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Research Article

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Determining PDL-1 Receptor Expression in Head and Neck Squamous Carcinoma with Various Clinicoetiopathological Correlation: A Large Central India Study

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ABSTRACT

Background: A multi-institutional study estimated that 65% of new head and neck cancers with locally advanced disease did not receive the benefit of optimal treatment, resulting in poor survival. The monoclonal antibodies anti-programmed death protein-1 (anti–PD–1) nivolumab and pembrolizumab are the first immune checkpoint inhibitors (ICIs) approved for the treatment of platinum-refractory HNSCC recurrent/metastatic (R/M).

Methods: More than a hundred blocks were collected, verified, sampled and sent for receptor status study. Results were analyzed and interpreted according to positivity for gender, locality, association with addiction history and site-wise distribution of primary cancer.

Results: PDL1 receptor status was analyzed in females and males based on biopsy tissue blocks from head and neck cancer patients. Among 100 blocks, 27% were from females and 73% from males. Approximately 70% of females and 65.75% of males showed positive PDL1 receptor status, with variations observed across anatomical sites. Negative expression ranged from 26% in females to 31.5% in males. The distribution of PDL1 expression across different anatomical sites revealed varying patterns, providing insights for potential immunotherapy strategies.

Conclusion: Conclusion: As compared to one of the largest sample sized studies of the European continent, the difference in the clinicopathological features can be attributed majorly to differences in the etiological factors as HPV infection dominates in the European continent while in the Indian and central Indian subcontinent per say, this can be attributed to tobacco chewing.

Key-words: International Agency for Research on Cancer, PDL1 Receptor, Head and Neck Cancers

INTRODUCTION

The International Agency for Research on Cancer (IARC) estimates that globally, 1 in 5 people develop cancer during their lifetime, and 1 in 8 men and 1 in 11 women die from the disease. These new estimates suggest that more than 50 million people are living within five years of a past cancer diagnosis. Globally, ageing populations and socio-economic risk factors remain among the primary factors driving this increase [1]. Head and neck squamous cell carcinomas (HNSCC) represent the sixth most common type of cancer, with 830,000 new cases and around 430,000 deaths each year worldwide [2].

The projected number of patients with cancer in India is 1,392,179 for the year 2020, and the common 5 leading sites are breast, lung, mouth, cervix uteri, and tongue. Trends in cancer incidence rate showed an increase in all sites of cancer in both sexes [3]. HNSCC is a spectrum of malignancies arising from the mucosal lining of the upper

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aerodigestive tract, with different localizations (concerning larynx, hypopharynx, oropharynx, nasopharynx, oral and nasal cavities, and paranasal sinuses) [4].

The incidence and types of cancers depend upon multiple factors such as geographic area, environmental, sociocultural and lifestyle-related factors such as tobacco chewing, (5) smoking, alcohol and various other factors. The most common cancer in Central India, as depicted by the present study, was oral cancer (31.8%), followed by cancer cervix (15.2%). Breast cancer was the third most common cancer in the present study. Tobacco chewing and smoking have been directly related to such a high prevalence of cancers especially head and neck cancers, which include oral and lung cancer in both males and females. [5]

The study underscores the urgency of improving therapeutic outcomes for head and neck squamous cell carcinoma (HNSCC), a disease often diagnosed at advanced stages with limited treatment options. It stresses the need for accessible and effective therapies to combat locoregional disease and distant metastases while preserving organ function and minimizing toxicity. Immune checkpoint inhibitors (ICIs) such as nivolumab and pembrolizumab offer promising avenues for treatment, particularly in platinum-refractory recurrent/metastatic HNSCC [6,7]. This assessment aims to enhance our understanding of immune checkpoint inhibitor efficacy in HNSCC and pave the way for improved treatment strategies tailored to the needs of patients in this region. Due to differences in etiological factors, most of the studies conducted in the Western world have been associated with etiological factors like HPV and its causation in head and neck cancer. In contrast, none of the studies conducted in central India have associated various etiologcal factors like tobacco chewing, smoking, and alcohol intake with an expression of PDL1 receptor in head and neck cancers [8,9]. As per our knowledge, this is one of the most unique studies as not only does it aim to determine the pdl1 expression in head and neck cancer patients, but it is also the only study of central India that does it for more than a hundred patients and association with various clinical parameters like subsites and lifestyle associated risk of head neck cancer and thus needs special attention [10].

MATERIALS AND METHODS

General study details- This study was conducted at a central Indian tertiary care hospital, the State Cancer Institute, Jabalpur, between April 2022 and September 2022. More than a hundred treatment-naive head and neck cancer patients who visited the outpatient department as a part of our institutional protocol were advised to have a biopsy from the respected site at our institute.

Patient demographics and relevant clinical data were obtained from the patients through proper counseling and interrogation. Details regarding tumor size, grade, and clinical stage were also recorded. A personal history of addiction to various substances like tobacco chewing, gutka, kheni, pan, smoking bidi, cigarettes, alcohol, and other commonly abused substances was also noted.

Inclusion criteria

- Biopsy proven cases of head and neck cancer
- Age >20 years to <70 years
- Treatment naïve cases
- Stage I to IV head and neck cancer

Exclusion criteria

- Cases proven only by fine needle aspiration cytology
- Non biopsy proven cancer
- Blocks not available for analysis
- Age <20 years and > 70 years
- Insufficient tissue
- Destruction of tissue

Methodology- The patient's paraffin blocks were collected, sampled, and sent for further procedure for PD-L1 DAKO procedure staining. The patients were given appropriate treatment according to the site and stage-specific NCCN guideline-directed treatment as followed in our institution using multiple disciplinary involvements of the department of radiation oncology, surgery and medical oncology [11]. The study was conducted after obtaining due approval from the institutional ethics committee.

PD-L1 DAKO Procedure- PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical assay using Monoclonal Mouse Anti-PD-L1, Clone 22C3 for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) head and neck cancer tissue using

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EnVision FLEX visualization system on Autostainer Link 48.

PD-L1 IHC 22C3 pharmDx contains the optimized reagents and protocol required to complete an IHC staining procedure of FFPE specimens using Autostainer Link 48. Following incubation with the primary monoclonal antibody to PD-L1 or the Negative Control Reagent (NCR), specimens are incubated with a Linker antibody specific to the primary antibody's host species. Then, they are incubated with a ready-to-use visualization reagent of secondary antibody molecules and horseradish peroxidase molecules coupled to a dextran polymer backbone. The enzymatic conversion of the subsequently added chromogen results in the precipitation of a visible reaction product at the site of the antigen. A chromogen enhancement reagent modifies the color of the chromogenic reaction. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope.

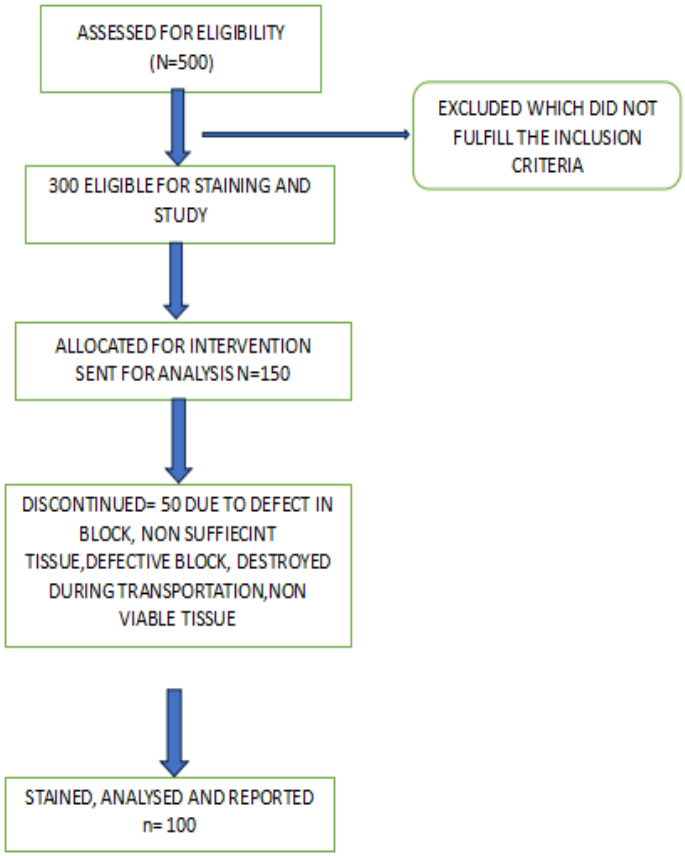

Fig. 1: Flow Chart of the whole method of this study

Procedure- All reagents should be equilibrated to room temperature (20-25°C) before immunostaining [11]. Likewise, all incubations should be performed at room temperature. Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining. All required steps and incubation times for staining are pre-programmed in the Dako Link software.

Staining Protocol

Step 1: Deparaffinization, Rehydration and Target Retrieval (3-in-1) Procedure Set PT Link (Code PT100/PT101/PT200) Preheat and Cool to 65°C. Set Heat to 97 °C for 20 minutes.

- Fill PT Link tanks with 1.5 L per tank of Target Retrieval Solution, Low pH, 1x working solution to cover the tissue sections.
- Preheat the Target Retrieval Solution to 65 °C.
- Immerse Autostainer racks containing mounted FFPE tissue sections into the pre-heated Target Retrieval Solution, Low pH (1x working solution) in PT Link tank. Incubate for 20 minutes at 97°C.
- When target retrieval incubation has been completed and the temperature has cooled to 65°C, remove each Autostainer slide rack with the slides from the PT Link tank and immediately place the Autostainer rack with slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing diluted, room temperature Wash Buffer (Code K8007).
- Incubate slides in diluted, room temperature Wash Buffer for 5 minutes.

Step 2: Staining Procedure After deparaffinization, rehydration and target retrieval (3-in-1) procedure, the Autostainer racks with slides are placed on Autostainer Link 48.

- The instrument was perform the staining process by applying the appropriate reagent, monitoring the incubation time and rinsing slides between reagents.
- The reagent times are preprogrammed in the Dako Link software.

Step 3: Counterstain Slides should be counterstained for 5 minutes with Hematoxylin (Link) (Code K8008).

The Hematoxylin incubation time is preprogrammed in the protocol [12].

Step 4: Mounting Non-aqueous, permanent mounting media is required.

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Definitions

IHC and Scoring

- ✓ PD-L1 staining/expression is defined as complete and/or partial, circumferential or linear plasma membrane asmic staining and exclusive cytoplasmic staining should be considered negative. staining of tumor cells of any intensity that can be differentiated from the background and distinct from cytoplasm.
- ✓ The Tumor Proportion Score is the percentage of viable tumor cells showing partial or complete membrane staining (≥ 1+) relative to all viable tumor cells present in the sample (positive and negative). Scoring is interpreted as follows.

1. No PD-L1 expression (TPS<1%)
2. Low pdl1 expression (TPS 1TO 49%)
3. High PDL1 expression (TPS > OR EQUAL TO 50 %)

The percentage of tumor cells that exhibit PDL.1 expression is recorded as PD-L1 tumor cell (TC) score.

- ✓ This assay is performed on the Dako Autostainer Link 48 platform with an automated staining protocol using a mouse monoclonal anti-PD-L1 antibody, clone 22C3.

The PD-L1 IHC 22C3 pharmDx assay is approved by the US Food and Drug Administration (FDA) as a companion diagnostic test for treatment with pembrolizumab in patients with advanced cancer.

Statistical Analysis- The study used SPSS 27 for effective analysis. The study recruited treatment-naive head and neck cancer patients aged 20-70. Biopsied tissue samples were stained for PD-L1 expression using PD-L1 IHC 22C3 pharmDx assay. The staining protocol involved deparaffinization, antigen retrieval, and counterstaining. Tumor Proportion Score categorized PD-L1 expression. Ethical approval was obtained for the study conducted at a central Indian tertiary care hospital. The study used ANOVA as a statistical tool. The level of significance was considered to be $p<0.05$.

Ethical Approval- The study obtained ethical approval from the Ethical Committee of Netaji Subhash Chandra Bose Medical College.

RESULTS

PDL1 Receptor Status in Females- In this study, out of 100 blocks submitted of biopsy tissue of previously histopathologically proven head and neck cancer, 27% of patients' blocks comprised women. Of these 27, approximately 70 percent of females reported positive (high and low receptor status) for PDL1 receptor status while approx. 26% reported negative for pdl1 receptor status. One out of the 27 blocks submitted did not have sufficient tissue for reporting.

PDL1 Receptor Status in Males- In this study, it has been found that 73% of the total blocks submitted for staining belonged to men. Of these 73, approximately 65.75% of males reported positive (high and low receptor status) for PDL1 receptor status, while approx. 31.5% reported negative for pdl1 receptor status (receptor status staining less than 1 % as shown by TPS). One out of the 73 blocks submitted 2 had insufficient tissue for reporting.

The receptor status was analyzed according to the distribution of blocks from various sites of primary cancer from which the block was made, rural-urban distribution, male-female distribution, and also according to the history of addiction provided by the patient.

This study showed that, out of the total 100 blocks of biopsy tissue, 27% of patients' blocks comprised of women, and the remaining 73% belonged to men. Of these 27, approximately 70 percent of females reported positive (high and low receptor status) for PDL1 receptor status, while 26% reported negative for pdl1 expression (Table 1, Fig. 2 and 3).

Table 1: PDL1 Receptor Status among males and females

	Male (n=73)	Female (n=27)
PDL Status=NO	23	7
PDL Status=HIGH	20	6
PDL Status=LOW	28	13
PDL Status=NIL	2	1

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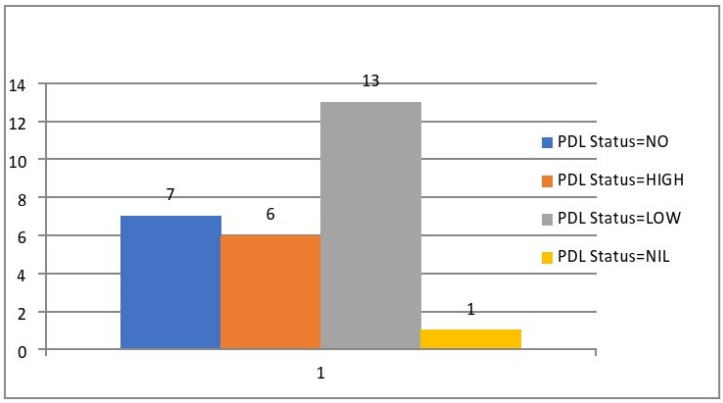

Fig. 2: PDL1 receptor status in Females

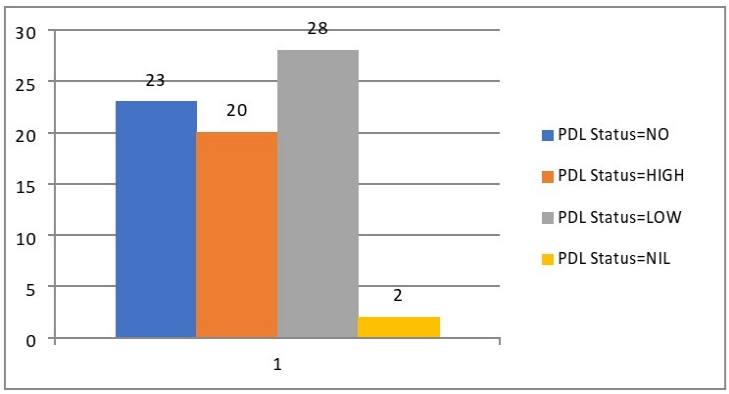

Fig. 3: PDL1 Receptor Status in Males

Table 2 presents the distribution of PDL-1 expression across different anatomical sites, including the oral cavity, oropharynx, hypopharynx, and larynx. In the oral cavity, out of a total of 83 cases, PDL-1 expression was observed in 19 cases (23.45%) at high levels, 35 cases (43.2%) at low levels, and was negative in 26 cases (31.32%). Similarly, in the oropharynx, from a total of 8 cases, PDL-1 was high in 3 cases (37.5%), low in 2 cases (25%), and negative in 3 cases (37.5%). In the hypopharynx, PDL-1 expression was only observed in 2 out of 3 cases (66.66%) at low levels, with no high expression detected. For the larynx, out of 6 cases, PDL-1 was high in 1 case (16.66%), low in 2 cases (33.33%), and negative in 3 cases (50%). These findings provide insights into the varying patterns of PDL-1 expression across different anatomical sites, which could have implications for understanding immune response and guiding immunotherapy strategies in these regions.

Table 2: Findings of PDL-1 concerning the site

Sites	Total number	PDL1 high	PDL1 low	PDL1 negative
Oral cavity	83(83%)	19(23.45 %)	35(43.2%)	26(31.32%)
Oropharynx	8(8%)	3(37.5%)	2(25%)	3(37.5%)
Hypopharynx	3(3%)	0%	2(66.66%)	1(33.33%)
Larynx	6(6%)	1 (16.66%)	2(33.33%)	3(50%)

DISCUSSION

PD-L1 is a cell surface glycoprotein that serves as the physiological ligand for PD-1. It is expressed by the immune effector cells and various tumor cells. PD-1 is a transmembrane receptor of the immunoglobulin superfamily that is expressed on activated T lymphocytes and even natural killer cells and B lymphocytes upon induction [12].

PD-1/PD-L1 interactions are critical in mediating mechanisms of immune tolerance and protecting against autoimmunity. In cancer, tumor cells can over-express PD-L1, providing a survival advantage to the tumor. It can evade host immune system by diminishing anti-

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tumor activity through anergy or apoptosis of antigen-specific T-cells [13,14].

PD-1 immunotherapy has gained immense recognition and acceptance, subject to realizing its scope in the management of various metastatic and recurrent carcinomas, sarcomas, and hematolymphoid neoplasms [14,15]. Pembrolizumab is recommended by US Food and Drug Administration (FDA) as a single agent or in combination with platinum-containing chemotherapy for metastatic or unresectable or recurrent HNSCC expressing PD-L1 with CPS =1.

The most reliable biomarker for patient selection before the institution of anti-PD-1 immunotherapy in HNSCC is PD-L1 (22C3) IHC performed on only the automated platform Dako link 48(16). IHC, a simple, standardized, and reliable diagnostic technique, is employed widely in histopathology laboratories. We exploited the manual LDT of IHC for PD-L1 using approved kits instead of the FDA-approved automated platform [15].

As can be deduced from the table, there is a wide variation of clinicopathological characteristics from the renowned European study and our study [16-18]. The study conducted in the European world corresponds to a higher percentage of patients presenting majorly with cancers of the oropharynx (59% and 40%, respectively). In comparison, the majority of patients presenting with oral cavity cancer (81%) in India and the central Indian subcontinent correspond majorly due to differences in the etiological factors [19]. While oral cavity cancer in India can be attributed to tobacco chewing, oropharyngeal cancers in the European subcontinent can be attributed to infection with HPV [20-22]. This study was limited due to sample collection size, and since it was a single-centered study, the diversity of results and analysis could not be adequately made.

CONCLUSIONS

Ours is a unique breakthrough prospective, nonrandomized study in that, according to available data, only limited studies have been conducted in the central India region. State Cancer Institute, situated in Jabalpur, is a medical hub and a medical homage to more than 20 districts in the surrounding region. More than a hundred blocks were collected, verified, sampled, and sent for receptor status study. As discussed above, results were analyzed and interpreted according to positivity for gender, locality, association with addiction history, and site-wise distribution of the primary cancer. As compared to one of the largest sample-sized studies of the European continent, the difference in the clinicopathological features can be attributed majorly to differences in the etiological factors as HPV infection dominates in the European continent while in the Indian and central Indian subcontinent per say, this can be attributed to tobacco chewing. For the central India region, only this study reports PDL1 receptor status in as large as 100 blocks collected for over a year in a single observational study. Hence, it deserves special attention.

CONTRIBUTION OF AUTHORS

Research concept- Mishi Tiwari, Rahul R Verma
Research design- Rahul R Verma, Rajesh Jain, Lalit Mohan Patel
Supervision- Shyamji Rawat
Materials- Mishi Tiwari, Rahul R Verma
Data collection- Mishi Tiwari, Rahul R Verma
Data analysis and Interpretation- Rahul R Verma, Rajesh Jain, Lalit Mohan Patel
Literature search- Rahul R Verma, Rajesh Jain
Writing article- Mishi Tiwari, Rahul R Verma, Rajesh Jain, Lalit Mohan Patel
Critical review- Shyamji Rawat
Article editing- Mishi Tiwari, Rahul R Verma, Rajesh Jain, Lalit Mohan Patel
Final approval- Shyamji Rawat

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