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Comparative analysis of PDL1 and cluster of differentiation 68 marker expression in oral squamous cell carcinoma patients: Correlation with depth of invasion and immunofluorescence through immunohistochemistry

Mehdi Shahabinejad, Siavash Bagheri Shirvan, Golnaz Fatemi, Farnaz Mohajer Tehran

Department of Oral and Maxillofacial Pathology, Oral and Maxillofacial Diseases Research Center, Faculty of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran

ABSTRACT

Background: Over the past 5 years, the use of immune checkpoint inhibitors in the treatment of head-and-neck squamous cell carcinoma (HNSCC) has increased. Both programmed death-ligand 1 (PD-L1) and cluster of differentiation 68 (CD68) are overexpressed in various carcinomas. Consequently, evaluating the expression of CD68 and PD-L1 in HNSCC lesions may lead to detecting a possible marker for HNSCC. This study aimed to evaluate the expression of PDL1 and CD68 markers in a patient with oral squamous cell carcinoma (OSCC) and examine its relationship with depth of invasion (DOI) and immunofluorescence (IF) through immunohistochemistry.

Materials and Methods: This cross-sectional study was conducted in the School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran, Department of Oral and Maxillofacial Pathology. Thirty-four paraffin blocks and demographic information of 15 female and 19 male OSCC patients were collected. Following sample preparations, immunohistochemical staining was performed. Subsequently, each tissue section was analyzed for tumor-infiltrating lymphocytes by CD68 marker and PD-L1 expression. Data analysis was conducted using SPSS software (version 25). Chi-square, Shapiro–Wilk, and independent *t*-analytical tests were employed for statistical assessments. $P < 0.05$ was remarked as statistically significant.

Results: CD68 and PDL1 expression in the squamous cell carcinoma (SCC) group was higher than the control group ($P < 0.001$). There was an increasing expression of PDL1 and CD68 as the grade of the disease progressed ($P < 0.001$ for each), as well as an increasing expression of IF and DOI.

Conclusion: The expression levels of CD68 and PDL1 were elevated in SCC tissues in comparison to the unaffected, healthy parts of the tissue section.

Key Words: Cluster of differentiation 68, diagnosis, immunofluorescence, oral cancer, programmed death-ligand 1

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Address for correspondence:
Dr. Siavash Bagheri Shirvan, Dental Research Center, Mashhad University of Medical Science, Azadi Sq., Mashhad, Iran.
E-mail: siavash.bagheri.shirvan@gmail.com

INTRODUCTION

Each year, approximately 880,000 new cases of head-and-neck squamous cell carcinoma (HNSCC)

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are diagnosed worldwide, representing a leading cause of death in some countries. At present, surgical or definitive radiotherapy can be used to treat early diagnosed cases effectively. Over the past three decades, overall survival rates for advanced cancer have improved only modestly and are only 50%–65% despite the addition of surgical resection with radiotherapy or chemotherapy.[1]

There has been an increase in the use of immune checkpoint inhibitors (ICI) in HNSCC treatment over the past 5 years.[1] A transmembrane protein termed programmed death-ligand 1 (PD-L1) is expressed in tumor cells and immune cells that infiltrate the tumor.[1] It inhibits cell-mediated immunity by interacting with the programmed cell death protein 1 (PD-1 or cluster of differentiation 279 [CD279], expressed on activated T-cells).[1] Evidence also exists that PD-L1 expression by tumor cells is associated with a poor prognosis in many carcinomas, including renal cell carcinoma, cervix squamous cell carcinoma (SCC), and hepatocellular carcinoma. In both invasive and noninvasive HNSCC tumors, PD-L1 is frequently overexpressed[2] and aimed at comparing clinicopathologic characteristics and outcomes associated with tumor PD-L1 expression have yielded conflicting results.[3]

Hsu *et al.* showed that higher PD-L1 expression is correlated with worse overall survival in nasopharyngeal carcinoma.[4] Nevertheless, in a cohort study of oropharyngeal SCC patients who had undergone surgery with adjuvant therapy or received definitive radiation with or without concurrent chemotherapy, no association was observed between PD-L1 expression and nodal disease, tumor, node, and metastasis stage, or other clinical parameters.[5]

CD68 is a highly glycosylated type I transmembrane glycoprotein that is primarily associated with the endosomal/lysosomal compartment.[6] CD68 belongs to the glycoprotein family lysosomal-associated membrane protein (LAMP) and shares structural similarities with LAMPs.[6] While the precise function of CD68 is not fully understood, its predominant localization within late endosomes suggests a potential role in peptide transport or antigen processing.[7]

CD68 is frequently employed as a pan-macrophage marker, and its expression has been linked to a poor prognosis in breast and hepatocellular carcinoma.[8] The most reliable tumor-associated macrophage (TAM) marker is CD68, expressed on both M1 and M2 phenotypes. According to Ni *et al.*, lymph node metastases and high tumor grade were associated with CD68 + TAM infiltration in SCC tumor stroma. Shortened overall survival was associated with a higher presence of TAMs, although TAMs were not identified as an independent predictor.[9]

Several studies have been conducted to evaluate the association between PD-L1 expression and various cancers; however, few studies have been conducted to investigate the effect of PD-L1 expression on SCC. To our knowledge, no study has examined the influence of PD-L1 and CD68 expression on immunofluorescence (IF) and depth of invasion (DOI) in SCC patients.

MATERIALS AND METHODS

Sample selection

A cross-sectional study was conducted with 34 oral SCC (OSCC) paraffin blocks, 15 females and 19 males, with healthy surgical margins (internal control group) collected from the School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran, Department of Oral and Maxillofacial Pathology. All participants provided written consent before the use of OSCC samples. OSCC samples were approved by the Mashhad University Ethics Committee (IR.MUMS.DENTISTRY.REC.1401.052). OSCC paraffin blocks with high quality and primary tumors showing no recurrence were included. Demographic and medical histories of all patients were gathered. Samples failing to meet quality standards, exhibiting unsuitable fixation, or containing necrotic or bloody regions were excluded from this study.

Tissue sample preparation

A comprehensive examination was performed for all patients, and demographic information was collected. After being fixed for 72 h in a neutral-buffered formalin solution, tissues were embedded in paraffin blocks. Sections of the paraffin blocks were then cut off to a thickness of 1 mm. Two sections were required for H and E staining to confirm the tumor samples' stage and grade.

Immunohistochemically staining

A 4-mm tissue section was placed on a glass slide and coated with poly-L-lysin. The slides were deparaffinized with xylene and rewashed in alcohol for 5 min, followed by 20 min at 98°C in Tris-ethylenediaminetetraacetic acid buffer (pH = 8). After cooling at RT for 20 min, the tissue samples were

washed with a Tris-buffered saline (TBS) buffer and placed on hydrogen peroxide 3% for 10 min to reduce background reaction. The slides were subsequently rinsed with TBS buffer once more. Using the primary PD-L1 and CD68 antibodies, samples were incubated with this antibody for 40 min and washed with TBS, where the procedure was performed based on protocol with optimal laboratory conditions. Afterward, the slides were incubated with a postprimary block solution (Leica Co, United Kingdom) for 20 min. The slides were then rewashed with TBS again. Next, Novolink Polymer (DAB included, Germany) was applied for 20 min. The slides were rewashed with TBS buffer and then incubated with diaminobenzidine peroxidase substrate (ref. MAD-021540Q-125). This was followed by washing the slides with tap water, putting them on hematoxylin for 3 min, rewashing them with tap water, rewashing them with graded alcohol, and examining them under the microscope.

Under a lens with ×100, each tissue section underwent analysis for tumor-infiltrating lymphocytes using the CD68 marker and for PD-L1 expression. Two separate oral and maxillofacial pathologists examined the samples with LABOMED LX400 microscope. The evaluation was based on the color of tumor infiltration staining, and CD68 and PD-L1 were assessed according to the following criteria: absence (fewer than five positive cell numbers: Score 0), low (5–25 positive cell numbers: Score 1), moderate (25–75 positive cell numbers: Score 2), and strong (more than 75 positive cell numbers: Score 3).[10]

Statistical methods

The demographic information of OSCC patients and the result of immunohistochemistry (IHC) staining were analyzed by SPSS software, version 22 (SPSS Inc., Chicago, IL, USA). The expression of PDL1 and CD68 markers and the staining strength in TME were compared by Chi-square, Shapiro–Wilk, and independent *t*-analytical tests. $P < 0.05$ was remarked statistically significant.

RESULTS

This study examined the incidence of PD-L1 and CD68 markers in 10 samples from the control group and 24 from the SCC group. A total of 34 patients were examined, including 15 females (44.1%) and 19 males (55.9%). There was no significant difference between the margin and SCC groups regarding average age ($P > 0.05$).

Expression of PD-L1 and cluster of differentiation 68 expression in squamous cell carcinoma and margin groups

Table 1 shows PD-L1 and CD68 expression in SCC and healthy unaffected margins [Figure 1]. There was a significant increase in the expression of PD-L1 and CD68 in the SCC group compared to the margins ($P < 0.001$).

Evaluating the relationship between cluster of differentiation 68 and PD-L1 expression with pathological grading

Table 2 represents correlations between PD-L1 and CD68 expression with pathological grading [Figure 2]. Expression of CD68 and PD-L1 significantly correlated with pathological grading ($P < 0.001$).

Table 1: Expression of programmed death-ligand 1 and cluster of differentiation 68 in study groups

Study group	Marker expression				Chi-square test (*P*)
	0	1	2	3	
PD-L1	1 (4)	6 (24)	5 (20)	13 (52)	<0.001
SCC margin	8 (80)	1 (10)	0	1 (10)	
CD68	1 (4)	0	3 (12)	21 (84)	<0.001
SCC margin	6 (60)	2 (20)	1 (10)	1 (10)	

PD-L1: Programmed death-ligand 1, SCC: Squamous cell carcinoma, CD68: Cluster of differentiation 68

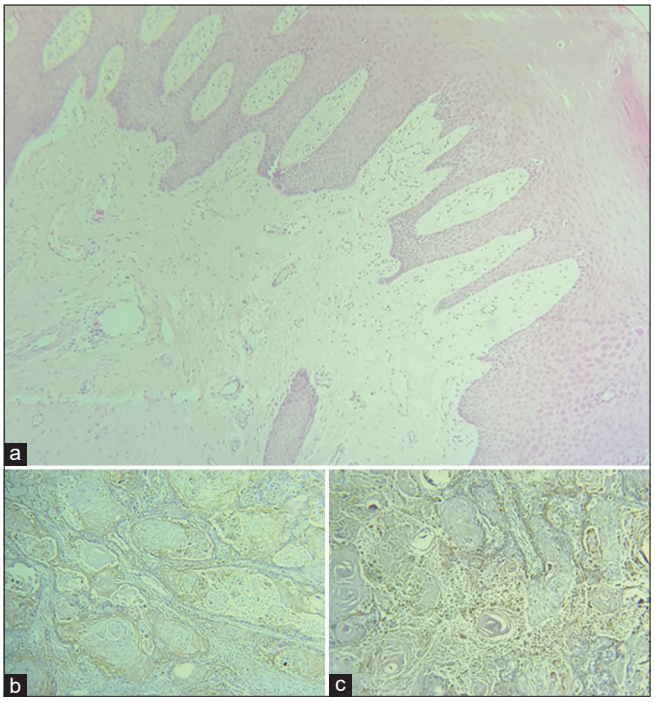

Figure 1: (a) Healthy margin without expression PD-L1 or cluster of differentiation 68 (CD68), (×100) (b) Grade I squamous cell carcinoma (SCC) sample with PD-L1 expression (×100) and c: Grade I SCC sample with CD68 expression (×100).

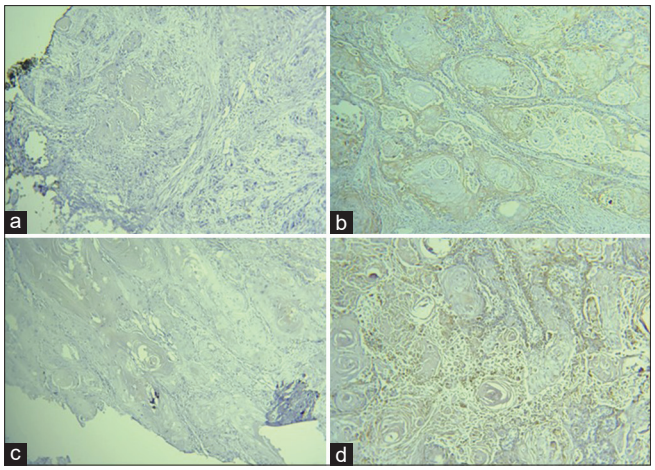

Figure 2: (a) Low-grade squamous cell carcinoma (SCC) and low expression of PD-L1 (score = 1) (×100), (b) High-grade SCC and high expression of PD-L1 (score = 3) (×100). (c) Low-grade SCC and low expression of cluster of differentiation 68 (CD68) (score = 1) (×100), (d) High-grade SCC and high expression of CD68 (score = 3) (×100).

Evaluating the relationship between cluster of differentiation 68 and PD-L1 expression with immunofluorescence

The correlation between PD-L1 and CD68 expression and IF is outlined in Table 3. The expression of PDL1 and CD68 was higher in the IF >5 group ($P < 0.001$).

Evaluating the relationship between cluster of differentiation 68 and PD-L1 expression with depth of invasion

Table 4 shows the relationship between PD-L1 and CD68 expression and the DOI. The expression of PDL1 and CD68 was higher in the DOI >5 group ($P < 0.001$).

DISCUSSION

This study's findings showed that PD-L1 and CD68 expression detected by IHC was higher in HNSCC tissue compared to unaffected, healthy parts of the tissue section. Expression of PD-L1 and CD68 was higher in HNSCC tissues with higher grades. This finding shows that CD68 and PD-L1 can act as a possible diagnostic and prognostic factor.

One of the most critical components of tumor progression is TAM. However, contradictory results have been reported in different studies investigating TAM. As a result, several have interpreted the proliferation of these macrophages as a positive factor and others as a negative factor during the treatment.[11-13] A higher density of TAMs has been linked to a reduction in the expression of epithelial markers such as E-cadherin, along with an elevation in mesenchymal markers such as vimentin, snail, and

Table 2: Relationship between a cluster of differentiation 68 and programmed death-ligand 1 expression with pathological grading

Marker expression	Grade					Chi-square test (*P*)
	0	1	2	3	4	
PD-L1						
0	8 (88.9)	0	0	1 (11.1)	0	<0.001
1	0	6 (100)	0	0	0	
2	0	1 (20)	2 (40)	1 (20)	1 (20)	
3	1 (7.1)	0	2 (14.3)	6 (42.9)	5 (35.7)	
CD68						
0	6 (86.7)	0	0	1 (14.3)	0	<0.001
1	1 (100)	0	0	0	0	
2	1 (25)	3 (75)	0	0	0	
3	1 (4.5)	4 (18.2)	4 (18.2)	7 (31.8)	6 (27.3)	

CD68: Cluster of differentiation 68; PD-L1: Programmed death-ligand 1

Table 3: Relationship between cluster of differentiation 68 and programmed death-ligand 1 expression with immunofluorescence

Marker expression	Invasive front			Chi-square test (*P*)
	0	<5	>5	
PD-L1				
0	8 (88.9)	0	1 (11.1)	<0.001
1	0	4 (66.7)	2 (33.3)	
2	0	3 (60)	2 (40)	
3	1 (7.1)	2 (14.3)	11 (78.6)	
CD68				
0	6 (86.7)	0	1 (14.3)	<0.001
1	1 (100)	0	0	
2	1 (25)	2 (50)	1 (25)	
3	1 (4.5)	7 (31.8)	14 (63.6)	

CD68: Cluster of differentiation 68; PD-L1: Programmed death-ligand 1

Table 4: Relationship between a cluster of differentiation 68 and programmed death-ligand 1 expression with the depth of invasion

Marker expression	DOI			Chi-square test (*P*)
	0	<4	>4	
PD-L1				
0	8 (88.9)	0	1 (11.1)	<0.001
1	0	6 (100)	0	
2	0	1 (20)	4 (80)	
3	1 (7.1)	3 (21.4)	10 (71.4)	
CD68				
0	6 (85.7)	0	1 (14.3)	<0.001
1	1 (100)	0	0	
2	1 (25)	3 (75)	0	
3	1 (4.5)	7 (31.8)	14 (63.6)	

DOI: Depth of invasion; CD68: Cluster of differentiation 68; PD-L1: Programmed death-ligand 1

slug. This suggests an influence on the mesenchymal–epithelial pathway.[14,15]

A study by Lin *et al.* found that tumor-related macrophage expression can be a predictor of distant metastasis and therapeutic outcome. Hypoxia can result in increased expression of tumor-related macrophages. Lin *et al.* found that tumor-related macrophage expression can predict distant metastasis and therapeutic outcomes. Hypoxia can result in increased expression of tumor-related macrophages.[16]

Insufficient oxygen and nutrients diffuse to surrounding tissue, causing hypoxia in solid tumors with a diameter over 2 mm. In these areas, monocytes migrate to the tumor area by secreting granulocyte-macrophage colony-stimulating factor and monocyte chemotactic protein 1.[17] Increased CD68 expression in the tumor stroma (instead of the tumor center) is associated with higher pathological grades, distant metastasis, and shorter survival rates.[9] In a separate study investigating patients with hepatic carcinoma, elevated expression of CD68 in the tissues surrounding the tumor, rather than within the tumor, was associated with lower survival rates and a poorer prognosis for the disease.

Tumor IF is a region, in which epithelium and mesenchyme cells interact and are responsible for determining the biological nature of tumors. An important factor related to the epithelium in the IF region is the attachment of epithelial cells, which is typically accomplished by the use of E-Cadherin molecules and their adapters (β-Catenins).[18,19] In addition to displacement, dispersion, and movement of tumor cells, these molecules alter the ability of epithelial cells to invade and metastasize to adjacent tissues, lymph nodes, and distant locations.[18] There are several epithelial–mesenchymal transition (EMT) factors found in the IF region, including collagen type IV, laminin, fibronectin, microRNAs, oncofetal antigens, podoplanin, and glucose transporter-1, which helps tumor cells avoid hypoxia. There are several studies suggesting that the biological margin of the tumor in the IF region may not match the surgical margin of the SCC. Epithelium and EMT may play a significant role in disease prognosis and treatment response.[20]

The present study showed that CD68 expression is higher in the group with a worse grade of disease, a higher IF, and a higher DOI than in the control group. Sun *et al.*[21] reported that CD68 expression was associated with clinical stage and distant metastasis in patients with pharyngeal SCC. In addition, CD68 expression was significantly higher in necrotic tissues. Patients' treatment prognosis and overall survival were lower in the group with high CD68 expression than in the group with low expression.

Another study conducted by Sun *et al.*[22] found that CD68 expression is significantly higher in esophageal SCC cancer tissues than in adjacent healthy tissues. A higher level of CD68 expression was also associated with more differentiation and angiogenesis. According to Lu *et al.*,[23] 92 patients with oral SCC with CD68 expression had larger tumor sizes, metastases, and more advanced stages of the disease. Moreover, Lo Muzio *et al.*[24] showed that tumor differentiation is associated with low CD68 expression.

In contrast to the present study, Kumar *et al.*[13] observed that increased CD68 expression was not associated with less differentiation. According to Bagul *et al.*,[25] CD68 expression did not correlate with tumor differentiation. The differences between the results of the present study and the studies of Bagul and Kumar can be attributed to the different tissues studied and the different methods examined (IHC in the present study and PCR in other studies). Compared to healthy tissues, tumors show a different expression of CD68 around and in their stroma, and CD68 plays a different role in tumor initiation and progression.[26]

As demonstrated in this study, the expression of PDL1 is higher than that of the healthy control group, and its increased expression correlates with a worse grade of the disease, higher IF, and higher DOI. Many human cancers have reported the expression of PDL1 in previous studies.[27,28] It has been shown that the expression of PDL1 by cancer cells leads to apoptosis of killer T-cells. PDL1 functions by reducing the activation process of T-cells. According to Cho *et al.*,[29] PDL1 expression was associated with apoptosis and a reduction in the number of immune T-cells in oral SCC patients.

In Maruse *et al.*,[30] the expression of PDL1 was higher in patients with oral SCC than in healthy individuals. In addition, in the study, PDL1 expression was associated with cervical lymph node metastasis and distant metastasis. In contrast to the present study, the grade of the lesion did not show any association. Cho *et al.*[29] showed that PDL1 expression was found to be 87% in oral SCC patients. However, they noted no correlation between PDL1 expression and clinical features of

malignancy. PDL1 expression was higher in poorly differentiated tumors than in well-differentiated tumors. Tsutsumi *et al.*[31] found a significant association between PDL1 expression near the invasive front and metastasis and worse clinical symptoms.

The current study found a significant increase in CD68 and PD-L1 expression in SCC tissues. Furthermore, these two markers exhibit a direct and significant correlation with the pathological grade of SCC. The higher the DOI, IF, and SCORE of SCC, the greater the expression of these two markers.

CONCLUSION

The current study evaluated 34 patients for the presence of CD68 and PDL1 markers. The results indicated that these two indicators are significantly higher in patients with SCC than in healthy individuals. Therefore, these two markers can be used to diagnose the disease. In addition, the occurrence of these two markers was related to the differentiation of the lesion and its invasion. Therefore, it can be assessed to determine the severity and progression of the disease. In similar studies, the presence of these two markers was found to be associated with overall survival and prognosis in patients with oral SCC. Consequently, they can gauge treatment response and evaluate patient prognosis.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Siavash Bagheri Shirvan, Golnaz Fatemi, Mahdi Shahabinejad, and Farnaz Mohajertehran. The first draft of the manuscript was written by Siavash Bagheri Shirvan, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.DENTISTRY.REC.1401.05.2).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, and financial or non-financial in this article.

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