

ORIGINAL ARTICLE

Expression of PD-L1, PD-L2, and inflammatory gene expression profile in locally advanced head and neck squamous cell carcinoma

M. H. Hong^{1†}, S. Park^{2†}, T. Vo³, J. Cho⁴, H. A. Jung², S.-H. Lee², S.-H. Kim⁵, H. Zhou³, D. Chirovsky³, Y. W. Koh⁵, S. O. Yoon⁶, A. L. Webber³, B. Gumuscu³, B. C. Cho^{1*} & M.-J. Ahn^{2*}

¹Division of Medical Oncology, Department of Internal Medicine, Yonsei Cancer Center, Severance Hospital, Yonsei University College of Medicine, Seoul; ²Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea; ³Merck & Co., Inc., Rahway, USA; ⁴Department of Pathology and Translational Genomics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul; Departments of ⁵Otorhinolaryngology; ⁶Pathology, Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea



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Background: The tumor immune microenvironment in cancer treatment response and resistance is of increasing interest. This retrospective study characterized and investigated programmed death-ligand 1 (PD-L1), PD-L2, and the immune gene expression signature and their association with clinical outcomes in locoregionally advanced head and neck squamous cell carcinoma (LA HNSCC).

Patients and methods: PD-L1 and PD-L2 expression on tumor and immune-infiltrating cells (positivity defined as combined positive score or immunohistochemistry proportion score ≥ 1) and T-cell-inflamed gene expression profile (Tcell_{inf}GEP) were evaluated in patients with LA HNSCC treated in South Korea from 2000 to 2015. Correlations among the three biomarkers and their associations with overall survival and recurrence-free survival were assessed.

Results: Among 366 patients, 38.8% had human papillomavirus-positive disease. PD-L1-positive, PD-L2-positive, and high Tcell_{inf}GEP (≤ -0.162) status were observed in 83.6%, 85.4%, and 73.2% of patients, respectively; 4.1% were posttreatment samples. Correlation between PD-L1 and PD-L2 scores was moderate ($r_{\text{Spearman}} = 0.50$), and each biomarker was slightly less correlated with Tcell_{inf}GEP (0.41-0.45). PD-L1 expression and high Tcell_{inf}GEP status were associated with human papillomavirus positivity. Higher levels of all biomarkers were observed in oral cavity and oropharyngeal cancers compared with other HNSCC sites. In a multivariable analysis that simultaneously adjusted for all three biomarkers, only high Tcell_{inf}GEP was significantly associated with longer overall survival (adjusted hazard ratio, 0.57; 95% confidence interval 0.33-0.98) and recurrence-free survival (adjusted hazard ratio, 0.41; 95% confidence interval 0.23-0.74).

Conclusion: High Tcell_{inf}GEP status, but not PD-L1 or PD-L2 expression, was independently associated with longer survival in patients with LA HNSCC. Results may have implications for evaluating therapies targeting programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) in HNSCC.

Key words: head and neck squamous cell carcinoma, PD-L1, PD-L2, gene expression profile, programmed cell death protein 1

*Correspondence to: Prof. Byoung Chul Cho, Division of Medical Oncology, Department of Internal Medicine, Yonsei Cancer Center, Severance Hospital, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seoul, Korea Tel: +82-2-2228-8126; Fax: +82-2-393-3652

E-mail: CBC1971@yuhs.ac (B. C. Cho).

Prof. Myung-Ju Ahn, Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81 Irwon-ro, Seoul, South Korea Tel: +82-2-3410-3438; Fax: +82-2-3410-1754

E-mails: silkahn@skku.edu or silk.ahn@samsung.com (M.-J. Ahn).

[†]Contributed equally as co-first authors.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common malignancy worldwide, with >740 000 cases of lip/oral cavity, oropharynx, hypopharynx, and larynx cancers diagnosed annually, and a corresponding 365 000 deaths.^{1,2} Although alcohol and tobacco consumption are the main risk factors, human papillomavirus (HPV) accounts for ~25% of HNSCC, mostly in the oropharynx.^{3,4} Approximately 60% of patients with HNSCC are diagnosed with locoregionally advanced (LA) disease,⁵ and $\geq 50\%$ will develop recurrence or distant metastasis.³ Historically, LA

HNSCC was managed through a multidisciplinary approach, including combinations of radiation, surgery, and chemotherapy or cetuximab.³

Recent therapeutics have targeted the interaction between programmed cell death protein 1 (PD-1) receptor and its ligands, programmed death-ligand 1 (PD-L1) and PD-L2, a key signaling pathway wherein cancer cells evade immune surveillance through inhibition of T-cell activation, cytokine production, and T-cell apoptosis.^{6,7} PD-1 inhibitors have shown efficacy in combination with chemoradiation in studies of LA HNSCC.⁸ PD-1 inhibitors pembrolizumab and nivolumab are approved for the treatment of recurrent or metastatic (R/M) HNSCC,^{9,10} with companion diagnostic PD-L1 IHC 22C3 pharmDx identifying patients with HNSCC eligible for treatment with pembrolizumab.¹¹

Aside from PD-L1, other features of the tumor-immune interface have been explored to better predict clinical benefit. PD-L2 expression was reported to have additional effects on response and survival to PD-1 inhibition in R/M HNSCC.¹² Recent studies show PD-L1 and PD-L2 expression are upregulated by interferon- γ (IFN- γ), and the T-cell-inflamed gene expression profile (Tcell_{inf}GEP), comprising IFN- γ -responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance, was predictive of clinical response to PD-1 blockade in multiple cancer subtypes.¹³

Real-world data characterizing and evaluating, in an integrative manner, the relationship of these biomarkers with clinical outcomes under conventional therapies, however, is scant in LA HNSCC. Prior studies of singular analytes in LA HNSCC have yielded conflicting results, with some showing an association between PD-L1 or PD-L2 expression and shortened¹⁴ or prolonged survival,¹⁵ whereas others showed no clinical impact.^{16,17} Discrepant results may be attributed to intertumoral/intratumoral heterogeneity and differences in immunohistochemical detection methods or scoring.¹⁸ Evidence also suggests that PD-L1 and PD-L2 scoring in both tumor and immune-infiltrating cells is more clinically relevant than scoring in tumor cells alone.^{12,19}

This study is intended to characterize the immune biomarker profile of the tumor microenvironment, including expression of PD-L1 and PD-L2 in tumor and immune cells and Tcell_{inf}GEP signature, and the association of these biomarkers with outcomes in Korean patients receiving standard-of-care primary treatment of LA HNSCC.

MATERIALS AND METHODS

Study design and patient population

Adults aged ≥ 18 years at diagnosis with histologically confirmed American Joint Committee on Cancer (AJCC) seventh edition stage III-IVB²⁰ squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx between 2000 and 2015 and who received primary curative treatment were retrospectively identified from Samsung Medical Center and Severance Hospital of the Yonsei University Health System. Patients with sufficient and evaluable tissue

for PD-L1, PD-L2, and Tcell_{inf}GEP assessment were included. Patients with a history of other primary cancers within 5 years of HNSCC diagnosis were excluded, except for those with basal cell carcinoma of the skin, superficial bladder cancer, squamous cell carcinoma of the skin, and *in situ* cervical cancer.

The study was conducted in accordance with Good Clinical and Pharmacoeconomics Practice according to a protocol approved by the ethics committees at both institutions. Given this analysis was retrospective, informed consent was not required.

Biomarker assessment

Immunohistochemistry (IHC) was carried out at a single central laboratory or sponsor-approved laboratory vendor per assay, on 4- μ m thick, formalin-fixed paraffin-embedded tumor tissue to determine PD-L1 and PD-L2 expression, using PD-L1 IHC 22C3 pharmDx (Agilent Technologies, Carpinteria, CA) and PD-L2 clone MEB123.3G2.038.¹² PD-L1 expression levels were presented as combined positive score (CPS), defined as the number of PD-L1-positive cells (tumor cells, lymphocytes, and macrophages) divided by the total number of tumor cells $\times 100$. CPS ≥ 1 was considered PD-L1-positive. PD-L2 expression levels were reported as the proportion of PD-L2-positive cells (tumor and immune cells) out of all cells. A proportion score ≥ 1 was considered PD-L2-positive. All samples were considered adequate for PD-L1 and PD-L2 quantification and interpretation if at least 100 viable tumor cells were present on non-decalcified tissue.^{21,22}

A Tcell_{inf}GEP signature comprising 18 genes indicative of a T-cell-activated tumor microenvironment was previously derived across multiple solid tumors and was associated with response to pembrolizumab.^{23,24} Tumor RNA, extracted from formalin-fixed paraffin-embedded slides, was analyzed on the NanoString nCounter system (Seattle, WA) using a research version of the Tcell_{inf}GEP assay. Tcell_{inf}GEP scores were calculated as a weighted sum of normalized expression values for the 18 genes.¹³ Tcell_{inf}GEP status was defined as low (< -0.318), intermediate (-0.318 to < -0.162), or high (≥ 0.162).¹³

HPV status was determined using p16 IHC clone E6H4 (Roche, Basel, Switzerland). HPV oncogene expression was assessed using a $>70\%$ cut point for tumor cells stained for p16 IHC.²⁵

Outcomes and statistical analysis

The primary endpoints were overall survival (OS), defined as the time from primary treatment initiation to date of death from any cause, and recurrence-free survival (RFS), calculated as the time from primary treatment initiation to date of locoregional recurrence, distant metastasis, or death from any cause, whichever came first. Data were censored when date of death was unknown at last follow-up.

Differences in patient and clinicopathologic characteristics between biomarker categories were evaluated using chi-square (χ^2) statistics. Statistical significance was based

on $P < 0.05$. Correlations between biomarkers were evaluated using the Spearman correlation coefficient. PD-L1 and PD-L2 scores were square-root transformed to minimize the outsized influence of large values.

The relationship between survival endpoints and biomarkers was analyzed using the Kaplan–Meier method with log-rank test. Crude and adjusted hazard ratios (aHRs) with 95% confidence intervals (CIs) were generated using multivariable Cox proportional hazards models. Model parsimony was established through stepwise variable selection, considering various demographic, clinicopathologic, tumor and treatment characteristics, and clinical site (Table 1).

RESULTS

Patient characteristics

Of 462 patients identified from Samsung Hospital and Severance Hospital with a diagnosis of HNSCC from 2000 to 2015, 96 (20.8%) were excluded or not assessable (Supplementary Figure S1, available at <https://doi.org/10.1016/j.esmoop.2024.103961>). Of the 366 patients with samples evaluated for ≥ 1 biomarker (193 from Samsung Hospital; 173 from Severance Hospital), 365, 364, and 302 had a PD-L1, PD-L2, and/or Tcell_{inf}GEP score, respectively.

Median age at diagnosis was 59 years (range, 23–86 years), 83.1% were male, 61.2% were current or former smokers (All tobacco), and 38.8% had HPV-positive tumors (Table 1). Most tumors were stage IVA/B (75.4%), and most arose in the oropharynx (36.1%) and lip/oral cavity (24.0%). Compared with Severance Hospital, Samsung Medical Center had proportionately more cancers of the larynx (29.0% versus 9.2%) and other ill-defined sites (17.1% versus 0.6%), but fewer oropharyngeal cancers (25.4% versus 48.0%).

Planned primary treatment most often included post-surgical radiation (52.2%) and concurrent chemoradiation (CCRT), either as a definitive therapy (5.2%) or following surgery (34.7%). Postsurgical chemoradiation was more prevalent (56.1% versus 15.5%, respectively) and post-surgical radiation less prevalent (34.1% versus 68.4%, respectively) at Severance Hospital than at Samsung Medical Center. Few patients (4.1%) had samples obtained following chemotherapy treatment.

Biomarker expression

Most patients expressed PD-L1 (83.6%), PD-L2 (85.4%), and high Tcell_{inf}GEP status (73.2%; Table 2). Compared with HPV-negative patients, HPV-positive patients had higher proportions of PD-L1 positivity (88.7% versus 80.0%, respectively) and high Tcell_{inf}GEP status (85.2% versus 65.5%, respectively). PD-L1, PD-L2, and high Tcell_{inf}GEP expression were found in greater proportions in oral cavity and oropharyngeal cancers (range, 72.2%–92.0%) than in laryngeal or hypopharyngeal cancers (range, 54.9%–77.5%). Likewise, overexpression of each biomarker was higher in patients with surgery followed by adjuvant radiation, with

or without chemotherapy (range, 73.9%–94.5%), than in those with other treatments (range, 35.7%–75.9%). PD-L2 positivity was slightly higher in patients treated at Samsung Medical Center (89.1%) compared with Severance Hospital (81.3%; Table 2).

PD-L1 and PD-L2 were moderately correlated with each other ($r_{\text{Spearman}} = 0.50$) but were slightly less correlated with Tcell_{inf}GEP ($r_{\text{Spearman}} = 0.41$ for PD-L1, $r_{\text{Spearman}} = 0.45$ for PD-L2; Figure 1A–C).

Survival outcomes

Univariate analyses showed no significant survival with PD-L1 expression (aHR, 0.79; 95% CI, 0.50–1.25) (Figure 2A). OS was significantly longer in patients with high Tcell_{inf}GEP status and PD-L2 overexpression versus low or non-expressers ($P \leq 0.01$; Figure 2B and C). Similarly, across all three biomarkers, longer RFS was seen in biomarker-high/biomarker-positive patients versus their biomarker-low/biomarker-negative counterparts ($P \leq 0.01$; Figure 2D–F).

In multivariable models of singular biomarkers, OS was significantly longer for PD-L2-positive versus PD-L2-negative tumors (aHR, 0.63; 95% CI 0.40–0.99), and for high Tcell_{inf}GEP status versus low Tcell_{inf}GEP status (aHR, 0.48; 95% CI 0.29–0.80) (Figure 2B and C). When all three biomarkers were simultaneously adjusted along with other explanatory covariates within a single parsimonious model, only high Tcell_{inf}GEP status remained significantly and independently associated with longer OS (aHR, 0.57; 95% CI 0.33–0.98; $P < 0.05$; Table 3).

A significant positive association was observed in multivariate analysis between RFS and high Tcell_{inf}GEP status (aHR, 0.47; 95% CI 0.27–0.81); however, no associations were observed for PD-L1 (aHR, 0.81; 95% CI 0.47–1.38) or PD-L2 expression (aHR, 0.92; 95% CI, 0.52–1.61) (Figure 2D–F). In a model that included all three biomarkers and other covariates, only high Tcell_{inf}GEP status remained significantly associated with RFS (aHR, 0.41; 95% CI 0.23–0.74; $P < 0.01$; Table 3).

DISCUSSION

In this retrospective study of Korean patients with LA HNSCC, PD-L1 was common (83.6%) and similar to that reported (84%–90%) in observational studies of patients with varying stages of HNSCC and using the same 22c3 antibody clone and CPS ≥ 1 cut point.^{26–28} Studies using other PD-L1 antibody clones but similar 1% positivity thresholds in tumor and immune cells in predominantly ($\geq 65\%$) LA HNSCC have reported varying prevalence of PD-L1 overexpression: 40% with clone EH33,²⁹ 71% with E1L3N,³⁰ and 90% with SP142.³¹

PD-L2 expression was also prevalent (85.4%) and moderately correlated with PD-L1 expression ($r_{\text{Spearman}} = 0.5$). This is comparable to the $>90\%$ prevalence and significant correlations reported in a study of archival HNSCC samples using the same PD-L2 antibody clone and 1% cut point in tumor and immune cells¹² and a study of LA HNSCC

Table 1. Baseline characteristics by clinic site				
Characteristics	Total (N = 366)	Samsung hospital (n = 193)	Severance hospital (n = 173)	χ^2 test P value ^c
	n (%)	n (%)	n (%)	
Age at LA HNSCC diagnosis, years				
Median	59	60	58	
≤65	273 (74.6)	140 (72.5)	133 (76.9)	0.3410
>65	93 (25.4)	53 (27.5)	40 (23.1)	
Sex				
Female	62 (16.9)	32 (27.5)	30 (17.3)	0.8464
Male	304 (83.1)	161 (72.5)	143 (82.7)	
Smoking status (All tobacco) ^a				
Current	131 (35.8)	61 (34.5)	70 (44.6)	0.1129
Former	93 (25.4)	50 (28.2)	43 (27.4)	
Never	110 (30.1)	66 (37.3)	44 (28.0)	
Unknown	32 (8.7)	16	16	
Smoking volume				
Never smokers	110 (30.1)	66 (34.2)	44 (25.4)	0.2280
<10 pack-years	13 (3.6)	5 (2.9)	8 (5.6)	
≥10 pack-years	190 (51.9)	100 (58.5)	90 (63.4)	
Unknown (among smokers)	21 (5.7)	6	15	
HPV status				
Negative	221 (60.4)	125 (65.6)	96 (55.8)	0.0605
Positive	142 (38.8)	66 (34.4)	76 (44.2)	
Unknown	3 (0.8)	2	1	
Primary HNSCC site				
Hypopharynx	40 (10.9)	14 (7.3)	26 (15.0)	<0.0001
Larynx	72 (19.7)	56 (29.0)	16 (9.3)	
Lip and oral cavity	88 (24.0)	41 (21.2)	47 (27.2)	
Oropharynx	132 (36.1)	49 (25.4)	83 (48.0)	
Other ill-defined sites in lip, oral cavity, and pharynx	34 (9.3)	33 (17.1)	1 (0.6)	
Stage at diagnosis ^b				
III	90 (24.6)	64 (33.2)	26 (15.0)	0.0001
IV A/B	276 (75.4)	129 (66.8)	147 (85.0)	
Histologic grade				
Well differentiated	87 (23.8)	53 (31.7)	34 (20.9)	0.0700
Moderately differentiated	185 (50.5)	85 (50.9)	100 (61.3)	
Poorly differentiated	58 (15.8)	29 (17.4)	29 (17.8)	
Unknown	36 (9.8)	26	10	
ECOG status				
0	116 (31.7)	86 (44.6)	30 (30.0)	0.0351
1	160 (43.7)	95 (49.2)	65 (65.0)	
2+	17 (4.6)	12 (6.2)	5 (5.0)	
Unknown	73 (19.9)	0	73	
Primary treatment (planned)				
Definitive CCRT	19 (5.2)	8 (4.1)	11 (6.4)	<0.0001
Surgery + CCRT	127 (34.7)	30 (15.5)	97 (56.1)	
Surgery + RT	191 (52.2)	132 (68.4)	59 (34.1)	
RT or surgery alone	29 (7.9)	23 (11.9)	6 (3.5)	
Tissue sample collected before chemotherapy				
No	15 (4.1)	1 (0.5)	14 (8.1)	0.0003
Yes	351 (95.9)	192 (99.5)	159 (91.9)	
Neoadjuvant chemotherapy				
No	350 (95.6)	193 (100.0)	157 (90.8)	<0.0001
Yes	16 (4.4)	0 (0.0)	16 (9.2)	

AJCC, American Joint Committee on Cancer; CCRT, concurrent chemoradiation; ECOG, Eastern Cooperative Oncology Group; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; LA, locoregionally advanced; RT, radiotherapy.

^aFormer smoker is one who quit >1 year before diagnosis; current smoker is one who currently smokes or quit <1 year before diagnosis.

^bAccording to AJCC seventh edition criteria.²⁰

^cP value is to test the difference in clinicopathologic characteristics between clinical sites; the unknown category was excluded from this calculation.

using EPR1161 clones at a 1% cut point in tumor cells.¹⁴ In contrast, Kogashiwa et al.³² reported no correlation between PD-L1 and PD-L2 in LA oral cavity cancer; however, any relationship may be challenging to detect given the low PD-L2 prevalence (23.8%) in their study.

High Tcell_{inf}GEP status was found in 73.2% of patients, with a modest correlation between Tcell_{inf}GEP and both PD-L1 and PD-L2 ($r_{\text{Spearman}} = 0.41$ and 0.45 , respectively). The KEYNOTE-012 trial of pembrolizumab in patients with R/M HNSCC used the same Tcell_{inf}GEP signature of IFN- γ -

Table 2. Baseline characteristics by biomarker status

Characteristics	PD-L1 expression ^c N = 365		χ ² test P value ^f	PD-L2 expression ^d N = 364		χ ² test P value ^f	Tcell _{in} GEP expression ^e N = 302			χ ² test P value ^f
	Negative	Positive		Negative	Positive		Low	Intermediate	High	
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	n (%)	
Overall	60 (16.4)	305 (83.6)	NA	53 (14.6)	311 (85.4)	NA	49 (16.2)	32 (10.6)	221 (73.2)	NA
Age, years										
≤65	44 (16.1)	229 (83.9)	0.7755	35 (12.9)	237 (87.1)	0.1154	33 (14.7)	31 (13.8)	160 (71.4)	0.0062
>65	16 (17.4)	76 (82.6)		18 (19.6)	74 (80.4)		16 (20.5)	1 (1.3)	61 (78.2)	
Sex										
Male	49 (16.2)	254 (83.8)	0.7612	44 (14.6)	258 (85.4)	0.9913	43 (17.1)	23 (9.2)	185 (73.7)	0.1582
Female	11 (17.7)	51 (82.3)		9 (14.5)	53 (85.5)		6 (11.8)	9 (17.6)	36 (70.6)	
HPV status										
Negative	44 (20.0)	176 (80.0)	0.0291	35 (15.9)	185 (84.1)	0.2247	35 (19.8)	26 (14.7)	116 (65.5)	0.0005
Positive	16 (11.3)	126 (88.7)		16 (11.3)	125 (88.7)		13 (10.7)	5 (4.1)	104 (85.2)	
Unknown	0	3		2	1		1	1	1	
Smoking status (All tobacco)										
Current	22 (16.8)	109 (83.2)	0.9285	18 (13.7)	113 (86.3)	0.3481	19 (17.9)	10 (9.4)	77 (72.6)	0.0203
Former	16 (17.4)	76 (82.6)		18 (19.6)	74 (80.4)		20 (26.3)	10 (13.2)	46 (60.5)	
Never	17 (15.5)	93 (84.5)		14 (12.7)	96 (87.3)		9 (9.1)	8 (8.1)	82 (82.8)	
Unknown	5	27		3	28		1	4	16	
Smoking volume ^a										
Never	17 (15.5)	93 (84.5)		14 (12.7)	96 (87.3)		9 (9.1)	8 (8.1)	82 (82.8)	
<10 pack-years	5 (38.5)	8 (61.5)	0.0979	1 (7.7)	12 (92.3)	0.5263	3 (27.3)	1 (9.1)	7 (63.6)	0.0689
≥10 pack-years	30 (15.9)	159 (84.1)		31 (16.4)	158 (83.6)		34 (22.2)	15 (9.8)	104 (68.0)	
Unknown (among smokers)	3	18		4	17		2	4	12	
Primary HNSCC location										
Hypopharynx	12 (30.8)	27 (69.2)	0.0001	11 (27.5)	29 (72.5)	0.0084	7 (22.6)	5 (16.1)	19 (61.3)	0.0012
Larynx	22 (30.6)	50 (69.4)		16 (22.5)	55 (77.5)		13 (25.5)	10 (19.6)	28 (54.9)	
Lip and oral cavity	12 (13.6)	76 (86.4)		7 (8.0)	81 (92.0)		11 (13.9)	11 (13.9)	57 (72.2)	
Oropharynx	11 (8.3)	121 (91.7)		14 (10.7)	117 (89.3)		13 (10.9)	3 (2.5)	103 (86.6)	
Other and ill-defined sites in lip, oral cavity and pharynx	3 (8.8)	31 (91.2)		5 (14.7)	29 (85.3)		5 (22.7)	3 (13.6)	14 (63.6)	
Histologic grade										
Well differentiated	17 (19.5)	70 (80.5)	0.1980	9 (10.3)	78 (89.7)	0.2493	15 (20.8)	8 (11.1)	49 (68.1)	0.6325
Moderately differentiated	31 (16.8)	153 (83.2)		32 (17.5)	151 (82.5)		23 (15.2)	17 (11.3)	111 (73.5)	
Poorly differentiated	5 (8.6)	53 (91.4)		7 (12.1)	51 (87.9)		6 (12.0)	4 (8.0)	40 (80.0)	
Unknown	7	29		5	31		5	3	21	
Stage at diagnosis ^b										
III	13 (14.6)	76 (85.4)	0.5919	12 (13.5)	77 (86.5)	0.7403	11 (14.9)	13 (17.6)	50 (67.6)	0.0809
IV A/B	47 (17.0)	229 (83.0)		41 (14.9)	234 (85.1)		38 (16.7)	19 (8.3)	171 (75.0)	
ECOG status										
0	13 (11.2)	103 (88.8)	0.2152	10 (8.6)	106 (91.4)	0.1508	14 (15.2)	9 (9.8)	69 (75.0)	0.9801
1	28 (17.5)	132 (82.5)		22 (13.8)	137 (86.2)		21 (16.3)	11 (8.5)	97 (75.2)	
2+	1 (6.3)	15 (93.8)		4 (23.5)	13 (76.5)		2 (12.5)	2 (12.5)	12 (75.0)	
Unknown	18	55		17	55		12	10	43	
Primary treatment regimen for locally advanced HNSCC										
Definitive CCRT	11 (57.9)	8 (42.1)	<0.0001	9 (47.4)	10 (52.6)	0.0001	6 (42.9)	3 (21.4)	5 (35.7)	0.0070
Surgery + CCRT	7 (5.5)	120 (94.5)		18 (14.3)	108 (85.7)		16 (13.9)	7 (6.1)	92 (80.0)	

Continued

Table 2. Continued

Characteristics	PD-L1 expression ^c N = 365		χ^2 test P value ^f	PD-L2 expression ^d N = 364		χ^2 test P value ^f	Tcell _{int} GEP expression ^e N = 302			χ^2 test P value ^f
	Negative	Positive		Negative	Positive		Low	Intermediate	High	
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	n (%)	
Surgery + RT	34 (17.8)	157 (82.2)		19 (10.0)	171 (90.0)		22 (14.4)	18 (11.8)	113 (73.9)	
RT alone or surgery alone	8 (28.6)	20 (71.4)		7 (24.1)	22 (75.9)		5 (25.0)	4 (20.0)	11 (55.0)	
Neoadjuvant therapy										
No	57 (16.3)	292 (83.7)	0.7986	47 (13.5)	301 (86.5)	0.0078	45 (15.7)	31 (10.8)	211 (73.5)	0.5011
Yes	3 (18.8)	13 (81.3)		6 (37.5)	10 (62.5)		4 (26.7)	1 (6.7)	10 (66.7)	
Tissue sample collected before chemotherapy										
No	4 (26.7)	11 (73.3)	0.2750	5 (33.3)	10 (66.7)	0.0353	5 (38.5)	2 (15.4)	6 (46.2)	0.0553
Yes	56 (16.0)	294 (84.0)		48 (13.8)	301 (86.2)		44 (15.2)	30 (10.4)	215 (74.4)	
Clinic site										
Samsung Hospital	32 (16.6)	161 (83.4)	0.9382	21 (10.9)	172 (89.1)	0.0345	24 (16.6)	13 (9.0)	108 (74.5)	0.6761
Severance Hospital	28 (16.3)	144 (83.7)		32 (18.7)	139 (81.3)		25 (15.9)	19 (12.1)	113 (72.0)	

AJCC, American Joint Committee on Cancer; CCRT, concurrent chemoradiation; CPS, combined positive score; ECOG, Eastern Cooperative Oncology Group; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; NA, not applicable; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; RT, radiotherapy; Tcell_{int}GEP, T-cell-inflamed gene expression profile.

^aFormer smoker is one who quit >1 year before diagnosis; current smoker is one who currently smokes or quit <1 year before diagnosis.

^bAccording to AJCC seventh edition criteria.²⁰

^cPD-L1 status: positive (CPS ≥ 1) and negative (CPS <1).

^dPD-L2 status: positive (proportion score ≥ 1) and negative (proportion score <1).

^eTcell_{int}GEP status: low (score of <-0.318), intermediate (score -0.318 to <-0.162), or high (score ≤ -0.162).

^fP value is based on comparison between subgroups of patient/tumor characteristics within the total population; the unknown category was excluded from this calculation.

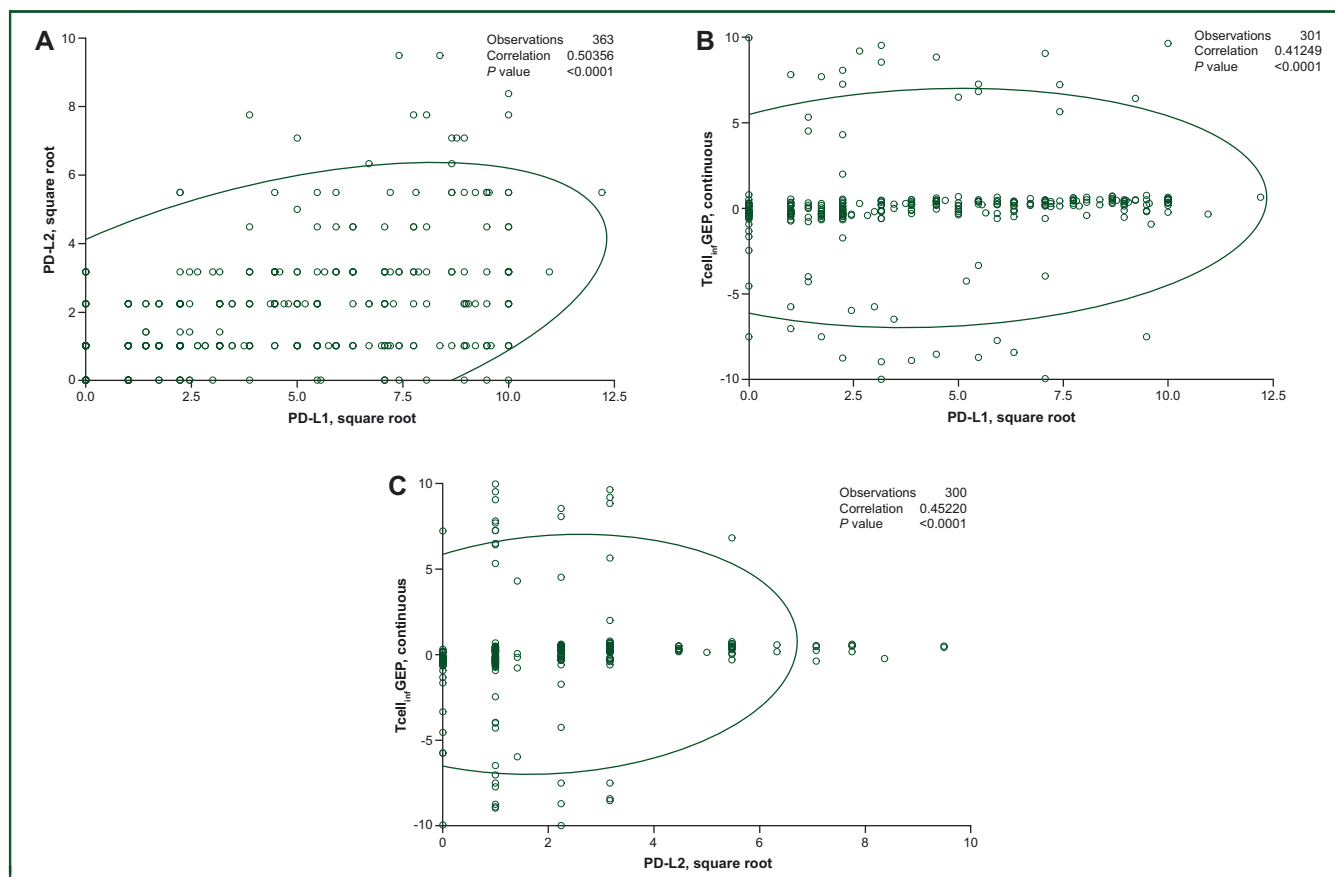


Figure 1. Correlation between (A) PD-L1 and PD-L2, (B) PD-L1 and Tcell_{infl}GEP, and (C) PD-L2 and Tcell_{infl}GEP scores. Both PD-L1 and PD-L2 scores have been square-root transformed to minimize outsized influence of large values. PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; Tcell_{infl}GEP, T-cell- inflamed gene expression profile.

responsive genes and also reported a high Tcell_{infl}GEP prevalence of 71% and a significant correlation with PD-L1 ($r_{\text{Spearman}} = 0.51$).³³ This correlation likely reflects the induction of PD-L1 and PD-L2 expression by IFN- γ , secreted from CD8+ T cells as part of the intratumoral T-cell adaptive immune response,¹³ rather than induction from an innate, oncogene-driven mechanism, such as activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways.³⁴

Overexpression of all three biomarkers was consistently higher in oral cavity/oropharyngeal cancers versus other HNSCC sites. Anatomic site-based differential in CPS ≥ 1 has been reported in other studies and may be a function of a relatively rich inflammatory environment in the tongue and oropharynx.^{27,35,36} Virus-induced chronic immune cell infiltration in HPV-associated oropharyngeal cancer may also explain why HPV-positive patients had greater levels of PD-L1 and Tcell_{infl}GEP in our study.^{37,38}

Biomarker-positive prevalence was also consistently higher in patients treated with postsurgical radiation, with or without chemotherapy, versus other treatment regimens. Cancer treatment could artificially modulate biomarker expression levels³⁹; however, few patients (4.1%) had samples obtained following systemic treatment in our study. Higher expression levels in surgically treated patients may be explained by an underestimation of CPS (and other

immune markers) in smaller biopsy samples versus resection specimens, which are more likely to harbor the tumor-invasive front, where immune activity is greatest.^{26,40}

Our results did not show PD-L1 to be associated with clinical outcome. Prior observational studies evaluating 22C3 clone at CPS ≥ 1 , including one study in Asia, have generally shown no association with survival endpoints in HNSCC with mixed stages of disease.^{27,36} Another study, however, reported varying results of association with PD-L1 based on the start of OS follow-up; no association was found when OS was defined from time of diagnosis, and a positive association was observed when OS was defined from time of tissue acquisition.²⁶ Two additional studies of patients in Asia, primarily with LA HNSCC, had included immune cells in the PD-L1 scoring methods, using clone SP142 at a 5% positivity threshold. Kim et al.⁴¹ reported PD-L1 in immune cells (but not tumor cells) was positively prognostic of OS in surgically treated patients, whereas Ngamphaiboon et al.³¹ showed no association between PD-L1 (as a composite of tumor- and immune-cell staining) and OS in patients who received definitive treatment.

PD-L2 was not independently associated with survival after adjustment for Tcell_{infl}GEP and PD-L1. Prior HNSCC studies using a variety of PD-L2 antibody clones, positivity thresholds, and cell types for scoring reported a negative⁴² or no association with survival.^{14,32} Inconsistent results across PD-L1 and PD-L2 studies could be attributed to

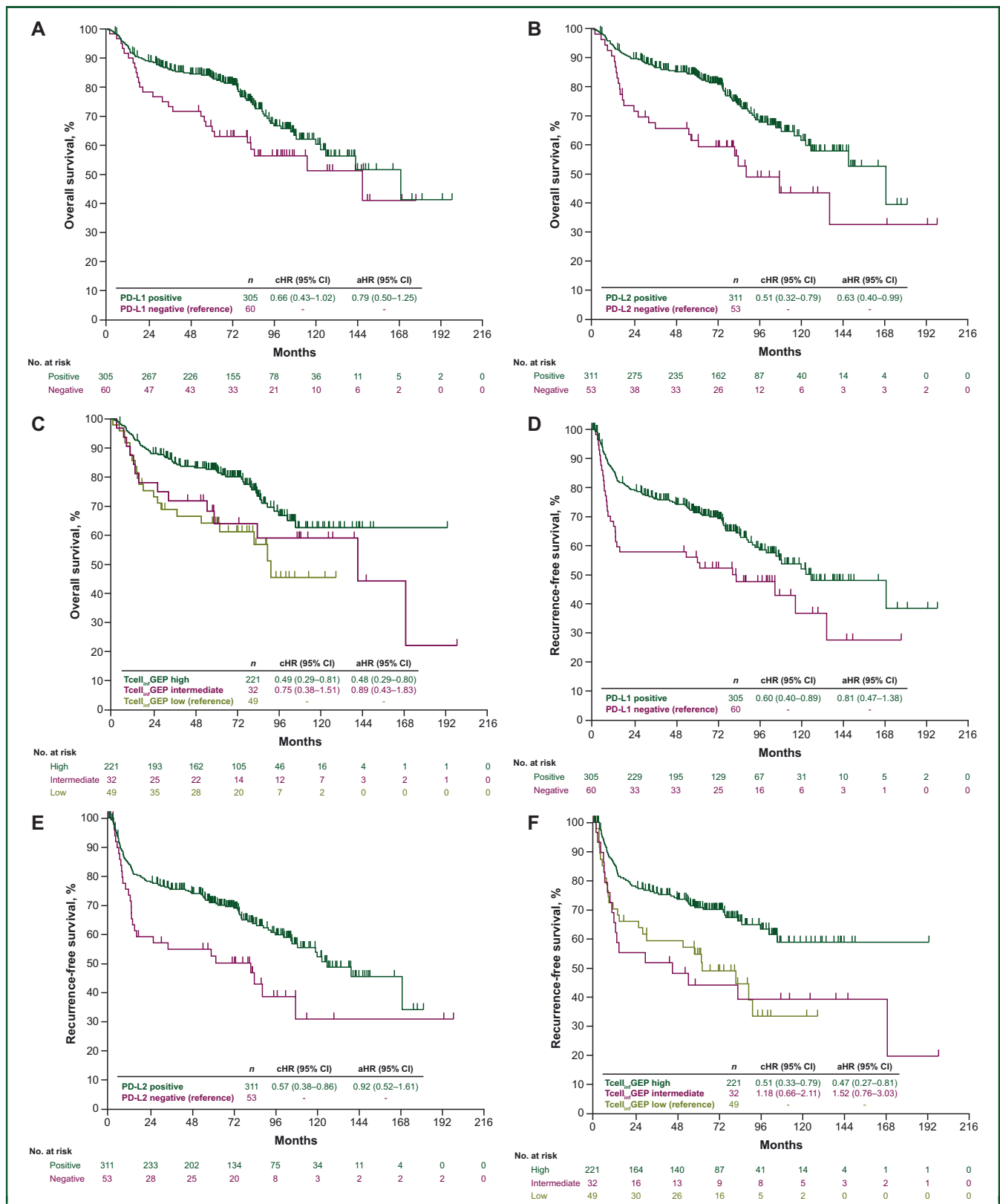


Figure 2. Kaplan–Meier curves of (A–C) overall survival and (D–F) recurrence-free survival, by singular biomarker status. Biomarkers were assessed singularly in separate models. aHR, adjusted hazard ratio; cHR, crude hazard ratio; CI, confidence interval; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; RFS, recurrence-free survival; Tcell_{infl}GEP, T-cell-inflamed gene expression profile.

	aHR (95% CI) for OS ^a	P value	aHR (95% CI) for RFS ^b	P value
PD-L1 negative (reference)	—	—	—	—
PD-L1 positive	0.69 (0.40-1.20)	0.193	0.75 (0.37-1.54)	0.434
PD-L2 negative (reference)	—	—	—	—
PD-L2 positive	0.74 (0.43-1.29)	0.290	1.21 (0.61-2.42)	0.581
Tcell _{inf} GEP-low (reference)	—	—	—	—
Tcell _{inf} GEP-intermediate	0.83 (0.40-1.76)	0.634	1.36 (0.66-2.80)	0.400
Tcell _{inf} GEP-high	0.57 (0.33-0.98)	0.043	0.41 (0.23-0.74)	0.003

aHR, adjusted hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; OS, overall survival; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; RFS, recurrence-free survival; Tcell_{inf}GEP, T-cell-inflamed gene expression profile.

^aParsimonious model adjusted for all three biomarkers plus age and clinical site.

^bParsimonious model adjusted for all three biomarkers plus ECOG, planned treatment, and clinical site.

tumor spatiotemporal heterogeneity [e.g. sample size (biopsy versus resection), tissue origin (primary versus metastatic), or staining pattern (membranous versus cytoplasmic)] or methodologic differences [e.g. variability in the antibodies, positivity cut-offs, or scoring criteria (tumor ± immune cells)].^{18,26}

After adjusting for the other biomarkers, only high Tcell_{inf}GEP status was independently associated with longer OS and RFS. This suggests the association of these immune biomarkers of the PD-1/L-1 axis with survival is mainly driven by high Tcell_{inf}GEP expression. This relationship may reflect a functionally active T-cell antitumor response, as represented by an elevated IFN-γ signature.³⁵ HNSCC is relatively rich in immune infiltrates; such tumors are likely to have more effective immune surveillance, and therefore, a better prognosis.³²

Key strengths of this study include the large sample size, detailed clinical information, and use of a single laboratory to mitigate assay variability. Limitations include the retrospective design, treatment regimen heterogeneity, and inconsistency in patient follow-up schedules, which could impact the timing of outcome assessment. Selection bias may have occurred, given patients were included based on tissue sample availability. We did not account for time-varying changes to therapy following the index primary treatment. Further, HPV DNA is not routinely collected in the clinical setting and therefore no additional HPV analyses were carried out outside of p16 IHC testing. During our study, only two patients received PD-(L)1 inhibitors after the index treatment, which could influence survival, albeit minimally. Additionally, although Korean patients are not representative of other races/ethnicities, no data suggest PD-L1 expression in HNSCC varies by race or ethnicity.

Conclusions

In this retrospective analysis, PD-L1, PD-L2, and Tcell_{inf}GEP expression were moderately correlated; however, only high Tcell_{inf}GEP status was independently associated with OS and RFS. Although additional studies are needed to confirm these associations, these results could provide a deeper understanding of the relationship between biomarkers of the tumor microenvironment and treatment response in HNSCC. Future studies may benefit from broadening the

profile of immune markers under evaluation and replicating analyses across different racial/ethnic populations.

DISCLOSURE

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DATA SHARING

Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ (MSD), is committed to providing qualified scientific researchers access to anonymized data and clinical study reports from the company's clinical trials for the purpose of conducting legitimate scientific research. MSD is also obligated to protect the rights and privacy of trial participants and, as such, has a procedure in place for evaluating and fulfilling requests for sharing company clinical trial data with qualified external scientific researchers. The MSD data sharing website (available at: http://engagezone.msd.com/ds_documentation.php) outlines the process and requirements for submitting a data request. Applications will be promptly assessed for completeness and policy compliance. Feasible requests will be reviewed by a committee of MSD subject matter experts to assess the scientific validity of the request and the qualifications of the requestors. In line with data privacy legislation, submitters of approved requests must enter into a standard data-sharing agreement with MSD before data access is granted. Data will be made available for request after product approval in the USA and European Union or after product development is discontinued. There are circumstances that may prevent MSD from sharing requested data, including country- or region-specific regulations. If the request is declined, it will be communicated to the investigator. Access to genetic or exploratory biomarker data requires a detailed, hypothesis-driven statistical analysis plan that is collaboratively developed by the requestor and MSD subject matter experts; after approval of the statistical analysis plan and execution of a data-sharing agreement, MSD will either perform the proposed analyses and share the results with the requestor or will construct biomarker covariates and add them to a file with clinical data that is uploaded to

an analysis portal so that the requestor can perform the proposed analyses.

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