using CCNE1 antibody. The amount of CCNE1 staining in nuclear tumour cells was used to assess CCNE1 expression. Level of intensity (A) of CCNE1 expression and percentage of number of positively stained nuclear cells (B) were assessed to get the final score.

Immunoreactive scoring system (IRS) has been used to get the Final score. The IRS score gives a range of 0-9 as a product of multiplication between positive cells proportion score (A) and staining intensity score (B) [27]. Levels of intensity was considered 0 = Negative, +1= weak, +2 = moderate, +3 = strong. Expression score was considered 0 = <10 % positive cells ,+1= 10-24% positive cells ,+2 = 25-49% positive cells ,+3 = >50% or more of positively stained cells (28).The value of IRS score ranged between 0-4 was considered as low CCNE1 expression and value of IRS score 6-9 was considered as high expression level of CCNE1.

Immunohistochemistry (IHC): Indirect immuno-histochemistry technique was adopted. Formalin-fixed and paraffin embedded tissues were deparaffinized. Antigen retrieval was accomplished by soaking the samples in citrate buffer solution and then heating them in a microwave oven at 95-100 degrees Celsius for 20 minutes. The slides were allowed to cool at ambient temperature, for 15 to 20 minutes. Phosphate buffer saline (PBS) and distilled water were used to wash the slides. Peroxidase blocking solution (PBS) was applied to the sections of the slides and incubated at room temperature for 10 minutes. Slides have been rinsed in PBS for 6 mins. To expose the colour of the antibody, chromogen substrate was added and slides were incubated in

peroxidase substrate solution. Slides were cleaned again after allowing colour to develop for less than 5 minutes. After that, slides were submerged in Haematoxylin counterstaining solution for 1-2 minutes. Slides were cleaned for another 15 minutes under running tap water. Alcohol was used to dehydrate tissue slides for 5 minutes. The slides were cleaned with three changes of xylene and a cover slip was applied with mounting solution before being kept at room temperature.

RESULTS

Data has been observed and analyzed using SPSS software (version 25). Descriptive analysis has been performed. Chi square test was used to analyze categorical data. The P-value less than or equal to 0.05 is considered as significant. The quantitative variables like age was measured as range, mean and standard deviation. The qualitative/ categorical variables like gender and intensity of CCNE1 expression and proportion of positively stained nuclear cells were measured as frequency and percentages and compared among three categories of OSCC.

In 90 cases of OSCC, average age of patients was 61.1 ± 10.2 years. Over all age range of patients was 40-79 years (min – max) which was statistically significant (p=0.02) (Table-I). Male to female ratio was 1.1:1 (p=0.003). The most common site of development of OSCC was Buccal mucosa (p=< 0.001). Moderate intensity of CCNE1 expression has been seen in 40% of total OSCC cases out of which 43% of the cases were belong to moderately differentiated OSCC and 20% was seen in poorly differentiated OSCC cases. Severe intensity of CCNE1 expression has been seen in 73.1% of poorly differentiated OSCC cases. P value was found < 0.001 which was statistically significant (Table-II). CCNE1 Expression score of +3 has been seen in 50% of moderately differentiated and 70% of poorly differentiated OSCC cases as shown in (Table-III). The results were found statistically significant (p-value 0.03). According to immunoreactive scoring system (IRS) value of CCNE1 expression levels was low in 42.2% of OSCC cases and high in 57.7% of OSCC cases. The high final score of 6 and 9 was found in 80% cases of poorly differentiated OSCC and the results were found statistically significant (p <0.001).

Figure-I shows the Heamatoxylin and Eosin staining of Poorly differentiated OSCC. Severe intensity and strong expression level (>50% or more positively stained nuclear cells) of CCNE1 expression has been seen in poorly differentiated OSCC cases (Figure-II).

Table-I: Frequency of three grades of OSCC cases in four age categories (n=30).

Age (years)	Well differentiated OSCC (n=30)	Moderately differentiated OSCC (n=30)	Poorly differentiated OSCC (n=30)	Total OSCC Cases (n=90)	p-value
Mean ± SD	61.6 ± 10.2	58.9 ± 10.3	59.8 ± 11.2	61.1±10.2	0.26*
Age categories					
Up to 50	5 (16.7%)	4 (13.3%)	9 (30.0%)	18 (20%)	0.02*
51 to 60	8 (26.7%)	15 (50.0%)	4 (13.3%)	27(30%)	
61 to 70	10 (33.3%)	8 (26.7%)	15 (50.0%)	33(36.6%)	
71 or above	7 (23.3%)	3 (10.0%)	2 (6.7%)	12(13%)	

Table-II: Frequency of OSCC cases showing association between three grades of OSCC and intensity ranges of CCNE1 (n=30).

Intensity of CCNE1	Well differentiated OSCC (n=30)	Moderately differentiated OSCC (n=30)	Poorly differentiated OSCC (n=30)	Total OSCC cases (n=90)	p-value
1 (Mild)	8 (26.7%)	7 (23.3%)	2 (6.7%)	17(18.8%)	<0.001*
2 (Moderate)	17 (56.7%)	13 (43.0%)	6 (20.0%)	36(40%)	
3 (Severe)	5 (16.7%)	10 (33.3%)	22 (73.3%)	37(41.1%)	

Table-III: Relative frequency of OSCC cases according to different scores of CCNE1 expression (n=30).

Score of CCNE1 Expression	Well differentiated OSCC (n=30)	Moderately differentiated OSCC (n=30)	Poorly differentiated OSCC (n=30)	Total OSCC cases (n=90)	p-value
0	2 (6.7%)	2 (6.7%)	0 (0.0%)	4(4.4%)	0.03*
1	7 (23.3%)	4 (13.3%)	1 (3.3%)	12(13.3%)	
2	13 (43.3%)	9 (30.0%)	8 (26.7%)	30 (33.3%)	
3	8 (26.7%)	15 (50.0%)	21 (70.0%)	44(48.8%)	

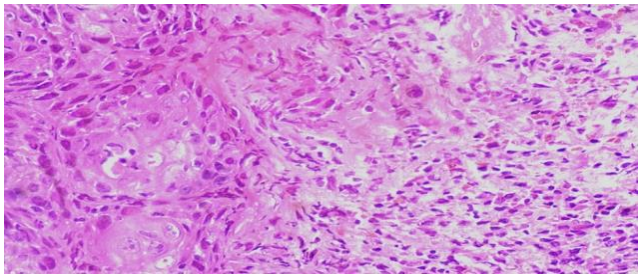

Figure-I: Poorly Differentiated OSCC (H&E Mag 10x).

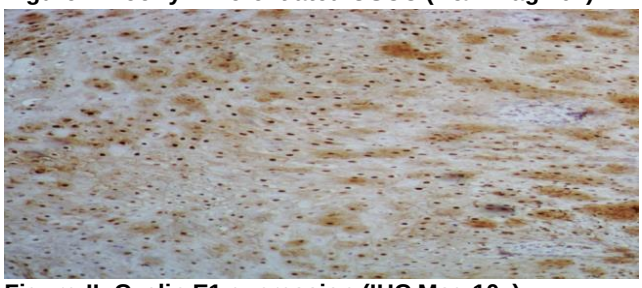

Figure-II: Cyclin E1 expression (IHC Mag 10x).

DISCUSSION

Dysregulation and abnormalities in Cyclins related to cell cycle have been linked to tumour development and progression in a variety of malignancies [29]. Understanding these changes in carcinogenesis may lead to the discovery of novel proteins that may be used as cancer diagnostic and prognostic markers, as well as possible targets for treatment methods in OSCC [30]. With this in mind, we designed this study to assess the impact of CCNE-1 expression levels in three histological grades of OSCC.

Out of 90 cases of OSCC, CCNE1 expression was present in almost all three grades of OSCC but highly expressed in cases of poorly differentiated OSCC. In this study numerous clinical and pathological indicator of the tumour progression such as age of patient, male to female ratio, tumour size, tumour site, histological grade has been assessed in already diagnosed cases of OSCC. In the present study the overall male to female ratio was 1.1:1 which shows a slight increase in the incidence of OSCC in men than women. Gustaf Adgren *et al*, reported increase ratio of men than woman in his research of OSCC [31]. Ali hashmi *et al*., 2018 also reported that there is increased incidence of OSCC in males than females because of increased consumption of Areca nut and Gutka [32]. On the other hand in a global study on epidemiology of OSCC data shows increased incidence of OSCC in females [33].

Present study suggests that the most common site of OSCC is buccal mucosa and the second most frequently affected site is tongue. These results are similar to the results reported by De Silva *et al*.,2018 that most common site of OSCC in south Asian population is Buccal mucosa and tongue (34). Awan *et al*., 2020 and Blanchard *et al*.,2017 also report buccal mucosa as most common site in OSCC , because of increased consumption of smokeless tobacco [30] [35]. Contrary to this Ehtesham *et al*., 2017 reported that OSCC mostly affects the border of tongue and floor of mouth in males, while buccal sulcus is the most commonly affected anatomical site in females [36].

In all three histopathological categories the tumor size was found ≤ 2 cm in majority of cases. Mean tumour size is 3.4 ± 6.6. The large size of tumor i.e. 5 cm or more was found more frequent in well differentiated 7 (23.3%) or moderately differentiated OSCC cases 5 (16.7%) as compared to poorly differentiated cases 1 (3.3%). However, the difference in size of tumors could not be proved statistically significant (p-value, 0.08).

In the present study the score +3 was found more common in moderately and poorly differentiated OSCC cases. The difference in expression score was found statistically significant (p-value,0.03). Significant research has not been done to assess the levels of CCNE1 expression in oral cancer or OSCC. Level of intensity of CCNE-1 has not been analysed in three grades of OSCC in previous studies. CCNE-1 antibody has been applied on several tumours and it is overexpressed in many malignancies and most of the work has done on breast cancers [37]. Present study is the first to assess intensity of CCNE1 in three histological grades of OSCC.

The expression score of CCNE1 was found 0 in 2 (6.7%) cases each of well differentiated and moderately differentiated OSCC cases compared to none in poorly differentiated cases. Moreover, expression score of 1 was found in 7 (23.3%) well differentiated OSCC cases. The score of 1 was found in most of well differentiated OSCC cases 13 (43.3%). Expression score 2 was found in 20% of moderately differentiated cases and 26.7 % of poorly differentiated cases. While expression score of 3 was found in 50 % of moderately differentiated cases and 70% of poorly differentiated OSCC cases. The difference in expression score was statistically significant (p-value, 0.03).

Significant research has not been done to assess the levels of CCNE1 expression in Oral Cancer or OSCC. There is only one study published in Europe in 2018 to evaluate the expression levels of Cyclins in OSCC and in that research along with Cyclin A2, B1 and CCND1 researchers have assessed the expression levels of CCNE1 in 134 samples of OSCC. Previous study only suggests the expression score of CCNE1 in OSCC but have not assessed the levels of CCNE1 in three histological

grades of OSCC. But various researches on other tumours of body have suggested that CCNE1 is highly expressed in poorly differentiated carcinoma.

Our results are in accordance with the previous researches of CCNE1 expression on several other tumours of body. Wu *et al*., 2019 have reported that CCNE1 is highly expressed in poorly differentiated Breast cancer [39]. Another research conducted by Li *et al*.,2018 on hepatocellular carcinoma have also suggested that CCNE1 is highly expressed in poorly differentiated carcinoma as compared to well differentiated carcinoma [40] . A study conducted in china by Xia *et al*., 2016 also reported high expression level of CCNE1 in poorly differentiated esophageal squamous cell carcinoma [41].

Final score between the range 0-4 was considered as low CCNE1 expression and final score 6-9 was considered as high level of CCNE1 expression. According to IRS scoring system 42.2% cases had low expression of CCNE1 and 57.7 % of cases had high levels of CCNE1 expression. P-value was found statistically significant ($p<0.001$).

In Pakistan our study is first to assess the levels of CCNE1 expression in three histological grades of OSCC. Higher expression of CCNE1 in poorly differentiated OSCC is in accordance with literature review on several other solid tumours of the body [42]. CCNE1 overexpression has been demonstrated to drastically shorten mitosis and cause chromosomal instability, both of which promote carcinogenesis [43].

In current study we show CCNE-1 overexpression to correlate with moderately differentiated and poorly differentiated OSCC. This elevated CCNE-1 expression has been attributed to gene amplification, transcription upregulation and disrupted deregulation [44]. This is in accordance with the crucial contribution of CCNE-1 in control of the cell Cycle and there by tumour proliferation [45].

CONCLUSION

It is concluded that increased expression levels of Cyclin E1 is significantly correlated with lack of differentiation in OSCC.

LIMITATIONS OF THE STUDY

Due to limited time and constrained financial resources, a larger study sample size could not be included.

RECOMMENDATION

It should be used as a predictive biomarker in oral potentially malignant disorders (OPMDs) to detect any cancerous lesion on time. Follow up studies will add to our knowledge that how CCNE1 related immunotherapy may help patients suffering from OSCC. Using more sensitive diagnostic techniques like PCR, ELISA, in situ hybridization can also give more reliable results. Other antibodies that work closely with CCNE1 like Cyclin A2 should be included in further studies.

AUTHOR CONTRIBUTION

Arooj Khurshid: Article write-up.
Sabeen Nasir: Review the article.
Sadaf Alam: Data analysis.
Madiha Insha: Assist in data collection.
Rabia Alamgeer: Assist in laboratory work.
Natasha Kamran: Assist in statistical analysis.

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