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Expression of programmed cell death-ligand 1 in oral squamous cell carcinoma and oral leukoplakia is associated with disease progress and CD8+ tumor-infiltrating lymphocytes

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ABSTRACT

Objective: In recent years, monoclonal antibodies targeting programmed cell death-ligand 1 (PD-L1) have become a promising cancer immunotherapy. However, the role of PD-L1 in oral squamous cell carcinoma (OSCC) and oral potentially malignant disorders (OPMDs), including oral leukoplakia (OLK), remains controversial. The aim of the present study was to investigate the expression level of PD-L1 in OSCC and OPMDs, and examine its relationship with CD8 expression and different clinicopathological features.

Method: Expression of PD-L1 and CD8 were conducted in 41 OSCC, 21 OLK, and 25 normal mucosa samples by immunohistochemistry. Then, the density of PD-L1 expression was measured, and its correlation with CD8 expression and different clinicopathological features was analyzed.

Results: PD-L1 protein was detected in 97.6% of OSCC, 61.9% of OLK, and 0% of normal tissues. PD-L1 was highly expressed in human OSCC tissue ($P < 0.0001$), when compared to both OLK and control tissues. PD-L1 positivity was significantly associated with CD8 density ($P < 0.0001$, $r = 0.8491$). The PD-L1 high expression OSCC group displayed a trend for improved overall survival (OS) and disease-free survival (DFS) compared to the low expression group, although the differences were not significant. Moreover, the expression level of PD-L1 in OSCC was positively correlated with the pathological grade ($P < 0.0001$), but it was independent of age, gender, smoking, drinking, tumor size, lymph node status, or recurrence ($P > 0.05$). Also, there was a significant upregulation of PD-L1 expression observed in the OLK group compared to the control group ($P < 0.0001$). PD-L1 positivity in OLK patients was associated with gender and smoking habits ($P < 0.05$), but it did not correlate with age, drinking, or dysplasia ($P > 0.05$).

Conclusion: The upregulation of PD-L1 may be associated with disease progress and CD8+ tumor-infiltrating lymphocytes in oral premalignant and malignant lesions.

1. Introduction

Among all pathological types of oral cancer, oral squamous cell carcinoma (OSCC) is the most common type; it accounts for about 90% of oral malignancies [1]. OSCC may develop from oral potentially malignant disorders (OPMDs), such as oral leukoplakia (OLK), erythroplakia, submucosa fibrosis, and lichen planus [2,3]. Among the OPMDs, OLK is the most common precursor of OSCC, with a malignant transformation rate ranging from 0.1% to 36% [4]. To date, the morbidity and mortality rates of OSCC remain high, and this could be attributed to a lack of valid therapeutic modalities and biomarkers for early diagnosis and prognosis prediction [5]. Therefore, there is an urgent need to identify specific biomarkers for both OPMDs and OSCC to prevent oral diseases from cancerization.

The immunosuppressive network between cancer cells and host immune cells may contribute to the ability of tumors to evade immune attacks [6]. Programmed cell death-ligand 1 (PD-L1) is often expressed on antigen-presenting cells and sometimes on tumor cells, and it is an important immunological checkpoint. The upregulation of PD-L1 could suppress T-cell activation and inhibit anti-tumor immune responses [7,8]. Recently, monoclonal antibodies targeting PD-L1 have shown great promise as a treatment for renal carcinoma, gastric cancer, breast cancer, and non-small cell lung cancer [8–11]. Previous studies have demonstrated that tumoral PD-L1 expression is correlated with the

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clinical outcomes of various types of cancer [12,13]. High expression of PD-L1 is strongly correlated with the infiltration of CD8+ tumor infiltrating lymphocytes (TILs) in the tumor microenvironment [14].

In recent years, several studies investigating PD-L1 expression in OSCC have been conducted [15–18]. However, the analysis method used to observe and score cell staining intensity involves the opinions of two investigators, and it is rather subjective [16,17]. Additionally, the conclusions about the relationship between PD-L1 expression and different clinicopathological parameters are inconsistent [15,16]. Moreover, there is limited data regarding PD-L1 expression in OLK, and the scoring methods that are based on human judgment are also subjective [19]. Therefore, we evaluated 41 cases of OSCC and 21 cases of OLK to determine the level of PD-L1 expression and its correlation with CD8 density using Image-Pro Plus software. In addition, we analyzed the association between PD-L1 expression and clinicopathological factors.

2. Material and methods

2.1. Participants and tissue specimens

Forty-one OSCC, 21 OLK, and 25 normal tissue specimens were used in this study; they were obtained from the Department of Oral Medicine at the School and Hospital of Stomatology, Wuhan University before treatment began. Histological diagnoses were made by two pathologists according to the World Health Organization criteria [20,21]. The experiments were approved by an ethics committee (No. 2013084), and signed informed consent was obtained from each patient. Tumors were staged according to the seventh edition of American Joint Committee on Cancer (AJCC) Cancer Staging Manual. Clinical data were obtained; these included gender, age, location, smoking, alcohol consumption, betel quid chewing, tumor stage, T, N, and M stages, recurrence, and survival. The clinical features of the subjects are listed in Table 1.

2.2. Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue sections were sectioned at a thickness of 4 μm, and they were routinely deparaffinized in xylene and rehydrated in graded alcohol solutions. Sections were then submerged into EDTA buffer (1 mM, pH = 8.0), and antigen retrieval was performed using microwaves. To attenuate non-specific protein binding, they were incubated in 5% BSA buffer for 1 h. The sections were then treated overnight at 4 ℃ in a moist chamber with primary antibodies for PD-L1 (clone SP142, abcam, Cambridge, United Kingdom; 1:100) and CD8 (clone EP1150Y, abcam, Cambridge, United Kingdom; 1:500). Endogenous peroxidase activity was eliminated by treating with 3% H_2O_2 for 20 min at 37 ℃. After treating with an HRP polymer-conjugated anti-mouse secondary antibody for 1 h at 37 ℃, the slides were visualized with diaminobenzidine (DAB) solution and counterstained with hematoxylin. For negative controls, the primary Ab was replaced with a rabbit IgG isotype (Sigma, St. Louis, MO, USA). Human tonsil tissues were used as internal positive controls for PD-L1 and CD8.

Table 1
Clinical features of the subjects.

Clinical features	OSCC	OLK	Control
Total number	41	21	25
Gender			
Male	27	13	12
Female	14	8	13
Age			
Range	33–79	24–75	13–58
Median	55	51	23
≤60	24	15	25
>60	17	6	0
Smoking			
Yes	26	11	8
No	15	10	17
Drinking			
Yes	28	11	7
No	13	10	18

2.3. Evaluation of positive immunostaining rate

The positive staining rate of immunohistochemical staining was evaluated and blindly scored by two pathologists. The proportion of PD-L1-expressing cells was scored as follows: 0, no staining; 1, ≤30%; 2, 31–60%; and 3, 61–100%. Cells were considered PD-L1-positive if the staining was notable in the cytoplasm, regardless of the intensity. Samples with less than 5% of tumor cells that stained positive were considered negative.

2.4. Quantitative analysis of positive staining density

To evaluate PD-L1 expression, the staining results were independently and blindly determined by two investigators. The positive staining was analyzed from at least three randomly selected areas at 400× magnification using integrated optical density (IOD) by Image-Pro Plus 6.0 (IPP, Media Cybernetics, Inc. Silver Spring, MD, USA) [22]. All of the images were taken using the same microscope and camera sets.

2.5. Statistical analysis

To determine the score for positive staining rate analysis and positive staining intensity analysis, the non-parametric Mann-Whitney *U* test was used for two groups and the Kruskal-Wallis H test was used for three groups. A two-tailed Spearman's rank correlation coefficient test was used to analyze the correlated expression of PD-L1 and CD8. Overall survival (OS) and disease-free survival (DFS) were calculated by the Kaplan–Meier method; differences were assessed by a log-rank test. Statistics were analyzed using SPSS 17.0 statistical software (SPSS, Inc. Chicago, IL, USA) and Graph Pad Prism version 6.0 (GraphPad Software Inc, La Jolla, CA). Results are presented as the mean ± standard error or median, as indicated. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of PD-L1 and CD8 protein in OSCC and OLK tissues

Primarily, PD-L1 was located in the membrane and cytoplasm of the cells (Fig. 1A). Positively stained OSCC vs OLK vs healthy tissues were scored as follows: score 0, 1 (2.44%) vs. 8 (38.10%) vs. 26 (100.00%); score 1, 14 (31.15%) vs. 8 (38.10%) vs. 0; score 2, 6 (14.63%) vs. 4 (19.05%) vs. 0; and score 3, 20 (48.78%) vs. 1 (4.76%) vs. 0 (Fig. 1B). There was a significant difference in positive staining scores across the three groups ($P < 0.0001$). Quantitative IOD analysis revealed significantly higher PD-L1 staining in OSCC (median = 156501 IOD) compared to OLK (median = 2363 IOD) ($P < 0.0001$) (Fig. 1C). The normal mucosa group was negative for PD-L1 staining (median = 0 IOD).

CD8+ TILs can be observed in PD-L1 positively stained tissues. CD8 was primarily located in the immune cells of OLK and OSCC, and it was higher in OLK and OSCC than in normal mucosa (Fig. 1A). The Spearman's rank correlation coefficient test and linear tendency test revealed that the protein expression of CD8 in OLK and OSCC significantly correlated with PD-L1 density ($P < 0.0001$, $r = 0.8491$) (Fig. 1D).

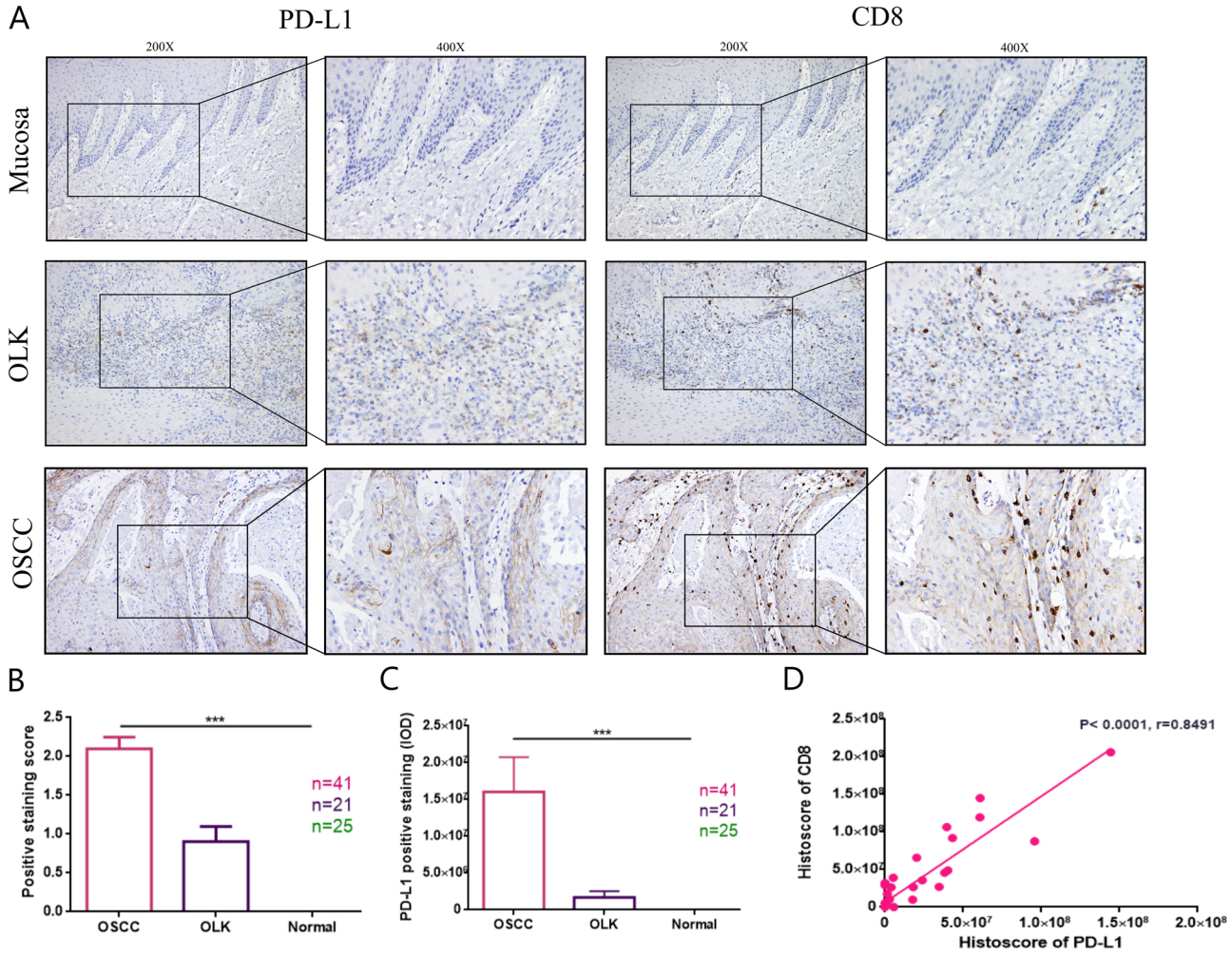

Fig. 1. The expression of PD-L1 and CD8 in OSCC, OLK patients and normal controls. (A) The expression of PD-L1 and CD8 in OSCC, OLK and normal tissue samples were detected by immunohistochemistry. Magnification: 200× (left), 400× (right). (B) Semi-quantitative analysis of PD-L1 expression in OSCC, OLK patients and controls using positively stained score as follows: 0, no staining; 1, ≤30%; 2, 31–60%; and 3, 61–100%. The score was significantly increased in OSCC and OLK compared with control group (Mann-Whitney *U* test, $P < 0.0001$). (C) Quantitative analysis of PD-L1 expression in OSCC, OLK patients and controls using IOD. The PD-L1 density was significantly stronger in both OSCC and OLK compared with normal control (Mann-Whitney *U* test, $P < 0.0001$). (D) Spearman rank correlation coefficient test of the correlation between CD8 and PD-L1 density. $P < 0.0001$, $r = 0.8491$. ***$P < 0.001$.

3.2. Association of PD-L1 expression with pathological features, tumor size, lymph node status, local recurrence, distant metastasis and survival

Further assessment of the relationships between PD-L1 and pathological features found that PD-L1 was significantly associated with the OSCC pathological grade (Kruskal-Wallis H test, $P < 0.05$) (Table 2, Fig. 2A, B). Grade III (Median = 23884685 IOD) OSCC exhibited intense PD-L1 immunoreactivity compared to Grade II (Median = 94709 IOD) and Grade I (Median = 2948 IOD) OSCC (Mann-Whitney *U* test, Grade II $P < 0.05$, Grade I $P < 0.01$) (Fig. 2B). The comparison of PD-L1 expression between Grade II and Grade I OSCC did not indicate a significant difference ($P = 0.0791$), but the PD-L1 density was higher in Grade II than in Grade I OSCC (Fig. 2B). Additionally, the PD-L1 immunoreactivity was independent of tumor size (T1–T4), lymph node status (N–, N+), local recurrence, and distant metastasis ($P > 0.05$; Table 2) in OSCC.

Based on PD-L1 density, samples were divided into low and high expression groups according to the median. Then, its correlation with OS and DFS of OSCC patients was analyzed. We found that the PD-L1 high expression group had a better OS and DFS than the low expression group, but these differences were not significant (OS $P = 0.6956$, DFS $P = 0.2095$).

In OLK samples, there was no significant difference in PD-L1 expression between lesions with epithelial dysplasia (n = 6) and lesions with no dysplasia (n = 15) ($P > 0.05$) (Table 3).

3.3. Association between PD-L1 protein expression and the clinical parameters of patients

The associations between PD-L1 protein expression and the clinical characteristics of patients with OSCC (Table 2) and OLK (Table 3) were investigated. Based on the WHO estimated life expectancy, the patients were divided into the following two groups according to age: the older group (>60 years old) and the younger group (≤60 years old) [23]. Patients that never smoked or drank alcohol are defined as “no” for “smoking” and “drinking”. Patients that currently smoke or drink and patients that used to smoke or drink are defined as “yes”. PD-L1 positivity was not associated with age ($P > 0.05$), gender ($P > 0.05$), smoking ($P > 0.05$), or drinking alcohol ($P > 0.05$) in OSCC patients (Table 2).

We further discussed the association between PD-L1 protein expression and the clinical characteristics of OLK patients. Interestingly, we found that male (Median = 268133 IOD) patients had higher PD-L1 protein expression compared to female (Median = 0 IOD; $P < 0.05$) patients (Table 3). There was a significant difference in PD-L1 positivity between smoking (Median = 464755 IOD) and non-smoking

Table 2
Association between PD-L1 protein expression and the clinicopathological characteristics of patients with OSCC.

Clinicopathological characteristic	No. of patients (N %)	IOD (Median)	P value
Gender			
Male	27 (65.8)	156,501	0.8569
Female	14 (40.2)	769,858	
Age (years old)			
≤60	24 (58.5)	55,412	0.0877
>60	17 (41.5)	5,293,394	
Smoking			
Yes	26 (63.4)	109,068	0.3092
No	15 (36.6)	484,031	
Drinking			
Yes	28 (68.3)	320,266	0.7483
No	13 (31.7)	94,709	
Grade			
I	11 (26.8)	2948	0.0104
II	21 (51.2)	94,709	
III	9 (22.0)	23,884,685	
Tumor size			
T1	8 (19.5)	156,501	0.9414
T2	15 (36.6)	61,634	
T3	13 (31.7)	1,461,304	
T4	5 (12.2)	875,390	
Lymph node metastasis			
Yes	22 (53.7)	964,519	0.1468
No	19 (46.3)	37,051	
Distant Metastasis			
Yes	4 (9.8%)	33,238	0.2774
No	37 (90.2%)	875,390	
Recurrence (local)			
Yes	8 (19.5)	34,831	0.1592
No	35 (80.5)	1,445,006	

(Median = 0 IOD) patients ($P < 0.05$) (Table 3). However, PD-L1 expression was independent of age and alcohol abuse in the OLK group ($P > 0.05$) (Table 3).

4. Discussion

An increasing number of studies have revealed that the protein level of PD-L1 expression is increased in several human malignancies, such as non-small cell lung carcinoma, breast cancer, small cell neuroendocrine carcinomas, and esophageal squamous cell carcinoma [24–27]. In the present study, our results demonstrate that PD-L1 expression is significantly higher in OSCC patients compared to OLK patients and healthy individuals. Furthermore, a trending increase in PD-L1 expression was observed in OLK patients compared to the normal control patients. Our results also demonstrated that the PD-L1 expression level was associated with tumor grade in the OSCC group, and it was associated with gender and smoking in the OLK group. PD-L1 positivity was closely correlated with CD8 density. The PD-L1 high expression group demonstrated better OS and DFS than the low expression group, but the differences were not significant.

To date, the prognostic value of PD-L1 has been studied in several premalignant lesions [28–31]. According to the immunohistochemical assessment of Saraggi et al., PD-L1 protein was significantly overexpressed in ampullary dysplastic lesions compared to the normal samples [28]. Consistently, PD-L1 protein has been found to be constitutively expressed in premalignant trophoblast subtypes of gestational trophoblastic diseases, independently from FIGO (International Federation of Gynecology Obstetrics) prognostic score, chemoresistance, or fatal outcomes [29]. The present study demonstrated that the density of PD-L1 in OLK was significantly higher than the control. Moreover, the percentage of PD-L1-positive OLK tissue samples was 61.9% compared to 0% in the control tissues, suggesting that PD-L1 has potential as a biomarker for OLK. To date, studies investigating the prognostic significance of PD-L1 in OLK lesions are limited. At present, the only research available has revealed that the expression of PD-L1 protein was higher in OLK tissues than in control tissues ($P < 0.05$), and it was similar to that observed in OSCC tissues ($P > 0.05$) [19]. Additionally, the expression level was positively correlated with the degree of epithelial dysplasia [19]. However, in the present study, the immunoreactivity of PD-L1 did not correlate with the grade of dysplasia. These inconsistent results may be due to the high percentage of severe dysplasia samples (18.7%) in their study [19].

Previous studies have indicated PD-L1 as a potential prognostic factor in several human cancers [32–34]. In non-small cell lung carcinoma, PD-L1 expression significantly correlated with better outcomes, independent of histology results [35]. Conversely, Muenst et al. demonstrated that PD-L1 expression was significantly associated with worse OS in breast cancer, and it was closely related to tumor size, tumor grade, and lymph node status [25]. Previous studies have resulted in controversial conclusions on the relationship between PD-L1 expression and tumor stage, tumor size, lymph node status, metastasis, and recurrence in OSCC [15–17]. In addition, the scoring method of cell staining intensity, which depends on the personal judgment of two researchers, is subjective [16,17]. In the present study, the results from the positive staining of cells revealed that 48.78% of OSCC tissue samples had a high (61%–100%) proportion of PD-L1-expressing cells. Furthermore, we utilized a reliable quantitative analysis method to measure the density of positive staining using Image-Pro Plus software that reduces the subjectivity of previous methods [36]. The integrated optical density (IOD) was blindly computed from at least three randomly selected areas by two independent investigators. As a result, a higher intensity of PD-L1 protein expression was observed in the OSCC group compared to the OLK group and the normal control group. Moreover, there was a significant positive correlation between the expression level of PD-L1 and the pathological grade, but it was independent of tumor size, lymph node status, local recurrence, and distant metastasis.

TILs in the tumor environment play an important role in the host immune response to cancer cells [14]. It has been shown that OSCC patients with infiltrating CD8+ TILs had less disease progression and better survival [37]. The correlation study of PD-L1 positivity and CD8 density suggested that PD-L1 was closely associated with CD8 expression in OLK and OSCC patients. Thus, PD-L1 may be a potential biomarker for CD8+ TILs. Furthermore, our study on prognosis showed that the PD-L1 high expression group had better OS and DFS than the low expression group. However, the differences were not significant, which may be due to the shorter follow-up time (19–57 months, Median = 33) than what was reported in the other studies [16]. Thus, further patient follow-ups are required for future research.

The relationship between the immunoreactivity of PD-L1 and clinical parameters, such as gender, age, smoking, and alcohol consumption, is a controversial topic. In colorectal cancers with high microsatellite instability, PD-L1-positive tumors were significantly associated with older age and the female gender [38]. However, there was no observed relationship between the expression of PD-L1 and age or gender in gastric cancer [39]. Calles et al. observed a higher intensity of PD-L1 expression more frequently in smokers with non-small cell lung cancer, and it was associated with more pack-years [40]. We assessed the clinical characteristics of the patients associated with the 41 OSCC and 21 OLK samples. PD-L1 positivity did not correlate with age, gender, smoking, or drinking alcohol. Interestingly, PD-L1 expression was significantly higher in male patients and smokers in the OLK group. These positive correlations in the OLK group could be because male gender and smoking are significant risk factors of OLK occurrence and malignant transformation [41].

In conclusion, this preliminary study revealed that PD-L1 expression in OSCC and OLK was closely associated with disease progress and CD8+ TILs. The PD-L1 high expression group had better OS and DFS than the low expression group, but these differences were not

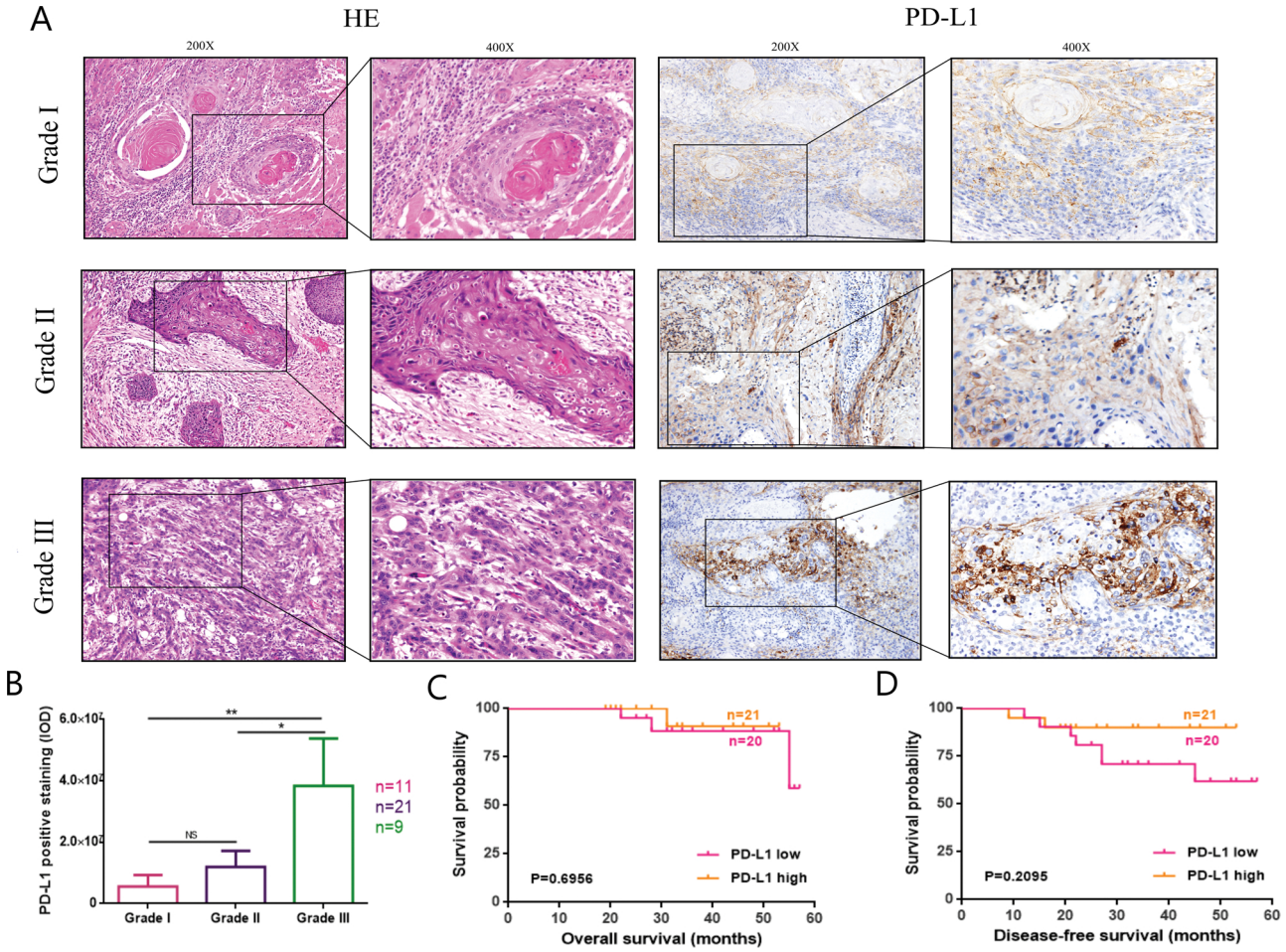

Fig. 2. PD-L1 expression was correlated with Grade of OSCC and survival. (A) The HE staining and immunohistochemistry of PD-L1 expression in OSCC tissues with different Grades (I-III). Magnification: 200× (left), 400× (right). (B) Quantitative analysis of PD-L1 expression in different Grades (I-III) of OSCC using IOD. Grade III OSCC exhibited intense PD-L1 immunoreactivity compared with Grade II and Grade I OSCC (Mann-Whitney *U* test, Grade II $P < 0.05$, Grade I $P < 0.01$). The comparison of PD-L1 expression between Grade II and Grade I OSCC did not indicate significant difference (Mann-Whitney *U* test, $P > 0.05$). **P < 0.01, *P < 0.05, NS: no significant.

Table 3
Association between PD-L1 protein expression and the clinicopathological characteristics of patients with OLK.

Clinicopathological characteristic	No. of individuals (N %)	IOD (Median)	P value
Gender			
Male	13 (61.9)	268,133	0.0436
Female	8 (38.1)	0	
Age (years old)			
≤60	15 (71.4)	1545	0.1586
>60	6 (28.6)	2,317,734	
Smoking			
Yes	11 (52.3)	464,755	0.0138
No	10 (47.6)	0	
Drinking			
Yes	11 (52.3)	25,869	0.2281
No	10 (47.6)	0	
Dysplasia			
Yes	15 (71.4)	1545	1
No	6 (28.6)	14116	

significant. Meanwhile, PD-L1 was positively correlated with tumor grade in OSCC patients and with males and smoking habits in OLK patients. Our study included a relatively large sample size with comprehensive patient information, and it provided a reliable quantitative method for density calculation. Nevertheless, further in vitro and in vivo studies on the mechanism of the PD-L1 pathway in OSCC and OLK are still needed.

Competing interests

The authors declare that they have no competing interests.

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