RESEARCH ARTICLE

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Comparison of PD-L1 Expression in Oral Squamous Cell Carcinoma and Premalignant Lesions of Oral Cavity

Saleha Saeed[1], Fozia Rauf[2]*, Fatima Iqbal[3], Abbas Saleem Khan[4], Amara Hayat Khan[5], Rabia Alamgeer[6]

Abstract

Objective: Objectives of this study were to compare expression of Programmed Death-Ligand 1(PD-L1) protein in oral squamous cell carcinoma (OSCC) and oral potentially malignant disorder (OPMD) cases; and to compare the PD-L1 protein expression in histological grades of OSCC and also in OPMD's with Dysplasia and without Dysplasia. **Materials & Methods:** In this study, 25 cases of Oral squamous cell carcinoms, 25 cases of Oral Potentially Malignant Disorders and 10 cases of non-neoplastic oral mucosa (control) cases were included. FFPE blocks of OSCC and OPMD cases were contributed by Department of Pathology, Histopathology Division,Pakistan Institute of Medical Sciences, Islamabad. Immunohistochemical staining of cases with PD-L1 monoclonal antibody (1:100; Dako) was carried out at Histopathology division , PMC Labs, Peshawar Medical College,Peshawar, Riphah International University, Islamabad . Epithelial cells (membranous and cytoplasmic) positivity was observed for PD-L1 Antibody. Data was analyzed in SPSS version20. For qualitative variables frequencies and percentages were calculated whereas for quantitative variables means and standard deviations were recorded. The Chi-square test was applied to evaluate the significant difference in categorical variables . p-value of ≤0.05 was taken as significant. **Results:** PD-L1 expression in OSCC cases turned out to be 48% (n=12/25) as compared to 8% of OPMD cases (n=2/25) with a significant p value of 0.002 and all non-neoplastic oral mucosa cases were negative. PD-L1 expression in high grade OSCC cases was quite high (61% n=11/18) as compared to low grade OSCC (14% n=1/7) cases with a significant p value of 0.035. **Conclusion:** A statistically significant increased PD-L1 expression was noted in OSCC as compared to OPMD. Expression of PD-L1 was more intense in high grade OSCC cases. The relation of PD-L1 expression to age ,gender or location of OSCC and OPMD cases , and presence of dysplasia in OPMD cases was statistically not significant.

Keywords: Oral squamous cell carcinoma-oral potentially Malignant disorders- programmed death-Ligand1

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Introduction

Squamous cell carcinoma of oral cavity is considered as 18thmost frequent cancer around the globe being responsible for 354,864 new cases and approximately 177,384 deaths per year (Bray et al., 2018). According to Globacan report 2018, oral cavity cancer is the 2nd most common in Pakistan. Lip and oral cavity cancer are categorized 1st in males according to the new number of cases and 2nd in females (Ferlay et al., 2018).

The well-known hazards causing oral squamous cell carcinoma include smoked and smokeless tobacco, betel grinding and alcohol use (Troiano et al., 2018). In developing countries like Pakistan, the main etiological agents are tobacco, areca nut, and betel quid which cause tumor of oral cavity as well as oral precancerous lesions (Sahaf et al., 2017). Nearly all squamous cell carcinomas of oral cavity emerge from premalignant disorders of oral cavity including clinical diseases for example erytheroplakia, leukoplakia, lichen planus, actinic cheilitis, chronic candidiasis and submucous fibrosis etc (Scully, 2014). Dysplastic changes are one of the signs of premalignant lesions with a malignant potential of up to 36% (Dave et al., 2019; Nankivell et al., 2013).

The increased frequency of OSCC and OPMDs in populations worldwide as well as dismal prognosis of high grade and end stage OSCC has compelled researchers to find various tissue and serum biomarkers that can be used to determine malignant potential and prognosis of oral lesions.

*[1]Department of Oral Pathology, Senior Lecturer, Faryal Dental College, Lahore, Pakistan. [2]Peshawar Medical College, Pakistan. [3]Department of Oral Pathology, Assistant Professor, Peshawar Dental College, Peshawar, Pakistan. [4]Department of Oral pathology, Peshawar Dental College, Riphah International University, Pakistan. [5]Department of Dental Education, Senior Lecturer, Peshawar Dental College, Peshawar, Pakistan. [6]Department of Oral Pathology, Lecturer, Sardar Begum Dental College, Peshawar, Pakistan. *For Correspondence: faraufg@gmail.com*

Immune checkpoint molecules like programmed death 1 receptor and its ligands are being studied extensively for their role in different cancers like breast cancer and lung cancer etc (Wang et al., 2017). Programed death 1 receptor (PD-1) is an inhibitory receptor which is found on various immune cells e.g., CD8+ T cells and natural killer cells. Programmed death ligand 1 attaches to PD-1 receptor. When programmed death ligand 1 is expressed on cancerous cells, it leads to the complexation of PD-1 and PD-L1 pathway causing exhaustion of T cell functions. In this way the cancer cells evade immune system by protecting themselves from the attack of CD8+ cytotoxic T cells (Dave et al., 2019).Immune checkpoint molecules have a targeted immunotherapy available that can block their action and has a promising role in cancer therapy (Kujan et al., 2020).

Immunological approaches blocking PD-1/PD-L1 pathway is good treatment strategy to avoid transformation of precancerous cells into malignancy. Immune checkpoint inhibitors (nivolumab and pembrolizumab) are being used for treating oral squamous cell carcinoma with promising results (Polverini et al., 2018; Saada-Bouzid et al., 2017).

There are not many studies in literature observing the expression of PD-L1 in OPMD cases in comparison to OSCC (Chen et al., 2019; Kouketsu et al., 2020). In Pakistani population, we have none comparing the PD-L1 expression in oral squamous cell carcinoma and oral premalignant lesions. Histopathological grading of OSCC and OPMD together with PD-L1 expression may prove to be a beneficial criterion for assessment of the biological behavior of OPMD and OSCC in our routine histopathology practice. This study is designed to compare the immunohistochemical expression of this biomarker PD-L1 in OPMD and OSCC cases to see whether the OSCCs have a higher expression of PD-L1 than OPMDs in our population.

Material and Methods

Study Design and Setting

Comparative Cross-Sectional Study was performed. Data was collected from Department of Pathology, Pakistan Institute of Medical Sciences Islamabad (PIMS), Pakistan. The laboratory work was conducted at Department of Pathology, Peshawar Medical College (PMC), Peshawar, Pakistan.

Sample Size

In this study, formalin fixed paraffin embedded tissue sections of 25 cases of oral squamous cell carcinoma, 25 cases of oral potentially malignant and 10 cases of non-neoplastic oral mucosa were included. All consecutive cases of oral squamous cell carcinoma and oral potentially malignant disorders were included in the study.

Ethical Approval

Approval of the study was taken from Board of advanced studies and Research (BASR) Riphah International University, Islamabad, Pakistan. The ethical consent of the study was given by the Institutional review board (IRB) of Prime Foundation Pakistan (IRB Approval No, PRIME/IRB/2019-192). Written approval was also taken from Head/In charge of the Lab and Departments of participating institutes. The data was recorded in Annexure.

Data Collection Tool

The OSCC and OPMD cases were retrospectively retrieved from electronic archives of Department of Pathology, Pakistan Institute of Medical Sciences, Shaheed Zulfiqar Ali Bhutto University, Islamabad and stained for immunohistochemistry at Department of Pathology, Peshawar Medical College, Riphah International University. The relevant clinical data such as age, gender of the patient, site of lesion, grades of OSCC, dysplasia and non-dysplasia in OPMDs.

Methodology

The hematoxylin & eosin (H&E) slides of OSCC, OPMDs and non-neoplastic cases were reviewed. The OSCC cases were graded by Bryne grading system (Bryne et al., 1991) and OPMD cases were graded for presence or absence of dysplasia. The selected formalin fixed paraffin embedded tissue blocks of OSCC, OPMD and non-neoplastic cases were cut 5µmeter on charged slides by Dako. These cases were stained with monoclonal antibody against PD-L1 by Dako (1:100). For Immunohistochemical staining of cases, first deparaffinization of formalin fixed paraffin embedded tissue was carried out. Antigen retrieval was done by inserting in citrate buffer and then heating in microwave oven at 95-100C for 20 minutes. Slides were allowed to cool at room temperature for 15-20 minutes. Slides were rinsed with distilled water and phosphate buffer saline (PBS). Peroxidase blocking solution was added to the sections of the slides incubated for 10 minutes at room temperature. Rinsed in PBS for 6 minutes. Primary antibody (PD-L1) was applied to sections on the slides and incubated for 60 minutes (1 hour) in humidified chamber at room temperature. After 1 hour, the slides were washed again. Biotinylated secondary antibody was applied to the sections and incubated for 30 minutes at room temperature. Rinsed in PBS for 6 minutes. Chromogen/substrate was applied, and sections were incubated in peroxidase substrate solution to reveal color of the antibody. Color was allowed to develop for less than 5 minutes, and slides were washed again. Counterstaining was performed by immersing slides in hematoxylin for 1-2 minutes. Slides were again rinsed in running tap water for about 15 minutes. Tissue slides were dehydrated through four changes of alcohol (95%, 95% 100% and 100%) 5 minutes each. Slides were cleared in three changes of xylene and cover slip was placed using mounting solution and were stored at room temperature. PD-L1 immunoreactivity scoring was defined according to cell intensity for all OSCC and OPMD cases. The following criteria was used to observe the intensity of PD-L1 stain.14 0=Negative 1=Weak staining 2=Moderate staining 3=Strong staining.

Statistical Analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) version 20. Statistical

results were given as mean and standard deviation for continuous variables for example age. Chi square test was used to compare categorical variables for example gender, site, grade and expression of PD-L1 marker. p value of less than and equal to 0.05 ($p \leq 0.05$) was considered statistically significant.

Results

PD-L1 Protein Expression in OSCC, OPMD and Non-Neoplastic Cases

The PD-L1 protein expression in membrane and cytoplasm of epithelial cells of 25 OSCC, 25 OPMD& 10 cases of non-neoplastic mucosa were observed.Positive PD-L1 protein expression was observed in 12/25(48%) cases of OSCC and 2/25(8%) cases of OPMD.There was a statistically significant difference in the positive staining between OSCC & OPMD (p=0.02) (Table 1).

PD-L1 Protein Expression in relation to OSCC Grades

Most of the OSCC cases were grade II, 44% (n=11/25) followed by grade III ,28% (n=7/25) and grade I ,28% (n=7/25). The OSCC cases were further sub grouped into low grade (grade I n=7/25) and high grade (grade II & grade III n=18/25 72%). PD-L1 protein expression in high grade OSCC cases was 61% (n=11/18) as compared to low grade OSCC cases 14% (n=1/7) with a significantp value of 0.035 (Table1).

PD-L1 Protein Expression in relation to OPMD

Out of 25 OPMD, 24% cases were of keratosis (n= 6), 44% were keratosis with lichenoid infiltrate (n= 11) and 32% were keratosis with dysplasia 32% (n= 8). Overall, 12/25 OPMD cases showed some degree of dysplasia, and 13/25 cases showed no dysplasia. 8% of OPMD showed epithelial positivity for PD-L1 (n= 2/25). One case of OPMD with dysplasia (n= 1/12 8.3%) was positive and one case without dysplasia was positive for PD-L1 (p>0.05) (Table 1).

Relation between PD-L1 Protein Expression and Demographic Parameters of OSCC & OPMD Cases

Mean age of OSCC cases was 57.4 yrs. (±13.4 SD) with age range of 23-82 years 0.32% were in their fifth decade (n=8/25). PD-L1 expression was not linked with age when compared between two groups of OSCC i.e., less than 60 yrs (n=16/25) and more than 60 yrs. of age (n=9/25) (p>.05). Mean age of the patients with OPMD cases was 60.8 yrs. (±10.9). 56% were in more than 60 years' age group (n=14/25). PD-L1 expression in OPMD cases was

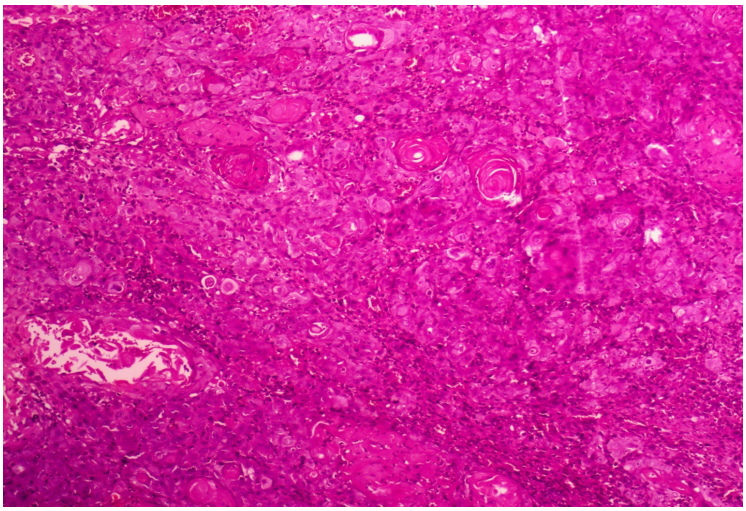

Figure 1. Moderately Differentiated Oral Squamous Cell Carcinoma(H&E stain , 40x)

Table 1. PD-L1 Expression in OSCC and OPMD Cases

	Total n (%)	n (%) PD-L1 Positive	n (%) PD-L1 Negative	p-value
OSCC	25 (100)	12 (48)	13 (52)	0.002
OPMD	25 (100)	2 (8)	23 (92)	
Grade				
OSCC				
Low grade	7 (28)	1 (14)	6 (85)	0.035
High grade	18 (72)	11 (61)	7 (39)	
OPMD				
Without dysplasia	13 (52)	1 (8)	12 (92)	0.95
With dysplasia	12 (48)	1 (8)	11 (92)	

Footnote: Comparing PDL-1 expression in OSCC and OPMD cases, it was found that OSCC shows a significantly higher expression of PDL-1 which indicates that PDL-1 has a significant role in tumor progression. Also statistically significant higher expression of PDL-1 in high grade OSCC (Grade II and grade III) shows that PDL-1 has a role in tumor progression.

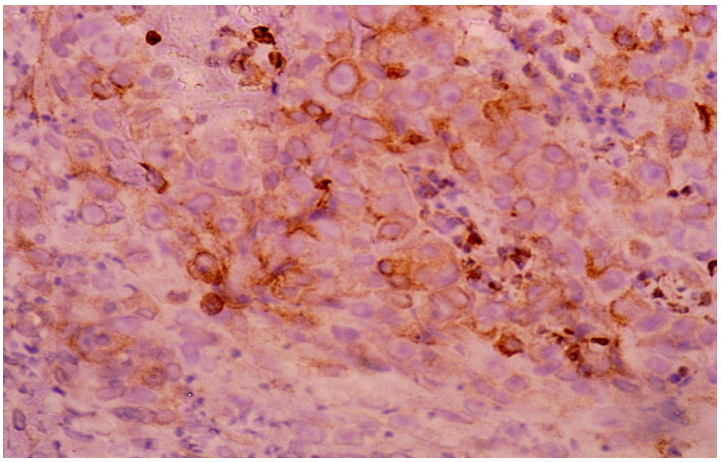

Figure 2. Expression of PD - L1 in Moderately Differentiated Squamous Cell Carcinoma(2+ Positive Membranous Staining ; 40x)

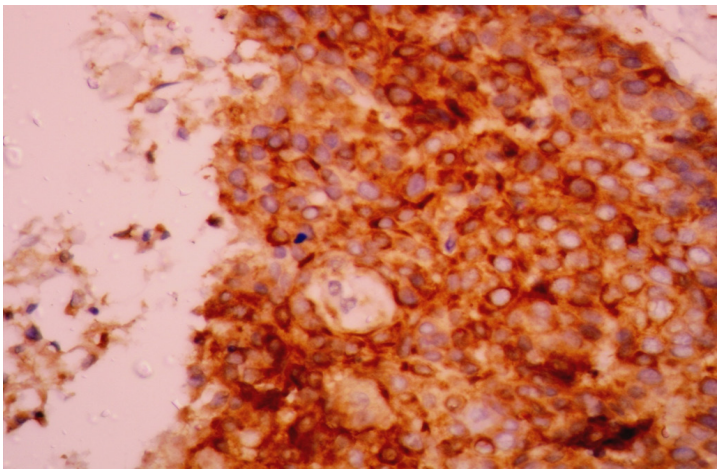

Figure 3. Expression of PD - L1 in Poorly Differentiated OSCC (3+ Positive Membranous Staining ; 40x)

not significantly correlated with age (p>0.05).

There was no difference in OSCC gender distribution of cases was 12 males and 13 females having M

F of 1:1.08. PD-L1 expression was not associated with gender (p>0.05). 13 cases of OPMD were male and 12 cases were female with M: F ratio of 1.08:1. No statistically significant difference was observed when both genders were compared for PD-L1expression. Tongue was the more frequent site in OSCC cases 64% (n=16/25)

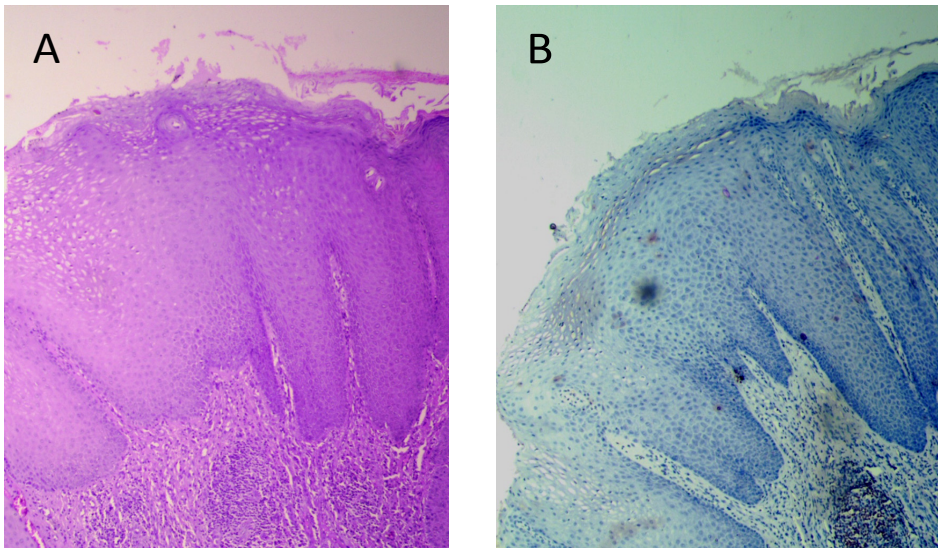

Figure 4. A, Hyperkeratosis without dysplasis of Buccal Mucosa (Incisional Biopsy; H&E 10x); B, Negative Expression of PDL – 1 in same case (10x).

followed by buccal mucosa and alveolar mucosa respectively. Buccal mucosa was the more frequent site for OPMD cases (n= 16/25 64%) followed by tongue and alveolar mucosa.

Discussion

Relation of PD-L1 Protein Expression in OSCC and OPMD

As per our findings, PD-L1 positivity was found more in OSCC cases (48%). Our results were in concordance with multiple studies which report 45% and 48.9% cases of OSCC positive for PD-L1 protein on IHC (Straub et al., 2016; Tojyo et al., 2019).

In the present research work, we found very low percentage of PD-L1 positive OPMD cases (8%) (Table 1). Our results contradict with multiple studies showing comparatively higher PD-L1 expression in OPMD cases i.e., 26.6%, 28% and 61.9% by Kouketsu et al., (2017) from Japan, Yagyuu et al., (2017) from Japan and Chen et al., 2019 from China respectively. Somestudies report that PD-L1 positivity in OPMDs e.g., Oral Leukoplakia is not associated with dysplasia and age but might be associated with gender and smoking (Chen et al., 2019).

In the present study, all control group cases were negative for PD-L1 protein expression. Our results are in line with the studies by Weber et al., (2017) from Germany. In the present study, there was a statistically remarkable difference in PD-L1 expression of OPMD (8%) and OSCC cases (48%) with a p value of 0.002 which suggests that PD-L1 is strongly correlated with tumor progression.Our results are in accordance with the study by Kouketsu et al., (2019) from Japan who observed PD-L1 expression in OSCC cases and oral epithelial precursor lesions by IHC showing positive expression in 67.9% (n=72/106) OSCC cases and 26.6% (n=21/79) oral epithelial precursor lesions (Kouketsu et al., 2020).

An international study by Chen et al., (2019) from China compared expression of programmed death ligand 1 protein in 41 OSCC cases, 21 oral leukoplakia cases and 25 cases of normal mucosa by immunohistochemistry and expressed high PD-L1 expression in 97.6% OSCC cases and 61.9%of oral leukoplakia cases respectively while normal mucosa group was negative for PD-L1. Their results were statistically significant because PD-L1 was highly expressed in OSCC cases (Chen et al., 2019). The findings of our study are also in line with an international study from Canada which analyzed PD-L1 expression in keratosis with high grade dysplasia progressing to squamous cell carcinoma cases (n=19) as compared to keratosis with dysplasia cases non progressing to OSCC (n=20) and OSCC (n=10) cases showing a remarkable rise in PD-L1 expression in oral lesions progressing to squamous cell carcinoma and OSCC cases as compared to non-progressing cases (Dave et al., 2020). However, in contrast to our results, Goncalves et al., (2017) from Brazil compared 80 cases of oral leukoplakia with 20 cases of OSCC and concluded that PD-L1 protein expression in both the groups was comparable but not statistically significant.

Relation of PD-L1 Protein Expression in Different Grades of OSCC and OPMD

In current research work, maximum number of positive PD-L1 cases were of high grade OSCC (61%) as compared to low grade OSCC (14%). A study by Chen et al., (2019) from China analyzed PD-L1 expression immunohistochemically in OSCC cases (n=41), oral leukoplakia cases (n=21) and normal mucosa cases (n=25) reported a positive correlation between PD-L1 expression and pathologic grades of OSCC. A meta-analysis by Cui and Su (2020) analyzing studies on PD-L1 expression in OSCC concludes significant association of PD-L1 expression with advanced grade of OSCC.

The results of present study are in contrast to the study by Maruse et al., (2018) from Japan who determined PD-L1 expression immunohistochemically in 97 OSCC cases out of which (n=62/97) were low grade and (n=35/97) were high grade, showing PD-L1 expression in high grade OSCC cases (28.8%) as compared to low grade OSCC cases (52.5%) (Maruse et al., 2018). Our results are in contrast to the study by Yoshida et al., (2018) who studied PD-L1 expression in (n=135) tongue OSCC cases out of which only (n=38/135; 28.1%) showed positive PD-L1 expression with 57.8% of low grade OSCC cases and 42.1% of high grade OSCC cases. Similarly, in contrast to our findings, a study by Qureshi et al., (2020) from Pakistan reported 40% of low grade OSCC cases positive for PD-L1 as compared to 28% high grade OSCC cases.

These results show the variation in PD-L1 expression from various geographical areas and need for further studies on various population groups. In the present study, when PD-L1 expression of OPMD with dysplasia was compared with no dysplasia, no significant statistical difference was observed (Table 1). Our findings are in accordance with Goncalves et al., (2017) from Brazil who observed the expression of PD-L1 in 80 cases of oral leukoplakia and concluded that expression of PD-L1 protein was independent of histopathological degree of dysplasia. Our results are in line with the study by Chen et al., 2019 from China who observed PD-L1 expression in OSCC cases (n=41) as compared to oral leukoplakia cases (n=21). In oral leukoplakia sample (n=21) there was no significant association between dysplastic epithelial lesions (n=6) and non-dysplastic lesions (n=15) ($p>0.05$) (Chen et al., 2019). However, our results are in contrast to those of Yagyuu et al., (2017) from Japan who observed PD-L1 expression in 120 OPMD cases and expression was higher in OPMD with high grade dysplasia (n=23/52 44%) as compared to OPMD with low grade dysplasia (n=11/68 16%) suggesting that PD-L1 expression was positively correlated with malignant transformation.

Our results are in contrast to another study conducted in Canada which showed higher PD-L1 expression in cases of dysplasia progressing to OSCC as compared to cases of non-progressing dysplasia to OSCC (Dave et al., 2020).

These differences in results point towards differences in biological behavior of OPMDs of various study groups that may be because of different causative agents of OSCC.

In Conclusion, a significant proportion of OSCC shows PD-L1 expression. Therefore PD-L1 blocking

immunotherapy may be used in those cases that show PD-L1 positivity on biopsy. Statistically significant higher expression of PD-L1 in higher grades of OSCC suggest a role of PD-L1 in progression of OSCC. PD-L1 expression was not dependent on age, gender and location of OSCC and OPMD cases and presence of dysplasia in OPMD

Author Contribution Statement

Dr. Saleha Saeed: Principal investigator as part of M Phil(Oral Pathology) Thesis research; Dr. Fozia Rauf: Supervisor of the research; Dr. Fatima Iqbal: Helped in data retrieval of OSSC cases; Dr. Abbas Saleem Khan: Helped in data Analysis; Dr. Amara Hayat Khan: helped in data retrieval of OPMD cases; Dr. Rabia Alamgeer: helped in literature search.

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Institutional Review Board Approval

Institutional Review Board of Peshawar Medical College , Riphah International University , Islamabad , approved the synopsis of this research through letter: Prime/IRB/2019-192

Ethical review board committee approval

IRB approval was given by the Ethical Review Committee, Institutional Review Board of Peshawar Medical College, Riphah International University, Islamabad via letter: Prime/IRB/2019-192.

Conflict of Interest

No conflict of interest was declared in this study.

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