

Expression of CXCR-4 and CD 133 and it's Correlation with Prognostic Pathologic Factors in Resectable Oral Squamous Cell Carcinoma: A Research Protocol

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ABSTRACT

Introduction: Cancer Stem Cells (CSCs), known for their self-renewal and resistance to therapy, drive tumour progression, metastasis, and recurrence. Markers such as Cluster of Differentiation 133 (CD133) and CXC Chemokine Receptor-4 (CXCR-4) are linked to poor prognosis in cancers, including Oral Squamous Cell Carcinoma (OSCC). CD133 promotes Epithelial-Mesenchymal Transition (EMT) and chemoresistance, while CXCR-4 enhances invasion via CXCL12 signalling. Their co-expression exacerbates outcomes; however, region-specific data, particularly from high-incidence areas like India, remain scarce.

Need of the study: The expression and prognostic correlation of CXCR-4 and CD133 in resectable OSCC enhance early detection, assess tumour aggressiveness, and may identify potential therapeutic targets.

Aim: This study aims to evaluate the immunohistochemical expression of CXCR-4 and CD133 in resectable OSCC and

analyse their correlation with key prognostic pathological factors, including tumour grade, tumour size (T-stage), Depth of Invasion (DOI), Lymphovascular Invasion (LVI), Perineural Invasion (PNI), lymph node metastasis (N-stage), and Lymph node metastasis and surgical margin status.

Materials and Methods: This observational, cross-sectional study will be conducted at Jawaharlal Nehru Medical College, DMIHER. Seventy-five OSCC tissue samples will undergo immunohistochemical analysis using monoclonal antibodies against CXCR-4 and CD133. The expression levels of these markers will be assessed semi-quantitatively. Subsequently, their correlation with key prognostic pathological factors will be analysed. Statistical analysis will be performed using SPSS version 27.0, with a p-value of less than 0.05 considered statistically significant.

Keywords: Biomarkers, Chemokine, Epithelial-mesenchymal transition, Gene expression regulation, Immunohistochemistry, Lymphatic metastasis, Tumour microenvironment

INTRODUCTION

The OSCC constitutes nearly 90% of all head and neck malignancies, making it a significant global health concern. Despite notable progress in diagnostic and treatment modalities, the five-year survival rate for OSCC has stagnated at approximately 50%, reflecting minimal improvement in long-term prognosis [1]. In India, the burden is particularly severe, with OSCC accounting for 7.6% of all cancers in 2021 and 10.2% in 2022, according to the Indian Council of Medical Research (ICMR) [1] and GLOBOCAN [2]. Key aetiological factors include chronic tobacco and alcohol consumption, along with infections by oncogenic strains of Human Papillomavirus (HPV), all of which contribute to cumulative genetic and epigenetic alterations in oral epithelial cells [3].

A critical factor in OSCC progression is the presence of CSCs—a small but potent subset of cells characterised by self-renewal, resistance to apoptosis, and tumour-initiating properties [2]. Among CSC biomarkers, CD133 and CXCR4 are notably linked to poor prognosis and aggressive tumour behaviour in OSCC [4].

CD133 (prominin-1), a transmembrane glycoprotein, serves as a recognised CSC marker in multiple malignancies, including OSCC [5]. Its overexpression in OSCC correlates with increased invasiveness, tumourigenicity, and poor prognosis [6]. CD133-positive cells also display resistance to chemotherapeutics such as cisplatin, promoting recurrence and treatment failure [7]. Mechanistically, CD133 is believed to regulate key signalling pathways that facilitate essential processes like EMT, thereby aiding cancer cell migration, invasion, and metastasis [8]. Immunohistochemical studies have detected

CD133 in primary OSCC lesions and subsequently in the metastatic lymph nodes, reinforcing its role in metastatic progression [8-10]. Moreover, its aberrant expression in preneoplastic oral epithelial changes suggests its involvement early in the carcinogenic process [10]. These findings highlight the potential of CD133 as a prognostic biomarker and a therapeutic target in the treatment of OSCC.

CXCR4, a chemokine receptor, plays an essential role in the progression of OSCC through its interaction with its ligand CXCL12, which activates the ERK signalling pathway and upregulates Matrix Metalloproteinases (MMP-9 and MMP-13). This promotes tumour cell migration, invasion, and lymph node metastasis [11,12]. Elevated CXCR4 expression has been strongly linked with cervical lymph node involvement, a key prognostic determinant in OSCC [13]. Its co-expression with CD133 identifies a sub-population of highly aggressive CSCs associated with poor clinical outcomes [4,13]. Both markers influence CSC behaviour through interactions with the tumour microenvironment, modulating stromal, immune, and signalling components to enhance tumour progression [14].

Signalling cascades such as WNT1 and NOTCH1, which are essential for CSC self-renewal, have been shown to exhibit crosstalk with CXCR4 pathways, thereby amplifying tumourigenic potential [15]. TRIM28 has also been identified as a regulatory molecule of CD133 expression, suggesting new avenues for targeted therapies in OSCC [16]. Recent studies increasingly support the therapeutic relevance of targeting CSC markers such as CD133 and CXCR4 in OSCC. Inhibition of CD133 has been shown to enhance chemosensitivity in CSCs, thereby improving treatment response in preclinical OSCC

models [6]. Similarly, blocking the CXCR4/CXCL12 signalling axis has demonstrated efficacy in reducing lymph node metastasis and tumour progression [13].

Despite these promising findings, translating CSC-targeted strategies into clinical practice remains challenging due to the inherent heterogeneity of CSCs and the variable expression of markers influenced by tumour stage, microenvironmental dynamics, and treatment exposure [17,18]. Personalised therapy, guided by detailed molecular profiling, is essential to overcome these barriers. CD133 and CXCR4 not only contribute to tumour initiation, progression, and metastasis but also confer resistance to standard therapies, positioning them as valuable prognostic markers and therapeutic targets [4]. However, region-specific studies remain scarce, particularly in high-incidence countries like India, underscoring the necessity for further investigations correlating these markers with clinicopathological parameters [1].

Despite the growing evidence of the prognostic value of CSC markers like CXCR4 and CD133 across various malignancies, data specific to OSCC—especially within the Indian population—remain limited. Given the region's high burden of OSCC and distinct aetiological factors, localised research is crucial for understanding tumour biology and guiding clinical decisions. The absence of consistent prognostic biomarkers often impedes accurate risk stratification and treatment planning. This study aims to evaluate the immunohistochemical expression of CXCR4 and CD133 in resectable OSCC and correlate these markers with clinicopathological prognostic factors. Identifying their expression profiles may enhance early diagnosis, assess tumour aggressiveness, and support the development of personalised therapies. The findings could contribute significantly to biomarker-driven prognosis and improve clinical outcomes for patients with OSCC.

REVIEW OF LITERATURE

The molecular complexity of OSCC contributes to its aggressive nature and poor clinical outcomes, prompting a shift towards biomarker-driven research. CSCs have emerged as pivotal contributors to OSCC progression, recurrence, and therapeutic resistance. CD133 and CXCR4 are critical markers linked to tumour aggressiveness, metastasis, and unfavourable prognosis in OSCC. This literature review explores their prognostic and therapeutic relevance, highlighting their potential as biomarkers and targets for more effective, individualised treatment approaches in oral cancer management.

Zhang Q et al., and Ma Z et al., demonstrated that CD133+ cells exhibit increased tumorigenicity and chemoresistance, making CD133 both a diagnostic and therapeutic target [7,19]. Similarly, Yu CC et al., found that silencing CD133 enhanced chemosensitivity, thereby confirming its role in drug resistance [6]. Moon Y et al., showed an association between CD133 and EMT, contributing to invasiveness [10]. The upstream regulation of CD133 by TRIM28, as shown by Kim YS et al., adds a molecular layer to its role [16]. Other studies correlated CD133 expression with markers such as Musashi-1 and Oct-4, as well as with poor differentiation and advanced tumour stages [10,20]. Caspa Gokulan R et al., observed a statistically significant correlation between CD133 and carcinogenesis, further validating its relevance [4].

Yu T et al., elucidated that CXCR4 promotes OSCC invasion by inducing the expression of MMP-9 and MMP-13 through the ERK pathway [11]. Its overexpression in OSCC was noted by Xia J et al., who found significantly higher cytoplasmic expression in OSCC compared to normal and precancerous tissues [12]. Uchida D et al., associated CXCR4 with lymph node metastasis and highlighted its upregulation at the infiltrating tumour edge [13]. The activity of the CXCL12-CXCR4/CXCR7 axis in early OSCC, as demonstrated by Chen N et al., implies its role in both early and advanced disease stages [21]. Cierpikowski P et al., and Lee JI et al., linked CXCR4

overexpression with poor prognosis, including reduced overall survival [15,22]. Vastrad SJ et al., further confirmed its role in maintaining stemness and resistance via key signalling networks [17].

The combined expression of CD133 and CXCR4 correlates strongly with aggressive tumour phenotypes. Caspa Gokulan R et al., observed that dual positivity was linked to poor differentiation, metastasis, and worse survival [4]. While Lu C et al., conducted their study in oesophageal Squamous Cell Carcinoma (SCC), their findings on CD133+/CXCR4+ cells exhibiting increased aggressiveness and chemoresistance are biologically relevant to OSCC due to similarities in epithelial origin [23].

Tripathi A et al., confirmed that a high availability of CSC markers, including CD133 and CXCR4, significantly correlates with unfavourable outcomes [24]. Singh A et al., supported this by demonstrating associations between CD133 expression and advanced-stage OSCC with nodal metastasis [19]. CD133-positive cells were also characterised by EMT and tumour sphere formation, as reported by Luna ECM et al., [5]. Guo Z et al., emphasised the importance of targeting the tumour microenvironment, including CXCR4 and CD133, to enhance therapeutic efficacy in Head and Neck Squamous Cell Carcinoma (HNSCC) [14]. Starska-Kowarska K highlighted the immune regulation of CSC dynamics, reinforcing the potential for immunotherapeutic strategies aimed at CSCs [25].

Research tools and models are essential for studying CSC-driven OSCC. Moya-Garcia CR et al., underscored the value of physiologically relevant *in vitro* models in validating CSC-targeted therapies [18]. Baillie R et al., advocated for the use of multiple CSC markers (CD133, CD44, ALDH1) to capture the heterogeneity of CSC populations in OSCC [3]. Epidemiological data from India further underscore the need for prognostic biomarkers to manage the high burden of OSCC effectively [1]. Although conducted in osteosarcoma, Mardani A et al., demonstrated similar findings regarding CD133/CXCR4 co-expression and tumour aggressiveness, supporting their conserved oncogenic roles across epithelial malignancies [26].

The literature consistently supports the notion that CD133 and CXCR4 are not merely surface markers but active contributors to OSCC progression, metastasis, and therapeutic resistance. Their expression profiles correlate with poor clinicopathological parameters, validating their use as prognostic and therapeutic targets. Investigating their co-expression in resectable OSCC, as proposed in the present protocol, may offer novel insights into tumour behaviour and recurrence risk.

Aim and Objectives

Aim: The aim of this study is to assess the expression of CXCR4 and CD133 in resectable OSCC and to determine their potential as pathological prognostic markers by correlating their expression with tumour grade, tumour size (T-stage), DOI, LVI, PNI, lymph node metastasis (N-stage), and surgical margin status.

Objectives: The following objectives will be pursued based on the identified research gap:

1. To perform immunoprofiling of tumour cells to assess the expression of CXCR4 and CD133.
2. To evaluate CXCR4 immunoexpression in OSCC tumour cells and analyse its correlation with tumour grade.
3. To evaluate CD133 immunoexpression in OSCC tumour cells and analyse its correlation with tumour grade.
4. To investigate the co-expression of CXCR4 and CD133 in OSCC tumour cells and assess their combined correlation with the aforementioned pathological factors.

Research Hypothesis

The present study hypothesises that CXCR4 and CD133 are overexpressed in the tumour cells of resectable OSCC and that their individual and combined expression levels will significantly correlate with established pathological prognostic factors, including

tumour grade, tumour size, DOI, LVI, PNI, lymph node metastasis and staging, and surgical margin status. It is anticipated that the co-expression of these CSC markers will be associated with more aggressive tumour characteristics and poorer clinical outcomes, thereby supporting their role as potential prognostic biomarkers in OSCC.

MATERIALS AND METHODS

Study design and setting: The proposed study will be an observational, cross-sectional investigation to be carried out at the Department of Pathology, Jawaharlal Nehru Medical College (JNMC), Datta Meghe Institute of Higher Education and Research (DMIHER). The study will span two years, commencing in December 2023 and concluding in November 2025. Ethical clearance has been granted by the Institutional Ethics Committee, with the approval reference number DMIHER(DU)/IEC/2024/142.

- The primary outcome of the study will be to evaluate the expression of CXCR-4 and CD133 in OSCC tissues using Immunohistochemistry (IHC).
- The secondary outcomes will include assessing the correlation of CXCR4 and CD133 expression with pathological factors {as per the College of American Pathologists (CAP) guidelines} [27].

Sample size calculation: A total of 75 patient records will be included in the study. In the proposed research study, the sample size calculation will be performed using the following formula (Cochran Formula):

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

Where:

- $Z=1.96$ (for a 95% confidence level),
- $P=10.2\%$ (incidence of lip and oral cavity, in India) [2],
=0.102
- $d=0.07$ (desired margin of error, 7%).

Substituting these values into the formula:

$$n = \frac{1.96^2 \cdot 0.102 \cdot (1-0.102)}{(0.07)^2}$$

$$n \approx 71.81 = 75$$

Thus, the calculated sample size of approximately 75 patients, based on a 10.2% incidence of OSCC, a 95% confidence level, and a 7% margin of error, is sufficiently close to the proposed target. Therefore, the sample size for the study can be approximated to 75 patients for practical and statistical purposes.

Study population and criteria: The study will include clinical and histopathological records of patients diagnosed with OSCC. These will comprise both biopsy samples and resected surgical specimens submitted to the Department of Pathology for histopathological examination. Patients across all age groups and genders will be included, covering all three of Broder's histological grades. Exclusion criteria will consist of incomplete clinical records, patients undergoing resection for recurrent OSCC, and those who have received pre-operative chemotherapy or monoclonal antibody treatment.

Data collection and sample handling: Data will be collected retrospectively from patient records, including findings from general and systemic examinations, basic laboratory tests (including tumour markers), and available radiological imaging reports. All specimens will undergo gross examination according to standard histopathological protocols. Tissue samples will be processed in an automated histokinette, embedded in paraffin wax, sectioned, and stained with Haematoxylin and Eosin (H&E) for microscopic evaluation. Histopathological assessment will be carried out following standard diagnostic criteria. Tumours will be staged according to the TNM classification system to determine their clinical extent [3].

Immunohistochemical procedure: IHC will be performed on selected paraffin-embedded tumour sections using commercially available monoclonal antibodies specific to CXCR-4 and CD133. Tissue sections will first undergo deparaffinisation in xylene, followed by rehydration through a graded alcohol series into Phosphate-Buffered Saline (PBS). Antigen retrieval will be performed using 0.1 M sodium citrate buffer in a microwave oven. To block endogenous peroxidase activity, sections will be treated with 3% hydrogen peroxide for 10 minutes. Primary antibodies will be applied and incubated overnight at 4°C, followed by PBS washing and secondary antibody treatment using streptavidin- HRP conjugate. Visualisation of the antigen-antibody complex will be achieved through chromogen staining, and the sections will then be counterstained with haematoxylin. Negative control slides, lacking primary antibodies, will be included for each IHC run to ensure specificity and quality control. The expression of CXCR-4 and CD133 will be evaluated based on the presence of cytoplasmic brown granules, and staining intensity will be scored semi-quantitatively.

Scoring system: Immunoreactivity will be assessed at 200× magnification across 3-5 randomly selected fields per slide. A semi-quantitative scoring system will be employed, where both the percentage of positively stained tumour cells and staining intensity will be considered. The intensity of staining will be categorised based on both nuclear and cytoplasmic staining, as follows [4]:

- **0 (Negative):** No staining or staining in <5% of cells
- **1+ (Mild):** Staining in 5-15% of cells
- **2+ (Moderate):** Staining in 16-25% of cells
- **3+ (Intense):** Staining in >25% of cells

For statistical analysis, expression will be dichotomised into negative (0 and 1+) and positive (2+ and 3+) groups. This standardised scoring method allows for reliable comparisons across samples and has been validated in previous studies evaluating CSC markers in OSCC [4].

STATISTICAL ANALYSIS

Statistical analysis will be conducted using SPSS software version 27.0. The Chi-square test will be applied to evaluate the association between CXCR4 and CD133 expression and clinicopathological parameters. Pearson's correlation coefficient will be used to determine the strength and significance of associations, with a p-value of <0.05 considered statistically significant.

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