SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 15: 2259-2265, 2018

PD-L1 expression is associated with p16^{INK4A} expression in non-oropharyngeal head and neck squamous cell carcinoma

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Received June 21, 2016; Accepted August 15, 2017

DOI: 10.3892/ol.2017.7564

Abstract. PD-L1 expression is critical in helping tumor cells evade the immune system. However, the level of PD-L1 expression in non-oropharyngeal head and neck squamous cell carcinoma (non-OPHNSCC) and its association with patient prognosis remains unclear. A retrospective clinicopathological analysis was performed on 106 patients with non-OPHNSCC diagnosed between 2007 and 2014. In the current study, tissue arrays from paraffin-embedded non-OPHNSCC samples obtained from patients were constructed, and PD-L1 and p16^{INK4A} expression were determined using immunohistochemistry. Systemic inflammatory factors, including C-reactive protein, serum white blood cell, neutrophil, monocyte and lymphocyte counts were also analyzed. The current study demonstrated that PD-L1 was overexpressed in 32.1% (34/106) and p16^{INK4A} in 20.8% (22/106) of patients. The expression of PD-L1 was associated with p16^{INK4A} expression (P<0.01) but was not associated with levels of systemic inflammatory factors. Tumor stage was determined to be a significant prognostic value (stage I/II vs. III/IV, P=0.03), however, PD-L1, p16^{INK4A} or other clinicopathological factors were not. The current study identified an association between PD-L1 and p16^{INK4A} expression in non-OPHNSCC. This may facilitate the development of anti-PD1/PDL1 therapies to treat patients with head and neck cancer.

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Key words: programmed cell death 1 ligand 1, p16^{INK4A}, head and neck squamous cell carcinoma, non-oropharyngeal

Introduction

Head and neck squamous cell carcinoma (HNSCC), the sixth most common type of cancer in the world, occurs at various sites, including the oral cavity, oropharynx, hypopharynx and larynx (1). The most common risk factors for HNSCC are tobacco use, betel quid chewing, alcohol consumption and human papillomavirus (HPV) infection (2). Previous studies have identified the distinct etiologies of HNSCC arising from different anatomical locations (3,4). In cancer arising from the oropharynx, such as oropharyngeal squamous cell carcinoma (OPSCC), HPV is the major causative factor and it has been reported that the expression of p16^{INK4A}, an important tumor suppressor protein encoded by the cyclin dependent kinase inhibitor 2A *(CDKN2A)* gene, is a biomarker for HPV infection and indicates good patient prognosis (5). By contrast, in cancer arising from the non-oropharyngeal head and neck region, such as non-oropharyngeal head and neck squamous cell carcinoma (non-OPHNSCC), the roles of HPV infection and p16^{INK4A} expression have not been clearly defined. The causes of non-OPHNSCC may be complex as environmental carcinogens, including alcohol, tobacco and betel quid serve a role in tumor initiation and progression (6). It has been demonstrated that p16^{INK4A} expression is a poor surrogate biomarker of HPV infection (7) and is controversial for its prognostic value in non-OPHNSCC (8). In Taiwan, a country with a high prevalence of betel quid chewing, the predictive value of p16^{INK4A} expression for HPV infection in non-OPHNSCC is low (9).

Inflammatory tumor microenvironments contribute to the carcinogenesis and progression of HNSCC (10); however, few studies have investigated the association between p16^{INK4A} expression and tumor inflammation or immunity. An association between p16^{INK4A} and inflammatory factors has been identified. A previous study demonstrated that the expression of p16^{INK4A} may be inhibited by Toll-like receptors (11). Furthermore, the expression of alternate reading frame protein, which is associated with macrophages surrounding the tumor, is correlated with p16^{INK4A} expression in pancreatic cancer (12).

In addition, environmental carcinogens damage normal mucosal cells in the upper aerodigestive tract due to repeated inflammation and are correlated with gene polymorphisms including *CTLA4* or *TNFα* that are important in determining the prognosis of patients with HNSCC (13,14). However, the role of $p16^{INK4A}$ in non-OPHNSCC remains unclear.

Programmed cell death 1-ligand 1 (PD-L1) is an immune modulatory molecule in cancer cells that inhibits cytotoxic T cell activity (15). The expression of PD-L1, which belongs to the B7 superfamily of proteins, can be induced in certain types of solid and hematological cancer. PD-L1 binds to programmed cell death protein 1 (PD-1) and cluster of differentiation 80 in T cells in the tumor microenvironment to modulate immunity. This is one of the mechanisms by which cancer cells evade the immune system (16). In non-OPHNSCC, interferon (INF)-α induces PD-L1 expression in cancer cells via the protein kinase D isoform 2 (PKD2) pathway to evade recognition by tumor antigen specific T cells (17). Studies have identified varying levels of PD-L1 expression in human HNSCC tissues, ranging from 40-100%; however, most of the data available pertain to OPSCC (18-20). PD-L1 expression may cause immune evasion of HPV, which in turn leads to malignant transformation. Furthermore, it has been reported that HPV-positive patients exhibit a higher expression of PD-L1 than HPV-negative patients with OPSCC (19). However, in patients with non-OPHNSCC, the expression of PD-L1 and $p16^{INK4A}$, as well as their association, remains unclear. Furthermore, the prognostic value of PD-L1 in HNSCC has not been clearly established, as its expression may not reflect the fluid interactions of PD-L1 to the dynamic immune response in the tumor microenvironment (21). To the best of our knowledge, the current study is the first to evaluate the expression of PD-L1 in non-OPHNSCC and its association with $p16^{INK4A}$ expression, as well as other clinicopathological characteristics. The prognostic role of PD-L1 was also evaluated.

Patients and methods

Patients. Between January 2007 and August 2014, 106 patients with non-OPHNSCC that was pathologically proven, at the Taipei Veterans General Hospital (Taipei, Taiwan) were retrospectively reviewed. Information regarding patient characteristics, including patient age, sex, history of betel quid chewing, tobacco use, alcohol consumption and treatment history was collected. Information about the pathological characteristics of perineural invasion, lymphovascular invasion, tumor emboli and extra-capsular spread was also collected. Cancer staging was established according to the 7th American Joint Committee on Cancer Staging Manual (22). The current study was approved by The Institutional Review Board of Taipei Veterans General Hospital (TVGHTPE-2017-08-002BC). Since the current study was retrospective, patient consent was waived.

Immunohistochemical (IHC) staining of PD-L1 and $p16^{INK4A}$. Tissue arrays (depth of 1.5 mm) were constructed as described previously (23). Xylene was used to deparaffinize the samples and serial dilutions of alcohol (100, 95, 75 and 50%) were used to rehydrate the array samples. Antigen retrieval was performed by placing samples in a citrate buffer (pH 6.0) and heating to 121˚C in an autoclave for 10 min. Following this, samples were bathed in the blocking agent, 3% bovine serum albumin (BSA), for 30 min at room temperature. Samples were then incubated overnight at 4˚C with primary antibodies, anti-PD-L1 (cat. no. 13684S; dilution, 1:200; Cell Signaling Technology, Inc., Danvers, MA, USA) and a monoclonal anti-mouse $p16^{INK4A}$ (cat. no. sc-81157; dilution, 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). By using MultiLink + HRP label kits (Super Sensitive™ IHC Detection Systems; BioGenex Laboratories, Inc., Fremont, CA, USA), samples were incubated with secondary antibody (a mix of anti-mouse and anti-rabbit IgGs conjugated to multiple biotin molecules) for 20 min at room temperature. Subsequently, a horseradish peroxidase (HRP)-conjugated streptavidin solution (Streptavidin/HRP complex; Multi-Link Biogenex, BioGenex Laboratories) was used for incubation for 20 min at room temperature. AEC substrates (cat. no. HK139-50K; ready to use; BioGenex Laboratories, CA, USA) was used for staining for 2 min at room temperature and the tissues were counterstained with hematoxylin for 1 min at room temperature. The sections were then examined by a light microscope (Eclipse 80i; Nikon Corporation, Tokyo, Japan).

Tumor cells exhibiting membranous and cytoplasmic staining were defined as positive for PD-L1 and those exhibiting nuclear and cytoplasmic staining were defined as positive for $p16^{INK4A}$. The distribution of staining was categorized as follows: 0, 0-5% staining; 1+, 5-20% staining; 2+, 20-50%; 3+, ≥51%. Cases were classified binarily as positive for PD-L1 when there was staining >5% (1+, 2+ and 3+) of cancer cells (20,24) and positive for $p16^{INK4A}$ when staining was >20% (2+ and 3+) (25). Staining was analyzed by two independent investigators (five random fields at magnification, x200).

Statistical analysis. The Mann-Whitney test was used to compare continuous variables and the χ^2 or Fisher's exact test was used to compare categorical variables between groups. Progression-free survival (PFS) was defined as the time period from diagnosis until disease progression. Overall survival (OS) was calculated from the time of diagnosis to mortality. Cox proportional analysis was also used to determine risk factors for disease progression and mortality. The log-rank test to compare Kaplan-Meier curves. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient clinicopathological characteristics. Of the 106 patients with non-OPHNSCC, there were 99 (93.4%) males and 7 (6.6%) females, with a mean age of 58.8±11.5 years. The tumor sites included the oral cavity (63.2%), hypopharynx (27.4%) and larynx (9.4%). A total of 33 patients (31.1%) were diagnosed as having stage I/II disease and 73 (68.9%) had stage III/IV disease. With respect to risk factors for HNSCC, 55 (51.9%) patients partook in chewing betel quid, 84 (79.2%) had used tobacco and 66 (62.3%) consumed alcohol. Regarding treatment, 40 (37.7%) patients received radical surgery alone and 50 (47.2%) patients received surgery followed by adjuvant therapy, consisting of chemotherapy (cisplatin 25 mg/m^2 IV weekly plus tegafur-uracil 400 mg daily for up to 7 weeks), radiotherapy (60-66 Gy) and concurrent chemoradiotherapy. A total of 16 (15.1%) patients

Table I. Demographic and clinical characteristics of the study population.

Characteristic	Case number (n=106) Number	%
Age (mean ± standard deviation)	58.8±11.5	
Male	99	93.4
Sites		
Oral cavity	67	63.2
Hypopharynx	29	27.4
Larynx	10	9.4
Stage		
I/II	33	31.1
III/IV	73	68.9
Betel quid chewing user		
Yes	55	51.9
No	51	48.1
Tobacco user		
Yes	84	79.2
No	22	20.8
Alcohol consumption		
Yes	66	62.3
No	40	37.7
Pathological characteristics		
PD-L1 expression	34	32.1
$p16^{INK4A}$ expression	22	20.8
Definite treatment		
Surgery	90	84.9
Surgery alone	40	37.7
Adjuvant therapy	50	47.2
CCRT	16	15.1
CCRT alone	6	5.7
IC followed by CCRT	10	9.4

CCRT, concurrent chemoradiotherapy; IC, induction chemotherapy; PD-L1, programmed cell death 1 ligand 1.

received definitive chemoradiotherapy (cisplatin 80 mg/m^2 on day 1 plus 5-fluorouracil 400 mg/m^2/day by continuous infusion on days 1-4, every 28 days for 2 cycles plus radiation 66-72 Gy); whereas 10 (9.4%) were administered induction chemotherapy (cisplatin 80 mg/m^2 on day 1 plus 5-fluorouracil 600 mg/m^2/day by continuous infusion on days 1-4 every 28 days for 2 cycles; or docetaxel 60 mg/m^2 plus cisplatin 75 mg/m^2 on day 1 plus 5-fluorouracil 850 mg/m^2/day by continuous infusion on days 1-4 every 28 days for 2 cycles; Table I). A total of 34 patients (32.1%) exhibited PD-L1 expression (Fig. 1A and B) and 22 (20.8%) exhibited $p16^{INK4A}$ expression (Fig. 1C and D).

Association between PD-L1 expression and clinicopathological characteristics. Positive $p16^{INK4A}$ expression was significantly higher in the group exhibiting positive expression of PD-L1

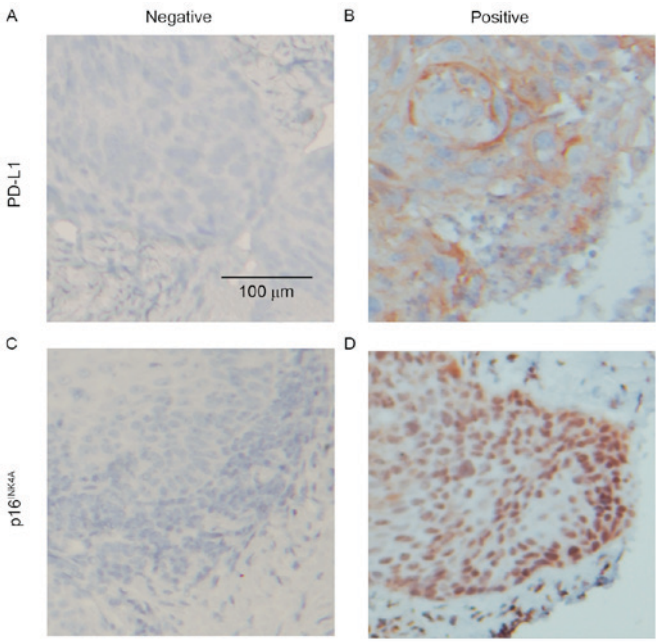


Figure 1. Immunohistochemical staining of PD-L1 and $p16^{INK4A}$ in representative cases. (A) PD-L1 negative; (B) PD-L1 expression; (C) $p16^{INK4A}$ negative; (D) $p16^{INK4A}$ expression. (all magnification, x400). PD-L1, programmed cell death 1 ligand 1.

compared with the group exhibiting negative expression of PD-L1 (38.2 vs. 12.5%; P<0.01; Table II). Furthermore, the mean age of patients exhibiting positive PD-L1 expression was significantly higher than those exhibiting negative PD-L1 expression (62.5±10.4 vs. 57.0±11.7; P<0.01; Table II). However, positive PD-L1 expression was not associated with clinical stage, oral habits or primary cancer sites (Table II). Since it has been demonstrated that PD-L1 is associated with the inflammatory tumor microenvironment (26), the association between PD-L1 and systemic inflammatory factors at diagnosis, including total white blood cell count, absolute neutrophil count, absolute lymphocyte count, absolute monocyte count, neutrophils/lymphocyte ratio and C-reactive protein levels, were investigated. However, there was no significant association between PD-L1 expression and any of the aforementioned inflammatory factors (Table II).

Risk factors for PFS and OS. Univariate Cox proportional hazards analysis demonstrated that only advanced cancer stage (III, IV) was a prognostic factor of OS (HR, 7.53; P=0.05). Neither oral habits, nor pathological characteristics, including PD-L1 and $p16^{INK4A}$ expression, were risk factors for disease progression and survival (Table III). Following adjustment for cancer stage, PD-L1 and $p16^{INK4A}$ expression did not qualify as independent risk factors.

Patients with early stage cancer (I or II) had a significantly better survival rate (P<0.05) than those with advanced stage cancer (III or IV; Fig. 2A). However, the differing status of PD-L1 and $p16^{INK4A}$ expression did not significantly affect the OS of patients (Fig. 2B and C).

Discussion

The results of the current study demonstrate that PD-L1 is expressed in a proportion of patients with non-OPHNSCC

Table II. Association between PD-L1 expression and patient clinicopathological characteristics.

	PD-L1 negative, n=72	PD-L1 expression, n=34	P-value
Age	57.0±11.7	62.5±10.4	0.01[a]
Stage			
I/II (%)	22 (30.6%)	11 (32.4%)	0.85
III/IV (%)	50 (69.4%)	23 (67.6%)	
Habits			
Betel quid chewing (%)	41 (59.4)	16 (48.5)	0.30
Tobacco use (%)	60 (87.0)	26 (78.8)	0.28
Alcohol consumption (%)	45 (67.2)	22 (66.7)	0.96
Sites			
Oral (%)	47 (65.3)	20 (58.8)	0.44
Hypopharynx (%)	20 (27.8)	9 (26.5)	
Larynx (%)	5 (6.9)	5 (14.7)	
Pathological characteristics			
p16 ^{INK4A} expression (%)	9 (12.5)	13 (38.2)	<0.01[a]
PNI (%)	21 (41.2)	18 (58.1)	0.14
LVI (%)	29 (58.0)	19 (61.3)	0.77
Tumor emboli (%)	15 (31.9)	15 (48.4)	0.14
ECS (%)	11 (59.4)	8 (61.5)	0.83
Systemic inflammatory factors			
WBC count (/cumm)	7,969±2,378	7,494±3,603	0.42
ANC (/cumm)	5,274±2,086	5,035±3,358	0.65
ALC (/cumm)	1,953±1,316	1,663±676	0.23
AMC (/cumm)	622±248	554±232	0.18
N/L	3.3±1.8	3.7±4.1	0.42
CRP (mg/dl)	6.8±5.5	8.7±6.8	0.25

All data are presented as the mean ± standard deviation, unless otherwise specified. [a]P<0.05; PNI, perineural invasion; LVI, lymphovascular invasion; ECS, extra-capsular spread; WBC, white blood cell count; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; AMC, absolute monocyte count; N/L, neutrophil lymphocyte ratio; CRP, C-reactive protein; PD-L1, programmed cell death 1 ligand 1.

and that PD-L1 expression is significantly associated with p16^{INK4A} expression. However, PD-L1 expression is not a prognostic factor for non-OPHNSCC. In the current study, 32.1% of subjects exhibited positive PD-L1 expression, comparable to the results of previous studies, which demonstrated that positive PD-L1 expression occurred in 19-66% of HNSCC cases (18,24,27) and 46-59% in OPSCC cases (19,20). Positive expression of PD-L1 was observed in 50% of larynx squamous cell carcinoma cases, a relatively high proportion, however the number of cases included in this study was relatively small (28). The variation in the level of PD-L1 expression may be attributed to the heterogeneity of subjects, a small sample size and the inclusion of different ethnic groups. In the current study, analysis of the levels of systemic inflammation factors demonstrated that they were not associated with PD-L1 expression, suggesting that the tumor microenvironment, not systemic inflammation, is an important factor influencing tumor immune evasion. The identification of PD-L1 has led to the development of PD-L1 antibodies to treat types of cancer that were previously considered to be immune-responsive, including non-small cell lung cancer and HNSCC (24). The results of the current study may provide information that may be important in the investigation of immune checkpoint blockage in non-OPHNSCC.

In the present study, it was demonstrated that there was an association between PD-L1 and p16^{INK4A} expression in cancer cells, which may be explained by the response of cancer cells to immune attack. It has been demonstrated that IFN-γ produced by inflammatory cells in the tumor microenvironment directly induces p16^{INK4A} expression and downstream retinoblastoma (Rb) protein hypophosphorylation in cancer cells, which leads to permanent growth arrest in tumors (29). This may be a general mechanism for arresting tumor progression. By contrast, in OPSCC, it has been suggested that p16^{INK4A} expression is caused by HPV infection that results in the inactivation by Rb by E7 oncoprotein (30). Furthermore, in non-OPHNSCC, IFN-γ induces cancer cells to express PD-L1 via the PKD2 pathway (17). Similar results have been reported in ovarian cancer, where IFN-γ stimulated PD-L1 expression, thus promoting tumor progression (31). The results of the current study identified the co-occurrence of senescence and immune evasion of cancer cells, which may be used to develop novel agents targeting non-OPHNSCC in the future.

Table III. Univariate analysis of progression and survival.

	PFS		OS	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age ≥60 years	1.10 (0.54-2.23)	0.79	1.24 (0.44-3.51)	0.68
Stage (III, IV)	1.31 (0.61-2.83)	0.50	7.53 (0.99-57.35)	0.05
Betel quid chewing	1.39 (0.68-2.84)	0.37	1.99 (0.63-6.36)	0.24
Tobacco use	1.85 (0.56-6.07)	0.31	2.41 (0.32-18.46)	0.40
Alcohol consumption	2.42 (1.00-5.92)	0.05	1.38 (0.43-4.40)	0.59
Pathological characteristics				
PD-L1 expression	1.29 (0.62-2.69)	0.49	1.24 (0.42-3.63)	0.70
$p16^{INK4A}$ expression	1.62 (0.67-3.80)	0.26	1.14 (0.39-3.37)	0.81
Close margin	1.35 (0.66-2.76)	0.42	0.57 (0.16-2.02)	0.38
PNI	1.87 (0.84-4.16)	0.13	2.94 (0.76-11.37)	0.12
LVI	1.22 (0.52-2.77)	0.63	1.54 (0.40-5.98)	0.53
Tumor emboli	1.42 (0.63-3.21)	0.39	1.98 (0.57-6.84)	0.28
ECS	2.52 (0.66-9.65)	0.18	2.21 (0.43-11.46)	0.34

PD-L1, programmed cell death 1 ligand 1; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; PNI, perineural invasion; LVI, lymphovascular invasion; ECS, extra-capsular spread.

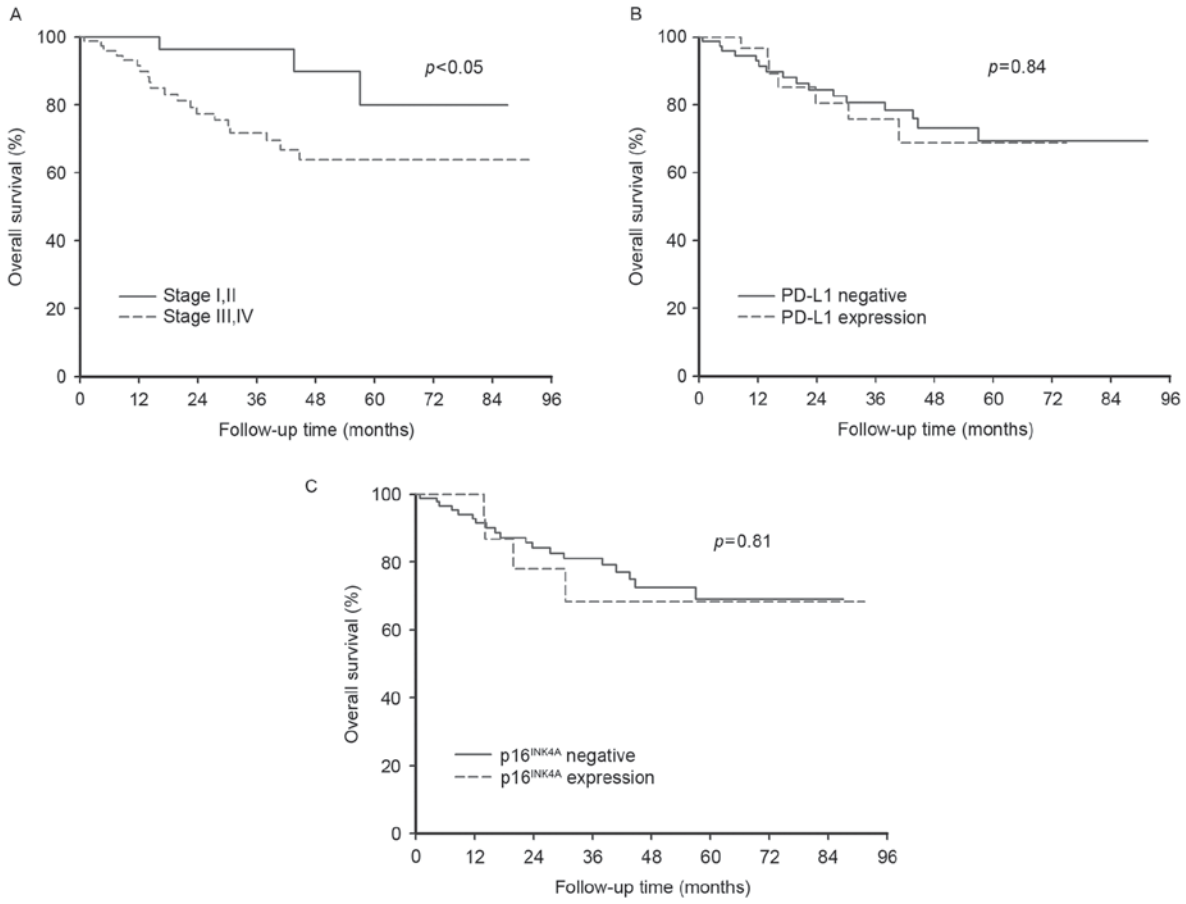


Figure 2. Kaplan-Meier analysis of overall survival stratified by (A) cancer stage, (B) PD-L1 expression and (C) $p16^{INK4A}$ expression. PD-L1, programmed cell death 1 ligand 1.

It remains unknown whether PD-L1 expression is associated with cancer stage and patient prognosis. The present study demonstrated that PD-L1 expression is not associated with non-OPHNSCC stage or sites of occurrence,

which is in accordance with the results of previous studies. Ukpo *et al* (20) reported that PD-L1 expression is not associated with nodal disease and tumor-node-metastasis stage. With regards to prognosis, previous studies have indicated that there is no correlation of survival rate with PD-L1 expression in oral squamous cell carcinoma (20,28), which is consistent with the results of the present study. The association between PD-L1 expression and patient outcomes is controversial; it has been demonstrated in lung cancer that PD-L1 expression is correlated with an improved outcome (32), however, this has not been the case in the other study (33). Such discrepancies may be due to the complex interactions that occur between tumor and immune cells in the tumor microenvironment. It has previously been established that PD-L1 expression helps cancer cells to evade immune attack, which may lead to tumor progression and poorer patient outcomes. However, the co-expression of PD-L1 and p16^{INK4A} may attenuate tumor growth and turn tumor cells into senescent cells, offsetting tumor aggression. Furthermore, immune evasion is not only determined by upregulation of PD-L1 but also by PD-1 expression in tumor-infiltrating T cells (18). Due to these factors, PD-L1 expression cannot be used as a prognostic factor in non-OPHNSCC.

There were several limitations of the present study. Although a significant association between PD-L1 and p16^{INK4A} expression was identified, the mechanism between immune checkpoint and senescence remains unclear. As well as the immune response, the expression of other genes or proteins may affect the expression of PD-L1 (34) and p16^{INK4A} (35). In addition, the patients included in the current study underwent different treatment strategies due to differences in cancer stage, which is a common selection bias of retrospective studies. Although adjustments for cancer stage were made, this bias may not have been fully corrected. Finally, there is no standard cutoff value of IHC expression to define PD-L1 and p16 positive. Having a different cutoff value may generate inconsistent results and further studies are required to establish standard values.

In conclusion, the present study identified an association between PD-L1 and p16^{INK4A} expression in non-OPHNSCC. The poor association between PD-L1 expression and clinical and prognostic status highlight the complex interactions between the tumor and its microenvironment. Further investigations into cancer cell senescence and immune evasion in microenvironment are required.

Acknowledgements

The current study was supported by the Ministry of Science and Technology (103-2314-B-010-034-MY3 to M.-H.Y.), Taipei Veterans General Hospital (V104-E8-001 to M,-H.Y.) and a grant from Ministry of Health and Welfare, Center of Excellence for Cancer Research (MOHW104-TDU-B-211-124-001 to P.-Y.C.). The current study was partly assisted by the Division of Experimental Surgery, Department of Surgery of Taipei Veterans General Hospital. The authors would like to acknowledge the support by the Biobank of Taipei Veterans General Hospital. The abstract of the current study was presented at the 2017 ASCO-SITC Clinical Immuno-Oncology Symposium in Orlando, US on Feb 24, 2017.

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