Human Pathology (2018) **80**, 28–39

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

In this issue

Clinical implication of programmed cell death-1 ligand-1 expression in tonsillar squamous cell carcinoma in association with intratumoral heterogeneity, human papillomavirus, and epithelial-to-mesenchymal transition☆,☆☆

Mi Jung Kwon MD, PhD[a,b,*], Young-Soo Rho MD, PhD[c], Eun Sook Nam MD, PhD[d], Seong Jin Cho MD, PhD[d], Hye-Rim Park MD, PhD[a], Soo Kee Min MD, PhD[a], Jinwon Seo MD[a], Ji-Young Choe MD, PhD[a], Eun Soo Kim MD, PhD[e], Bumjung Park MD, PhD[f], Mineui Hong MD, PhD[g], Kyueng-Whan Min MD, PhD[h]

[a]*Department of Pathology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Dongan-gu, Anyang-si, Gyeonggi-do 14068, Republic of Korea*
[b]*Research Institute for Complementary & Alternative Medicine, Hallym University, Hwaseong, Gyeonggi-do, 445-170, Republic of Korea*
[c]*Department of Otorhinolaryngology-Head and Neck surgery, Ilsong Memorial Institute Head and Neck Cancer Center, Kangdong Sacred Heart Hospital, Hallym University College of Medicine, Seoul 134-701, Republic of Korea*
[d]*Department of Pathology, Kangdong Sacred Heart Hospital, Hallym University College of Medicine, Seoul 134-701, Republic of Korea*
[e]*Department of Radiology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Dongan-gu, Anyang-si, Gyeonggi-do 14068, Republic of Korea*
[f]*Department of Otorhinolaryngology-Head and Neck surgery, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Dongan-gu, Anyang-si, Gyeonggi-do 14068, Republic of Korea*
[g]*Department of Pathology, Kangnam Sacred Heart Hospital, Hallym University College of Medicine, Yeongdeungpo-gu, Seoul 150-950, Republic of Korea*
[h]*Department of Pathology, Hanyang University Guri Hospital, Hanyang University College of Medicine, Guri-si, Gyeonggi-do 11923, Republic of Korea*

Received 15 January 2018; revised 8 March 2018; accepted 29 March 2018

☆ Competing interests: none.

☆☆ Funding/Support: This research was supported by the Hallym University Research Fund (HURF-2017-38) and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A1B03935447) to M. J. Kwon.

* Corresponding author at: Department of Pathology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, 22, Gwanpyeong-ro 170beon-gil, Dongan-gu, Anyang-si, Gyeonggi-do 14068, Republic of Korea.
E-mail address: mulank@hanmail.net (M. J. Kwon).

https://doi.org/10.1016/j.humpath.2018.03.025

Keywords:
Programmed cell death-1 ligand-1;
Tonsil;
Squamous cell carcinoma;
Human papillomavirus;
Epithelial-to-mesenchymal transition

Summary Programmed cell death-1 ligand-1 (PD-L1), essential for immune evasion, is a potential candidate for pathogenesis and therapeutic target of human papillomavirus (HPV)–positive tonsillar squamous cell carcinomas (TSCCs). MET/hepatocyte growth factor signaling and transcription factors involved in epithelial-to-mesenchymal transition (EMT) upregulate PD-L1, which can contribute to clinical outcome. Intratumoral heterogeneity of PD-L1 expression is of clinical importance in selection bias due to false-negative patient enrollment. However, the clinicopathological features, prognostic value, and coexpressed molecules of PD-L1 remain unclear in TSCCs. PD-L1 expression was evaluated via immunohistochemistry using a specific monoclonal antibody (SP142) between whole-tissue and tissue microarray (TMA) sections of 79 tumors (5% cutoff value with weak staining). Expressions of EMT markers (TWIST1, Snail, and SNIP1) and MET/hepatocyte growth factor were also analyzed. Staining of the TMA sections showed 78.5% concordance rate to the whole section. PD-L1 positivity and its intratumoral heterogeneity were 29.1% and 15.2% of TSCCs by whole section, respectively. PD-L1 positivity was prevalent in females, HPV-positive tumors without base of tongue invasion, and SNIP1-overexpressed tumors. SNIP1 overexpression, unmethylated *TWIST1*, smoking, and poorly differentiated tumors were predictive for PD-L1 overexpression. PD-L1 positivity was a favorable independent prognostic factor. Subgroup analyses according to the coexpression of PD-L1 with HPV, SNIP1, or unmethylated *TWIST1* indicated the best clinical outcome than any other subgroups. In conclusion, intratumoral heterogeneity of PD-L1 expression was frequent, warranting a caution in punching TMA cores. A combined analysis of PD-L1 with EMT and HPV may define a characteristic subset of patients and prognostic group.

1. Introduction

Tonsillar squamous cell carcinoma (TSCC) is the most common oropharyngeal cancer, accounting for approximately 70%-80% of all cancers in this region, and represents a highly aggressive malignancy with early lymphatic dissemination and dismal prognosis with a 5-year survival rate of 37% and poor functional outcome [1-3]. Human papillomavirus (HPV) became an important molecular predictive and prognostic factor in oropharyngeal squamous cell carcinomas (SCCs) because HPV-associated oropharyngeal SCCs respond better to radiochemotherapy and have a better prognosis than HPV-negative tumors [4,5]. However, few new effective targeted therapies have been introduced for patients with advanced head and neck SCC in the last decade. Because of the limited treatment options, programmed cell death-1 ligand-1 (PD-L1, also known as B7-H1 or CD274) has recently attracted particular attention as a potential therapeutic target.

Evasion of the immune system is essential for cancer development, progression, and resistance to treatment [6]. PD-L1 is one of these immune surveillance molecules and is expressed in tumor cells; PD-L1 binds to programmed death 1 (PD-1) that is expressed in T cells, B cells, dendritic cells, and natural killer T cells to suppress anticancer immunity and to enable neoplastic growth [6]. Increased PD-L1 expression is associated with poor prognosis in various cancers [6-8]. Clinical trials of PD-L1 inhibitors have been characterized by overall response rates of up to 50% and durable benefits in responding patients with PD-L1–positive head and neck cancers [9]. Recently, because the PD-L1/PD-1 axis has shown to be related to HPV-positive head and neck SCC [10,11], we aimed to focus on aberrant PD-L1 overexpression in tonsil cancer. Because the tonsillar region is the most common among HPV-positive oropharyngeal cancers [4], the TSCC may be a suitable model to evaluate the clinical impact of PD-L1 in HPV-positive cancers. The deep invagination of tonsil crypts makes it susceptible to the collection of bacteria and foreign material, and the tonsillar lymphoid stroma makes the robust lymphohistiocytes chronically exposed to high concentrations of foreign antigen [4], resulting in ongoing basal immune activation in the tonsillar crypts driving PD-L1 expression [11]. Nevertheless, few studies have investigated the pathological role of PD-L1 in association with HPV in TSCCs [10,11]. PD-L1 upregulation is also caused by the activation of transcriptional factors that trigger epithelial-to-mesenchymal transition (EMT) as well as mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways [6,12-14]. We previously demonstrated the EMT-related transcription factors (TWIST1, Snail, and SMAD nuclear interacting protein-1 [SNIP1]) as well as MET/hepatocyte growth factor (HGF) in mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways are involved in tumor aggressiveness of tonsil cancers [15,16]. EMT-related transcription factors and MET/HGF may be the potential candidates for PD-L1 upregulation in TSCCs.

Some studies have reported the presence of intratumoral heterogeneity of PD-L1 expression in lung, head and neck, and colorectal cancers [17-19]. Intratumoral heterogeneity may cause a selection bias for PD-L1–positive patient enrollment and subsequently an incorrect response rate. PD-L1

expression is usually assessed by tissue microarray (TMA) methods in research settings [17,19,20] or by small biopsy specimens in the clinical practice [21], which can produce false negatives. The frequency and characteristics of the heterogeneity of PD-L1 expression remain unexplored in tonsil cancers. Few studies have evaluated whether TMA could be used to evaluate whole-tissue sections of TSCC.

Herein, we investigated the PD-L1 intratumoral heterogeneity via whole-tissue sections and the reliability of TMA sections of PD-L1 expression in comparison with whole-tissue sections. With further reliable results on PD-L1 expression, we determined the prognostic significance of PD-L1 expression in TSCCs, especially depending on HPV, MET/HGF, and EMT.

2. Materials and methods

2.1. Patients and tissues

The patient cohort had been included partly or entirely in previously published studies [5,15,22]. The present study was conducted using formalin-fixed, paraffin-embedded tissues obtained from 79 patients with primary TSCC who underwent surgery at Ilsong Memorial Institute of Head and Neck Cancer, Kangdong Sacred-Heart Hospital, between 1997 and 2010. The selection criteria included the following patients: (1) those who underwent primary resection; (2) those with no prior treatment; and (3) those with available complete medical records, including pathologic slides and paraffin blocks of resected specimens. Clinical information and follow-up data were retrospectively obtained from the medical records, pathology report files, and radiological study results. Surgical resection was followed by postoperative radiotherapy in 16 patients and chemo/radiotherapy in 34 patients. Twenty-nine patients were treated with surgery alone. All patients underwent neck dissection on at least one side. No patient was treated with immunotherapy.

Histopathological characteristics were independently reviewed by 2 pathologists. Diagnosis and histological differentiation were evaluated according to the World Health Organization classification [23]. Staging was based on the American Joint Committee on Cancer staging system (seventh edition) [24]. Alcohol consumption, smoking, and the tumor growth pattern at the invasive front were categorized as previously described [25]. This retrospective study was conducted with the approval of the institutional ethics committee of Kangdong Sacred Heart Hospital (IRB No.14-2-57).

2.2. TMA construction and immunohistochemistry

For TMA construction, all hematoxylin and eosin–stained slides were reviewed, and representative areas were carefully selected. Each paraffin-embedded block relevant to hematoxylin and eosin slides was punched out by using a TMA manufacture tool (Quick-Ray; Unitma, Seoul, South Korea) and placed into a recipient paraffin block. Three tissue cores (3 mm in diameter) were obtained separately from each tumor specimen.

Using 4-μm–thick whole-tissue and TMA sections, immunohistochemistry was performed on an automated immunostainer according to the manufacturer's protocol (BenchMark XT; Ventana Medical Systems, Tucson, AZ). Antigen retrieval was performed for 92 minutes with CC1, and the antibody was incubated for 120 minutes in 37°C in an autostainer. Signal visualization was achieved with the Optiview DAB IHC detection kit and Optiview Amplification kit (Ventana). The primary antibody was PD-L1 (rabbit anti-human PD-L1 monoclonal, 1:25, clone SP142; Ventana). PD-L1 expression was evaluated on the cell membrane with/without cytoplasmic staining in tumor cells. The proportion of PD-L1–positive cells was estimated as a percentage of total tumor cells.

The staining intensity and percentage of positive tumor cells were scored. PD-L1 was scored as 0 (no staining or any staining less than 5% of cells), 1+ (weak staining in more than 5% of the tumor cells), 2+ (moderate staining in more than 5% of the tumor cells), or 3+ (strong staining in more than 5% of the tumor cells). Consistent with several previously published reports, an IHC score of $\geq$1+ was considered positive [11,17,26-28]. Using whole-tissue sections, one single section of tissue exhibiting areas of PD-L1 expression alternating with areas of no expression with no clear morphological differences in the areas was considered having heterogeneous PD-L1 expression [19]. The immunohistochemical results of p16, p53, MET, HGF, TWIST1, Snail, and SNIP1 had been previously analyzed, and their results in the previously published data [5,15] were reused in this study to associate with PD-L1 overexpression.

2.3. Detection for HPV and *TWIST1* promoter methylation

Genomic DNA was extracted from 10-μm–thick sections of 10% neutral formalin-fixed, paraffin-embedded tumor tissue blocks using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). PANArray HPV chip test (PANAGENE, Daejeon, South Korea), a chip-based peptide nucleic acid probe-based assay for detecting amplified HPV DNA of 32 genotypes (19 high- and 13 low-risk HPV types), was used for determination of HPV status according to the manufacturer's instructions, validated with excellent specificity and sensitivity with a perfect agreement (99.7%) of HPV genotypes to type-specific sequencing and very low frequency of false-positive or false-negative results even for multiple HPV infections [5,15]. *TWIST1* promoter methylation status was evaluated by quantitative real-time methylation-specific polymerase chain reaction as described in the article from our group [15].

2.4. Statistical analysis

Comparison of the results obtained with the whole sections with those derived from the TMA sections was

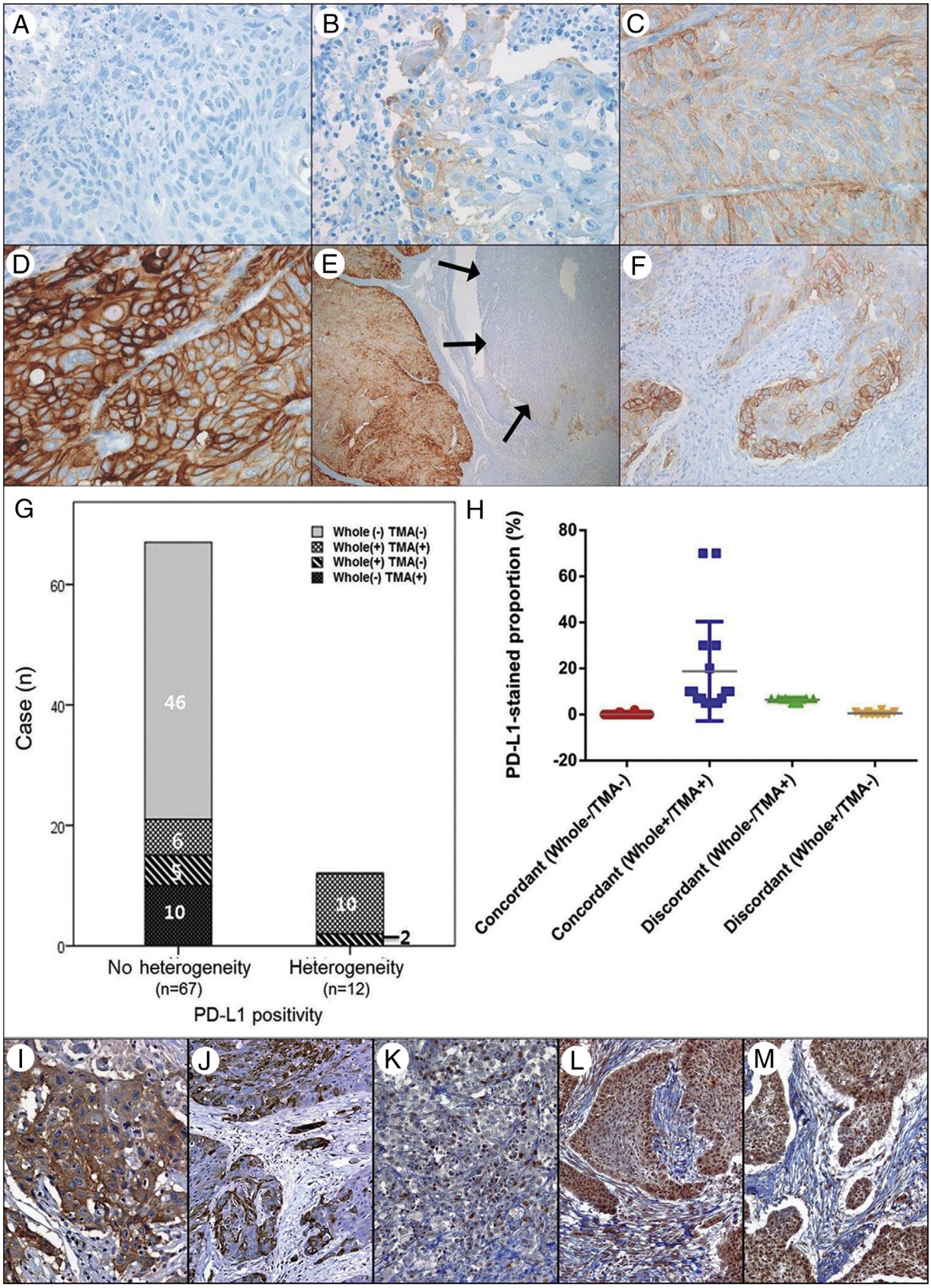

Fig. 1 Representative images of PD-L1 expression interpreted as 0 (A), 1+ (B), 2+ (C), and 3+ (D) in immunohistochemistry. E, Intratumoral heterogeneity of PD-L1 expression is noted. No PD-L1 expression in tumor sheets (arrows) is seen adjacent to strong homogeneous PD-L1 expression in TSCC. F, Among heterogeneously PD-L1–stained cases, PD-L1 expression is stronger in the peripheral tumor sheets or tumor invasive front. G, Among the 12 heterogenous PD-L1 expression cases, 2 PD-L1–positive cases in the whole section are changed into PD-L1 negativity in the TMA section. H, Concordant whole+/TMA+ cases show the highest percentage of PD-L1–positive tumor cells, whereas the discordant whole+/TMA− or whole−/TMA+ cases show low percentage of PD-L1–positive cells. Representative images of MET expression (I), HGF expression (J), TWIST1 expression (K), Snail expression (L), and SNIP-1 expression (M) (A-D, I: original magnification, ×200; E: ×40; F, J-M: ×100).

studied by assessing the concordance rate with the kappa (κ) statistic. The κ value was evaluated to measure the degree of agreement between the 2 sets of results (κ value: ≤0.2, slight agreement; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; ≥0.81, almost perfect). The nonparametric Mann-Whitney *U* test was used to examine differences in the percentage of PD-L1–positive tumor cells between the concordant and discordant cases. The χ^2 test or Fisher exact test was used for discrete variables as appropriate. *Overall survival* (OS) was defined as the interval from the first day of surgery until death or the end of follow-up. *Disease-free survival* (DFS) was defined as the interval from the first day of surgery until tumor progression, death, or the end of follow-up. Survival differences between individual groups were calculated using the Kaplan-Meier method with the log-rank test. We used the Cox proportional hazards model for the multivariate analysis of OS and DFS. OS and DFS rates were analyzed up until January 2011. SPSS version 18 (SPSS Inc, Chicago, IL) was used for all statistical analyses. A *P* of <.05 was considered statistically significant.

3. Results

3.1. PD-L1 expression and intratumoral heterogeneity

PD-L1 expression was predominantly localized to the cell membrane of tumor cells, with the expression occurring in the cytoplasm in some cases, whereas no nuclear immunoreactivity was found. A few scattered patterns of PD-L1 expression were observed in the stromal lymphocytes of the tumor invasive front or normal tonsillar crypt epithelial cells and lymphocytes. PD-L1 positivity was identified in 23 cases (29.1%) analyzed by whole sections: 56 tumors were scored as 0, 12 as 2+, and 11 as 3+ (Fig. 1A-D).

We observed 12 cases (52.2%) exhibiting intratumoral heterogeneity of PD-L1 expression through 23 PD-L1–positive cases obtained from the whole sections, which were 15.2% of the 79 TSCCs (Fig. 1E). Six cases showed stronger PD-L1 expression in the peripheral tumor sheets or tumor invasive front (Fig. 1F).

3.2. Comparison of PD-L1 positivity between whole-tissue and TMA sections

Although whole sections were used to identify PD-L1 positivity in 29.1% (23/79), the 3-mm core TMA sections revealed PD-L1 positivity in 34.2% (27/79) of the samples (Table 1). Between the 2 methods, 62 concordant cases (78.5%) and 17 discordant cases (21.5%) for the PD-L1 results were identified (Fig. 1G). This 78.5% concordance rate between whole sections and TMA core sections showed statistically significant moderate agreement (κ = 0.475) (*P* < .001): there was no difference between the 2 techniques in terms of which tumors were negative and which were positive. The concordant cases comprised 16 (69.6%, 16/23) whole+/TMA+ cases (PD-L1 positivity confirmed by whole section and also identified as PD-L1 positive by TMA section) and 46 (82.1%, 46/56) whole−/TMA− cases. The whole+/TMA+ cases tended to show higher proportion of PD-L1–positive cells than whole −/TMA− or other discordant cases (*P* < .001) (Fig. 1H). As a result, the sensitivity and specificity of TMA sections for PD-L1 positivity were 69.6% and 82.1%, respectively.

On the other hand, discordant cases consisted of 7 (41.2%, 7/17) whole+/TMA− cases and 10 (58.8%, 10/17) whole−/TMA+ cases. Among the whole+/TMA− cases, 2 cases (11.8%, 2/17) were due to the intratumoral PD-L1 heterogeneity, and 5 cases (29.4%, 5/17) showed less than 5% of PD-L1 expressed tumor cells despite moderate or strong staining intensities. In addition, the whole−/TMA+ cases showed more than 5% of tumor cells with weak staining intensity in TMA sections, which could be attributed to the fact that a small portion of TMA cores was overestimated compared to stained tumor cells. Among the heterogeneous PD-L1 expression, the 2 PD-L1–positive cases in the whole section (16.7%) were changed to PD-L1 negativity in TMA sections. Therefore, we decided to use the PD-L1 results obtained from the whole-tissue sections instead of the TMA results in the analysis of clinicopathological correlations with PD-L1 expression.

3.3. Clinicopathological features related to PD-L1 expression

The associations of clinical and pathological features with PD-L1 overexpression are summarized in Table 2. PD-L1

Table 1 Comparison of PD-L1 immunohistochemical expression and the PD-L1 heterogeneity between whole sections and TMA sections

Whole section	N = 79 (%)	TMA		C, %	κ	*P*
		Positive	Negative			
		n = 26(%)	n = 53(%)			
Positive	23 (29.1)	16 (20.3)	7 (8.9)	78.5	0.498	<.001 *
Heterogeneity	12 (52.2)	10 (83.3)	2 (16.7)			
Negative	56 (70.9)	10 (12.7)	46 (58.2)			

Abbreviation: C, concordance rate.
* Statistically significant, *P* < .05.

Table 2 Association of PD-L1 expression with patient characteristics with tonsil cancers and EMT markers

Clinicopathological variables	Total	PD-L1 expression		*P*
	N = 79 (%)	Positive	Negative	
		n = 23 (29.1%)	n = 56 (70.9%)	
Sex				<.001 *
Male	68 (86.1)	13 (56.5)	55 (98.2)	
Female	11 (13.9)	10 (43.5)	1 (1.8)	
Age (y)				.057
≤60	56 (70.9)	20 (87.0)	36 (64.3)	
>60	23 (29.1)	3 (13.0)	20 (35.7)	
Smoking (pack-y)				.209
<20	23 (29.1)	9 (39.1)	14 (25.0)	
≥20	56 (70.9)	14 (60.9)	42 (75.0)	
Alcohol (drink/wk)				.396
<14	32 (40.5)	11 (47.8)	21 (37.5)	
≥14	47 (59.5)	12 (52.2)	35 (62.5)	
Tumor location				.552
Right side	52 (65.8)	14 (60.9)	38 (67.9)	
Left side	27 (34.2)	9 (39.1)	18 (32.1)	
pT status				.396
pT1-2	47 (59.5)	12 (52.2)	35 (62.5)	
pT3-4	32 (40.5)	11 (47.8)	21 (37.5)	
pN status				1.000
pN0	18 (22.8)	5 (21.7)	13 (23.2)	
pN1-2	61 (77.2)	18 (78.3)	43 (76.8)	
AJCC stage				.494
I-II	11 (13.9)	2 (8.7)	9 (16.1)	
III-IV	68 (86.1)	21 (91.3)	47 (83.9)	
HPV status				.046 *
Positive	28 (35.4)	12 (52.2)	16 (28.6)	
Negative	51 (64.6)	11 (47.8)	40 (71.4)	
Differentiation				.214
Keratinizing	44 (55.7)	10 (43.5)	34 (60.7)	
Nonkeratinizing	35 (44.3)	13 (56.5)	22 (39.3)	
Tumor differentiation				.200
Well/moderate	53 (67.1)	13 (56.5)	40 (71.4)	
Poor	26 (32.9)	10 (43.5)	16 (28.6)	
BOT invasion				.045 *
Present	36 (45.6)	6 (26.1)	30 (53.6)	
Absent	43 (54.4)	17 (73.9)	26 (46.4)	
Soft palate invasion				.450
Present	30 (38.0)	7 (30.4)	23 (41.1)	
Absent	49 (62.0)	16 (69.6)	33 (58.9)	
Pterygoid invasion				.667
Present	7 (8.9)	1 (4.3)	6 (10.7)	
Absent	72 (91.1)	22 (95.7)	50 (89.3)	
PPW invasion				.746
Present	13 (16.5)	3 (13.0)	10 (17.9)	
Absent	66 (83.5)	20 (87.0)	46 (82.1)	
Tumor-stromal border				.806
Pushing	42 (53.2)	13 (56.5)	29 (51.8)	
Infiltrative	37 (46.8)	10 (43.5)	27 (48.2)	
Lymphatic invasion				.790
Present	56 (70.9)	17 (73.9)	39 (69.6)	
Absent	23 (29.1)	6 (26.1)	17 (30.4)	
ILN status				.056
Metastasis	66 (83.5)	22 (95.7)	44 (78.6)	

(*continued on next page*)

Table 2 (*continued*)

Clinicopathological variables	Total	PD-L1 expression		*P*
	N = 79 (%)	Positive	Negative	
		n = 23 (29.1%)	n = 56 (70.9%)	
No metastasis	13 (16.5)	1 (4.3)	12 (21.4)	
CLN status				.215
Metastasis	14 (17.7)	2 (8.7)	12 (21.4)	
No metastasis	65 (82.3)	21 (91.3)	44 (78.6)	
P16 expression				.044 *
Positive	31 (39.2)	13 (56.5)	18 (32.1)	
Negative	48 (60.8)	10 (43.5)	38 (67.9)	
P53 expression				.276
Positive	21 (26.6)	4 (17.4)	17 (30.4)	
Negative	58 (73.4)	19 (82.6)	39 (69.6)	
MET				1.000
Positive	31 (39.2)	9 (39.1)	22 (39.3)	
Negative	48 (60.8)	14 (60.9)	34 (60.7)	
HGF				1.000
Positive	53 (67.1)	16 (69.6)	37 (66.1)	
Negative	26 (32.9)	7 (30.4)	19 (33.9)	
TWIST1				.217
Positive	17 (21.5)	7 (30.4)	10 (17.9)	
Negative	62 (78.5)	16 (69.6)	46 (82.1)	
Snail				.264
Positive	27 (34.2)	10 (43.5)	17 (30.4)	
Negative	52 (65.8)	13 (56.5)	39 (69.6)	
SNIP1				.025
Positive	46 (58.2)	18 (78.3)	28 (50.0)	
Negative	33 (41.8)	5 (21.7)	28 (50.0)	
TWIST1 gene status [a]		1.59 ± 2.55	52.17 ± 220.14	.040 *
Methylated	22 (27.8)	3 (13.0)	19 (33.9)	
Unmethylated	57 (72.2)	20 (87.0)	37 (66.1)	

Abbreviations: AJCC, American Joint Committee on Cancer; CLN, contralateral cervical lymph node metastasis; ILN, ipsilateral cervical lymph node; PPW, postpharyngeal wall.

[a] Two-tailed *t* tests of mean ± SD.

* Statistically significant, $P < .05$.

Table 3 Clinicopathological factors affecting PD-L1 overexpression by multivariate analysis

	PD-L1 expression		*P*
	OR	95% CI	
Gender (male *vs* female)	1.267E3	12.011-1.337 × 10^5	.003 *
Age (y) (<60 *vs* ≥60)	0.210	0.013-3.447	.274
Alcohol (drink/wk) (<14 *vs* ≥14)	1.138	0.104-12.450	.916
Smoking (pack-y) (<20 *vs* ≥20)	12.443	1.053-147.003	.045 *
Differentiation (W/M *vs* poorly)	18.722	1.307-268.162	.031 *
pT stage (T1-2 *vs* T3-4)	3.358	0.377-29.931	.278
pN stage (N0 *vs* N1-2)	3.358	0.377-29.931	.278
HPV (negative *vs* positive)	47.184	1.980-1.124 × 10^3	.017 *
MET (negative *vs* positive)	3.542	0.525-23.922	.194
HGF (negative *vs* positive)	4.249	0.241-74.808	.323
TWIST1 (negative *vs* positive)	0.523	0.051-5.381	.586
Snail (negative *vs* positive)	1.432	0.148-13.843	.757
SNIP1 (negative *vs* positive)	18.244	1.027-324.003	.048 *
TWIST1 methylation (negative *vs* positive)	0.013	0.000-0.623	.028 *

Abbreviations: M, moderately differentiated; W, well differentiated.

* Statistically significant, $P < .05$.

Table 4 Univariate and multivariate analyses of OS and DFS of patients with TSCC by univariate analysis

	OS				DFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex	0.345	.145			0.300	.098		
Male *vs* female	(0.082-1.441)				(0.072-1.249)			
Age (y)	2.635	.006 *	2.140	.062	2.154	.023 *	3.369	.005 *
<60 *vs* ≥60	(1.321-5.253)		(0.961-4.763)		(1.110-4.182)		(1.450-7.831)	
Alcohol (drink/wk)	0.794	.510			0.544	.065		
<14 *vs* ≥14	(0.399-1.578)				(0.285-1.037)			
Smoking(pack-y)	2.317	.062			2.828	.020 *	2.802	.038 *
<20 *vs* ≥20	(0.958-5.606)				(1.179-6.781)		(1.057-7.427)	
Differentiation	2.282	.017 *	1.802	.131	1.845	.065		
W/M *vs* poorly	(1.160-4.488)		(0.840-3.869)		(0.964-3.531)			
pT stage	2.702	.005 *	4.270	<.001 *	2.391	.008 *	2.904	.010 *
T1-2 *vs* T3-4	(1.357-5.378)		(1.937-9.414)		(1.253-4.561)		(1.292-6.530)	
pN stage	2.025	.147			2.628	.045 *	6.316	.001 *
N0 *vs* N1-2	(0.780-5.254)				(1.023-6.752)		(2.088-19.103)	
HPV	0.264	.003 *	0.252	.009 *	0.475	.045 *	0.685	.421
Absent *vs* present	(0.108-0.641)		(0.090-0.707)		(0.230-0.983)		(0.278-1.690)	
PD-L1	0.274	.009 *	0.262	.020 *	0.226	.002 *	0.233	.010 *
Negative *vs* positive	(0.096-0.781)		(0.090-0.707)		(0.080-0.640)		(0.077-0.704)	
MET	0.703	.350			0.550	.104		
Negative *vs* positive	(0.336-1.472)				(0.267-1.132)			
HGF	4.376	.002 *	2.095	.168	3.046	.006 *	2.158	.118
Negative *vs* positive	(1.753-10.888)		(0.732-5.991)		(1.380-6.725)		(0.823-5.656)	
TWIST1	0.943	.890			0.827	.650		
Negative *vs* positive	(0.410-2.168)				(0.364-1.879)			
Snail	0.621	.224			0.744	.410		
Negative *vs* positive	(0.288-1.339)				(0.368-1.503)			
SNIP1	0.546	.085			0.412	.008 *	1.067	.874
Negative *vs* positive	(0.275-1.086)				(0.213-0.797)		(0.480-2.371)	
TWIST1 methylation	2.368	.016 *	1.318	.502	2.513	.006 *	1.048	.904
Negative *vs* positive	(1.175-4.776)		(0.589-2.950)		(1.310-4.820)		(0.488-2.254)	

Abbreviations: HR, hazard ratio; CI, confidence interval; HPV, Human papillomavirus; W, well-differentiated; M, Moderately-differentiated.
* Statistically significant, $P < .05$.

overexpression was associated with females ($P < .001$) and a lack of base of tongue (BOT) invasion ($P = .045$). There was a borderline significant tendency of association between PD-L1 positivity with younger age (≤60 years) and ipsilateral cervical lymph node metastasis ($P = .057$ and $P = .056$, respectively).

HPV was detected in 28 (35.4%) of the 79 patients, all of which were high-risk HPV genotype 16. High MET and HGF expression was observed in 31 (39.2%) cases and 53 (67.1%) of the TSCCs, respectively (Fig. 1I-J). TWIST1, Snail, and SNIP1 overexpressions were observed in 17 (21.5%), 27 (34.2%), and 46 (58.2%) of the TSCCs, respectively (Fig. 1K-M). PD-L1 positivity was associated with HPV, p16 overexpression, SNIP1 overexpression, and *TWIST1* hypermethylation ($P = .046$, $P = .046$, $P = .025$, and $P = .040$, respectively). On the other hand, no significant differences were observed between PD-L1 positivity and protein expressions of p53, MET, HGF, TWIST1, or Snail.

3.4. Clinicopathological factors affecting PD-L1 overexpression

The multivariate analyses revealed that females, smoking, poorly differentiated tumors, HPV positivity, high SNIP1 expression, and a lack of *TWIST1* hypermethylation were the independent predictive factors affecting PD-L1 overexpression ($P = .003$, odds ratio [OR] 1.267E3, 95% confidence interval [CI] 12.011-1.337E5; $P = .045$, OR 12.443, 95% CI 1.053-147.003; $P = .031$, OR 18.722, 95% CI 1.307-268.162; $P = .017$, OR 47.184, 95% CI 1.980-1.124E3; $P = .048$, OR 18.244, 95% CI 1.027-324.003; and $P = .028$, OR 0.013, 95% CI 0.000-0.623; respectively) (Table 3).

3.5. Prognostic significance of PD-L1 overexpression

We analyzed the prognostic relevance of PD-L1 expression and clinicopathological parameters for OS and DFS (Table 4).

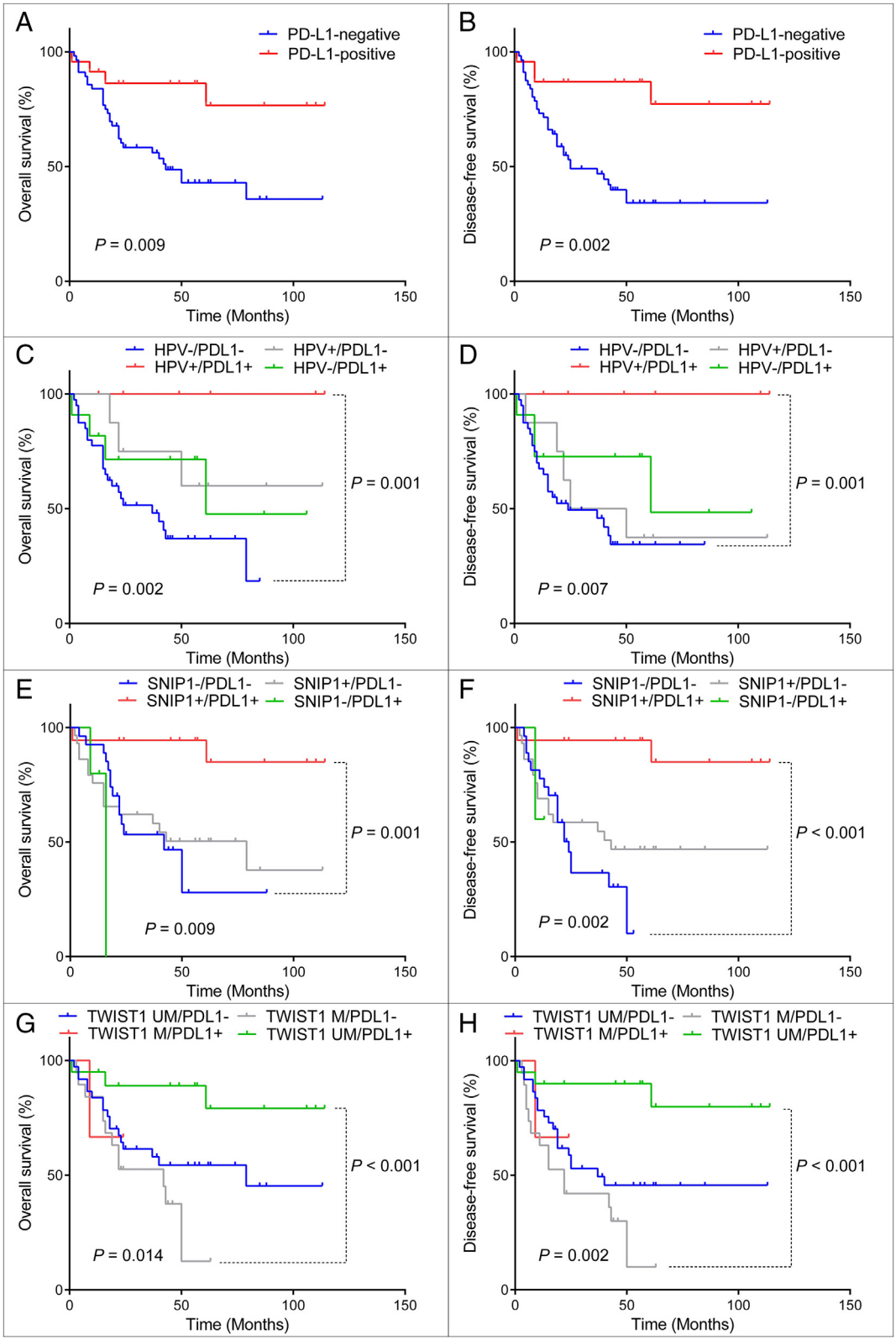

Fig. 2 PD-L1 positivity is associated with better OS (A) and DFS (B) in overall 79 patients with TSCC. C-H, Combined analyses of PD-L1 expression and HPV status, SNIP1 expression status, and *TWIST1* methylation status demonstrate that patients with HPV-negative/PD-L1–negative tumor, SNIP1-negative/PD-L1–negative tumor, or *TWIST1* methylated(M)/PD-L1–negative tumor have the worst OS and DFS, whereas those with HPV-positive/PD-L1–positive tumor, SNIP1-positive/PD-L1–positive tumor, or *TWIST1* unmethylated(UM)/PD-L1–positive tumor have the most favorable prognosis.

Kaplan-Meier survival analysis showed that patients with PD-L1 positivity had better OS and DFS (mean ± SD, 94.47 ± 8.76 months and 94.84 ± 8.63 months, respectively) than those with PD-L1 negativity (58.58 ± 6.58 months and 51.57 ± 6.47 months, respectively) (P = .009 and P = .002, respectively) (Fig. 2A and B). PD-L1 positivity was confirmed as an independent favorable prognostic factor affecting OS and DFS using multivariate analyses (P = .020, hazard ratio [HR] 0.262, 95% CI 0.090-0.707; P = .010, HR 0.233, 95% CI 0.077-0.704).

3.6. Subgroup analysis of prognosis

The subgroup survival analyses according to HPV, SNIP1, and *TWIST1* methylation showed statistically significant survival differences of both OS and DFS (P = .002 and P = .007; P = .009 and P = .002; P = .014 and P = .002, respectively) (Fig. 2C-H). The HPV-negative/PD-L1−negative (HPV−/PD-L1−), SNIP1−/PD-L1−, or *TWIST1*-methylated(M)/PD-L1− subgroups had the worst survival outcomes in OS and DFS, whereas the HPV+/PD-L1+, SNIP1+/PD-L1+, or *TWIST1*-unmethylated(UM)/PD-L1+ subgroups had the most favorable prognosis (P = .001 and P = .001; P = .001 and P < .001; P < .001 and P < .001, respectively).

4. Discussion

The present study showed the association of PD-L1 expression with a better OS and DFS in patients with TSCC, which demonstrated PD-L1 as an independent favorable prognostic factor for both OS and DFS. This result was in agreement with the majority of the published studies that demonstrated favorable prognoses of PD-L1 expression in head and neck SCCs [9,10,26,29]. On the other hand, some studies have reported poor prognosis [20,30] or no prognostic significance of PD-L1 expression [17,21,31]. In the present study, high PD-L1 expression was prevalent in females and HPV-positive tumors without BOT invasion, all of which are favorable clinical factors in TSCC and ultimately appear to influence favorable patients' clinical outcomes. A similar higher frequency of PD-L1 positivity in females has been described in head and neck cancers [17,20,26,32]. The variable prognostic value across the studies may potentially be depending on using validated antibodies, determining appropriate cutoff levels for antigen positivity, specimen material (TMA versus whole section), and confounders that upregulate PD-L1. In the current study, PD-L1 positivity was observed in 29.1% of the cases, which is within the range of 18.3%-91% in oral SCCs [10,17,20,21,26]. To facilitate clinical relevance, the present study specifically on tonsil cancers has used the SP142 anti-PD-L1 antibody via an autostainer under a validated protocol, which is a US FDA–approved antibody and the companion diagnostic tool for atezolizumab for advanced urothelial cancers [33]. PD-L1 positivity in the specimens was defined based on a 5% expression threshold with more than weak staining, validated in the previous studies [11,17,26-28,33].

The correlation between HPV and PD-L1 positivity and their prognostic relevance is also controversial. HPV-positive oropharyngeal cancers have highly exhibited PD-L1 expression than those of HPV-negative tumors [10,11], whereas frequent PD-L1 expression has also been reported regardless of the presence of HPV [21]. In the present study, PD-L1 expression was strongly associated with HPV as well as p16 overexpression, a surrogate marker for high-risk HPV infection, where one-half (42.8%) of HPV-positive TSCCs exhibited PD-L1 overexpression. The frequency of PD-L1 expression in HPV-positive TSCCs in our study is compatible to the range of 46%-71% observed in HPV-associated oropharyngeal cancers [11,21]. The present study demonstrated that the HPV+/PD-L1+ subgroup had the most favorable prognosis, whereas the HPV−/PD-L1− subgroup had the worst survival outcomes, consistent with the previous tonsil cancer study [10], which may suggest an antitumor effect of PD-L1 expression in HPV-positive TSCCs. Significantly increased expressions of IFN-γ and CD8+ cytotoxic T cell mRNA are noted in PD-L1−positive tonsil cancers than in PD-L1−negative tumors [11]. PD-L1 expression is induced by IFN-γ secreted from tumor-infiltrating immune cells in response to HPV [11]. IFN-γ seems to be an important mechanism by which CD4+ Th1 cells kill tumor cells and prevent or suppress the development of cancers, and IFN-γ also increases the infiltration of CD8+ cytotoxic T cells into the tumor [11,34]. By these mechanisms, HPV infection can spontaneously regress in uterine cervical cancers [34].

We found a close relationship between PD-L1 overexpression and EMT; SNIP1 expression and intact *TWIST1* promoter status were drivers that upregulate PD-L1 expression, and both of them exhibited a similar pattern in survival outcomes. PD-L1+/EMT+ (SNIP1+/PD-L1+ or *TWIST1*-UM/PD-L1+) was the best prognostic subgroup. EMT expression together with PD-L1 expression in tumor cells may result in susceptibility to immune attacks against the tumor. The mechanism detailing how PD-L1 and SNIP1 expressions or intact *TWIST1* promoter status affects survival has not been fully elucidated. SNIP1 has a crucial role in cellular growth and tumorigenesis as a suppressor for TGF-β signaling that promotes the migration and invasion of cancer cells. Because SNIP1 directly blocks TGF-β signaling, SNIP1 attenuated TGF-β–induced cell migration [13]. In contrast, Ock et al [12] reported poor survival rates in PD-L1+/EMT+ patients with head and neck SCCs. The discrepant conclusion may be due to the specifically enrolled tonsil cancer cohort and transcriptional factors affecting EMT used in our study, which are different from the nonspecific head and neck cancer cohort and EMT markers (E-cadherin and vimentin) in previous studies. On the other hand, we did not observe any correlation between MET/HGF and PD-L1 expressions, indicating that MET and HGF seem to be of limited importance on PD-L1 expression in TSCCs.

A modest concordance rate (78.5%) was obtained between whole section and TMA section for PD-L1 expression. TMA

section staining exhibited low sensitivity (69.6%) and relatively high specificity (82.1%), indicating that one-third of the cases that may benefit from PD-L1 inhibitor therapy would not be identified via the TMA method. The discrepancy of PD-L1 positivity in whole-tissue and TMA sections was due to intratumoral heterogeneity or overestimating the percentage and intensity of focal PD-L1–positive cells in TMA sections, which are previously described [17-19]. The low percentage of PD-L1–stained cells and intratumoral heterogeneity caused PD-L1 negativity to be undetected by the TMA sections. Likewise, the false-negative PD-L1 results may be sufficiently encountered in clinical practices using small needle biopsy specimens. In the present study, 15.2% intratumoral heterogeneity of PD-L1 expression was observed in the tonsil cancers. Satgunaseelan et al [17] also reported the intratumoral heterogeneity in 30% of oral SCCs. The reason that a proportion of PD-L1–negative patients also benefits from anti–PD-1 therapy in clinical trial may be due to the intratumoral heterogeneity [35]. In fact, 16.7% of the PD-L1–positive cases with intratumoral heterogeneity were misdiagnosed as PD-L1 negative in the TMA analysis in the present study, primarily warranting a caution in punching TMA cores in clinical research. Notably, prominent PD-L1 staining was observed at the periphery of the tumor sheets, in contrast to few staining in the center of tumor sheets. This PD-L1 expression pattern is described in head and neck SCCs [11,30,36] and uterine cervical cancer [34]. It could be attributed to the fact that PD-L1 is more correlated with mesenchymal features than epithelial features of EMT [12,13]. Another explanation may be that PD-L1 tends not to be expressed uniformly within HPV-positive head and neck or uterine cervical SCCs but rather at CD8+ lymphocyte infiltrations [11,34]. Therefore, the TMA cores should be punched out in the tumor invasive front to overcome the false negativity due to intratumoral heterogeneity.

The single-institutional retrospective study, not a trial-based correlative study; the limited number of patients; use of old specimens; and absence of data regarding the latest American Joint Committee on Cancer eighth edition classification may be limitations of the current study. Nevertheless, certain trends were identified from the results. Intratumoral heterogeneity of PD-L1 expression is common in TSCCs, warranting a caution in punching TMA cores in clinical research. Females with HPV-positive tumors without BOT invasion represent a subgroup of patients expected to exhibit PD-L1 expression. HPV, SNIP1 expression, and unmethylated *TWIST1* affect PD-L1 overexpression, of which coexpressions may be potentially used as a favorable prognostic indicator in tonsil cancers.

Acknowledgments

We thank Ms Hye Kyung Lee for her technical assistance performing immunohistochemical staining.

References

[1] Cohan DM, Popat S, Kaplan SE, et al. Oropharyngeal cancer: current understanding and management. Curr Opin Otolaryngol Head Neck Surg 2009;17:88-94.
[2] Lim YC, Koo BS, Lee JS, et al. Distributions of cervical lymph node metastases in oropharyngeal carcinoma: therapeutic implications for the N0 neck. Laryngoscope 2006;116:1148-52.
[3] Jaber JJ, Moreira J, Canar WJ, et al. A 25-year analysis of veterans treated for tonsillar squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2009;135:1147-53.
[4] Betiol J, Villa LL, Sichero L. Impact of HPV infection on the development of head and neck cancer. Braz J Med Biol Res 2013;46:217-26.
[5] Kwon MJ, Kim DH, Park HR, et al. Frequent hepatocyte growth factor overexpression and low frequency of c-Met gene amplification in human papillomavirus–negative tonsillar squamous cell carcinoma and their prognostic significances. Hum Pathol 2014;45:1327-38.
[6] Kwon MJ, Kim KC, Nam ES, et al. Programmed death ligand-1 and MET co-expression is a poor prognostic factor in gastric cancers after resection. Oncotarget 2017;8:82399-414.
[7] Kim R, Keam B, Kwon D, et al. Programmed death ligand-1 expression and its prognostic role in esophageal squamous cell carcinoma. World J Gastroenterol 2016;22:8389-97.
[8] Shin SJ, Jeon YK, Kim PJ, et al. Clinicopathologic analysis of PD-L1 and PD-L2 expression in renal cell carcinoma: association with oncogenic proteins status. Ann Surg Oncol 2016;23:694-702.
[9] Chow LQ, Burtness B, Weiss J, et al. A phase IB study of pembrolizumab (PEMBRO;MK-3475) in patients (pts) with human papillomavirus (HPV)–positive and negative head and neck cancer (HNC). Ann Oncol 2014;25:v1-v41.
[10] Hong AM, Vilain RE, Romanes S, et al. PD-L1 expression in tonsillar cancer is associated with human papillomavirus positivity and improved survival: implications for anti-PD1 clinical trials. Oncotarget 2016;7:77010-20.
[11] Lyford-Pike S, Peng S, Young GD, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res 2013;73:1733-41.
[12] Ock CY, Kim S, Keam B, et al. PD-L1 expression is associated with epithelial-mesenchymal transition in head and neck squamous cell carcinoma. Oncotarget 2016;7:15901-14.
[13] Chen L, Gibbons DL, Goswami S, et al. Metastasis is regulated via microRNA-200/ZEB1 axis control of tumour cell PD-L1 expression and intratumoral immunosuppression. Nat Commun 2014;5:5241. https://doi.org/10.1038/ncomms6241.
[14] Balan M, Mier y Teran E, Waaga-Gasser AM, et al. Novel roles of c-Met in the survival of renal cancer cells through the regulation of HO-1 and PD-L1 expression. J Biol Chem 2015;290:8110-20.
[15] Kwon MJ, Kwon JH, Nam ES, et al. TWIST1 promoter methylation is associated with prognosis in tonsillar squamous cell carcinoma. Hum Pathol 2013;44:1722-9.
[16] Okada T, Suehiro Y, Ueno K, et al. TWIST1 hypermethylation is observed frequently in colorectal tumors and its overexpression is associated with unfavorable outcomes in patients with colorectal cancer. Genes Chromosomes Cancer 2010;49:452-62.
[17] Satgunaseelan L, Gupta R, Madore J, et al. Programmed cell death-ligand 1 expression in oral squamous cell carcinoma is associated with an inflammatory phenotype. Pathology 2016;48:574-80.
[18] Nakamura S, Hayashi K, Imaoka Y, et al. Intratumoral heterogeneity of programmed cell death ligand-1 expression is common in lung cancer. PLoS One 2017;12:e0186192.
[19] El Jabbour T, Ross JS, Sheehan CE, et al. PD-L1 protein expression in tumour cells and immune cells in mismatch repair protein-deficient and -proficient colorectal cancer: the foundation study using the SP142 antibody and whole section immunohistochemistry. J Clin Pathol 2018;71:46-51.

[20] Lin YM, Sung WW, Hsieh MJ, et al. High PD-L1 expression correlates with metastasis and poor prognosis in oral squamous cell carcinoma. PLoS One 2015;10:e0142656.
[21] Kim HS, Lee JY, Lim SH, et al. Association between PD-L1 and HPV status and the prognostic value of PD-L1 in oropharyngeal squamous cell carcinoma. Cancer Res Treat 2016;48:527-36.
[22] Kwon MJ, Kang SY, Nam ES, et al. HIPK2 overexpression and its prognostic role in human papillomavirus–positive tonsillar squamous cell carcinoma. Biomed Res Int 2017;2017:1056427.
[23] Slootweg PJ, Eveson JW. Tumours of the oral cavity and oropharynx. In: Barnes L, Eveson JW, Reichart P, et al, editors. World Health Organization classification of tumours. Pathology and genetics of head and neck tumours. Lyon. France: IARC Press; 2005. p. 166-75.
[24] Edge SB, Byrd DR, Compton CC, et al. Pharynx. In: Edge SB, Byrd DR, Compton CC, et al, editors. AJCC cancer staging manual. 7th ed. Chicago, IL: Springer; 2010. p. 41-56.
[25] Lee DJ, Kwon MJ, Nam ES, et al. Histopathologic predictors of lymph node metastasis and prognosis in tonsillar squamous cell carcinoma. Korean J Pathol 2013;47:203-10.
[26] Kogashiwa Y, Yasuda M, Sakurai H, et al. PD-L1 expression confers better prognosis in locally advanced oral squamous cell carcinoma. Anticancer Res 2017;37:1417-24.
[27] Thompson RH, Kuntz SM, Leibovich BC, et al. Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 2006;66:3381-5.
[28] Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012;366:2443-54.
[29] Oliveira-Costa JP, de Carvalho AF, da Silveira da GG, et al. Gene expression patterns through oral squamous cell carcinoma development: PD-L1 expression in primary tumor and circulating tumor cells. Oncotarget 2015;6:20902-20.
[30] Chen TC, Wu CT, Wang CP, et al. Associations among pretreatment tumor necrosis and the expression of HIF-1alpha and PD-L1 in advanced oral squamous cell carcinoma and the prognostic impact thereof. Oral Oncol 2015;51:1004-10.
[31] Cho YA, Yoon HJ, Lee JI, et al. Relationship between the expressions of PD-L1 and tumor-infiltrating lymphocytes in oral squamous cell carcinoma. Oral Oncol 2011;47:1148-53.
[32] D'Incecco A, Andreozzi M, Ludovini V, et al. PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients. Br J Cancer 2015;112:95-102.
[33] Balar AV, Galsky MD, Rosenberg JE, et al. Atezolizumab as first-line treatment in cisplatin-ineligible patients with locally advanced and metastatic urothelial carcinoma: a single-arm, multicentre, phase 2 trial. Lancet 2017;389:67-76.
[34] Mezache L, Paniccia B, Nyinawabera A, et al. Enhanced expression of PD L1 in cervical intraepithelial neoplasia and cervical cancers. Mod Pathol 2015;28:1594-602.
[35] Herbst RS, Soria JC, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014;515:563-7.
[36] Yu GT, Bu LL, Huang CF, et al. PD-1 blockade attenuates immunosuppressive myeloid cells due to inhibition of CD47/SIRPalpha axis in HPV negative head and neck squamous cell carcinoma. Oncotarget 2015;6:42067-80.