Immunohistochemical Expression of Programmed Death Ligand-1 in Tumour Milieu of Oral Cancer

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Abstract

Objectives: To determine the immunohistochemical expression of PD-L1 in oral squamous cell carcinoma and to correlate it with clinicopathological parameters.

Methods: This cross-sectional study comprised of 140 cases diagnosed with oral squamous cell carcinoma (OSCC), which were recruited by purposive sampling technique. Demographic details and clinical features were acquired through a questionnaire and consent was taken prior to the selection of cases. The diagnosis of OSCC was confirmed by biopsy and immunohistochemical staining was done by applying PD-L1 antibody. The positive expression of PD-L1 was defined as at least 5 % of tumour cells exhibiting membranous staining at any intensity.The obtained data were then analysed statistically through SPSS version 25.

Results: Mean age of the participants was 48.91 ± 11.7 years. A majority of them were males(103; 74%) as compared to females (37; 26%). Most of the participants (64; 46%) belonged to the Urdu speaking ethnic group. Majority of them were habitual of using chewable tobacco products i.e. Pan, Gutka and Betel nut (89; 64%). The most common site affected by OSCC in the study was buccal mucosa (75; 54%). Majority of the cases had histologically moderately differentiated grade of OSCC (86; 61%). Out of 140 samples, PD-L1 positivity was observed in (87; 62.1%) of cases. A statistical significance was noted for PD-L1 with moderate differentiation of tumour. (P value: 0.006). The association of PD-L1 with other variables such as age, gender, ethnicity, sites or habits was statistically non-significant.

Conclusion: PD-L1 positivity was observed in OSCC patients, which is a subject of cancer immunotherapy in modern day oncology. Its significance with tumour grade is indicative of progressing tumour severity; although further studies need to be conducted in this domain to draw definitive conclusions.

Keywords: Programmed cell death 1 ligand 2 protein, B7-H1 antigen, carcinoma, squamous cell.

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Introduction

Oral squamous cell carcinoma (OSCC) is a subset of head and neck cancers which ranks 6th globally and account for up to 40% of all cancers in developing regions such as South East Asia. In this region, the prevalence of OSCC in Pakistan varies from 7.0 - 9.9% and it is reported as the 2nd most common malignancy in thecountry[1].

OSCC occurs between 3rd to 7th decades and a major portion of affected cases are men as compared to women[2,3]. Although female disposition towards oral cancers is also observed in various regions, a general trend towards male predominance is noted globally. In southern Pakistan, studies have reported a constant increase in the number of patients suffering from oral cancer. Most of the patients present at a later stage, which reduces their life span. The recent updates have also shown younger individuals carrying high risk for be-

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ing affected byoral cancer due to social practices and habits.

Multiple etiological factors such as habits of alcohol consumption, smoking and use of chewable tobacco products such as Paan, Gutka, Betel nut and Naswar serve as major contributors in developing OSCC. In the western world, the important elements of risk include smoking and alcohol abuse. Viral infection by HPV 16 and 18 are also the causative agents for developing this cancer. Genetic components, nutritional deficiencies and weak immune system also play an intrinsic role in causing oral cancer.

Clinically, the lesions of OSCC progress into exophytic masses and develop into non-healing ulcers. Additional symptoms may also consist of profusely bleeding ulcerative lesions, burning sensation of the mucosa of oral cavity, trismus, loosening of teeth, dysphagia, odynophagia and achalasia, which is accompanied by weight loss in severe cases.

The most common sites for OSCC lesions include buccal mucosa and tongue whereas other sub sites include lip, floor of the mouth and gingivae[4]. In the west, tongue and floor of the mouth are frequently involved sites. On the contrary, in Pakistan, cheek is mostly affected which is possibly because of keeping the tobacco products in close contact with buccal mucosa for an extended duration.

The diagnosis of OSCC is largely based on clinical evaluation, which can be aided by radiological examination using CT, MRI or PET scans. However, the gold standard for diagnosis of OSCC remains to be biopsy sample evaluation and the subsequent histopathological examination. The disease is associated with a poor prognosis, with an overall 5-year survival rate of 60%. Unfortunately, two-thirds of the tumours present with locally advanced or metastatic disease (stages III and IV)[5]. The reason for this is mainly late diagnosis and late presentation at the clinic for evaluation of oral lesions. Also, lack of awareness and oral screening programs in our region are a major concern for health facilitators. Particularly are the remote areas where even basic necessities are not provided and people are not aware of health related hazards in their environment as well as behaviours.

The present management of OSCC involves a multidisciplinary approach involving a panel of surgeons and oncologists with treatment modalities including surgery, radiation therapy and chemotherapy. However, the increasing burden of OSCC can be reduced by developing novel management therapies. In this regard, cancer immunotherapy has enthused excitement for its broader potential in treating cancers including those of the head and neck[6, 7].

Quite recently, PD-L1, an immune checkpoint inhibitor has gained significant popularity as it achieved a Nobel Prize in physiology and medicine for its potential as an immunotherapeutic agent.

PD-L1 (programmed death ligand 1), a transmembrane protein, is a negative immune checkpoint regulator that inhibits T cell proliferation whereby limiting T cell activity by engaging PD-1 receptor. Researches have explored its importance in many human cancers including head and neck carcinoma and breast carcinoma reporting a substantial translational worth[8]. Immunotherapies with PD-L1 monoclonal antibodies have been recently approved by FDA for treatment of non-small-cell lung carcinoma (NSCLC) whereas phase III clinical trials are currently underway in melanoma, breast cancer and urothelial cancers[9].

Studies of PD-L1 in OSCC have yielded diversified results. Some studies report it as a prognostic marker and some as a marker of early detection and malignant transformation[10].

Due to reported ambiguity in results, definite conclusions have not been drawn and it seems mandatory to conduct studies identifying the significance of PD-L1 in oral cancer.

PD-L1 in oral malignant lesions can explain its potential as biomarker for therapeutic intervention

and patient selection for immune therapy. This provides a rationale to conduct researches in our region of different population dynamics and prevailing risk of having oral cancer.

Thus, in this study we determined the expression of PD-L1 in biopsies bearing oral squamous cell carcinoma via immunohistochemical analysis, and related it to the clinicopathological parameters.

Subjects and Methods

This cross-sectional study comprising of 140 cases of OSCC was conducted at histopathology department, Ziauddin hospital, Karachi from December 2018 to August 2019. Sample size was calculated by keeping prevalence(p) at 10% (0.01), confidence interval at 95%, Z score at 1.96 and Absolute precision (d) at 0.05 by using the formula: $n= z^2 P (1-P)/d^2$

Samples were recruited using purposive sampling technique respectively. Demographic details and clinical features of patients diagnosed with OSCC were collected and noted down through a questionnaire, and consent was taken prior to the selection of cases. The diagnosis of OSCC was confirmed by experienced panel of histopathologists. Patients of OSCC who consented to be a part of this study, irrespective of age, sex and race were included. Malignancies other than oral squamous cell carcinoma and metastatic tumours to oral cavity were excluded from the study. For laboratory procedures, selection of most appropriate formalin-fixed paraffin-embedded biopsy blocks was done bearing copious amounts of tumour. Nearly 4 µm thick sections were cut to be stained with Hematoxylin and Eosin (H & E) for evaluation via light microscopy. WHO/Broder's classification was used to divide the tumour according to grades of differentiation. Immuno histochemical staining was done by applying PD-L1 monoclonal antibody (Cell marque Clone ZR3) according to the manufacturer's protocol.

PD-L1 status was evaluated using a light microscope and was analysed according to a scoring method adopted from related studies. A 4-tiered grading system was used and scores 0, +1, +2,+3 were given for the proportion of stained tumour cells respectively (Score 0: 0-<5%, +1: ≥5%- ≤30%, +2: ≥31%- ≤60%, +3: ≥61%- ≤100%). Staining was grouped as no staining, weak staining, moderate staining and strong staining. The positivity of PD-L1 expression is defined as at least 5 % of tumour cells exhibiting membranous staining at any intensity. This cut off value of 5% has been used in the majority of clinical trials in head and neck cancers. Scoring was done of viable tumour cells that exhibited complete, circumferential or partial linear plasma membrane staining at any of the mentioned intensities. Percentage of viable cells was also determined in the entire specimen. Immune cells, normal cells, necrotic cells and cytoplasmic staining were not taken into account for microscopic analysis and were excluded from scoring. Squamous lung tissue was taken as a positive control. The interpretation was carefully done and the slide specimens that did not show satisfactory staining were considered invalid. 4 x objective magnification was used to examine tumour areas on the slides. This was followed by using 10-40 x magnification to score and determine the percentage of viable tumour cells in the specimen.

The study was carried out after the approval of Ethics Review Committee (ERC). Ref: 0490918MAPAT.

For statistical analysis, SPSS version 25 was used. Quantitative variables such as age were expressed as mean and standard deviation. Qualitative variables such as gender, ethnicity, habits, sites and grades of OSCC were expressed as frequency and percentages. Chi square test was used to find association of PD-L1 with these variables. P value less than 0.05 was taken as significant.

Results

Out of 140 participants, PD-L1 positivity was observed in 62.1 % of cases (n= 87) and majority of cases showed moderate staining with a TPS (tumour proportion score) of +3 upon immunohistochemical analysis. Fig 1. shows H & E stained

specimen slide sections along with PD-L1 immuno stained counterparts at different intensities.

Majority of study subjects were males (74%, n= 103) as compared to females (26%, n= 37) which also comprised of the major portion of PD-L1 positive cases (76%, n= 66).The mean age of participants was 48.91 ± 11.7 years. Majority of them belonged to Urdu speaking ethnic background, (46%, n= 64).

For most affected anatomical locations, tumours arising from the buccal mucosa were most frequent (54%, n= 75), followed by tongue (14%, n= 19), lip (9%, n= 13) and other intra oral sites. Majority of participants had habits of chewing tobacco products including Paan, Gutka, Betel nuts (64%, n= 89). There were 24% of participants who smoked (n= 33) and remaining were involved in other habits like alcohol intake or naswar. According to differentiation, moderately differentiated tumours were present in larger number amongst all OSCC patients (61%, n= 86). This was followed by well differentiated tumours (29%, n= 41) and poorly differentiated tumours (9%, n= 13). Upon finding association of PD-L1 with these variables, a statistical significance was noted for PD-L1 status with moderately differentiated tumours as shown in Table 2. The p values for association of PD-L1 with other variables such as age, gender, ethnicity, sites or habits were non-significant in our results.

Discussion

The concept of immunoediting is listed as one of the important and newly established hallmarks of cancer. Through this, the cancer cells escape detection and normal immune surveillance mechanisms in the host's body and create a tumour microenvironment for their sustenance. It has been reported that cancer cells do this by overexpressing the PD-L1 protein, which is an immune checkpoint inhibitor, resulting in T cell exhaustion. Immunotherapy has its foundation on the fact that using anti PD-L1 treatment strategies could help restore T cell activity and have cytotoxic effects on tumour cells.

Exploring the role of PD-L1in OSCC has piqued interest in various regions due to its emerging translational significance. Despite ongoing research, the results have shown disparity owing to variations in methodologies applied. OSCC is prevalent in our region and the expression levels of PD-L1 have not been investigated in this population subset up to date.

In the present study, we found 61.2 % positivity of PD-L1 in 140 OSCC subjects. This percentage of positive cases is within the range of results provided in other studies i.e. 46-87%[5]. PD-L1 positivity was considered by adopting a cut off value of 5% with positive membranous staining. Literature research has shown variable results. Most studies have considered membranous and cytoplasm staining collectively or separately with variable cut off values for PD-L1 scores such as 1%, 10% or 5%.[11-13]. However, keeping up with the biological role of PD-L1, majority of studies and clinical trials have taken membranous staining into account with a cut off value of 5% for tumour cell proportion.

In our study the majority of subjects were males, which is in agreement with other studies that have shown that OSCC is a male predominant disease[14]. The reason of this is more frequent exposure of this gender to different risk factors like addictive use of tobacco and alcohol. Moreover, males as compared to females have far more access to tobacco products as they are better off in the society hence, involved into smoking and smokeless tobacco.

Several studies have mentioned that OSCC is common in older age group or from 3rd to 5th decades. Most cases in our study belonged to 5th to 6th decade age group. In the present study, PD-L1 positivity was observed more in males (n= 66) than females (n= 21).This is in agreement with a study by Lin et al that reported increased PD-L1 positivity in male gender as compared to females. The association of PD-L1 with age or gender was non-significant in our study. (p=0.838, p=0.276). In contrast, studies also found higher expression of PD-L1 in fe-

males and some also showed no gender related significance of PD-L1[15,13,16].

Table 1. Distribution of PD-L1 in all cases of oral squamous cell carcinoma (OSCC)

PD-L1 Expression	Distribution % (n)
Positive	62.1(87)
Negative	37.9(53)
Total	n= 140

Table 2. Association of PD-L1 with clinicopathological and demographic features of all study participants

OSCC Patients n= 140	Percentage %(n)	PD-L1 positive n	PD-L1 negative n	p- value
Gender				
Male	74(103)	66	37	0.27
Female	26(37)	21	16	
Mean age of participants: 48.91 ± 11.7				
Age Groups				
21-30	03(04)	03	01	
31-40	17(24)	16	08	
41-50	32(45)	25	20	
51-60	31(44)	29	15	0.83
61-70	11(16)	09	07	
71-80	05(07)	05	02	
Ethnicity				
Urdu Speaking	46(64)	38	26	
Pathan	18(25)	18	07	
Sindhi	07(10)	05	05	
Punjabi	06(08)	05	03	0.79
Balochi	08(11)	08	03	
Memon	11(15)	08	07	
Others	05(07)	05	02	
Habits				
Smoking	24(33)	22	11	0.34
Alcohol	04(05)	02	03	0.27
Gutka	27(38)	22	16	0.32
Betel nut	14(19)	13	06	0.36
Naswar	09(13)	09	04	0.41
Pan	23(32)	19	13	0.43
Sites of OSCC				
Buccal Mucosa	54(75)	45	30	0.35
Tongue	14(19)	11	08	0.43
Lip	09(13)	11	02	0.06
Labial Mucosa	06(08)	06	02	0.36
Palate	06(09)	04	05	0.21
Floor of mouth	04(06)	03	03	0.41
Alveolar Ridge	07(10)	07	03	0.43
Grades of OSCC				
Well Differentiated	29(41)	21	20	0.06
Moderately Diff.	61(86)	61	25	0.006*
Poorly Diff.	09(13)	05	08	0.063

P- Value for Association of PD-L1 with clinicopathological and demographic features.
*Chi-square test * Represents significant association

Ethnic background of a study population is essential to consider as it carries variable genetic ancestry and a diversified cultural impact. Oral cavity cancers are mostly prevalent in Karachi, which is a hub of multiple ethnicities. These include immigrants from India, the Urdu speaking community, and natives like Sindhis, Punjabis and Balochis. Pathans, Memons and Christians also form a subset of the Karachi population. All these ethnicities are habitual of different types of smoking and chewable habits. The majority of participants in our study belong to Urdu speaking sect. This finding was also observed in other studies conducted in this city[16]. Most participants are habitual users of chewable tobacco products as this habit has been intertwined in their culture from Indo Pak region since pre partition era[3].

Tobacco usage holds a hazardous risk for the development of most cancers. This is because tobacco contains many carcinogenic compounds. In our society tobacco is smoked in the formed of rolled cigarettes (bidi), cigar and water pipe smoking (sheesha). Reverse smoking has similarly proven to be as hazardous as other habits. Chewing betel nuts, paan and using naswar is also not uncommon. Most of these habits are unfortunately

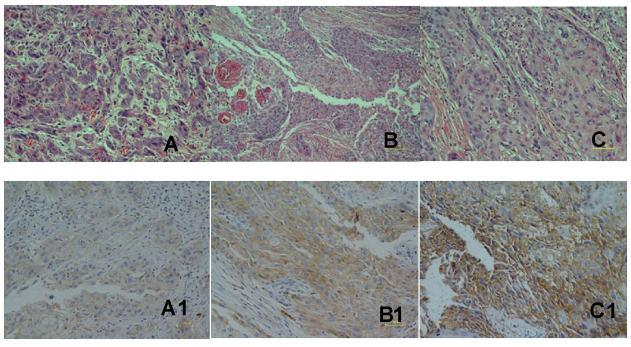

Fig 1. Photomicrographs (A,B,C) showing H & E stained sections of OSCC Tissue; 10x Magnification and PD-L1 Immuno stained sections (A1,B1,C1) exhibiting (A1) Weak, (B1) Moderate and (C1) Strong immune reactivity; H&E with membranous staining (arrows).

popular among women too. In Karachi, women are mostly habitual of consuming paan containing tobacco additives. Such habits have exposed our population subset towards higher risk of carcinogenesis.

In our study, we correlated expression of PD-L1 with habitual risk factors. Interestingly, we found majority of females consuming chewable tobacco products and males involved into habits of smoking. However, the association of PD-L1 with these habits was non-significant. Studies have shown variation of PD-L1 expression in relation to habitual risk factors of OSCC. According to a multivariate analysis, Lin et al correlated increased expression of PD-L1 with a poor prognosis in smoker men[17]. Studies have also reported no significance of PD-L1 expression level with smoking and alcohol intake[18]. As the majority of studies have correlated PD-L1 expression with smoking and alcohol consumption, our study is the first to report an association of PD-L1 with chewable tobacco products including betel nuts, paan and naswar.

Buccal mucosa is the usual intra oral site affected by oral malignancies in Pakistan and India[19,20]. Similar finding is reported in our study. Tobacco derived products are placed here where they exert an oncogenic effect. Studies conducted for association of PD-L1 expression with various anatomical sites have shown variable results. Troeltzsch et al reported a high expression of PD-L1 in structures related to the mandible or tongue in comparison with soft palate or Maxilla. On the other hand, Satgunaseelan reported higher PD-L1 expression in tumours arising in buccal and lingual mucosa than floor of the mouth or gingivae. One of few studies showed no relation of PD-L1 with anatomical subsites[21]. Likewise, inour study, we have not found any statistical significance of PD-L1 levels with any intra oral sub sites; however, we found a larger number of tumours affecting the buccal mucosa.

Tumour grade is one of the important histopathological parameter that helps predict the treatment and prognosis of tumours. According to WHO, there are three grades of tumours which are classified on the basis of degree of differentiation of tumour cells. The three grades are namely, well differentiated, moderately differentiated and poorly differentiated tumours. Well differentiated cancers have better prognosis than poorly differentiated cancers. In this study, out of the three tumour grades, we found a major portion to be of moderately differentiated tumours. This finding agrees with related studies in our region that report more occurrences of moderately differentiated oral cancers diagnosed at the time of presentation[3]. We also reported a statistical significance with positive PD-L1 expression and moderately differentiated carcinoma.

Literature reveals few studies correlating PD-L1 expression with tumour grade. One of these associated PD-L1 expression with poorly differentiated oral cancer lesions[21]. Cho et al in 2011 stated that well differentiated tumours had lower expression of PD-L1 in comparison with moderately differentiated tumors[22]. Much recently, Maruse et al reported that positive expression of PD-L1 associated with nodal metastasis and dismal prognosis but not with histological grade[23]. In our study, we report a strong significance of PD-L1 expression in moderately differentiated tumour grade (p value: 0.006). Moderately differentiated tumours correspond to worsening prognosis in oral cancer.

OSCC is present in majority of people who are consumers of chewable tobacco products with buccal mucosa being the commonest site of infestation by this tumour.

The role of PD-L1 is essential to investigate as it has proven to be of importance in the new era of immune therapeutics. Elaborated work needs to be done in this domain. Oral cancers are on the rise and the issue needs to be addressed globally and where the access to basic health facilities is denied[24]. Treatment by immune checkpoint inhibitors could turn the tides for oral cancer management, which are an endemic in southeastern Asia including Pakistan.

This, to the best of our knowledge, is the first research conducted in our population subset to investigate the role of PD-L1 in oral squamous cell carcinoma and its correlation with clinicopathological features along with habitual predisposing factors.

Although we successfully established relation of PD-L1 with certain parameters, we present some limitations. The value of PD-L1 in studies hitherto have shown plentiful of discordance in results. This, in accordance with our findings, warrants further research emphasizing the role of PD-L1 comprising of larger sample size, control groups for comparisons, and to standardize the scoring methods in PD-L1 assays. Lastly, limited resources prevented us from applying much robust techniques for improved sensitivity in validating our results. Studies to investigate the prognostic role of PD-L1 in oral cancers and in premalignant lesions could lead to a better insight of PD-L1 in this tumour entity and for treatment options via immunotherapy.

Conclusion

The outcome of our study revealed that PD-L1 has relevance with moderately graded tumours and the relation of PD-L1 with age, gender, habits and sites is not significant.This is the first study that has associated PD-L1 expression with habits of chewable products as well as smoking, alcohol and naswar. Its significance with histopathological grades of oral squamous cell carcinoma is indicative of progressing tumour severity and dismal prognosis. This knowledge could prove useful in future research and management of oral cancer in the light of immunotherapy.

Conflict of Interest

The authors declare no conflict of interest, and all authors have studied and approved the final manuscript.

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