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Research Protocol

Pathology Section

Tumour-infiltrating Lymphocytes and its Association with Prognostic Pathological Parameters in Resectable Oral Squamous Cell Carcinoma: An Observational Ambispective Study Research Protocol

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ABSTRACT

Introduction: Tumour-infiltrating Lymphocytes (TILs) have emerged as important components of the tumour microenvironment and are recognised as significant prognostic indicators in several human malignancies. In Oral Squamous Cell Carcinoma (OSCC), increasing evidence suggests that the density, distribution and immune phenotype of infiltrating lymphocytes may influence tumour progression, nodal metastasis and overall biological behaviour. However, limited structured data are available from the Indian population correlating specific lymphocyte subsets with established pathological prognostic parameters in resectable OSCC.

Need of the study: Despite the high burden of OSCC, prognostication continues to rely mainly on conventional parameters such as tumour grade, tumour size, nodal status and Tumour-Node-Metastasis (TNM) staging. The tumour immune microenvironment represents a potentially valuable adjunct prognostic marker that may refine risk stratification and improve therapeutic planning. Evaluating the density and localisation of CD4+, CD8+ and CD57+ lymphocytes within the tumour parenchyma and at the invasive front may provide additional insight into tumour-host immune interactions and disease behaviour.

Aim: To evaluate the immune profile of TILs identified by CD4, CD8 and CD57 expression in resectable OSCC and to determine their association with pathological prognostic parameters.

Materials and Methods: The present study will be an observational ambispective cross-sectional study and will take place in the Department of Pathology, Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research, Acharya Vinoba Bhave Rural Hospital, Wardha, Maharashtra, India. Patients with a resectable OSCC scheduled for surgical excision will be included based on predefined inclusion and exclusion criteria. Demographic and clinical information e.g., age, sex, occupation, risk habits, tumour site, tumour size and nodal status will be recorded. Histopathological parameters such as grade of tumour, T stage, N stage and TNM stage will be documented. Tissue sections fixed in paraffin blocks (formalin) will be subjected to histological immuno-enzymatic tests (antibodies against CD4, CD8, CD57) for determination of tumour infiltrating lymphocytes in the intratumoural region and at the invasive front. Data will be analysed by using Statistical Package for Social Science (SPSS). Associations between TIL subsets and pathological prognostic parameters will be assessed by the use of appropriate statistical tests at $p<0.05$ as threshold for statistical significance.

Keywords: Cytotoxic T cells, Head and neck malignancy, Immunohistochemistry, Natural killer cells, Prognostic markers, Tumour microenvironment

INTRODUCTION

The OSCC for vast majority of body cancers; the Global Cancer Observatory (GLOBOCAN) reported incidence of OSCC in India in the year 2020 is 10.3%.The Indian Council of Medical Research (ICMR) Cancer Registry reported incidence of OSCC in India in the year 2021 is 7.6% [1,2]. The OSCC for its prognosis is largely been gauged through the tumour size, grade, the local tumour spread, nodal metastasis and distant metastasis [3].

It has been observed that, the molecular landscape of OSCC holds numerous pathogenetic mechanism that makes its behaviour more aggressive. This mechanism contributes to the pathogenesis of OSCC and its aggressive behaviour in the selected cases. Therefore, the researchers publish the article not only on the molecular mechanism that makes the squamous cell carcinoma grow but also on the mechanism wherein the OSCC is inhibited for its progression. In consideration to pathologic prognostic parameters one such area in past half decade that upsurged and generated the interest of the researcher is immune local microenvironment in the resectable primary OSCC [4-6]. The tissue infiltrating immune cells therefore are being studied as a prognostic marker in OSCC.

A meta-analysis published by Hadler-olsen E, Wirsing AM, reached to the conclusion that the presence of CD57+ cells and CD163+ M2 macrophages in the tumour of the oral cell cancer, are promising predictors of good survival of oral cancer patients [6]. Brummel K et al., in their review of tissue infiltrating lymphocytes concluded that more the number of it within the tumour, especially CD8+, CD4+ CD57+ and within the infiltrating border of the tumour, better is the prognosis and survival of the patients of OSCC [7].

There are numerous mechanisms suggested for the action of the tissue infiltrating lymphocytes. One such mechanism suggested in recent time is the action of tissue infiltrating lymphocytes through PDL1 ligands [8-11]. Further, more the PDL1 presence has also been linked with immunotherapy for blocking PD1-L1 access.

Objectives

- **Primary objective:** The primary objective of this research protocol is to evaluate the density and distribution of TIL subsets identified by CD4, CD8 and CD57 expression in resectable OSCC and to determine their association with key pathological prognostic parameters, namely tumour grade, pathological T stage, pathological N stage and overall TNM stage.
- **Secondary objectives:** The secondary objectives are to compare the compartment-wise distribution of CD4+, CD8+ and CD57+ lymphocytes within the intratumoural compartment and at the invasive tumour front and to examine whether each individual TIL subset (CD4+, CD8+ and CD57+) demonstrates differential correlations with pathological grade, depth/extent of invasion as reflected by T stage, nodal metastasis as reflected by N stage and composite TNM stage.

Hypothesis

Null hypothesis (H_0): There is no statistically significant association between the density of CD4+, CD8+ and CD57+ TILs in resectable OSCC (in the tumour parenchyma and/or at the invasive front) and pathological prognostic parameters including tumour grade, T stage, N stage and TNM stage.

Alternate hypothesis (H_1): There is a statistically significant association between the density of CD4+, CD8+ and/or CD57+ TILs in resectable OSCC (in the tumour parenchyma and/or at the invasive front) and one or more pathological prognostic parameters including tumour grade, T stage, N stage and TNM stage.

REVIEW OF LITERATURE

The following is an abridged review of key studies that support the objectives of the present research work and highlight the prognostic relevance of TILs in OSCC:

Caruntu A et al., evaluated the prognostic potential of TILs in resectable OSCC, with particular emphasis on CD4-positive, CD8-positive and CD57-positive lymphocyte subsets [4]. The study included 90 cases and the density of CD4, CD8 and CD57 immune cells was assessed in two tumour compartments, namely the intratumoural region and the invasive front. Immunohistochemistry was performed using monoclonal antibodies against CD4, CD8 and CD57. Quantitative analysis was conducted by identifying hotspots and counting positive lymphocytes across 10 adjacent high-power fields. Additionally, the distribution pattern of immune cells was categorised as nodular, diffuse or absent. The authors reported significant correlations between CD4/CD8 lymphocyte density and pathological prognostic parameters. A statistically significant difference was observed between compartments, with high intratumoural CD4/CD8 density showing significance (p=0.0086) and infiltration at the invasive front showing stronger significance (p=0.0011). The study concluded that tumour-infiltrating immune cells are associated with improved patient outcomes and show meaningful relationships with prognostic pathological parameters.

Fang J et al., assessed the prognostic significance of tumour-infiltrating immune cells in OSCC and studied immune markers including CD8+, CD4+, T-bet, CD68+ and CD57+ cells [5]. The study involved 78 OSCC patients and immune cell infiltration was evaluated in three tumour compartments: tumour epithelium, tumour stroma and advancing tumour margin. Immunohistochemistry was performed using monoclonal antibodies and quantitative analysis involved counting the total number of each immune cell type in stromal areas across 10 adjacent high-power fields. The authors reported that a favourable prognosis was associated with higher CD57+ expression, which correlated with absence of lymph node metastasis (p=0.005) and early clinical stage disease (Stage I/II vs III/IV; p=0.016). Higher CD8 expression was also associated with absence of nodal metastasis (p=0.033) and no history of alcohol intake (p=0.046). Furthermore, patients aged above 60 years exhibited higher T-bet abundance. The study concluded that stromal CD57 and CD8 expression are independent predictors of survival in OSCC and demonstrate significant correlation with lymph node status.

Zhou C et al., conducted a study aimed at developing and validating a seven-immune-feature-based prognostic score for OSCC [8]. The TILs evaluated included CD3+, CD4+, CD8+, CD45RO+, CD66b+, CD68+ and FOXP3+ immune cells. The study comprised 269 OSCC cases drawn from three independent cohorts. Immunohistochemical analysis was performed on 4 µm thick paraffin sections using specific antibodies for each immune marker. Quantitative assessment was performed by evaluating the localisation and density patterns of these immune cells in two distinct tumour regions, namely the centre of tumour and the invasive margin. The authors observed that CD4+, CD8+ and CD45RO+ TILs were significantly more abundant compared to FOXP3+ lymphocytes and CD66b+ neutrophils, irrespective of tumour compartment. The study concluded that the immune-based prognostic score, when combined with clinicopathological parameters, can effectively predict survival in OSCC patients following curative resection.

Soopanit T et al., investigated the prognostic relevance of PD-L1 expression and CD8+ TILs in OSCC [12]. The study included 64 patients and the authors analysed associations between PD-L1 expression, CD8+ infiltration and clinicopathological outcomes. Immunohistochemistry was performed using IgG antibody against PD-L1 and monoclonal primary antibody for CD8. Quantitative analysis was conducted in three regions: intraepithelial, stromal and tumour periphery. CD8 expression was calculated as the percentage of CD8+ lymphocytes relative to tumour cell counts, while PD-L1 expression was calculated as the number of PD-L1-stained cells divided by viable tumour cells ×100. The study reported that PD-L1 positivity was significantly associated with high TIL density, particularly in tumours showing perineural invasion (p=0.041). The authors concluded that high CD8+ TIL density is linked to favourable outcomes, whereas the prognostic significance of PD-L1 expression in OSCC remains inconclusive.

MATERIALS AND METHODS

The present study will be an observational ambispective cross-sectional study being conducted in the Department of Pathology at Institute of Higher Education and Research, Acharya Vinoba Bhave Rural Hospital, Wardha, Maharashtra, India, for a duration of 24 months from 1st March 2024 until 28th February 2026. The study involves both retrospective and prospective recruitment of patients diagnosed with resectable OSCC who undergo surgical resection and histopathological evaluation.

During the study period eligible archived cases will be analysed retrospectively and newly diagnosed cases will be enrolled prospectively for immunohistochemical and statistical evaluation.

The study has received approval from the Institutional Ethics Committee of Institute of Higher Education and Research (IEC Ref. No.: DMIHER(DU)/IEC/2024/146). The study will be conducted in accordance with the ethical principles of the Declaration of Helsinki (2013) and applicable institutional guidelines. Patient confidentiality will be maintained throughout the study and no personal identifiers will be disclosed. For cases included retrospectively, data will be collected from available records and archived specimens and consent waiver will be obtained from the IEC wherever applicable. For prospectively recruited subjects, written informed consent will be obtained prior to enrolment in the study.

Sample size calculation: The sample size was calculated using Daniel's formula (1999) [13] based on the prevalence of lip and oral cavity cancer in India (10.3%) as reported by GLOBOCAN [1].

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

Where:

Z = 1.96 (standard normal deviate at 95% confidence level)

p = 0.103 (prevalence of OSCC in India = 10.3%)

d = 0.07 (margin of error = 7%)

$$n = \frac{(1.96)^2 \times 0.103 \times (1-0.103)}{(0.07)^2}$$

Thus, the calculated sample size is 73 patients, which will be included in the study.

Study Procedure

The following details will be recorded for all participants:

- Demographic details including name, age, sex, address, occupation and unit of care;
- Presenting complaints and relevant clinical history.

Clinical evaluation: A detailed assessment will be carried out and recorded, including:

- **Disease-specific history:** oral hygiene practices, smoking, gutka chewing, tobacco chewing and related habits.
- **General and systemic examination:** relevant findings including clinical features suggestive of OSCC.
- **Local/intraoral examination:** site of lesion, tumour size, number, morphology, location, fixity, involvement of adjacent structures, skin involvement and neck nodal status.
- **Radiological findings:** X-ray, ultrasonography, Computed Tomography (CT) scan and Magnetic Resonance Imaging (MRI) findings (wherever available).
- **Basic investigations:** complete blood count and relevant biochemical parameters; tumour biomarkers where available.

Inclusion criteria: diagnosed with OSCC at any anatomical site within the oral cavity and undergoing surgical resection will be recruited according to the following criteria:

- Cases diagnosed as OSCC (all three Broder's grades) on biopsy, irrespective of age and sex;
- Patients who undergo surgical resection and whose specimens are submitted for histopathological evaluation to the Surgical Pathology Section, Department of Pathology, JNMC, irrespective of the type of surgery.

Exclusion criteria:

- Patients with inadequate clinical records;
- Patients undergoing surgical resection for recurrent OSCC;
- Patients who have received preoperative chemotherapy or monoclonal antibody-based therapy.

Immunohistochemistry: The tissue samples were processed using routine automated histokinette procedures to obtain paraffin blocks, which were then sectioned at a thickness of 4-5 µm using a microtome. These sections were stained with haematoxylin and eosin following standard protocols. Immunohistochemical evaluation of the tumour sections was also performed using conventional methods. Paraffin-embedded tissues were cut into 4 µm sections, followed by heating at 60°C for 60 minutes for dewaxing, with subsequent deparaffinisation in xylene. The slides were then rehydrated through graded alcohols and subjected to antigen retrieval by microwaving in citrate buffer (pH 6.0) for 10 minutes at low power. The sections were incubated with primary antibodies CD4 (1:250), CD8 (1:150) and CD57 (1:150). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 20 minutes, after which the slides were treated with a secondary antibody and DAB chromogen at 37°C for 30 minutes. Visualisation was achieved using 3,3′-diaminobenzidine, followed by counterstaining with haematoxylin.

Commercially available monoclonal antibodies against CD4, CD8 and CD57 will be used as study markers. Immunohistochemical staining was interpreted as membranous positivity in lymphocytes. TILs were assessed in two compartments: intratumoural region and invasive front. Evaluation will be performed in areas of highest lymphocytic density (hotspots) by counting positive cells in ten high-power fields (×400) and the mean value was calculated.

TIL density was graded semi-quantitatively as low (≤10% positive cells), moderate (11-40%) and high (>40%) based on previously described methods in OSCC studies [4,6]. For statistical analysis, cases will be categorised into low and high groups using median values as cut-off where applicable.

The cut of criteria will be:

a. Low group: Low + Moderate (≤40%)

b. High group: High (>40%)

The following parameters will be studied: age, sex, tumour site, tumour grade (Broder's grading), pathological T stage, pathological N stage, overall TNM stage American Joint Committee on Cancer (AJCC) [14], lymphovascular invasion, perineural invasion and compartment-wise density of CD4+, CD8+ and CD57+ TILs.

Outcomes

- **Primary outcome:** The first outcome will be the evaluation of the density and distribution of TIL subsets (CD4+, CD8+ and CD57+) in the intratumoural compartment and at the invasive tumour front in resectable OSCC specimens.
- **Secondary outcomes:** The secondary outcomes will be the evaluation of the association of the TIL density and histopathological prognostic parameters of tumour grade, T stage, N stage and overall TNM stage. In addition, the study will determine the association of TIL subsets with clinical parameters including tumour site, size and nodal status and characterise the immune profile of the tumour microenvironment in OSCC based on the distribution of CD4+, CD8+ and CD57+ lymphocytes.

STATISTICAL ANALYSIS

Data will be analysed using SPSS version 27.0. Categorical variables will be presented as frequencies and percentages and continuous variables as mean±standard deviation. The Chi-square test will be used to assess associations between categorical variables. Associations between CD4+, CD8+ and CD57+ TIL density and pathological parameters (tumour grade, T stage, N stage, TNM stage) will be evaluated using the Chi-square test or Fisher's exact test. Comparisons of means will be performed using Student's t-test. A p-value <0.05 will be considered statistically significant.

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