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Original Research Article

A Study of Immunohistochemical Expression of PD-1 and Its Ligand (PD-L1) In Head and Neck Squamous Cell Carcinoma

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Abstract:

Introduction: Head and neck squamous cell carcinoma (HNSCC) is a common malignancy with high rates of recurrence and poor survival in advanced stages. Immune checkpoint molecules, particularly programmed cell death protein-1 (PD-1) and its ligand PD-L1, have emerged as important therapeutic targets. Their expression in relation to human papillomavirus (HPV)–associated tumors, assessed by p16 immunohistochemistry, remains variable and incompletely understood.

Materials and Methods: This observational, cross-sectional analytical study included 30 cases of primary HNSCC. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues for PD-1, PD-L1, and p16. PD-L1 expression was assessed in tumor cells based on percentage, intensity, and H-score, while PD-1 expression was evaluated in intratumoral lymphocytes. p16 immunostaining was used as a surrogate marker for HPV infection. Statistical analysis was done to assess concordance between markers.

Results: PD-L1 expression was observed in 66.6% of cases, while PD-1 expression in tumor-infiltrating lymphocytes was seen in 76.6% of cases. p16 positivity was noted in 20% of tumors. No statistically significant association was found between PD-L1 and p16 expression (p=0.33), or between PD-1 and p16 expression (p=0.66). Concordance between PD-1 and PD-L1 expression was approximately 70%, which was also not statistically significant (p=0.127).

Conclusion: A substantial proportion of HNSCC cases express PD-1 and PD-L1 irrespective of p16 status. These findings suggest that immune checkpoint inhibitor–based immunotherapy may be beneficial in both p16-positive and p16-negative advanced or metastatic HNSCC. Larger studies are required to validate these observations and to refine biomarker-based patient selection for immunotherapy.

Keywords: Immunohistochemistry, Biomarkers in HNSCC, PD-1, PD-L1.

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Introduction

Head and neck cancer is the third most common malignancy across the globe and over 600,000 new cases diagnosed each year [1,2]. Smoking, alcohol, betel nuts, tobacco etc. are major risk factors for the development of squamous cell carcinoma of head and neck [3,4]. Despite multimodal treatment more than half of these patients will recur locoregionally or distantly. In HNSCC survival after disease progression or metastasis is relatively short compared to other cancers [5].

Programmed cell death protein (PD-1) is a transmembrane immune checkpoint receptor. It has two ligands PD- L1 and PD- L2. PD-1 is majorly expressed on the T-cells of the immune system, whereas PD-L1 is on the cancer cells and antigen presenting cells. One study has revealed that PD-1 expression on lymphocytes is significantly correlated with cancer prognosis [6]. Recently, cancer immunotherapy with immune checkpoint inhibitors has been the focus of many studies targeting PD-1 and its ligand PD-L1. Clinical trials with monoclonal antibodies to PD-1 and PD-L1 have shown impressive response rates in patients, particularly for melanoma, non-small cell lung cancer, and renal cell carcinoma and bladder cancer. Thus, for metastatic HNSCC still needs enough trials to strengthen the outcomes. Recent advancement has indicated that the expression of immune- inhibitory checkpoints like PD-1/PD-L1 and CTLA-4 act as potent mediators for the balance and escape phases of cancer immune editing.

Currently, many studies are being conducted to establish the value of PD-1 pathway inhibitors in other cancer types. Considering that only some

patients will be able to take advantage of such expensive therapies. So, there is need to expedite biomarker development to focus therapy on patients who will be most likely benefit and be the chosen candidates for FDA approved agents. Recent studies have demonstrated that circulating tumor cells (CTCs) express PD-L1. So, it has potential against patient stratification for anti-PD-1/PD-L1 therapy. An application of CTCs in HNSCC could be those patients with high baseline PD-L1 expressions be given monotherapy and patients with low PD-L1 expressions selected for a combination therapy for better outcomes [7]. The reasons of unresponsive to immunotherapy are attributed to multiple factors, such as (i) insufficient infiltration of activated CD8+ T-cells into the non-inflamed types of tumor microenvironment that retards anti PD-1 targetability [8], (ii) variable population of CD8+ T-cells from edge to core of the tumor due to tumor heterogeneity, hypoxia and variability in mutations of specific oncogene pathway [9]. Thus, there is need for a highly sensitive assay for identification biomarker expression of a patient population to determine feasibility of PD-1/PD-L1 therapy [10]. In general, toxicities with anti-PD-1/PD-L1 mAbs appear to be less common and less severe when compared with anti-CTLA-4 mAbs. Recent studies reported that the PD-1: PD-L1 axis is highly related to HPV-positive rather than HPV-negative HNSCC. PD-1 is expressed on effector T cells in both HPV-positive and -negative tumors, whereas the expression level appears to be more in HPV-positive HNSCC. It suggests that PD-1 expression on cytotoxic T cells is relevant and may play an important role in HPV-OSCC [11,12]. However, the clinical relevance of PD1 expression in HNSCC remains unclear. Therefore, the development of new therapeutic methods of high curative value as well as low toxicity and side effects, especially for patients with advanced HNSCC is needed. The study was conducted with basic aim to know the percentage of HNSCC that express PD-1 and PD-L1 and is there any difference in expression of these markers in HPV positive and HPV negative tumors.

Material and Methods

This Observational, cross sectional and analytical study was conducted in the Department of Pathology, UCMS and GTB Hospital, Delhi from November 2019 to April 2021. Clearance from the institutional ethics committee was duly obtained. In this study 30 cases of primary HNSCC were taken as per the inclusion and exclusion criteria.

Methodology: Immunostaining for PD-1 and PD-L1was performed on paraffin embedded formalin fixed tissues using monoclonal antibodies as per the manufacture's protocol. Tonsil was taken as positive controls and primary antibody was omitted for negative control. p16 immunostaining was performed in a similar fashion except, citrate buffer (pH 6.0) was used for antigen retrieval. Cervical squamous cell carcinoma was taken as positive control.

Criteria for PD-L1immunostaining: Positive: ≥ 1% of tumor cells showing membranous positivity with or without cytoplasmic staining. Negative: No reactivity or some tumour cells with cytoplasmic positivity alone.

Pattern of Staining: The pattern of staining with PD-L1 was classified as, membranous or both membranous and cytoplasmic based on the predominant staining pattern.

Fraction of Cells Showing Positivity: The percentage of cells showing positive PD-L1 expression were noted.

Evaluation of Staining: Each slide was evaluated for PD-L1 immunoreactivity and was assigned an immunohistochemical staining score using a semi quantitative scoring system. Based on extent/proportion of staining and intensity of staining. (Negative: 0, Weak: 1, Moderate: 2 and strong: 3). For negative slides (<1% of cells stained), the H score was considered zero.

H score: 0 for <1% -Negative, > 1% - positive

Formula for H-score in positive cases: 3 x percentage of strongly staining cells + 2 x percentage of moderately staining cells+ 1x percentage of weakly staining cell. Final score: 0 to 300.

Criteria for PD-1 immunostaining:

Pattern of Staining: Brown staining in cytoplasm was considered as positive.

Fraction of Cells Showing Positivity: PD-1 expression in lymphocytes was evaluated intratumorally.

Evaluation of Staining: PD-1 expression in lymphocytes was evaluated intratumorally, based on the number of positive TILs counted in three high-power fields (HPF) (400X magnification) of highest density. Stratification was made for negative expression (0-5), weak (6-15), moderate (16-30) or strong (>30) infiltration patterns.

Results

PD-L1: Pattern of staining in the primary HNSCC: Out of 30 cases of HNSCC studied, 20 (66.6%) were positive for PD-L1, while 10 (33.3%) were negative. The cases were divided according to the percentage of tumour cells showing PD-L1 staining into the following categories (Table 1)

Table 1: Percentage of PD-L1 expression in primary HNSCC

Categories	No of cases(n=30)
<1% (negative)	10 (33.3%)
1-49%	13 (43.3%)
50-75%	04 (13.3%)
76-100%	03 (10%)

The staining intensity of PD-L1 was graded into weak (1+), moderate (2+) and strong (3+) (Table 2).

Table 2: Showing intensity of PD-L1 staining in the primary HNSCC

Staining intensity	No of positive cases(n=20)
Weak (1+)	13 (65%)
Moderate (2+)	04 (20%)
Strong (3+)	03 (15%)

The following criteria was used for the evaluation of PD-L1 expression in primary HNSCC (Table 3).

Table 3: PD-L1 Immunostaining Score:

H Score (A X B) (Extent of staining x Intensity of staining)	Number of Cases(n=30)
0	10 (33.3%)
1-50	08 (26.6%)
51-100	06 (20%)
101-200	02 (6.6%)
201-300	04 (13.3%)

The Intratumoural PD-1 expression in TILs ranged from 0-100/3 hpf (mean 19/3 HPF). Out of 30 cases of HNSCC studied, 23 (76.6%) were positive for PD-1, while 07 (23.3%) were negative. The cases were divided into the following categories (Table 4)

Table 4: PD-1 expressing TILs infiltration in the primary HNSCC

Categories	No of cases(n=30)
Negative	07 (23.3%)
Weak	09 (30%)
Moderate	10 (33.3%)
Strong	04 (13.3%)

p16 Immunohistochemistry: Of the 30 cases of HNSCC, 06 (20%) were positive for p16, while 24 (80%) were negative.

p16: Intensity of Staining: The staining intensity of the p16 was graded as weak, moderate and strong by comparing the intensity of staining in the test cases with the positive control. Out of these 06 cases with p16 positivity, 05 (83.3%) showed moderate staining and 01(16.6%) case showed strong staining.

Amongst the 20 cases with positive PD-L1 expression, 03(15%) cases showed p16 positivity and 17 (85%) were negative for p16 staining.

10 out of 30 cases with negative PD-L1 expression, 03(30%) were positive and 07(70%) were negative for p16 staining (Table 5).

Table 5: Agreement between PD-L1 and p16 staining in primary HNSCC

PD-L1 staining	p16 positive	p16 negative
Positive(n=20)	03(15%)	17(85%)
Negative(n=10)	03(30%)	07(70%)

Percentage of concordance between PD-L1 and p16 is around 33.33% which is statistically not significant (p=0.33). Amongst the 23 cases with positive PD-1 expression, 05(21.7%) cases showed p16 positivity and 18 (78.2%) were negative for p16 staining. 07 out of 30 cases with negative PD-1 expression, 01(14.2%) was positive and 06(85.3%) were negative for p16 staining (Table 6).

Table 6: Agreement between PD-1 and p16 staining in primary HNSCC

PD-1 staining	p16 positive	p16 negative
Positive(n=23)	05(21.7%)	18(78.2%)
Negative(n=07)	01(14.2%)	06(85.3%)

Percentage of concordance between PD-1 and p16 is around 36.6% which is statistically not significant (p=0.66). Amongst 30 cases, 17 (56.6%) out of 20 cases that were PD-L1 positive showed PD-1 positivity while 03(10%) cases were PD-1 negative. 04(13.3%) cases were neither PD-L1 positive nor PD-1 positive (Table 7).

Table 7: Agreement between PD-1 and PD-L1 in primary HNSCC

Staining	PD-1 positive	PD-1 negative
PD-L1 positive (n=20)	17 (56.6%)	03 (10%)
PD-L1 negative (n=10)	06 (20%)	04 (13.3%)

Percentage of concordance between PD-1 and PD-L1 is around 70 % which is statistically not significant (p=0.127)

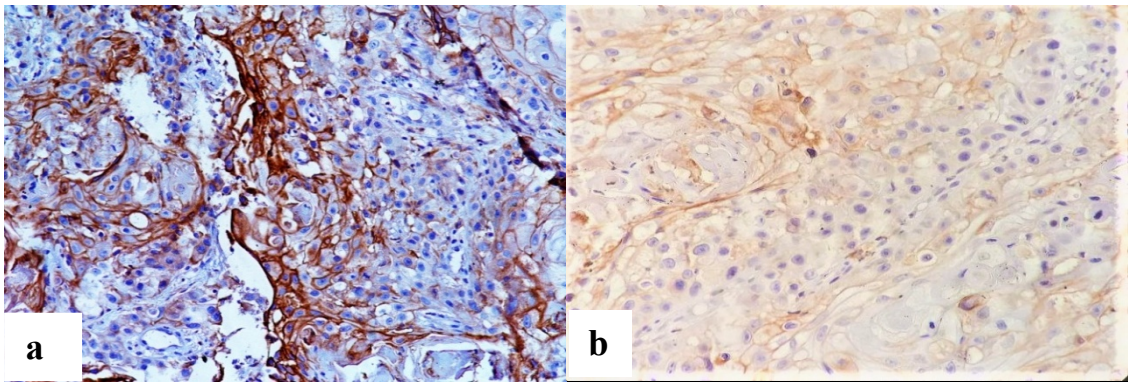

Figure 1(a):PDL1 expression in primary HNSCC. Intense membraneous staining of 50% of tumor cells (IHC x 400). (b)PDL1 expression in primary HNSCC. Moderate intensity of membraneous staining of the tumor cells (IHC x 400).

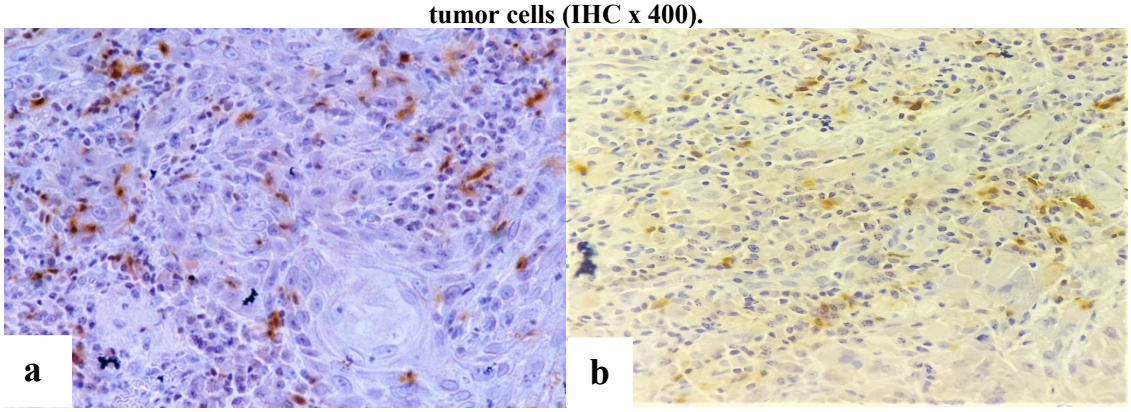

Figure 2: (a) Strong positivity (>30cells/3hpf) of PD-1 in TIL in primary HNSCC (IHC X 400).(b)Moderate positivity (16-30cells/3hpf) of PD-1 in TIL in primary HNSCC (IHC X 400).

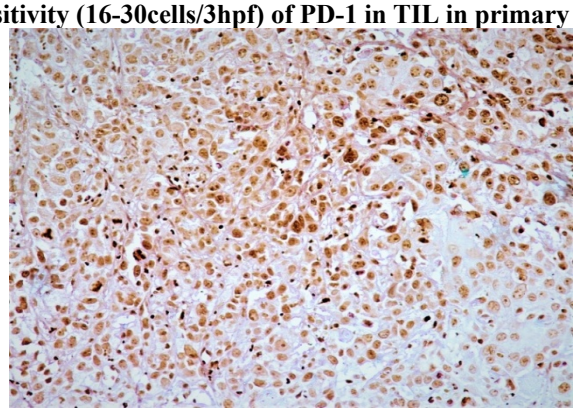

Figure 3: p16 IHC in HNSCC. Moderate to intense nuclear staining in >70% of tumour cells

Discussion

The most common staining pattern observed in our study was both membranous and cytoplasmic positivity which agrees with the study done by Schneider et al [13] However, Chen SW et al [14] considered only membranous staining as positive in HNSCC. Out of 30 cases of HNSCC studied, 20 (66.6%) were positive for PD-L1. Chen SW et al [14] found 64 (67.3%) out of 95 cases positive for

PD-L1 which is nearly in concordance with our study. The study conducted by Schneider et al [13] in HNSCC showed 45 (36%) out of 125 cases with positive PD-L1 expression. The most common staining pattern observed in our study was cytoplasmic positivity which agrees with the study done by Chen SW [14].

However, Paulsen EE et al [15] considered membranous staining as positive in NSCLC for PD-1. Out of 30 cases of HNSCC studied, 23 (76.6%) were positive for PD-1. Contrary to that, Paulsen EE et al [15] found 506 (94.4%) out of 536 cases positive for PD-1 in NSCLC. The other study conducted by Chen SW et al [14] in HNSCC showed 51 (67.3%) out of 95 cases positive for PD-1 expression. In the pioneering study by Murthy V et al [16], out of 170 cases, 34 (20%) showed p16 positivity in HNSCC which is in concordance with our study. In contrast, studies done on HNSCC by Badoual et al [17] and Kim et al [18] found 50% and 67% p16 positivity respectively. In our study, concordance between PD-L1 and p16 was not found to be statistically significant (p=0.333). This is in concordance with the study done by Badoual et al [17] as they found HPV positivity in 32/64 (50%) cases and PD-L1 positivity in 34(51.5%) out of 64 cases. They also did not find any statistically significant correlation between p16 and PD-L1 immuno-expression. Kim et al [18] had assessed HPV status and PD-L1 expression in 133 cases of HNSCC and found no significant correlation of PD-L1 expression with either p16 positive or negative tumours. The results are contrary to the study done by Chen SW [14] as they found that 74.60% of PD-L1 positive tissues were positive for p16 positive tissues (p=0.035). Thus, in this study p16 positivity showed a positive relationship with PD-L1 expression. The concordance between PD-1 and p16 in primary HNSCC was not found statistically significant (P=0.66). The results are similar with the study done by Schinder et al [13] as they found 24/112 cases (21.4%) positive for both PD-1 and p16 and 85/112 (75.8%) negative for p16.12 out of 112 cases with negative PD-1 expression,01 (8.3%) was positive and 10 (83.3%) were negative for p16 staining (p=0.4554). The study done by Chen SW [14] was contrary to this study, as they found that 63.49% of PD-1 positive tissues were positive for p16 positive tissues (p=0.007). The percentage of concordance between PD-1 and PD-L1 in primary HNSCC was around 70% which is statistically not significant (P=0.127). These observations are contrary to the study done by Chen SW [14] among the 64/95 PD-L1-positive patients, 68.75% were PD-1-positive (P< 0.001), indicating an obviously positive correlation between PD-L1 and PD-1. The conflicts in value may be attributed to the difference in racial, genetic and geographical heterogeneity. There is also a possibility of difference in sample size, antibodies and their cutoff value.

Conclusion

This study was designed to assess and compare the expression of PD-1, PD-L1, and p16 by IHC in primary HNSCC. Immunohistochemical method was found to be useful for detection of PD-L1and PD-1. Although the result of the study needs further validation, these results suggest that immunotherapy in PD-L1 and PD-1 may be useful in both p16 positive and p16 negative advanced and metastatic HNSCC.

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