Original Article

Immunohistochemical study of Programmed Cell Death Ligand 1 (PDL1) expression by combined positive score using 22C3 clone in head and neck squamous cell carcinomas, its correlation with clinicopathological features and outcome

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Abstract

Context: Programmed cell death ligand 1 (PD L1) is a transmembrane protein that is highly expressed in neoplastic cells. Therapy with immune checkpoint inhibitors target PD-1/PD-L1 blockade-inducing tumour regression. Immunohistochemistry (IHC) for PD-L1 expression enables patient selection for immunotherapy and can be considered as a potential predictive biomarker for immunotherapy in head and neck squamous cell carcinoma (HNSCC).

Aims: To determine the PDL1 expression in HNSCC, to correlate with clinicopathological features and outcome.

Settings and Design: We retrospectively analysed 59 cases of HNSCC at our Tertiary Hospital between January 2017 and November 2018 and followed up until death/Nov 2022 for Overall survival.

Methods and Material: IHC analysis of PD-L1 using Combined Positive Score (CPS) with antibody clone 22C3 in 59 cases of HNSCC was performed. PD-L1 expression was correlated with clinicopathological features and outcomes.

Statistical Analysis Used: Pearson Chi-square test was used to analyse the correlation between PD-L1 expression and clinicopathological parameters using SPSS20.0. Survival curves were calculated by Kaplan–Meier method, and differences were analysed by log-rank test.

Results: A total of 25 cases (42.4%) had positive PDL expression (CPS ≥ 1). 16/25 cases (27.1%) belonged to CPS (≥ 1, <10). An almost-perfect interobserver agreement was noted by two pathologists for PD-L1 IHC expression. No statistically significant correlation was noted between PD-L1 score and clinicopathologic features.

Conclusions: Detection of PD-L1 status gives further insight into frequency of PD-L1 expression in Indian HNSCC patients to possibly improve clinical treatment strategies, ensuring that our patients get the maximum therapeutic benefit of immunotherapy.

Keywords: 22C3 clone, combined positive score, head and neck squamous cell carcinoma, immunohistochemistry, immunotherapy, PDL1

Access this article online

Quick Response Code:

Website:
https://journals.lww.com/JPAT/

DOI:
10.4103/jomfp.jomfp_339_23

How to cite this article: Gangadhar P, Ilanthodi S, Shetty R, Shenoy KK, Philipose TR. Immunohistochemical study of Programmed Cell Death Ligand 1 (PDL1) expression by combined positive score using 22C3 clone in head and neck squamous cell carcinomas, its correlation with clinicopathological features and outcome. J Oral Maxillofac Pathol 2024;28:29-36.

INTRODUCTION

In the era of targeted therapy, the immune modulatory checkpoint proteins have evolved as novel treatment strategies in head and neck squamous cell carcinoma (HNSCC).[1] Programmed cell death 1 (PD-1) is a transmembrane protein that is frequently expressed on the surface of T lymphocytes. Programmed cell death ligand 1 (PD L1) is a transmembrane protein that is highly expressed in neoplastic cells. PD-L1 binding to PD-1 forms an immunological checkpoint, which impairs the proliferative potential and function of the respective lymphocytes.[2]

High expression of PD-L1 on tumour cells contributes to an immunosuppressive microenvironment and disruption of antitumoral immune response. HNSCC is known to have immunosuppressive activity; however, significance of PD-L1 expression in HNSCC is still not fully elucidated, unlike in other malignancies.[3]

Most patients of HNSCC receive aggressive multimodal therapeutic regimens consisting of combinations of radiation, chemotherapy and surgery.[4,5] PDL1 is a promising novel predictive biomarker identified in cancer immunotherapy.[6] However, comprehensive data about its expression in HNSCC and therefore a rational basis for antiPDL1/PD1 therapy is lacking.[3]

In 2019, the US Food and Drug Administration (FDA) approved pembrolizumab as first-line treatment for patients with Recurrent/Metastatic HNSCC.[7] FDA also approved a companion diagnostic device for measuring the combined positive score (CPS) by using PD-L1 immunohistochemistry (IHC) 22C3 pharmDx kit, to select patients for pembrolizumab monotherapy.[8]

Individuals whose tumours express PD-L1 as defined by a CPS ≥1 can receive pembrolizumab monotherapy, but individuals without expressivity receive pembrolizumab in combination with other standard agents.[9,10]

Extensive search on the database did not reveal any Indian studies for the expression of PDL1 in HNSCC by FDA-approved companion diagnostic kit using 22C3 Clone with the CPS as the standard.

Conflicting data are available from western literature regarding the expression of PDL1 and the outcome.[11,12] Few studies show[13,14] high expression as a strong predictor of poor outcomes. However, this is hitherto an unexplored area in the Indian scenario. Therefore, data in the Indian context would be invaluable to accurately analyse the expression of PD-L1 with CPS using a companion diagnostic test.

In our research, we aimed to give further insight into frequency of PD-L1 expression in Indian HNSCC patients as defined by a CPS of ≥1 by using an FDA-approved companion diagnostic kit as the standard, and correlated the associations between PD-L1 expression and clinicopathologic features to possibly improve clinical treatment strategies ensuring that our patients get the maximum therapeutic benefit of immunotherapy.

SUBJECTS AND METHODS

Patient population
We retrospectively analysed 59 cases of HNSCC and followed up until death/November 2022 after obtaining clearance from the Institutional Ethical Committee.

Inclusion criteria
Consecutive resected specimens from the oral cavity squamous cell carcinomas (OSCC) and consecutive biopsies from oropharyngeal squamous cell carcinomas (OPSC), hypopharyngeal squamous cell carcinomas (HPSC) and laryngeal squamous cell carcinomas (LSCC), who were diagnosed and had undergone treatment at our Tertiary care Hospital between January 2017 and November 2018 were included in the study.

Exclusion criteria
Patients with <100 viable tumour cells or unavailable formalin-fixed paraffin-embedded (FFPE) samples, unavailable medical records and patients who have received neoadjuvant chemotherapy were excluded from the study.

Clinical data were obtained from the medical records.

Immunohistochemical analysis of PD-L1

Assessment of PD-L1 staining.

Procedure and evaluation
FFPE blocks were retrieved, H&E slides were assessed for adequacy.

Immunohistochemical staining for PDL1 was outsourced to an national accreditation board for testing and calibration laboratories (NABL) accredited laboratory. IHC was performed on the Dako Autostainer Link 48 staining platform by using monoclonal antibody 22C3 pharmDx (Dako, Agilent Technologies, Carpinteria, CA, USA) as per guidelines provided by the manufacturer.

Scoring of PDL1[15,16]

IHC was evaluated using CPS at 200× magnification by using the following formula:

$$CPS = \frac{\text{No. of PD-L1 stained cells} \begin{pmatrix} \text{TCs; lymphocyte; macrophage} \end{pmatrix}}{\text{Total no. of viable TCs}} \times 100$$

CPS ≥1 was scored as positive with partial or complete membrane staining. CPS <1 or no expression in tumour or immune cells was scored as negative. Clinically relevant cut-offs for positive staining (≥1) were taken as 1-10, 10-20, and ≥20 for CPS were used in our study. Two trained pathologists assessed the PD-L1 expression with CPS and tumour proportion score (TPS) evaluation in a blinded fashion without knowledge of clinical data. TPS is the percentage of viable tumour cells (TCs) showing staining to all viable TCs.

Statistical methods

The statistical analysis was performed by using SPSS20.0. Demographic variables are expressed in percentage. The contingency of the categorical variables was observed by using Chi-square/Fisher's exact test. The interobserver agreement for the PD-L1 test was calculated through the overall per cent agreement, and intraclass correlation coefficient (ICC) was calculated for interobserver reliability. A *P* value <0.05 was considered statistically significant. Survival curves were calculated by the
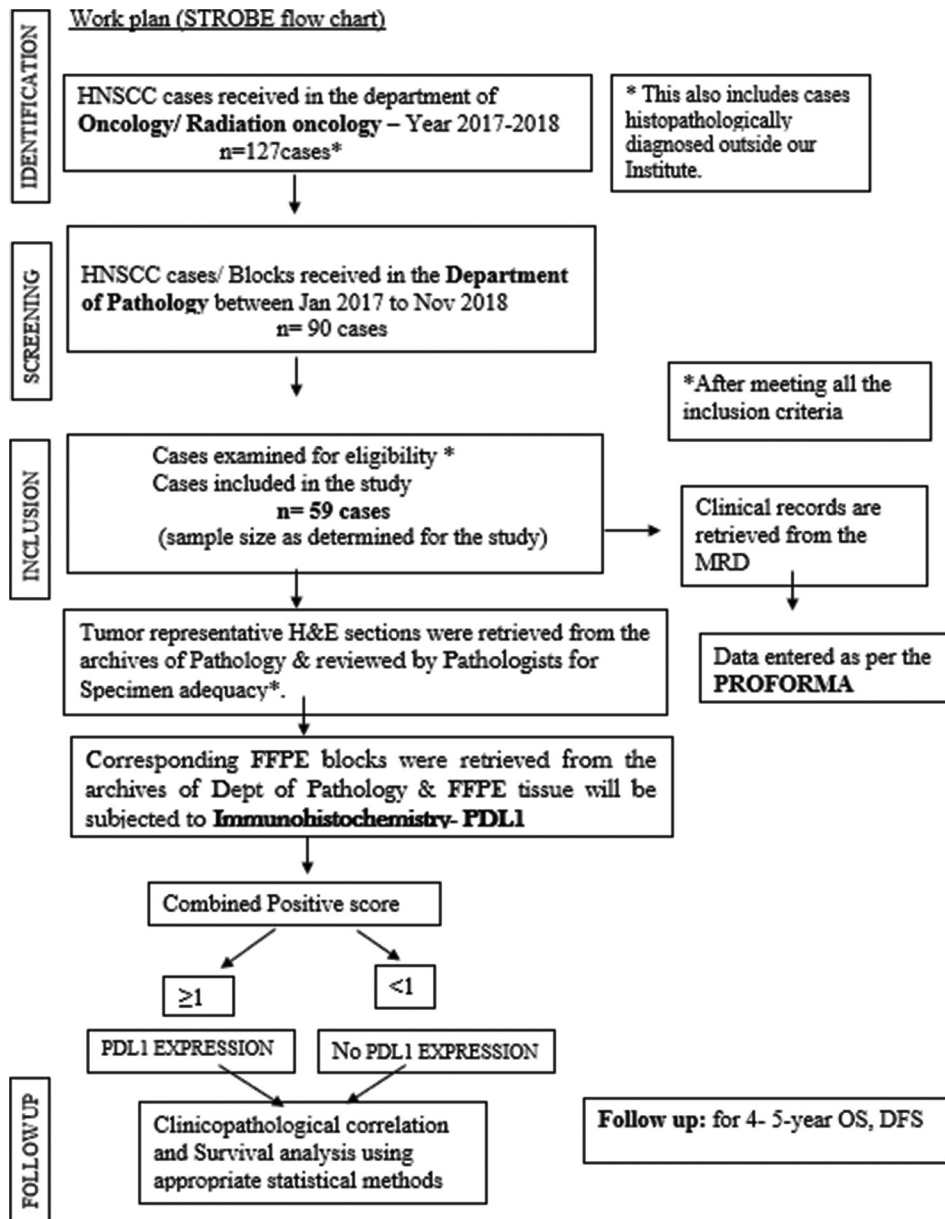
Kaplan-Meier method, and differences were analysed by log-rank test, and *P* values <0.05 were considered statistically significant.

Overall survival (OS) was defined as the interval from the day of surgery to the day of death from any cause.

RESULTS

PD-L1 expression and patient baseline clinical characteristics

A total of 59 cases of HNSCC were studied, comprising 44 cases (54.57%) of OSCC, 6 cases (10.16%) of OPSC, 1 case (1.6%) of HPSC and 8 cases (13.55%) of LSCC. Among the 44 cases of oral SCC, the majority were in buccal mucosa 26/44 (59%), followed by tongue in 12/44 cases (27.27%), with one case (2.27%) each in the gingivobuccal sulcus, and floor of the mouth and lip.

The average age of the patient was 54.95 ± 12.39 years. Patients' characteristics, clinicopathological parameters and PDL1 expression are summarised in Table 1.

Representative images of PD-L1 expression in CPS <1 (Negative), CPS ≥1 (positive) are depicted in Figure 1. All photomicrographs are taken at ×200, H&E stain.

Association between the clinicopathological characteristics and PD-L1 expression

The analysis shows no significant association between clinicopathological parameters and PDL expression. No statistically significant correlation was noted between low and high PD-L1 scores with clinicopathological parameters, as tabulated in Table 2.

Analysis shows an excellent reliability in the scores between the two raters with ICC = 0.979 and *P* < 0.001.

Overall survival

In the present study, the cumulative survival proportion does not appear to differ considerably between the PDL-positive and negative groups. A log-rank test for the PDL positive and negative groups did not show a statistically significant difference with Chi-square = 0.171 and *P* = 0.697, respectively.

DISCUSSION

PD-L1 is a potent biomarker in various types of tumours; hence, there are various IHC diagnostic assays with different antibodies, thresholds and algorithms to detect its expression.[17] In such a scenario, the reliability and reproducibility of testing PDL1 expression would invariably be called into question. Furthermore, the interobserver agreement among different pathologists and different protocols for the PD-L1 assessment in HNSCC has not been sufficiently studied.[18] In addition, controversies exist about the antibodies implemented to detect PD-L1 expression. In this context, a comparison of antibodies was found in two studies,[19,20] showing that the PD-L1 status can be influenced by the choice of assay.

PD-L1 testing was performed using IHC 22C3 pharmDx in the investigated study cohort, which is the only FDA-approved companion diagnostic (CDx) used to identify patients with HNSCC for treatment with KEYTRUDA®.[21] On the basis of the demonstration of an overall per cent agreement ranging from 95.7% to 97.8% in evaluating CPS > 1 and 92.1% to 97.3% for CPS > 20, the FDA-approved 22C3 clone on the Autostainer platform as a CDx.[18] Furthermore, the present study conducted using the FDA-approved clone also shows excellent reliability in the scores between the two raters with ICC = 0.979 and *P* < 0.001.
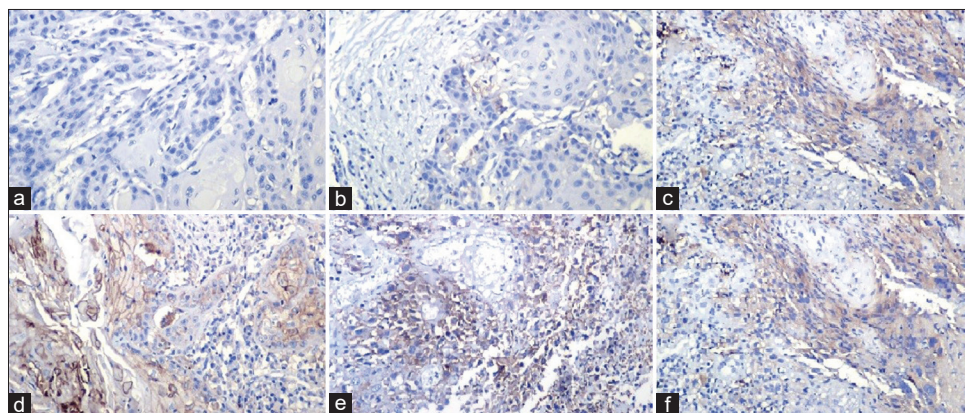

Figure 1: (a) Photomicrograph depicting PD-L1 Immunohistochemical staining CPS < 1, negative, OSCC. (b) TPS <1, CPS: 2 (>1, <20) OSCC. (c) CPS: 15, LSCC (d) CPS: 50, OSCC. (e) CPS: 30, OSCC. (f) CPS: 6 LSCC. All photomicrographs are taken at ×200, H&E stain

Table 1: Patients' characteristics and the PD-L1 expression

	Frequency	Percentage
Gender		
Female	10	16.9
Male	49	83.1
Smoking		
Absent	35	59.3
Present	24	40.7
Betal nut		
Absent	5	8.5
Present	54	91.5
SCC		
SCC, Conventional	58	98.3
Verrucous SCC	1	1.7
Alcohol		
Absent	17	28.8
Present	42	71.2
Differentiation		
MD	16	27.1
WD	43	72.9
Tumour Stage		
T1	7	11.9
T2	25	42.4
T3	10	16.9
T4	17	28.8
Nodal stage		
N0	32	54.3
N1	13	22.0
N2	12	20.3
NX	2	3.4
Metastatic stage		
M1	1	1.7
MX	58	98.3
AJCC stage		
I	2	3.4
II	16	27.1
III	15	25.4
IV	25	42.4
NA	1	1.7
Treatment		
CRT	9	15.3
S only	3	5.1
S+CRT	38	64.4
S+RT	9	15.3
PDL expression (CPS)		
Negative	34	57.6
Positive	25	42.4
PDL expression based on score		
<1	34	57.6
>1, <10	16	27.1
>1, 10–20	6	10.2
>1, 20–50	2	3.4
>1, >50	1	1.7
Outcome		
Alive	25	42.4
Dead	30	50.8
NA	4	6.8

MD - Moderately differentiated, WD - Well differentiated,
CRT - chemotherapy and radiotherapy, S+CRT- surgery + chemotherapy
and radiotherapy, S+RT - surgery+radiotherapy, S only - Surgery only

The inter-observer concordance rate in our study is comparable to that investigated by Downes *et al.*[22] reflecting reliable reporting of IHC.

Results from phase 1 of the blueprint PD-L1 IHC assay comparison project[23] showed the most popular

Table 2: Association analysis between clinicopathologic features and PD-L1 expression

Characteristics	Negative	Positive	Chi square	P
Sex				
Female	6	4	0.028	0.868
	17.6%	16.0%		
Male	28	21		
	82.4%	84.0%		
Smoking				
Absent	20	15	0.008	0.928
	58.8%	60.0%		
Present	14	10		
	41.2%	40.0%		
Betelnut				
Absent	2	3	0.695	0.404
	5.9%	12.0%		
Present	32	22		
	94.1%	88.0%		
SCC type				
SCC, Conventional	33	25	0.748	0.387
	97.1%	100.0%		
VSCC	1	0		
	2.9%	0.0%		
Alcohol				
Absent	12	5	1.643	0.2
	35.3%	20.0%		
Present	22	20		
	64.7%	80.0%		
Grading				
MD	11	5	1.112	0.292
	32.4%	20.0%		
WD	23	20		
	67.6%	80.0%		
Tumour stage				
T1	5	2	4.098	0.251
	14.7%	8.0%		
T2	16	9		
	47.1%	36.0%		
T3	3	7		
	8.8%	28.0%		
T4	10	7		
	29.4%	28.0%		
Nodal stage				
N0	20	12	0.721	0.868
	58.8%	48.0%		
N1	7	6		
	20.6%	24.0%		
N2	6	6		
	17.6%	24.0%		
NX	1	1		
	2.9%	4.0%		
Metastasis				
M1	1	0	0.748	0.387
	2.9%	0.0%		
MX	33	25		
	97.1%	100.0%		
AJCC stage				
I	2	0	4.952	0.292
	5.9%	0.0%		
II	11	5		
	32.4%	20.0%		
III	6	9		
	17.6%	36.0%		
IV	14	11		
	41.2%	44.0%		
NA	1	0		
	2.9%	0.0%		

Contd...

Table 2: Contd...

Characteristics	Negative	Positive	Chi square	P
Treatment				
CRT	7	2	4.624	0.202
	20.6%	8.0%		
S only	1	2		
	2.9%	8.0%		
S+CRT	19	19		
	55.9%	76.0%		
S+RT	7	2		
	20.6%	8.0%		

Table 3: Interrater reliability using ICC

| ICC	95% Confidence Interval		P
	Lower Bound	Upper Bound	
0.979	0.964	0.987	P<0.001

Figure 2: Overall survival

commercially available antibody clones and concluded that in addition to the FDA-approved 22C3, SP263 can be reliably used for PD-L1 typing. While SP-142 gave variable and often weaker staining results and should be avoided.

Literature review showed an Indian study[5] which used locally developed laboratory tests (LDTs) instead of the more expensive CDx. However, LTDs not only require an appropriate validation for their employment in different clinical settings but also require harmonisation for both analytic and pre-analytic factors. Hence, the present study is the only Indian study to detect PDL1 in HNSCC using the gold standard CDx approved by FDA.

In our research, we used CPS, which is an FDA-recommended scoring system and a necessary inclusion if PD-L1 is to be a useful predictive biomarker for HNSCC.[8] We also compared CPS with TPS and concluded that CPS is superior to TPS. There were 9 cases (15.2%) which were negative in TPS (<1) but were positive in CPS (>1). This difference is due to the inclusion of only stained TCs for scoring in TPS as compared to both TCs as well as immune cells in CPS [Table 3]. Hence, by using CPS, a greater number of patients become eligible for PDL1 therapy.

The expression of PDL1 varies from 30% to 80% in previous studies.[8,24] In the present study, PD-L1 expression was seen in 42.4% of cases, similar to a study by Blatt S *et al.*[14] with 43.6% PD-L1 positivity. Out of 42.4% in the present study, more than 63.9% of the cases belonged to CPS of >1<10 category. The literature review of different studies is provided in Table 4. These studies have evaluated PDL1 using different clones and have used variable cut-off scores.

PD-L1 expression in our data was relatively lower than most of the aforementioned studies. The difference in
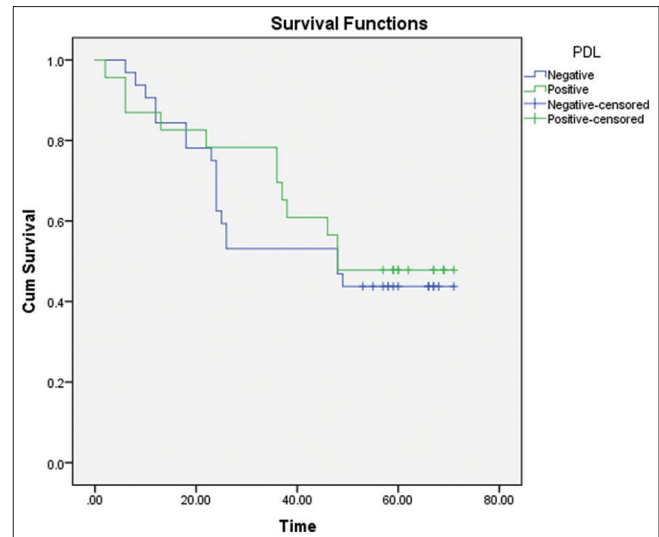
expression may be due to the intratumor heterogeneity[28] and the varying protocols used. This scoring system has a prognostic role, as demonstrated by the results of the KEYNOTE 048 trial[25], which reported a better response to immunotherapy treatment for patients with CPS ≥ 20 compared to those with CPS ≥ 1.

The demographic characteristics of the investigated study cohort showed that the majority of the cases were in males with an average age of 54.95 ± 12.39 years [Figure 2]. Furthermore, the analysis shows no significant association between patient characteristics and clinicopathological parameters with PDL expression ($P > 0.05$). These observations coincide with previous studies.[3,8,14]

However, a few studies showed high PD-L1 expression in OSCC compared to OPSC, HPSC and LSC.[8]

No significant influence on the differentiation of tumour/lymph node metastasis/early tumour stages (T1/2) versus late tumour stages (T3/4) on the expression pattern of PD-L1 could be observed in the present study. However, few of the studies demonstrated a positive association between PD-L1 expression with a higher grade of tumour, early T stage and lymph node metastasis.[8]

In addition, no association between PD-L1 expression and patient outcome could be found in the present study. This was concordant with previously investigated studies,[14,8] which concluded that PD-L1 expression was not seen as a prognostic factor for OS and disease free survival (DFS). Levels of PD-L1 at either end of the spectrum seemed not to have any influence on OS. On the contrary, few studies[29,30] drew completely divergent conclusions,

Table 4: Literature review of different studies

Reference	Tumour site	Patient cohort	PDL1 cutoff	PDL1 immunopositivity	Clone	Platform
Mishra P S, 2019[5]	HNSCC	93	≥1	47.3%	22C3	Manual
			≥50	16.1%		LDT
Downes *et al.*, 2019[22]	HNSCC	27	≥1%	6778%	22C3	Dako
			≥25%	1922%		
KEYNOTE-048 study, 2019[25]	HNSCC		≥1	85%	22C3	Dako
			<20	42%		
			≥20	43%		
Schneider *et al.*, 2018[26]	HNSCC	125	≥5%	36%	5H1	–
Balermpas *et al.* 2017[27]	HNSCC	161	≥5%	39.1%	22C3	Dako
Wusiman *et al.* 2022[8]	HNSCC	119	≥1	89.9%	22C3	Dako
			≥20%	43.7%		
Our study 2023	HNSCC	59	≥1	42.4%	22C3	Dako
			≥20%	5.08%		

which showed a high expression of PD-L1 (>50%) demonstrating favourable outcomes with significantly fewer local and distant recurrences. In contrast, studies[3,12,16] have also shown a strong correlation between high PD-L1 expression, tumour size, clinical stage, regional metastases and a worse OS. In summary, there is contrary evidence about the prognostic value of PD-L1 expression in HNSCC.

There are certain shortcomings in this study; the retrospective manner of this study may implicate a recall bias. Secondly, the number of patients in the analysis of specific tumour sites in this study was small, which may lead to statistical bias.

In conclusion, despite certain limitations, our study holds extreme relevance as it is the first study from India to comprehensively investigate and stratify a cohort of HNSCC based on PDL1 immunohistochemical expression using the gold standard FDA-approved clone Pharm DX 22C3 with CPS. PDL1 expression in HNSCC cases was independent of all the clinicopathological parameters. No influence of PD-L1 expression on OS and DFS could be found in this study.

Financial support and sponsorship

Conflicts of interest
There are no conflicts of interest.

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