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Original Article

Role of HPV status and PD-L1 expression in prognosis of laryngeal squamous cell carcinoma

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Abstract: Purpose: Human papillomavirus (HPV) infection has been recognized as a cause of head and neck squamous cell carcinomas (HNSCC). Laryngeal squamous cell carcinoma (LSCC) is one of the most common pathologic types of HNSCC. Clinical trials show that there are differences in response to immunotherapy according to HPV status. It was reported that a high level of programmed cell death-ligand 1 (PD-L1) is correlated with better survival in HPV-positive head and neck cancer. In this study, we investigated the expression of PD-L1 in HPV-positive and HPV-negative LSCC to determine its prevalence and prognostic value. Methods: 52 cases of LSCC were collected from Tangshan Head and Neck Disease Pathology Research Base. PCR-reverse dot blot hybridization and RNAscope in situ hybridization were used to detect HPV status. PD-L1 expression was evaluated by immunohistochemistry and all cases were followed up for survival. SPSS24.0 was used for data entry and statistical analysis. Kaplan-Meier method and Log-rank time series analysis were used for single factor analysis. Multivariate analysis was performed using Cox proportional hazard regression model, and HR and 95% CI were calculated. Results: Of the 52 LSCC patients, 32.7% (17/52) were HPV-positive by RNAscope in situ hybridization, and 51.9% (27/52) of patients were positive for PD-L1 expression by immunohistochemistry. Regression analysis showed that with a median follow-up period of 69 months, smoking and late stage were associated with poor overall survival (OS), whereas HPV positivity and PD-L1 expression showed a better overall survival outcome. Conclusion: Smoking status, tumor stage, HPV status, and PD-L1 expression in tumor cells may represent useful prognostic biomarkers in patients with LSCC.

Keywords: Human papillomavirus, programmed death ligand 1, Laryngeal squamous cell carcinoma, prognosis

Introduction

Laryngeal cancer is a common head and neck cancer and more than 90% of them are squamous cell carcinoma [1]. Evidence shows that tobacco and alcohol are the main risk factors for LSCC [2]. Patients with HPV tend to be younger and have a favorable response to clinical treatment [3]. Related studies have reported that HPV-positive patients with HNSCC have a better clinical prognosis than HPV-negative ones [4]. The latest clinical trials have shown that there are differences in response to immunotherapy between HPV-positive and HPV-negative HNSCC cases, suggesting that the immune escape mechanisms of HPV-positive and HPV-negative head and neck tumors may be different [5, 6]. However, the clinical significance of programmed death ligand 1 (PD-L1) in laryngeal carcinoma is unclear. The purpose of this study was to investigate the expression of PD-L1 in LSCC and analyze its correlation with HPV status and prognosis.

Patients and methods

Cases description

From January 1st, 2010 to December 31st, 2016, 52 LSCC cases from patients aged 37 to 73 years, with a median age of 55 years were selected at Tangshan Head and Neck Disease Pathology Research Base, including 30 males and 25 females. All the patients signed the informed consent of the ethics committee of Tangshan Union Hospital. According to the eighth edition of AJCC TNM staging manual, the clinical stage were I and II in 21 cases, III and IV

in 31 cases. There were 12 cases of high differentiation, 20 cases of moderate differentiation, and 20 cases of poor differentiation. All sections were reviewed by two senior pathologists. According to the eighth edition of AJCC TNM staging manual, the tumors were divided into stages I to IV: stage I + II in 24 cases, and stage III + IV in 25 cases.

Inclusive and exclusive criteria

The inclusion criteria were as follows: 1. Pathologic examination confirmed squamous cell carcinoma with total or partial laryngectomy. 2. Patients were not treated by chemotherapy or radiotherapy. 3. Patients lacked distant metastasis and other primary tumors. 4. Patients had an estimated survival time of more than 6 months. 5. Patients or family members were informed and signed informed consent. The exclusion criteria were: 1. Patients with recurrent tumors or other tumors. 2. Patients who had received chemo-radio therapy or with a history of mental illness.

PCR-DNA reverse dot blot hybridization to detect HPV DNA

The specimens were fixed with 4% neutral formalin and sectioned with routine H&E staining. Histologic observation was performed before HPV DNA reverse dot blot hybridization. Paraffin sections were put into EP tube for dewaxing, and 0.5 ml was centrifuged at 14000 r/min (r = 5 cm) at room temperature for 1 min. DNA was extracted with HPV detection kit after discarding the supernatant, and 1 μl DNA sample was used as template for PCR. The positive control showed color signals at the corresponding HPV genotypes and IC sites of the hybrid film strips, while the negative quality control only showed color signals at the IC sites. HPV genotypes were determined according to the chromogenic position.

RNAscope in situ hybridization to detect HPV E6 and E7 mRNA

The E6 and E7 mRNA of HPV16/18 were detected by RNAscope Kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China). Sample preparation, probe hybridization, signal detection, and slide restaining was carried out according to the kit instructions. The positive signal was dot-shaped brown-yellow granules in cytoplasm. HPV16 mRNA positive laryngeal carcinoma tissue was used as a positive control.

Tumor PD-L1 expression by immunohistochemistry

The specimens were sectioned at 3 μm. We baked slices in oven at 65°C for 2 hours, dewaxed with xylene, hydrated with ethanol and washed with phosphate-buffered saline (PBS) for three times. We incubated the repaired section in 3% hydrogen peroxide at room temperature for 10 minutes to block the activity of endogenous peroxidase. Slides were rinsed with PBS for three times and 3% goat serum was used as blocking solution, placed at 37°C for 1 hour and the primary antibody (diluted at 1:200, Beijing Zhongshan Golden Bridge Bio-technology Co., Ltd.) was added. The slices were placed in the refrigerator at 4°C overnight. After rewarming, PBS rinse was done for three times; the secondary antibody was added and placed in 37°C environment for 30 minutes. After PBS was rinsed, streptavidin labeled-horseradish peroxidase was added and incubated at room temperature for 15 minutes. Then DAB solution was added, hematoxylin was stained again, dehydrated, transparent, and known positive samples of PD-L1 was used as positive control, while PBS replaced the primary antibodies as the negative control. PD-L1 positivity was defined as membranous staining in 20% of tumor cells. PD-L1 expression on tumor cells was evaluated using the intensity score and proportion score. The criteria of staining intensity was as follows: no staining was identified as 0, weak staining as 1, moderate staining as 2, and strong staining as 3.

Survival analysis

All patients were followed up for more than three years or until death. Kaplan- Meier method and log-rank test were used in univariate analysis. HR and 95% CI were calculated by multivariate Cox proportional hazard regression model, and the relationship between clinical covariate variables (age, clinical stage, tumor site and smoking status) and survival of LSCC patients was evaluated.

Statistical analysis

The statistical analyses were performed with PASW Statistics 24.0. The differences in pa-

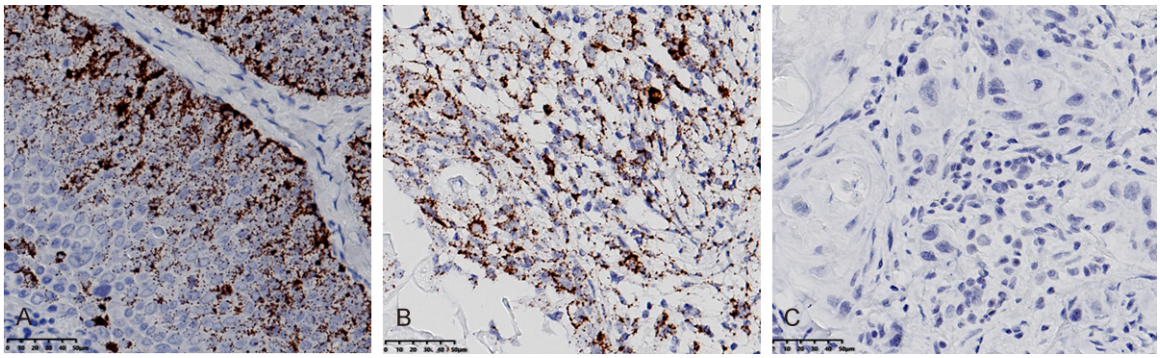

Figure 1. Expression of high-risk HPV mRNA in LSCC (RNAScope method ×100). A. Brown granules in the cytoplasm of HPV 16 positive cells. B. Positive control. C. Negative control.

Table 1. Clinicopathologic characteristics of 52 cases of LSCC [n (%)]

Clinical Features	HPV16/18 mRNA+	X^2	*P*	PD-L1+	X^2	*P*
Gender						
Male	11 (29.7)	0.512	0.474	19 (51.4)	0.321	0.571
Female	6 (40.0)			9 (60.0)		
Age						
≤50 y	11 (40.7)	1.653	0.199	17 (63.0)	2.742	0.098
>50 y	6 (24.0)			10 (40.0)		
Smoking Status						
Smoker	6 (21.4)	3.498	0.061	11 (39.3)	3.881	0.049
Non-smoker	11 (45.8)			16 (66.7)		
Drinking						
Drinker	8 (38.1)	0.082	0.775	11 (47.8)	0.277	0.598
Non-drinker	9 (31.0)			16 (55.2)		
Differentiation						
Well	4 (33.3)	0.117	0.943	6 (50.0)	0.123	0.940
Moderate	6 (30.0)			11 (55.0)		
Poor	7 (35.0)			10 (50.0)		
TNM staging						
I and II	5 (29.4)	1.263	0.261	8 (38.1)	2.698	0.100
III and IV	12 (70.6)			19 (61.3)		

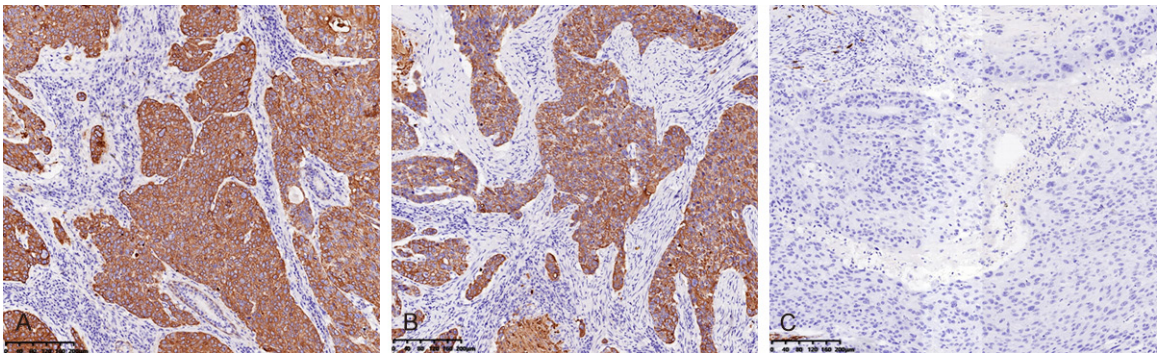

Figure 2. Immunohistochemical expression of PD-L1 in LSCC tissues (SP method ×100). A. Immunohistochemical detection of PD-L1 in LSCC tissue. B. Positive control. C. Negative control.

Table 2. Single factor analysis of prognosis in 52 patients with LSCC

Factors	n	Median overall survival (Month)	Statistics	*P*
Gender				
Male	37	68	3.227	0.199
Female	15	75		
Age				
≤50 y	27	77	0.155	0.694
>50 y	25	78		
Smoking Status				
Smoker	28	74	1.156	0.282
Non-smoker	24	73		
Drinking				
Drinker	23	73	0.005	0.941
Non-drinker	29	76		
Differentiation				
Well	12	77	0.099	0.952
Moderate	20	72		
Poor	20	67		
TNM staging				
I and II	21	76	6.624	0.010
III and IV	31	72		
HPV				
positive	17	100	5.671	0.017
negative	35	67		
PD-L1				
positive	17	85	4.664	0.031
negative	35	66		

Table 3. Factors influencing survival rate of LSCC patients and their evaluation

Variable		Assignment
X_1	Gender	Male = 0, Female = 1
X_2	Age	≤50 y = 0, >50 y = 1
X_3	Smoking	No = 0, Yes = 1
X_4	Drinking	No = 0, Yes = 1
X_5	HPV	HPV- = 0, HPV+ = 1
X_6	Differentiation	Well = 1, Moderate = 2, Poor = 3
X_7	Stage	I + II = 0, III + IV = 1
X_8	PD-L1	PD-L1- = 0, PD-L1 + = 1
T	Survival time	Month
Y	Survival outcome	Survival or Delete = 0, Death = 1

tients' gender, age, smoking, drinking, tumor differentiation, and TNM stage between HPV and PD-L1 were evaluated by Chi-square test test or Fisher exact probability method. The test level was $\alpha = 0.05$. Kaplan-Meier method and log-rank time series analysis were used for univariate analysis. Cox proportional hazard regression model was used for multivariate analysis. HR and 95% CI were calculated to evaluate the relationship between clinical features and overall survival of patients.

Results

Detection of HPV DNA in LSCC tissues

Among 52 LSCC patients, 13 cases were HPV-DNA positive. The overall prevalence rate of HPV was 25.0% (13/52). Among the 13 HPV-DNA positive cases, 76.9% (10/13) were HPV 16, 15.4% (2/13) HPV 31 and 7.7% (1/13) HPV 56. 75.0% (39/52) were HPV-DNA negative.

HPV status by RNAscope In situ hybridization

In the 52 LSCC specimen, HPV 16/18 E6/E7 mRNA positive rate was 32.7% (17/52), including 82.4% (14/17) were HPV 16 mRNA positive and 17.6% (3/17) were positive for HPV-18 mRNA (**Figure 1**). All HPV-mRNA positive cases were also positive for HPV DNA whereas 38% (5/13) of the HPV-DNA positive cases did not express the respective mRNA. 67.3% (35/52) were HPV 16/18 E6/E7 mRNA negative. HPV status was not significantly correlated with patients' gender, age, smoking, drinking, or tumor stage ($P>0.05$, **Table 1**).

Association of PD-L1 expression and clinical characteristics

Regarding PD-L1 expression in tumor cells, the positive staining rate was 51.9% (27/52). The positive rate of PD-L1 in HPV-positive patients was 88.2% (15/17), higher than that of HPV-negative ones (34.2%, 12/35, $P = 0.001$). The positive rate of PD-L1 in non-smoking LSCC patients was 88.2% (15/17), higher than that of smoking cases (34.2%, 12/35, $P = 0.001$). The expression of PD-L1 was irrelevant to gender, age, drinking, differentiation, or tumor stage (**Figure 2**).

Survival outcome

In a logistic regression model with survival outcome as the dependent variable and sex, age, smoking, drinking, tumor differentiation degree, stage, HPV status, and PD-L1 expression as the independent variables, statistically sig-

Table 4. Cox regression analysis of survival factors in 52 LSCC patients

Factors	*B*	S.E	Wald	*P*	Exp (B)	HR (95% *CI*)
HPV	-2.807	0.644	19.009	<0.01	0.060	0.017~0.213
PD-L1	-1.117	0.376	8.822	<0.01	0.327	0.157~0.684
Smoking	1.183	0.321	13.588	<0.01	3.265	1.740~6.125
Staging	1.681	0.347	3.868	0.049	1.977	1.002~3.899

nificant independent variables (*P*<0.1, **Table 2**) were selected and included in a multivariate logistic regression analysis. Results showed that the regression coefficient of smoking and tumor staging were positive, HR>1, indicating that smoking and late tumor staging were risk factors for survival of LSCC patients. The regression coefficient of HPV status and PD-L1 expression were negative, indicating that HPV and PD-L1 are protective factors affecting the prognosis of LSCC patients (**Tables 3**, **4**). Cox multifactor regression analysis showed that HPV (HR = -2.807, 95% CI: 0.017~0.213), PD-L1 (HR = -1.117, 95% CI: 0.157~0.684), smoking (HR = 1.183, 95% CI: 1.740~6.125) and tumor staging (HR = 2.681, 95% CI: 1.002~3.899) were related to the overall survival outcome of patients (*P*<0.05). Patients who were HPV-negative, loss of PD-L1 expression, smoking, and late stage suggest a poor prognosis and need to be followed up closely (**Figure 3**; **Tables 2**-**4**).

Discussion

Laryngeal cancer is a common head and neck malignant tumor and most of them are squamous cell carcinoma (LSCC). The occurrence and development of LSCC are closely related to many factors. At present, surgery is the main treatment for laryngeal cancer. However, some patients still have postoperative recurrence. According to the results of clinical trials, the prognosis of patients with HPV-positive HNSCC is obviously improved. Some scholars have analyzed that the better prognosis of HPV-positive tumors is mainly due to the increased sensitivity to chemoradiotherapy [7, 8]. Williams *et al*. [9] found that the proliferation rate of HPV-positive tumor cells expressing E6/E7 protein in immunocompetent mice was significantly slower than that in HPV-negative ones, but the same experimental results were not found in immunodeficient nude mice. Therefore, it is speculated that the better prognosis of HPV-positive tumors may be related to the adaptive immunity of the body. This study included 52 HPV-positive and HPV-negative LSCC patients. The log-rank test and Cox proportional hazard regression model showed that HPV status was related to the overall survival outcome of LSCC patients, indicating that HPV-positive patients showed better OS than HPV-negative cases, which further confirmed that HPV status was an independent factor for good prognosis of LSCC in part of the Chinese population (HR = -2.807, 95% CI:0.017~0.213, *P*<0.01).

Programmed death-1 (PD-1) and its ligand, programmed death protein ligand-1 (PD-L1), are important immune checkpoint of the body, which can bind to PD-1 on cytotoxic T cells to deplete its function, thus assisting tumor cells to escape immune surveillance [10, 11]. It was also reported that PD-L1, known as B7-H1, is a glycoprotein as a receptor expressed on the surface of immunocytes. It gets exhausted inducing some tumor cells' apoptosis by binding to PD-L1 expressed on tumor cells. Immunotherapy, especially for PD-1/PD-L1 immune checkpoint inhibitors, has shown good prospects in the treatment of a variety of tumors, and has also achieved good results in HNSCC treatment [12, 13]. Sato *et al*. [14] found that the PD-1 inhibitor nivolumab is more effective in PD-L1-positive HNSCC patients. As a result, it is necessary to detect the PD-L1 expression in laryngeal carcinoma tissues, which can provide baseline data for the application of PD-1/PD-L1 in LSCC.

Some studies have examined the expression of PD-L1 in HNSCC; however, the clinicopathologic features associated with PD-L1 expression in LSCC tissues remain largely unknown [15-17]. Earlier findings have shown that overexpression of PD-L1 is relevant to tumor metastasis and poor prognosis, including renal, skin, lung, and pancreatic cancers. However, in head and neck tumors, PD-L1 overexpression is not consistent with the prognosis [18-21]. In this

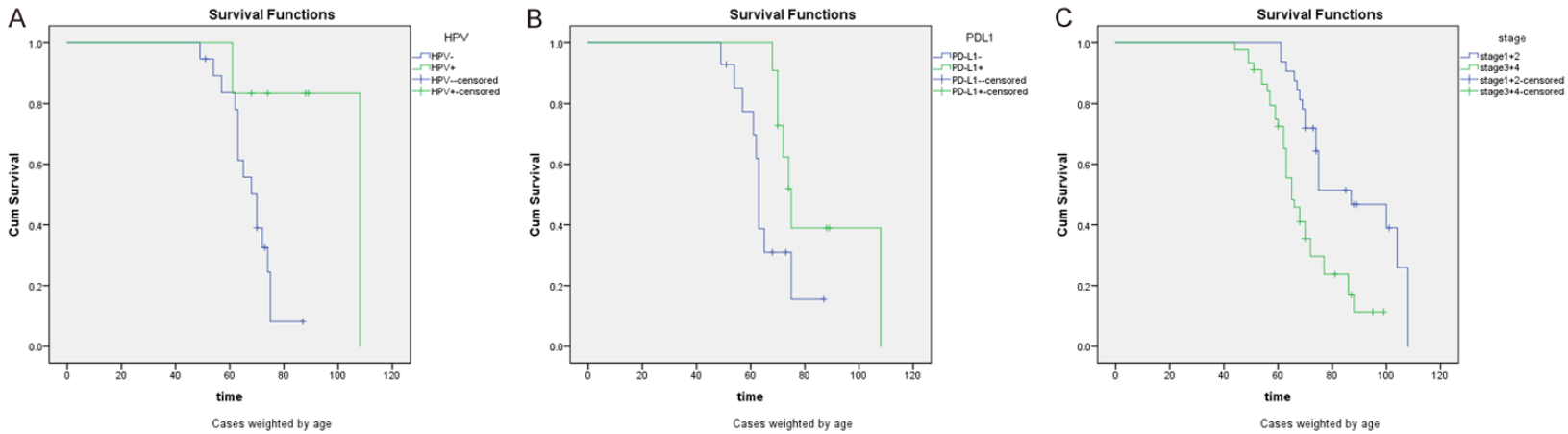

Figure 3. Kaplan-Meier survival analysis showing the impact of clinical features on overall survival. A. Correlation of HPV status with OS of patients with LSCC. $F = 5.671$, $P = 0.017$. B. Correlation of PD-L1 expression with OS of patients with LSCC. $F = 4.664$, $P = 0.031$. C. Correlation of tumor stage with OS of patients with LSCC. $F = 12.924$, $P<0.001$.

study, Kaplan-Meier curves highlighted the worse OS of the patients with late stage compared with those with early stage. Similarly, the patients with negative HPV status and negative PD-L1 demonstrated significantly worse OS compared with the positive groups. Cox regression analysis revealed the lower death risk for the patients with non-smoking (HR = 1.183, 95% CI: 1.740~6.125, P<0.01), early stage (HR = 2.681, 95% CI: 1.002~3.899, P<0.01), HPV and PD-L1 positive, which confirmed that PD-L1 expressions was an independent factor for good prognosis of LSCC in part of the Chinese population (HR = -1.117, 95% CI: 0.157~0.684, P<0.01). Such inconsistencies may be related to the following reasons. First, virus-associated tumors are caused by virus-associated oncoproteins which usually with low or moderate mutation load. Many kinds of virus-related tumors show a stronger immune response and overexpression of PD-L1 [22]. Second, tumor-specific antigen and tumor associated antigen activated the immune system after activation of tumor-specific antigen and tumor-associated antigen, PD-L1 expression was up-regulated in tumor cells. PD-L1 expression may represent a previous endogenous anti-tumor immune response that slowed down but did not completely stop tumor growth, thus PD-1/PD-L1 inhibitors could be used to reactivate it [23, 24]. Third, some studies suggest that PD-L1 overexpression mechanism is different, including dynamic IFN or oncogene activation. The PD-L1 overexpression induced by IFN elevation is a part of acquired immunity, which is usually associated with immune cell infiltration, showing localized overexpression. PD-L1 overexpression associated with oncogene activation usually lacks lymphocyte infiltration and presents diffuse overexpression [25]. PD-L1 overexpression induced by different mechanisms may be related to different efficacy of immune checkpoint inhibitors.

The correlation between PD-L1 expression and HPV infection in HNSCC is not clear. Some studies reported that PD-L1 expression was not associated with HPV infection, while others found that PD-L1 expression was higher in HPV-positive head and neck tumors [26, 27]. This study provides evidence that non-smokers with LSCC tend to be PD-L1 positive and PD-L1 expression was significantly correlated with the HPV status. The positive rate of PD-L1 in HPV-positive LSCC patients was significantly higher than that in HPV-negative cases (P = 0.001). These conflicting results may be due in part to a lack of uniformity in analytical methods, which include variations in immunohistochemical testing between observers, the lack of standardized antibodies to determine PD-L1 expression, and various differences in the thresholds that define positive expression. At the same time, the expression of PD-L1 in tumor cells may change dynamically at different stages of the disease, and the detection results may be influenced by the time of biopsy. Furthermore, the tumor is heterogeneous and the expression of PD-L1 may be different in different sites of the same lesion.

In conclusion, non-smoking, early stage, and being HPV-positive and having PD-L1 expression are independent prognostic factors for LSCC patients. PD-L1 expression is higher in HPV-positive LSCC patients, and PD-L1 positive cases tend to be non-smokers. The better prognosis of HPV-positive laryngeal cancer patients may be related to a different immune microenvironment.

Disclosure of conflict of interest

None.

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