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Research Protocol

Pathology Section

Utility of Programmed Cell Death Ligand One (PDL1) Expression for Assessing Tumour Behaviour in Comparison to Histopathological Grading and TNM Staging in Oral Squamous Cell Carcinoma Patients: A Research Protocol for a Cross-sectional Study

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ABSTRACT

Introduction: Conventional prognostic indicators such as histopathological grade and TNM staging often fail to fully capture the heterogeneity in tumour behaviour. Recent advances in molecular pathology have highlighted the role of immune checkpoint markers, particularly Programmed Cell Death Protein 1 (PD-1)/Programmed Cell Death Ligand-1 (PD-L1), in tumour progression and immune evasion. Evaluating PD-L1 expression in Oral Squamous Cell Carcinoma (OSCC) may therefore provide important insights into prognosis and therapeutic strategies.

Need of the study: Although PD-L1 has been recognised as a predictive biomarker in several malignancies, its diagnostic and prognostic utility in OSCC, especially within the Indian population, is inadequately studied. Given the high incidence of OSCC in India and the limited effectiveness of conventional prognostic tools, assessing PD-L1 expression in relation to histological grading and TNM staging is essential. Such findings may refine risk stratification, improve patient outcomes, and aid in integrating immunotherapy into treatment protocols.

Aim: To evaluate the role of PD-L1 expression as an immunohistochemical marker in assessing tumour behaviour in OSCC and compare its utility with histopathological grading and TNM staging.

Materials and Methods: The present two-year observational cross-sectional study will be conducted from April 2024 to April 2026 in the Department of Pathology, Jawaharlal Nehru Medical College, Sawangi (Meghe), in collaboration with Oral Surgery, Sharad Pawar Dental College and Hospital, Sawangi, Wardha, Maharashtra. Ninety-five histopathologically confirmed OSCC cases will be included. Formalin-fixed paraffin-embedded sections will undergo Haematoxylin and Eosin (H&E) staining for histological grading (CAP guidelines) and staging (AJCC 8th edition). PD-L1 immunohistochemistry will be performed using clone 22C3 (Dako), and expression scored by Combined Positive Score (CPS), with CPS <1 negative and CPS ≥1 positive. Tonsillar carcinoma will serve as the positive control and omission of secondary antibody as the negative control. Associations between PD-L1 expression, grade, and TNM stage will be tested by Chi-square, with $p<0.05$ considered significant.

Keywords: Combined positive score, Histopathological grade, Immunohistochemistry, Immune evasion, Prognostic indicator

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is the most prevalent oral malignancy, with 389,485 new cases and 188,230 deaths from lip and oral cavity cancers reported worldwide in 2022 [1]. Clinically, oral cancer typically presents as a persistent ulcer, lesion, or abnormal growth within the oral cavity, affecting regions such as the lips, tongue, palate, cheeks, sinuses, the floor of the mouth, and oropharynx [2].

In India, oral cancer is among the top three most common malignancies, contributing to over 30% of the total cancer burden [3]. Annually, the country accounts for nearly one-fourth of global cases, with an estimated 77,000 new diagnoses and 52,000 related deaths [4]. The incidence is particularly high across South and Southeast Asian nations, positioning oral cancer prevention as a pressing global health priority. Patient survival outcomes are strongly influenced by both sociodemographic and clinical determinants [5,6].

Survival outcomes differ significantly between regions. In developed nations, the five-year relative survival rate for oral cancer is approximately 68%, whereas in India it averages around 50% following surgical and radiotherapeutic management, considerably lower than global standards. Although cancers of the lip and oral cavity contribute only about 0.32% of worldwide cancer-related deaths, survival varies greatly across regions, and patients often experience a marked decline in quality of life even after treatment [2]. Over the past three decades, reductions in oral cancer burden have been more pronounced in high-income countries, while Low- and Middle-Income Countries (LMICs) continue to shoulder a disproportionately higher impact [7].

Oral Squamous Cell Carcinoma (OSCC) develops through a multistep, multifactorial process and may arise de novo or from Oral Potentially Malignant Disorders (OPMDs) such as leukoplakia, erythroplakia, Oral Submucous Fibrosis (OSMF), and Oral Lichen Planus (OLP) [8]. Reported malignant transformation rates vary

widely: oral leukoplakia (0.13-34%) [9], erythroplakia (14–50%) [10], proliferative verrucous leukoplakia (68-100%) [11], OSMF (7-30%) [12], and OLP (1.09%) [13]. These variations underscore the need for reliable biomarkers to improve early detection, treatment planning, and prognostication [14].

Molecular alterations often precede phenotypic changes, offering opportunities for earlier diagnosis and risk stratification. Advances in molecular pathology have enabled the use of biomarkers for prognosis and therapy, aiming to improve patient outcomes. Among emerging approaches, immune checkpoint inhibitors targeting the Programmed Cell Death Protein 1 (PD-1)/Programmed Cell Death Protein Ligand 1 (PD-L1) pathway show promise, although their role in OSCC and OPMDs remains uncertain [15].

Programmed Cell Death Protein 1 (PD-1) is a transmembrane receptor commonly present on activated T lymphocytes, while its ligand, PD-L1, is a transmembrane protein frequently overexpressed on tumour cells. Interaction between PD-1 and PD-L1 establishes an immune checkpoint that suppresses lymphocyte proliferation and function. Overexpression of PD-L1 in malignant cells promotes an immunosuppressive tumour microenvironment, thereby weakening the host's antitumour immune response. Head and Neck Squamous Cell Carcinoma (HNSCC) is recognised for its inherent immunosuppressive properties; however, the precise clinical and prognostic significance of PD-L1 expression in HNSCC remains less clearly defined compared to other cancers [16].

As per the aforementioned details, the heterogeneity in clinical behaviour of OSCC, even within the same histopathological grade or stage, highlights the limitations of conventional prognostic indicators such as histological grading and TNM staging alone [17]. Molecular alterations that occur early during malignant transformation may provide more reliable insights into tumour biology and prognosis. Immune checkpoint pathways, particularly the PD-1/ PD-L1 axis, have emerged as critical regulators of tumour immune evasion [18]. PD-L1 expression on tumour cells contributes to the suppression of host immune response and has been implicated as a predictive biomarker in several malignancies [19]. However, its prognostic and diagnostic value in OSCC remains inadequately explored, especially in the Indian population. Therefore, evaluating PD-L1 expression in OSCC and correlating it with histopathological grade and TNM staging may provide a more comprehensive understanding of tumour aggressiveness, assist in patient risk stratification, and potentially guide the integration of immunotherapy into treatment protocols. This study is thus essential to bridge existing knowledge gaps and to identify biomarkers that could enhance diagnosis, prognosis, and therapeutic planning in OSCC patients. Hence, the present study aims to evaluate the role of PD-L1 expression as an immunohistochemical marker in assessing tumour behaviour in OSCC, and to compare its utility with conventional histopathological grading and TNM staging.

Primary objective:

- To assess PD-L1 expression in histopathologically confirmed cases of OSCC.

Secondary objectives:

- To compare PD-L1 expression with the histopathological grade of OSCC.
- To correlate PD-L1 expression with TNM staging of OSCC.

Null Hypotheses: There is no significant expression of PD-L1 in histopathologically confirmed cases of OSCC and of TNM staging of OSCC.

Alternative Hypotheses: There is a significant expression of PD-L1 in histopathologically confirmed cases of OSCC and with TNM staging of OSCC.

REVIEW OF LITERATURE

Lin YM et al., (2015) demonstrated that high PD-L1 expression in OSCC was associated with distant metastasis and poor prognosis. The study further identified PD-L1 as an independent prognostic marker, particularly in male and smoker patients, highlighting its role in tumour progression and the potential utility of PD-L1–targeted immunotherapy [14]. Kitichotkul K et al., (2022) evaluated 85 OSCC cases and reported PD-L1 expression in 25.9% of samples, independent of HPV/p16 status and other clinicopathological features. The findings suggest that nearly one-fourth of OSCC patients may be candidates for anti-PD-L1 immunotherapy, regardless of viral or molecular markers [20]. Blatt S et al., (2022) studied 161 patients with OSCC (n=78) and OPSCC (n=83), reporting PD-L1 positivity in 43.6% of specimens. Although no correlation with survival outcomes was found, qualitative differences in expression between OSCC and OPSCC were observed, indicating site-specific expression patterns without direct prognostic significance [21].

Greeshma LR et al., (2023) assessed PD-1/PD-L1 expression in oral leukoplakia, OSCC, and normal mucosa. The study revealed a progressive increase in PD-L1 expression from premalignant to malignant lesions and a strong correlation with PD-1 expression in tumour-infiltrating lymphocytes. Interestingly, PD-L1-positive fibroblasts were more frequent in leukoplakia than in OSCC, suggesting dynamic changes in the tumour microenvironment during malignant transformation [22]. Pachpande PS et al., (2023) compared PD-L1 expression in 32 OPMD and 32 OSCC cases. PD-L1 positivity was observed in 100% of OSCC cases and 93.7% of OPMD cases, with significantly higher expression in OSCC (mean = 4.59 ± 1.965) than in OPMD (mean = 2.03 ± 1.204). The findings suggest that higher PD-L1 expression may help identify patients at greater risk of malignant transformation [15].

Gangadhar P et al., (2024) retrospectively analysed PD-L1 expression in 59 HNSCC cases using CPS (clone 22C3) and reported PD-L1 positivity in 42.4% of cases. Although no statistically significant correlation with clinicopathological features or outcomes was found, the study emphasised the importance of evaluating PD-L1 status in Indian HNSCC patients to improve therapeutic strategies, particularly for immunotherapy [16]. Srivastava P et al., (2025) evaluated PD-L1 expression in 35 OSCC patients by comparing biopsy and resection specimens. Tumour Proportion Score (TPS) positivity was 20% at ≥1% cutoff, while CPS positivity was 22.9%. Significant correlations were observed between TPS and disease stage (p=0.01) and between CPS, perineural invasion (p=0.04), and male sex (p<0.05). The study highlighted the value of resection specimens over biopsies for accurate PD-L1 assessment and suggested its relevance in guiding immunotherapy, particularly in advanced disease [23].

Taken together, the literature demonstrates that PD-L1 expression is consistently linked to tumour progression, higher histopathological grade, and specific clinicopathological features in OSCC. While some studies support its prognostic role, others indicate variability across tumour sites and stages, emphasising the need for further large-scale validation.

MATERIALS AND METHODS

Study Design and Setting

The present observational, cross-sectional, prospective study will be conducted over two years from April 2024 to April 2026 in the Histopathology and Immunohistochemistry Division, Department of Pathology, Jawaharlal Nehru Medical College, Sawangi (Meghe), in collaboration with the Department of Oral Surgery, Sharad Pawar Dental College and Hospital. Ethical clearance was obtained from the Institutional Ethics Committee, and informed consent will be taken from all participants. Ethics

approval has been obtained to proceed with the current study. The Institute Ethical Committee had provided approval for the study under reference number DMIHER(DU)/IEC/2024/145 dated 1st March 2024. The trial has been registered with the Clinical Trial Registry of India (CTRI) under the approval number CTRI/2025/01/079571, dated 27th January 2025.

Inclusion criteria:

- Histopathologically confirmed cases of OSCC.
- All operated cases of OSCC.
- OSCC cases arising de novo.
- Composite resection specimens including lesion + suitable Modified Radical Neck Dissection (MRND) + flap reconstruction.

Exclusion criteria:

- Non-neoplastic lesions of the oral cavity.
- Recurrent OSCC.
- Patients with prior chemotherapy or radiotherapy.

Sample Size: Sample size was calculated using Daniel's formula (1999) [24] for prevalence studies:

Where:

$$n - \frac{Z(1-\alpha/2) \times p(1-p)}{(d^2)}$$

- Z = 1.96 (95% confidence interval)
- P = 1% (prevalence of OSCC = 0.01)
- d = 3% (margin of error = 0.03)

N= sample size

Z (1-α/2) = 1.96 (value of standard normal variate corresponding to the level of significance α of 0.5)

d = specified precision on either side of the mean (10% or 0.1)

p = reporting PD-L1 positivity in 43.6% of specimens in the previous study by Blatt S et al., (2022) [21].

Blatt S et al., (2022) studied 161 patients with OSCC (n=78) and OPSCC (n=83), reporting PD-L1 positivity in 43.6% of specimens [21].

Taking d=10%, the total sample size calculated is 94.6.

$$N = \frac{(1.96)^2 \times 0.44 \times (1-0.44)}{(0.1)^2}$$

Calculating the squared terms:

$$(1.96)^2 = 3.8416$$

$$(0.1)^2 = 0.01$$

Calculating the term (1-0.44):

(1 – 0.44) = 0.56

Now, multiplying the terms:

N = 3.8416 × 0.44 × 0.56 / 0.01

N = 3.8416 × 0.2464 / 0.01

N = 0.9466 / 0.01

N = 94.66

So, the value of N is approximately 94.66.

N is approximately 94.66. (Rounding up to the nearest whole number, the required sample size is 95)

= 95 patients will be part of the study

Materials required

- Formalin-fixed, paraffin-embedded tumour blocks.
- Grossing instruments (tray, scalpel, forceps, tape).
- Automated tissue processor.
- H&E staining reagents.
- PD-L1 antibody (clone 22C3).
- Glass slides (7.5×2.5 cm).
- Research microscope.

Data collection and clinical evaluation: A detailed clinical history and thorough physical examination will be carried out for all newly diagnosed OSCC patients. Resected oral cancer specimens, received from the Department of Oral and Maxillofacial Surgery, will be processed and examined in the Department of Pathology, J.N.M.C. Each specimen will undergo gross inspection and dissection, with representative sections obtained from the tumour mass, lymph nodes, and surgical margins. Following routine tissue processing, the sections will be stained with Haematoxylin and Eosin for microscopic evaluation. Histological grading of tumours will be performed in accordance with the CAP guidelines [25], while tumour staging will be classified using the TNM system as per the AJCC [26]. Expression of PD-L1 will be evaluated through IHC [27] and correlated with TNM staging.

Laboratory procedures: All resected OSCC specimens will be processed in the Department of Pathology, J.N.M.C. Following fixation in 10% neutral buffered formalin, tissues will undergo routine paraffin embedding and sectioning.

Haematoxylin and Eosin (H&E) staining [28]: Sections will be cut at 3-4 µm thickness and deparaffinized in xylene, followed by rehydration through graded alcohols to water. They will then be stained with Harris's hematoxylin for 5-10 minutes and washed in running water to achieve "bluing." Differentiation will be carried out using 1% acid alcohol for 5-10 seconds, followed by re-washing. Slides will then be dehydrated with 90% ethanol and absolute alcohol, cleared in xylene, and mounted with DPX. These slides will be examined microscopically, and histological grading of tumours will be performed according to CAP guidelines [25], while staging will be assessed using the AJCC 8th edition TNM classification [26].

Immunohistochemistry (IHC) for PD-L1 [27]: From each case, an appropriate paraffin block containing sufficient tumour tissue and adjacent normal tissue will be selected. Sections (3-4 µm) will be mounted and deparaffinised, followed by antigen retrieval using a low pH buffer (pH 6.0) for 53 minutes at room temperature [Table/Fig-1] [28]. After blocking non-specific binding with serum,

Step	Procedure	Details
1. Specimen preparation	Sectioning	Tissue specimens will be sectioned at a thickness of 3 µm.
2. Deparaffinisation and rehydration	Xylene and graded alcohols	Sections will be deparaffinised thrice in xylene (10 min each), followed by rehydration through graded alcohols to water.
3. Target retrieval	Low pH (6.0) buffer	Retrieval will be performed for 53 minutes at room temperature.
4. PD-L1 antibody and staining	Primary antibody	Mouse monoclonal PD-L1 antibody (clone 22C3, 1:200 dilution, Dako) will be applied for 60 minutes at room temperature using an autostainer.
5. Counterstaining and microscopy	Haematoxylin	Sections will be counterstained with haematoxylin and examined under a microscope at 200× magnification.
6. Evaluation	Combined Positive Score (CPS)	PD-L1 expression will be assessed by two examiners using CPS = (PD-L1 positive tumour cells + lymphocytes + macrophages ÷ total tumour cells) × 100. Positive staining is defined as any partial or complete linear membrane staining in tumour cells or cytoplasmic/membrane staining in MICs. Neutrophils, eosinophils, plasma cells, and MICs related to non-neoplastic structures will be excluded.

7. Expression groups	Negative / Positive	Negative PD-L1 expression: CPS <1; Positive PD-L1 expression: CPS ≥1.
8. Controls	Internal and external controls	Positive control: PD-L1-positive tonsillar carcinoma. Negative control: sections without secondary antibody.

[Table/Fig-1]: Steps for Programmed Death-Ligand 1 (PD-L1) Immunohistochemistry (IHC) analysis on tissue specimen [27,28].

slides will be incubated with a primary mouse monoclonal PD-L1 antibody (clone 22C3, dilution 1:200, Dako) for 60 minutes at room temperature. Staining will be performed using an Autostainer. Following incubation with the secondary antibody, visualisation will be achieved using 1% DAB substrate. Counterstaining with haematoxylin will then be performed, after which slides will be dehydrated, cleared, and mounted.

Evaluation of PD-L1 expression [23]: Stained slides will be examined under a light microscope at 200× magnification. PD-L1 expression will be evaluated independently by two examiners using the Combined Positive Score (CPS). CPS is calculated as:

Combined Positive Score (CPS)=PD-L1 positive tumour cells, lymphocytes and macrophages/total viable tumour cells*100

Any convincing partial or complete linear membrane staining of tumour cells, regardless of intensity, will be considered positive. Membranous or cytoplasmic staining in Mononuclear Inflammatory Cells (MICs) within the tumour nests or stroma will also be considered positive. Neutrophils, eosinophils, plasma cells, and inflammatory cells related to non-neoplastic structures or ulcerated areas will be excluded.

For interpretation, cases will be classified into two groups: negative (CPS <1) and positive (CPS ≥1). Consensus scoring by two examiners will be used. Tonsillar carcinoma, known to be PD-L1 positive, will serve as the positive control, while slides without secondary antibody will act as negative controls.

STATISTICAL ANALYSIS

Data will be analysed using SPSS version 27.0. The chi-square test will be used to assess associations between PD-L1 expression, histological grade, and TNM staging. Statistical significance will be considered at $p<0.05$. The correlation of PD-L1 expression with TNM staging of OSCC (r value) will be calculated using the Pearson Correlation Coefficient test.

Ethical considerations: Ethical approval for the present study has been obtained from the Institutional Ethics Committee, Datta Meghe Institute of Higher Education and Research, and departmental permission was secured prior to the commencement of the research. Patient confidentiality will be strictly maintained throughout the study, with all relevant clinical and research data recorded using a standardised proforma. Additionally, written informed consent will be obtained from all study participants before their enrollment in the study.

Outcomes

All newly diagnosed OSCC patients will undergo detailed clinical evaluation, followed by histopathological and immunohistochemical analysis of resected specimens. Tumour tissues, lymph nodes, and surgical margins will be processed with standard H&E staining for histological grading as per CAP guidelines and staged according to AJCC 8th edition.

Primary outcome: PD-L1 expression will be assessed by IHC using clone 22C3 antibody, with results evaluated independently by two examiners based on the CPS. Cases will be categorised as PD-L1 negative (CPS <1) or positive (CPS ≥1).

Secondary outcome: The secondary outcomes will focus on the distribution of histological grades, TNM stages, and PD-L1 expression patterns, and their statistical correlations, providing insights into the prognostic significance of PD-L1 in OSCC.

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