ABSTRACT

Purpose: Immune checkpoint proteins programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) are important therapeutic targets for head and neck cancer. This large-scale case study aimed to analyze tongue squamous cell carcinomas (SCCs) and evaluate the correlation between PD-L1 expression and clinical prognosis. So far, this study is the largest case study on PD-L1 expression in tongue SCCs.

Methods: This is a case-control study that analyzed 121 tongue SCCs. Paraffin-embedded sections and clinical data were obtained retrospectively and immunohistochemistry with PD-L1 was performed.

Results: 11.6% contained ≥50% of PD-L1-positive cells, 57.1% of these cases had a poor prognosis with nodal metastasis. Among cases of T1/2 primary lesions with nodal metastasis, cases of high PD-L1 expression had a significantly shorter disease-free survival than cases of no PD-L1 expression ($p=0.018$). The hazard ratio for high PD-L1

expression was 3.21 (95 per cent CI, 1.26-8.72) compared with no PD-L1 expression after adjusting for other factors.

Conclusions: These data indicate that PD-L1 upregulation in tongue SCCs is associated with a more advanced stage and shorter disease-free survival. PD-1/PD-L1 inhibitors might hence constitute potential adjuvant therapy for tongue SCCs with PD-L1 upregulation.

1. Introduction

Head and neck cancers occur in more than 600,000 new individuals each year globally [1], 90% of which are squamous cell carcinoma [2]. Patients often present with locally advanced tumor with lymph node metastasis and are treated with combinatorial multimodal therapy including surgery, radiotherapy, and chemotherapy. However, despite recent advancements in treatment, the long-term survival rate of head and neck cancer patients is still under 50%, decreasing to 19% if patients are diagnosed at a very advanced stage [3]. Notwithstanding platinum-based chemotherapy with cetuximab, the so-called EXTREME regimen, and newer molecular targeted drugs (e.g., zalutumumab and afatinib), median overall survival is still less than 1 year in patients with recurrent or metastatic cancers [4, 5].

Cancer immunotherapy is reportedly an effective treatment modality in multiple tumor types including head and neck cancers [6]. Immune checkpoints including programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) play important roles in the formation of "immune privilege" regions, viral persistence, tumor development, and immune evasion [3]. Therefore, blockade of the PD-1/PD-L1 axis reactivates dysfunctional or exhausted T cells via restoration of tumor-specific immunity, aimed at eliminating cancer cells [3]. Nivolumab, a commercially available human monoclonal anti-PD-1 antibody reportedly active against recurrent/metastatic head and neck squamous cell carcinoma (HNSCC) and has been approved clinically in several countries after a phase III clinical trial [7]. Among patients with platinum-refractory HNSCC, Nivolumab prolonged survival and resulted in fewer toxic effects than did standard therapy. Although cancer immunotherapy has great potential to treat HNSCC, the prognostic value of PD-L1 in HNSCC is unclear because only ~ 20% of metastatic/recurrent HNSCC patients would respond to Nivolumab and studies have reported inconsistent results regarding PD-L1 expression, thereby indicating an unpredictable immune response.

In this study, we evaluated PD-L1 expression in tongue SCCs, which are not usually associated with Human Papilloma Virus or Epstein-Barr Virus infections. The association of PD-L1 expression with patient clinicopathological characteristics and prognosis were analyzed. To our knowledge, this study is the largest case study on PD-L1 expression in tongue SCCs.

2. Patients and methods

The study was approved by Bioethics Committee of Okayama University (approval number 2241; project title: Prognostic factors in head and neck tumors). In total, 121 patients with tongue SCCs, who initially underwent surgery between November 2003 and October 2015 at Okayama University Hospital (Okayama,

Japan), Red Cross Society Himeji Hospital (Himeji, Japan) and Okayama medical Center (Okayama, Japan) were included. All cases were treated without PD-1/PD-L1 treatment including Nivolumab, which is the only immunotherapeutic agent for HNSCC as of 2018 after being approved by the Japanese government in 2017. The histological grade of the tumor was determined in accordance with the WHO classification and the extent of the tumor was classified in accordance with 7th edition of TNM classification established by the American Joint Committee on Cancer and the International Union Against Cancer (AJCC/UICC) [8]. Neoadjuvant chemotherapy has been administered to the patients whose waiting time for surgery had been expected to be longer than 4 weeks. As for surgical strategy in management of early-stage T1/2 cases, prophylactic neck dissection was performed for T2N0 to resect occult metastasis. Paraffin-embedded surgical specimens and clinical data were obtained retrospectively. Serial 4-μm-thick sections were cut from each tissue block and stained with hematoxylin and eosin. Paraffin-embedded sections of each sample were used for immunohistochemical staining with anti-PD-L1 antibody (1:200, E1L3N; Cell Signaling, Boston, MA, USA) using the automated Bond III stainer (Leica Biosystems; Wetzlar, Germany). Immune-reactive tumor cells were quantified and evaluated by 10% increments ranging from 0 to 100% of the total tumor cell population. The quantification process was performed by investigators blinded to the clinical data of patients. Sensitivity analysis using COX proportional models indicated that cut-off values of ≥30%, 40%, 50% and 60% showed similar hazard ratios, then we set 50% as representative cut-off value (Table 1, Figure 1); 0–40% patients were labeled as negative and ≥50% patients were labeled as positive.

Student's *t*-test was performed to compare the average age, and Fisher's exact test was performed to compare categorical variables between groups. Disease-free survival (DFS) was calculated from the time of surgery to cancer recurrence or death from any cause. Generalized Wilcoxon's Test was performed to compare Kaplan-Meier curves. Multivariate analyses were performed to test the independent significance of variables, using the Cox proportional hazards model. P-values less than 0.05 indicated statistical significance. Analyses were performed using BellCurve for Excel version 2.14 (Social Survey Research Information Co., Ltd.) and STATA version 15.1 (Stata, College Station, Texas, USA).

3. Results

The median age of the patients, including 79 men and 42 women, was 63 (23–96) years. All the patients were treated surgically, including 44 patients treated with neoadjuvant chemotherapy: 34 patients

treated with Nedaplatin + Fluorouracil; 3, Docetaxel + Cisplatin+ Fluorouracil; 2, Docetaxel; 2, Tegafur/Gimeracil/Oteracil; 1, Docetaxel + Cetuximab; 1, Cisplatin; 1, Nedaplatin + Tegafur/Gimeracil/Oteracil (duplicates were allowed). Only one patient was previously administered radiation therapy using the CyberKnife system (Accuray Inc., Germany) (Table 2). The surgical margins were negative in all the 121 cases. Seven cases with marked extra-nodal invasion of the metastases received radiotherapy (4 cases, 63/28fr) or chemoradiotherapy/bioradiotherapy (Carboplatin+Fulurouracil: 1; S-1: 1; Cetuximab: 1, 60Gy/30fr). We have checked the influence of storage period of the sample to the PD-L1 appearance rate, and found that there was no correlation between these 2 subjects (R-squared = 0.0074). PD-L1 expression levels in tumor cells were 0% in 97 patients (80.2%), 10–40% in 10 patients (8.3%), and ≥50% in 14 patients (11.6%) (Figure 1). Following sensitivity analysis, 0-40% expression was labeled as negative (107 [88.4%] patients), and ≥50% patients were labeled as positive (14 [11.6%] patients).

While primary tumor size did not differ significantly between the PD-L1-positive and PD-L1-negative groups (p=1), lymph node metastasis occurred at a significantly higher rate in the PD-L1-positive group than in the PD-L1-negative group (57.1% vs. 27.1%, respectively; p=0.031). Consequently, individuals in the PD-L1-positive group displayed a significantly more advanced stage than those in the negative group (p=0.018). In addition, median disease-free survival (DFS) was significantly shorter in the positive group (8 months) than in the negative group (62 months) (p=0.038. Figure 2).

Since majority of the subjects were T1/2 patients in this study (n=99, 81.8%), we analyzed the prognosis focusing on this population (Table 3). With respect to this population, 87 patients (87.9%) were labeled as negative and 12 patients (12.1%) were positive, and the median DFS in the positive group (8 months) was significantly shorter than the negative group (108 months) (p=0.0091. Figure 3). In this population, lymph node metastasis occurred at a significantly higher rate in the PD-L1-positive group than in the negative group (58.3% vs. 14.9%, respectively; p=0.0021) (Table 3). Thus, PD-L1 expression may be a significant risk factor for lymph node metastasis. In addition, to evaluate the effect of PD-L1 expression on prognosis at the same clinical stage, 20 (T1/2) patients with lymph node metastasis (stage III/IV) were divided into 2 subgroups based on PD-L1 expression: 7 (35%) PD-L1-positive patients and 13 (65%) PD-L1-negative patients. Median DFS was significantly shorter in the positive group (6 months) than in the negative group (17 months) (p=0.018. Figure 4).

For multivariate analysis of DFS, confounding variables were selected based on a priori hypothesis from a preliminary investigation. Stratified analysis on sex showed that hazard ratios (HR) of male and female were 1.8 and 0.9 respectively. Since those inverse ratios implied the presence of effect modification and only 2 female cases were PD-L1 positive, the analyses were done restricted to male. The adjusted HR for positive PD-L1 expression was 3.21 (95 percent CI, 1.26-8.72) compared with negative (Table 4). When restricted to T1/2 patients, adjusted HR for positive PD-L1 decreased to 2.32 in fully adjusted model (95 percent CI, 0.80-6.79) and age-adjusted model still presented statistically significant HR (Table 5).

4. Discussion

Before comparing the association between patient prognosis and PD-L1 expression, it is essential to standardize assay conditions including the antibody for immunohistochemistry (IHC), which should be specific and show low background levels. The Blueprint PD-L1 IHC Assay project compared 5 monoclonal antibodies (clone 22C3, 28-8, SP142, SP263, and 73-10) in lung cancer and reported that 22C3, 28-8, and SP263 showed comparable results, while fewer positive cells were detected using SP142 [9]; this should be avoided. Scognamiglio and Chen assessed 3 monoclonal PD-L1 antibodies (clone SP263, SP142, and E1L3N) in normal tonsils as the positive control and reported that all the three antibodies yielded the expected positive staining pattern [6, 10]. Herein, E1L3N was selected based on these studies, showing comparable results with previous studies.

The rate of PD-L1 positivity in oral SCCs have reportedly been different, probably owing to differences in the IHC conditions and tissue samples; recent case studies have reported that 18.5–64.9% of cases were characterized by PD-L1 expression in ≥5% of tumor cells with monoclonal antibodies [10-14]. Regarding the threshold to determine the potential for the application of PD-1/PD-L1 treatment, no decisive value for PD-L1 positivity rate in tumor cells has been suggested for HNSCCs. In clinical trials involving the combinatorial administration of Nivolumab and Pembrolizumab for recurrent/metastatic HNSCCs, the overall response rates (ORR) were 17.0–17.9% among patients with PD-L1 expression ≥1%, and the efficacy improved to 27.1–27.9% upon selecting for PD-L1 expression levels of ≥10–50% [7, 15]. Considering the positive correlation observed between PD-L1 expression and the efficacy of the blockade of the PD-1/PD-L1 axis in clinical trials, setting threshold for the PD-L1 positivity rate to segregate the population seems difficult and could be an arbitrary value. In clinical trials involving Pembrolizumab to treat non-small-cell lung cancer, showing tumor heterogeneity and HNSCC, PD-L1 expression in ≥50% tumor cells was reported as the threshold on the basis of

receiver operating characteristic curve analysis, and patients with ≥50% PD-L1 expression displayed improved Pembrolizumab efficacy [16-18]. In the present study, a threshold value of ≥50% was established to select patients with PD-L1 upregulation following sensitivity analysis, thus indicating that 11.6% of tongue SCCs are characterized by PD-L1 upregulation, concurrent with previous reports on oral SCCs. Although chemotherapy may have promoted the positive conversion of PD-L1 upregulation [19], the present data suggest that neoadjuvant chemotherapy did not significantly upregulate PD-L1 (Table 2). However, the largest case study reported that among 305 cases of oral SCCs, 43.6% showed PD-L1 upregulation, using a polyclonal rabbit anti-PD-L1 antibody [20], thereby suggesting that the positive population seems too high when compared with other studies, thus highlighting the uncertainty of using an unspecific polyclonal antibody as opposed to monoclonal antibodies and the lack of quantitative data on expression levels.

Besides the efficacy or application of PD-1/PD-L1 inhibitor treatment for recurrent/metastatic HNSCCs, recent studies have reported that PD-L1 expression may be a potential prognostic marker for primary HNSCC. With the large number of patients, PD-L1 expression in HNSCCs was significantly correlated with lymph node metastasis and unfavorable outcomes [11, 13, 14]. The present results indicate that PD-L1 upregulation in tumor cells in tongue SCCs is associated with a more advanced stage, lymph node metastasis, and shorter DFS.

To put focus on T1/2 patients, which consisted of 81.8% of the population in this study, univariate and multivariate analyses restricted to these patients were performed; PD-L1-positive patients showed significantly shorter DFS than PD-L1-negative group. Although HR of PD-L1 expression was not statistically significant in fully adjusted model, PD-L1 expression seemed to have negative influence on the prognosis in T1/2 patients. To compare the prognosis of tongue SCCs between PD-L1-positive and -negative patients at the same stage, we considered cases of T1/2 with lymph node metastasis, revealing that PD-L1-positive patients had a shorter DFS than PD-L1-negative patients. These results suggest that primary tongue SCCs with PD-L1 upregulation result in a more severe clinical outcome with nodal metastases and recurrence.

However, recurrent/metastatic HNSCCs with PD-L1 upregulation showed a better response rate upon blockade of the PD-1/PD-L1 axis in clinical trials. In general, R/M HNSCC patients have poor clinical outcomes; this is evident from the finding that median OS in the trial was 5.1 months with standard therapy and 7.5 months with Nivolumab, and OS extended to 8.7 months upon selecting only patients with ≥10% PD-L1 expression [7]. Furthermore, PD-L1-negative patients presented survival benefits; however, considering the

relatively low response rate, severe prognosis, and the high cost of drugs, patients with PD-L1 downregulation may have benefit more from receiving the other treatment first.

There are limitations to this study. First, even though no significant difference was observed in PD-L1 expression between the group that had received neoadjuvant chemotherapy and the others, it is possible that chemotherapy may have promoted PD-L1 upregulation [19]. Second, we excluded female cases from the multivariable analyses due to its small number of cases. Hanna et al. demonstrated that tumor PD-L1 expression is associated with improved survival and lower recurrence risk in young women with oral cavity SCC [21]. However, the present study included only 2 out of 42 female cases were PD-L1 positive, which was too small to statistically analyze the effect of PD-L1 expression among women. Third, the number of T3/4 cases was small compared to that of T1/2. Further study is required to evaluate the association of PD-L1 expression with prognosis in T3/4 cases.

In summary, the present study analyzed PD-L1 expression in 121 tongue SCCs, hence being the largest case study at this time, suggesting that 11.6% were characterized by ≥50% of PD-L1 expression levels, and these populations showed an increased frequency of lymph node metastasis and shorter disease-free survival when restricted to male. These results suggest that tongue SCCs with high PD-L1 expression levels may need adjuvant therapy and PD-1/PD-L1 treatment may be a favorable treatment alternative to counter the poor outcome. Further studies are required to evaluate changes in PD-L1 expression in tumors during treatment and metastatic progression.

Disclosure statement

All authors declare that: (i) no support, financial or otherwise, has been received from any organization that may have an interest in the submitted work; and (ii) there are no other relationships or activities that could appear to have influenced the submitted work.

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Figure captions

Fig.1 Representative images of PD-L1 expression in tongue squamous cell carcinoma. (A) Less than <10%, (B) 10–49%, and (C) ≥50% of tumor cells expressed PD-L1

Fig.2 Kaplan-Meier analysis of disease-free survival (DFS) based on PD-L1 expression levels. DFS was significantly shorter in the PD-L1-positive group(p=0.038)

Fig.3 Kaplan-Meier analysis of disease-free survival (DFS) based on PD-L1 expression in T1/2 cases. DFS was significantly shorter in the PD-L1-positive group(p=0.0091)

Fig.4 Subgroup analysis based on PD-L1 expression in T1/2 cases with lymph node metastasis. Kaplan-Meier analysis of disease-free survival (DFS) based on PD-L1 expression. T1/2N+ cases showed significantly shorter DFS when PD-L1 expression was positive(p=0.018)

Figure 1

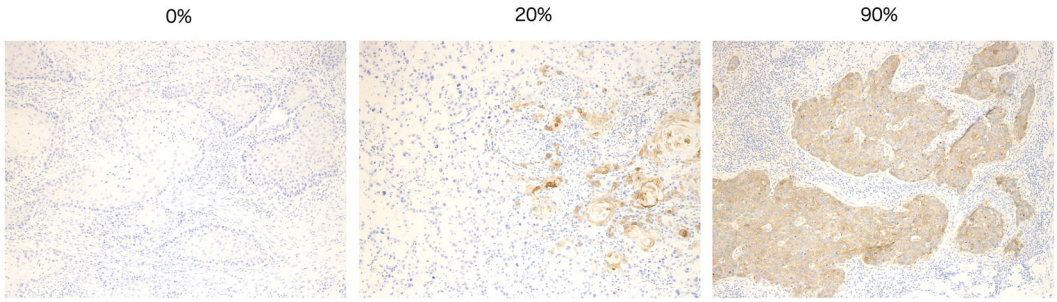

Figure 2

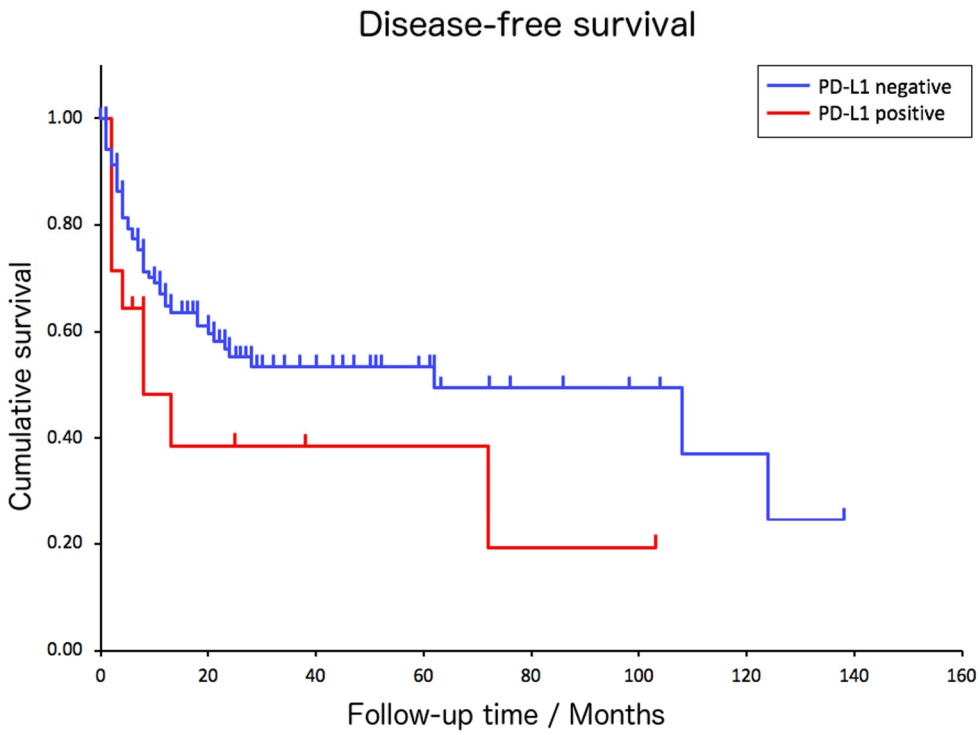

Figure 3

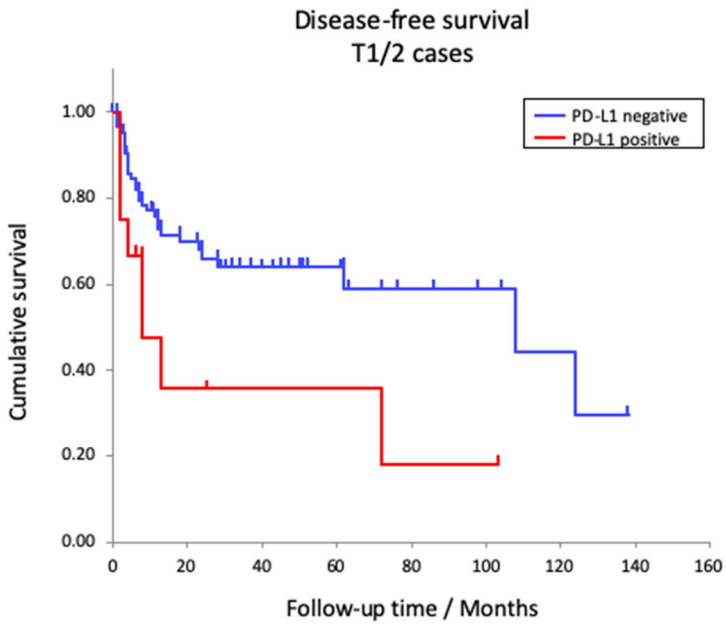

Figure 4

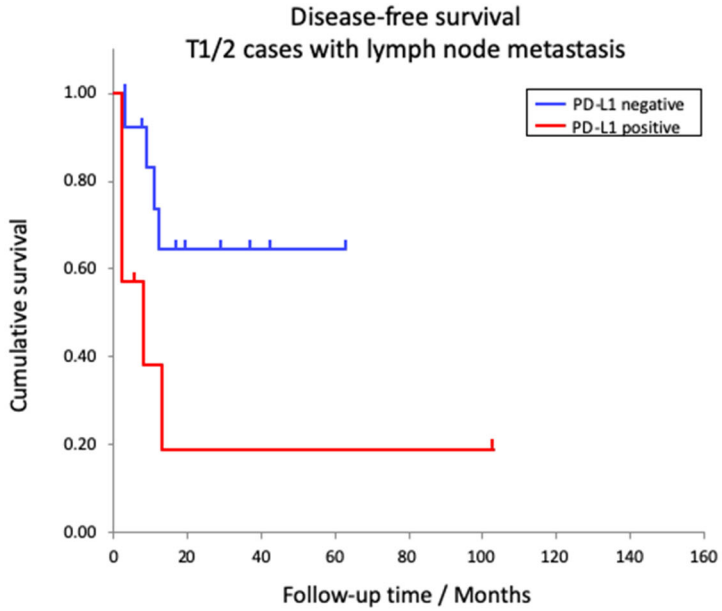

Table 1. Sensitivity analysis using Cox proportional hazards models for disease-free survival.

	fully adjusted		
PD-L1 cut off	HR	(95% CI)	P-value
<10, 10>=	1.89	(0.84-4.24)	0.123
<20, 20>=	1.33	(1.00-1.76)	0.049
<30, 30>=	3.49	(1.47-8.32)	0.005
<40, 40>=	3.31	(1.26-8.72)	0.016
<50, 50>=	3.31	(1.26-8.72)	0.016
<60, 60>=	2.99	(1.00-8.94)	0.051
<70, 70>=	0.61	(0.08-4.83)	0.643
<80, 80>=	1.02	(0.13-7.93)	0.983
<90, 90>=		not estimated	

Abbreviations: CI: confidence interval, HR: Hazard ratio

Table 2. Association of PD-L1 expression with clinicopathological characteristics of the study patients. All data are presented as mean ± standard deviation values.

	total		PD-L1 expression				
			negative		positive		
	n=121		n= 107		n=14		P-value
Age	59.0±17.4		58.1±17.7		58.1±16.2		p=0.844
Sex							p=0.135
Male	79	65.3%	67	62.6%	12	85.7%	
Female	42	34.7%	40	37.4%	2	14.3%	
Local							p=1
T1/2	99	81.8%	87	81.3%	12	85.7%	
T3/4	22	18.2%	20	18.7%	2	14.3%	
Nodal meta							p=0.031*
N-	84	69.4%	78	72.9%	6	42.9%	
N+	37	30.6%	29	27.1%	8	57.1%	
Stage							p=0.018*
I/II	79	65.3%	74	69.2%	5	35.7%	
III/IV	42	34.7%	33	30.8%	9	64.3%	
Tabacco use							p=0.137
Yes	62	51.2%	52	48.6%	10	71.4%	
No	52	43.0%	49	45.8%	3	21.4%	
Alcohol consumption							p=0.557
Yes	57	47.1%	49	45.8%	8	57.1%	
No	57	47.1%	52	48.6%	5	35.7%	
Neoadjuvant chemotherapy							p=0.137
Yes	44	36.4%	36	33.6%	8	57.1%	
No	77	63.6%	71	66.4%	6	42.9%	

Table 3. Association of PD-L1 expression with clinicopathological characteristics of the study patients restricted to the T1/2 population. All data are presented as mean ± standard deviation values.

	total		PD-L1 expression				
			negative		positive		
	n=99		n= 87		n=12		P-value
Age	59.3±16.8		59.3±17.1		59.2±15.8		p=0.978
Sex							p=0.052
Male	64	64.7%	53	60.9%	11	91.7%	
Female	35	35.4%	34	39.1%	1	8.3%	
Nodal meta							p=0.002*
N-	79	79.8%	74	85.1%	5	41.7%	
N+	20	20.2%	13	14.9%	7	58.3%	
Tabacco use							p=0.054
Yes	49	52.7%	40	48.8%	9	81.8%	
No	44	47.3%	42	51.2%	2	18.2%	
Alcohol consumption							p=0.206
Yes	49	52.7%	41	50.0%	8	72.7%	
No	44	47.3%	41	50.0%	3	27.3%	
Neoadjuvant chemotherapy							p=0.075
Yes	26	26.3%	20	23.0%	6	50.0%	
No	73	73.7%	67	77.0%	6	50.0%	

Table 4. Multivariable analyses of disease-free survival restricted only to male.

Disease-free Survival (Restricted only to male)									
	age adjusted			Model 1			fully adjusted		
	HR	(95% CI)	P-value	HR	(95% CI)	P-value	HR	(95% CI)	P-value
PD-L1 (positive vs negative)	2.04	(0.92-4.50)	0.077	3.02	(1.26-7.25)	0.013	3.21	(1.26-8.72)	0.016
Age (per 10 year increment)	1.14	(0.91-1.43)	0.266	1.30	(1.00-1.69)	0.054	1.39	(1.05-1.85)	0.023
Local (T1/2 vs T3/4)				5.62	(2.03-15.56)	0.001	6.18	(2.17-17.63)	0.001
Nodal meta (N- vs N+)				0.67	(0.23-1.93)	0.458	0.62	(0.22-1.80)	0.388
Neoadjuvant chemotherapy				1.27	(0.42-3.86)	0.667	1.44	(0.46-4.51)	0.533
Alcohol consumption							1.22	(0.56-2.68)	0.619
Tabacco use							0.61	(0.25-1.46)	0.269

Abbreviations: CI: confidence interval, HR: Hazard ratio

Table 5. Multivariable analyses of disease-free survival restricted only to male in the T1/2 population.

Disease-free Survival (Restricted only to male in the T1/2 population)									
	age adjusted			Model 1			fully adjusted		
	HR	(95% CI)	P-value	HR	(95% CI)	P-value	HR	(95% CI)	P-value
PD-L1 (positive vs negative)	2.58	(1.07-6.24)	0.036	2.43	(0.92-6.42)	0.072	2.32	(0.80-6.79)	0.123
Age (per 10 year increment)	1.17	(0.89-1.54)	0.263	1.16	(0.88-1.54)	0.294	1.23	(0.92-1.65)	0.168
Nodal meta (N- vs N+)				1.56	(0.41-5.96)	0.520	1.53	(0.37-6.37)	0.557
Neoadjuvant chemotherapy				0.71	(0.20-2.51)	0.593	0.72	(0.18-2.91)	0.646
Alcohol consumption							2.45	(0.83-7.29)	0.106
Tabacco use							0.52	(0.17-1.54)	0.236

Abbreviations: CI: confidence interval, HR: Hazard ratio