Original Research paper

The expression of PD-L1 within Stages of Oral Squamous Cell Carcinoma: Immunohistochemical Analysis.

ABSTRACT

Aims: To determine the immunohistochemical expression of PD-L1 in oral squamous cell carcinoma and to find an association of PD-L1 with stage and clinicopathological parameters of oral squamous cell carcinoma.

Study Design: Cross-sectional study.

Place and Duration of Study: Ziauddin Medical University, Karachi, 1 Year duration during 2018-2019.

Methods: A total number of 140 biopsy confirmed cases of oral squamous cell carcinoma were recruited in the study. Immunohistochemical expression of PD-L1 was evaluated and associated with the clinicopathological parameters of OSCC. The data was statistically analyzed through Descriptive statistics and Chi square test by using SPSS v.20.

Results:

Out of 140 participants, 74% were males (n=103) and 26% were females (n=37). PD-L1 positivity was observed in 62.1 % of cases (n=87). The Mean age of the participants was 48.91 ± 11.7 years. The most common site of cancer involvement was buccal mucosa and majority of participants were habitual of consuming chewable products i.e. Pan, Gutka and betel nut (89; 64%). Stage III and IV tumours comprised a major portion of OSCC cases in our study. (52; 37%), (56; 40%). A statistically significant *p*-value was noted for the association of PD-L1 with stage II and IV tumours. (P-values: 0.029, 0.001)The association of PD-L1 with other variables such as age, gender, ethnicity, sites or habits was not statistically significant.

Conclusion: This study concludes that the statistical significance of PD-L1 expression with tumuor stage is suggestive of worsening prognosis and might have detrimental effects as tumour progresses in advanced stage. PD-L1 positivity in OSCC patients could be useful in future research in the light of cancer immunotherapy which has shown success in the field of oncology.

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Keywords: Programmed Death Ligand -1(PD-L1); Squamous cell carcinoma; Immunotherapy; Immune checkpoint protein;

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INTRODUCTION

Melanesia and South East Asian countries have observed a dramatic increase in the prevalence of oral squamous cell carcinoma (OSCC), ranking it as 16th most common cancer worldwide as per GLOBOCON 2018. (1) Pakistan has a reported prevalence of 10 % and oral cancers are the 2nd most prevalent

malignancy in this country with 18,881 new cases reported, in both sexes and all age groups in the year 2018. (1)

Oral cancer is present amongst 3rd to 7thdecade age groups and most diagnosed cases are of the male population as compared to the female gender.(2, 3). A multitude of causative factors result in carcinogenesis and development of this tumour. (4) These factors maybe Extrinsic, such as alcohol consumption or use of tobacco and addictive chewable products, or Intrinsic, which mainly involve genetic elements, viral infections, nutritional deficiencies and most importantly deficiencies in the immune system that result in immune suppression and cancer development. (4) The habits of using tobacco products and chewable items such as Gutka, Paan, chalia are common in the population subset of Pakistan.

Oral cancers begin as premalignant lesions and over a period of time result in bleeding, painful ulcers appearing as exophytic growth with life threatening conditions. (5) (6) (7)Oral inspection and radiological techniques aid in diagnosis, however, histopathological examination remains the gold standard for diagnostic purpose. (8) Oral cancer bears poor prognosis, having a 5-year survival rate of 60% according to literature. Almost two-thirds of oral tumours are associated with regional or distant metastasis i.e. in stages III and IV respectively. Metastasis of OSCC usually involves ipsilateral cervical lymph nodes and distant metastases to lungs and esophagus (9) Tumour grade and stage serve as tools to determine prognosis. Regardless of myriads of treatment modalities, the overall survival and prognosis remains to be poor up to date. Around 30 % of affected individuals die because of recurrence and distant metastatic spread.

Advances in treatment have recently focused on the use of immune checkpoint inhibition to treat cancers. Programmed death ligand 1(PD-L1) has yielded promising results in management of various carcinomas including Non small cell lung carcinomas, Melanoma and those of the head and neck. . (10, 11) (12-14). (15, 16) (17, 18)

PD-L1 is an immune checkpoint protein which negatively modulates immune cell responses by involving the PD-1 receptor which is present on T lymphocytes. The PD-L1 overexpressing tumour cells result in exhaustion of T cells which enable tumour growth, proliferation and escape from immune surveillance

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mechanism. PD-L1 has been linked with poor prognosis in some studies and its expression level has been reported to be higher with advanced stage cancers. (19) (20) (21)

Blocking this PD-L1/PD-1 pathway by Anti PD-L1 therapy may restore T- cell function and anti tumour activity. This concept has brought the novelty of this biomarker to usefulness in the field of cancer immunotherapy. Studies are rapidly being undertaken to explore the expression of PD-L1 in oral malignancies and have presented a series of diversified results warranting further research in this domain. In such phase of ongoing researches we aimed to determine the relation of PD-L1 expression with the stages of oral cancer along with clinical parameters.

METHODS

This was a cross sectional study which was carried out at Ziauddin hospital during the years 2018 to 2019. A total number of 140 cases of OSCC were recruited in the study by purposive sampling technique and Consent was obtained preceding the selection of cases. The clinical parameters and demographic characteristics of patients diagnosed with OSCC were collected through a questionnaire. The diagnosis of cases was based on clinical and histopathological evaluation. Patients diagnosed with OSCC, irrespective of gender, age and ethnicity were included in the study and patients with other malignancies and tumours with a metastatic spread to oral sub sites were excluded.

Following this, laboratory procedures were carried out. Paraffin- embedded formalin-fixed biopsy blocks were selected. Around 4 µm tissue sections were cut and stained with Hematoxylin and Eosin (H & E) for observation through light microscope. Tumour staging was done by applying TNM staging system given by AJCC 7th Edition. .(22)

Immunohistochemical Analysis

Monoclonal antibody for PD-L1 (Cell marque Clone ZR3) was applied for immunohistochemistry as per the instructions given by the manufacturer. (Cell Marque, catalog No. 438R-25). Evaluation of PD-L1 staining was done via light microscopy and this was followed by scoring system mentioned in similar studies. A four tiered Grading method was adopted and different scores were labelled for the stained

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cancer cells percentage. Score 0 was labelled as no staining with 0- <5% of tumour cell percentage. Score +1 was labelled as weak staining with ≥5%- ≤30% tumour cell percentage. Likewise, scores +2 and +3 were labelled as moderate and strong staining with tumour cell percentage of ≥31%- ≤60%, and ≥61%- ≤100% respectively. PD-L1 positive expression was defined as at least 5 % of cancer cells showing membranous staining at any of the mentioned intensities. The cut off figure of 5% has been applied in the clinical trials involving Head and cancers.(23, 24) SPSS version 20 was used for statistical evaluation

The percentage of viable tumour cells that showed complete, circumferential or partial linear plasma membrane staining at any intensity were considered for scoring. Immune cells, normal cells, necrotic cells and cytoplasmic staining were excluded. Squamous lung tissue was taken as a positive control. 4X objective magnification was used to examine tumour zones on the slides and 10-40x magnification was used to score viable tumour cells in the entire specimen.

Statistical Analysis

Quantitative data were expressed as mean and standard deviation, whereas, qualitative data were expressed as frequency and percentages. The association of PD-L1 with all variables was determined by applying the Chi square test. *P* value of less than 0.05 was taken as significant. The study was carried out after the approval of Ethics Review Committee (ERC).

RESULTS

Out of 140 participants, around 74% were men (n=103) in contrast to women (26%, n=37). PD-L1 positivity was found in 62.1 % of samples (n=87) and the major portion of PD-L1 positive cases belonged to males. (76%, n=66). The majority of cases showed moderate staining with a score of +3 for Tumour proportion Score (TPS). (Table 1 & 2)

Table 1. Distribution of cases according to Staining Intensity of PD-L1.

Staining Intensity	Distribution % (n)

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No Staining	37% (53)
Weak Staining	13%(18)
Moderate Staining	26%(36)
Strong Staining	24%(33)

Table 2. Distribution of case according to Scoring of PD-L1.

TPS Scores		**Distribution %(n)**
0	**0 - <5 %**	37%(53)
+1	**≥ 5 % - ≤30 %**	21%(29)
+2	**≥ 31 % - ≤60 %**	19%(26)
+3	**≥61 % - 100 %**	23%(32)

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The expression of PD-L1 via immunohistochemistry according to various intensities in tissue specimens of OSCC is shown in Figure 1 and Control Tissue in Figure 2. The mean age of participants was

calculated to be 48.91 ± 11.7 years. The clinical and demographic details of all participants are shown in Table.3

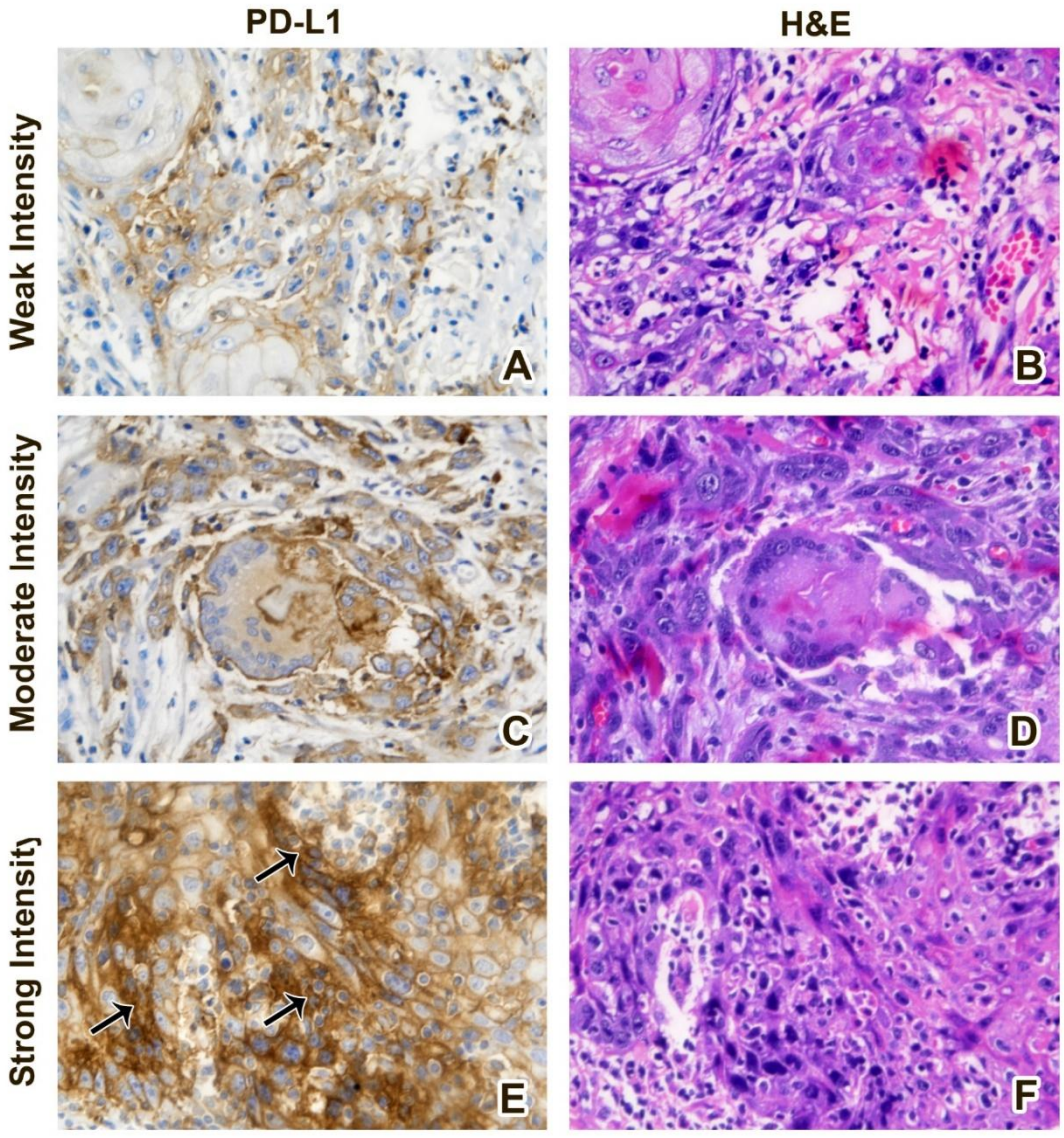

Figure 1. Photomicrograph (B,D,F) showing H & E stained sections of OSCC Tissue;40x Magnification and PD-L1 Immuno stained sections (A,C,E) exhibiting (A) Weak, (C) Moderate and (E) Strong immune reactivity; 40 x Magnification, showing Strong membranous staining (arrows).

OSCC Patients n = 140	Percentage %(n)	PD-L1 positive %(n)	PD-L1 negative %(n)	*p*- value*

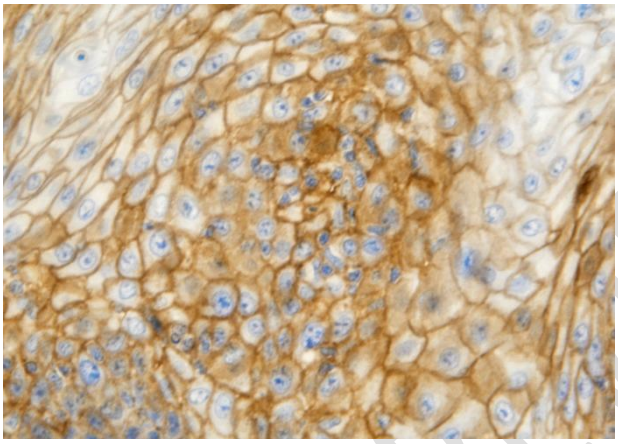

Figure 2. Photomicrograph of squamous cell carcinoma (SCC) of human lung control exhibiting membranous immunoreactivity. (40x magnification)

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Table 3. The association of PD-L1 with clinicopathological parameters of all study participants.

Gender Male	74(103)	76(66)	70(37)	0.27
Female	26(37)	24(21)	30(16)	
Mean age of participants: 48.91 ± 11.7				
Age Groups 21-30	03(04)	03(3)	2(01)	0.83
31-40	17(24)	18(16)	15(08)	
41-50	32(45)	29(25)	38(20)	
51-60	31(44)	33(29)	28(15)	
61-70	11(16)	10(09)	13(07)	
71-80	05(07)	6(05)	4(02)	
Ethnicity Urdu Speaking	46(64)	44(38)	50(26)	0.79
Pathan	18(25)	21(18)	13 (07)	
Sindhi	07(10)	6 (05)	9 (05)	
Punjabi	06(08)	5 (05)	6 (03)	
Balochi	08(11)	9 (08)	6 (03)	
Memon	11(15)	9 (08)	13 (07)	
Others	05(07)	6 (05)	4 (02)	
Habits Smoking	24(33)	25 (22)	21 (11)	0.54
Alcohol	03(05)	2 (02)	6 (03)	0.29
Gutka	27(38)	25 (22)	30 (16)	0.52
Betel nut	14(19)	15 (13)	11 (06)	0.54
Naswar	09(13)	11 (09)	8 (04)	0.58
Pan	23(32)	22 (19)	24 (13)	0.71
Sites of OSCC Buccal Mucosa	54(75)	52 (45)	57 (30)	0.57
Tongue	14(19)	13 (11)	15 (08)	0.68
Lip	09(13)	13 (11)	4 (02)	0.79
Labial Mucosa	06(08)	7 (06)	4 (02)	0.44
Palate	06(09)	4 (04)	9 (05)	0.26
Floor of mouth	04(06)	3 (03)	5 (03)	0.53
Alveolar Ridge	07(10)	8 (07)	6 (03)	0.59
Stages of OSCC Stage I Stage II Stage III Stage IV	 10 (14) 13 (18) 37 (52) 40 (56)	 9 (08) 8 (07) 32 (28) 51 (44)	 11 (06) 21 (11) 45 (24) 23 (12)	 0.68 0.029* 0.12 0.001*

*Chi-square test; * Represents significant association

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Most of these participants were of Urdu speaking ethnicity (46 %, n=64). Buccal mucosa was the most frequent anatomical location affected by tumour (54%.n=75). This was followed by 14% cases of the region of tongue (n=19), lip region (9%, n=13) and other anatomical sub sites. Majority of individuals were habitual of using chewable items including Gutka, Paan, and chalia or Betel nut. (64%, n=89). 24% of participants were smokers (n=33) and rest of them were habitual of alcohol consumption or using Naswar. According to TNM staging, in our study stage III and IV formed a major portion of cases (52; 37%), (56; 40%). This was followed by stage II oral cancers (18; 13%) and stage I (14; 10%) respectively. Statistical analysis was done to find an association of PD-L1 with all the parameters studied in our research. A significant *p* value was observed for PD-L1 in relation to stage II and IV tumours as shown in Table 3. There was no statistically significant *p* value reported for the association of PD-L1 with the rest of the parameters such as gender, age, ethnical background, anatomical sub sites and habits.

DISCUSSION

The immune checkpoint inhibitor, PD-L1, has emerged as a novel biomarker due to its increasing translational significance in various malignancies. (25, 26). Regardless of continuing research, the results have shown inconsistency over time probably due to difference in methodologies used. Oral cancer is a prevalent cancer in Pakistan and PD-L1 expression levels have not been explored in this population subset as yet. Moreover, the low socio economic status has compromised the health care system which could enable easy access and treatment for a multitude of cancer types prevalent in the region. This has resulted in poor prognosis and reduced survival rates in patient population.

In the current study we observed 61.2% PD-L1 positive cases of OSCC. This value of positive PD-L1 percentage falls within the range of 46-87%, which is the range mentioned in the results of related studies.(9) Such a range of variable percentage of PD-L1 positivity is probably due to using different cut off points, considering cytoplasmic and/or membranous staining for scoring or probably the use of different immune assays for PD-L1 with different antibody detection methods.(9).

Literature has mentioned studies that have adopted varying cut off points for PD-L1 scoring as well as interpreting cytoplasmic and membranous staining collectively or independently for scoring purpose.(27-

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29). In our study, positivity of PD-L1 was defined by taking on a 5% cut off value exhibiting exclusive membranous staining which is in accordance with the physiological function of PD-L1.Also, majority of researches as well as clinical trials have undertaken membranous positivity of PD-L1 with a 5% cut off point for proportion of stained tumour cells.

As per our findings, the majority of participants were men which is in line with most international studies that have reported that oral cancer is predominantly diagnosed in males. (30-32). Male preponderance is likely due to exposure of males to etiological factors like regular usage of tobacco products and alcohol intake. (33). The age of most participants in our study was between to 5^{th} to 7^{th} decades. These findings are consistent with findings in other studies.(34, 35). Variable lifestyle in all ages, poor oral hygiene and habits play a key role in development of OSCC in South Asian countries(36). In the current research, PD-L1 positivity was mostly seen in male gender (n-66) than in females. (n=21).This agrees with Lin et al that has reported higher expression of PD-L1 in males. In contrast, some studies have also reported increased expression of PD-L1 in female gender, whereas, few studies have not reported any significance of PD-L1 linked with gender.(37, 38) , (39, 40). Although predominance in males and early onset of cancer was observed, we did not find a statistical significance of PD-L1 expression with age and gender which is in agreement with some studies. (37, 38).

Ethnicity of a population carries a diversified genetic lineage and cultural influence. In Pakistan, most oral malignancies are in south of Karachi in which multiple ethnicities reside such as Urdu speaking community, natives like Sindhis, Punjabis, Balochis and Pathans etc. All these ethnicities are habitual of different types of smoking and chewable habits.(3) Major portion of the study participants belong to Urdu speaking background. This finding is reinforced by other regional studies.(41) (32, 42-44) Most participants use chewable products and tobacco as this habit has been an element of the Indo Pak culture. (3) (45)

As far as significance of PD-L1 with ethnicity is concerned, we did not find a significant statistical link of PD-L1 expression with this parameter. This is in parallel with studies showing little or no evidence of the importance of PD-L1 with racial predisposition or ethnic consideration in study participants. These studies included Asiatic population, Caucasians and African Americans as their study participants. (46, 47)

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Most of our study participants were smokers and consumers of Gutka, and Paan. This finding was also reported in regional studies.(31, 32, 41) However, the link of these habits with PD-L1 was not significant in our research. The overall literature to link the expression of PD-L1 with habitual risk factors of OSCC like smoking, alcohol usage and chewing carcinogenic products, is weak and conclusions have not been drawn. (37). (21).

The most common site of oral cavity affected by oral malignancies in Indo- Pak region is the buccal mucosa which is similarly reported in our study.(48, 49). Products consisting of tobacco and other additives are placed in the buccal vestibule eliciting a carcinogenic effect. Troeltzsch et al reported high PD-L1 expression in structures connected to tongue or mandible as compared with soft palate or Maxillary structures. On the contrary, Satgunaseelan reported increased PD-L1 expression in cancer originating in bucco lingual region than from the floor of mouth or gingival structures. Our study, showed no statistical link of PD-L1 in relation with anatomical locations affected by OSCC, which is in agreement with a study conducted in Japan. (50). However, we noted most cases of oral tumours involving the buccal mucosa which is attributed to the chewable habits prevalent in our society.

Tumour stage is an essential tool to determine prognosis of cancers. (51) The stages I to IV are according to the worsening prognosis and severity of cancer. Early detection of tumours is effective for improved survival therefore early stage cancers confer better outcomes.(52).

Our study reports higher prevalence of stage III & IV tumours which is in line with local studies that have reported a higher prevalence of advanced stage of oral cancers. (3, 31, 32) (21).

Some studies also report early stage presentation of oral cancers i.e. stage I and stage II.(32, 53)

In this study we found a significant *p* value for the association of PD-L1 with stage II and stage IV tumours. The significant association of PD-L1 with late stage agrees with previous studies, suggesting that tumour aggressiveness is linked to tumour immune resistance.(24, 40). However, some previous studies also reported no prognostic significance of PD-L1 with oral cancers.(40)

We believe that the significant association of PD-L1 with stage II tumours in our study is probably due to limited number of stage II cases of OSCC in our sample set.

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Advanced stage of presentation is due to lack of awareness and illiteracy amongst the people who are socio economically poor and with compromised living standards with lack of professional care set ups for routine checkups and early diagnosis.

Prolonged delays in presentation of oral cancers is associated with late stage disease. (54). It would therefore, be wise to inform the general public about tumour symptoms which would enable earlier visits to health care institutions. (55)

Extensive research needs to be done on PD-L1 as it is essential in the area of cancer immune therapeutics. (51) Treatment approach by incorporating immune checkpoint inhibitors could be a new turn for oral cancer management in economically compromised countries. This, to our information is the initial study carried out in the sub group of Pakistani population to find an association of PD-L1 with the clinicopathological characteristics of oral squamous cell carcinoma.

Although we observed positive findings, a few limitations and recommendations of the study have been reported. Elaborated research stressing the mechanistic role of PD-L1 consisting of a larger sample size and inclusion of a control group would have provided an opportunity to determine and compare the role of PD-L1 in progression of a dysplastic lesion to carcinoma, Studies to explore the value of PD-L1 in oral pre-cancerous lesions could also give a better understanding of PD-L1 in this cancer. Limited budget prevented us from utilizing other immune checkpoint proteins in immune assay along with PD-L1 for analysis and comparison of results in our study. Moreover, inclusion of blood samples might as well be helpful in comparing the data with regards to PD-L1 evaluation in peripheral blood and tissue specimens.

CONCLUSION

To conclude our findings, we support the literature stating that oral cancer is present in individuals who consume tobacco and chewable items and that buccal mucosa is the most frequent site involved in this tumour in the Pakistani population subset.

We have observed that PD-L1 is quite frequently expressed in OSCC biopsy specimens, which could have a meaningful impact in OSCC .The analysis reported that PD-L1 is linked with advanced stage of the disease which suggests increasing tumour severity and poor prognosis. This knowledge could prove useful in initiating future research and management of oral cancers in the light of immunotherapy.

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Immune checkpoint therapy could be a novel application in the management of oral cancer which is a prevailing disease of concern in South Asian countries including Pakistan

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