Pathology Section

Assessment of Clinically Occult Nodal Metastasis by Evaluation of Histological Parameters and Immunohistochemical Markers (Cyclin D1 and E-cadherin) in Early Stage Oral Squamous Cell Carcinoma: A Cross-sectional Study

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ABSTRACT

Introduction: Oral Squamous Cell Carcinoma (OSCC) is a prevalent malignancy, particularly in South Central Asia and Melanesia. Despite advancements in diagnosis and treatment, the optimal approach for early stage OSCC (T1-T2, N0) remains debated. While Elective Neck Dissection (END) helps identify occult metastases, it may also contribute to unnecessary morbidity in patients without nodal involvement. Thus, accurate predictive markers for occult nodal metastasis are crucial.

Aim: The present study aimed to assess clinically occult metastasis by evaluation of various histopathological parameters and Immunohistochemistry (IHC) markers (E-cadherin and cyclin D1) in early stage OSCC.

Materials and Methods: The present prospective cross-sectional observational study was conducted at Mahatma Gandhi Medical College and Hospital, Jaipur, Rajasthan, India. The study included 50 biopsy-confirmed early stage (T1/T2, N0) OSCC patients from June 2023 to May 2024. Histopathological examination followed College of American Pathologists (CAP) and American Joint Committee on Cancer (AJCC) 8th edition guidelines. Immunohistochemistry (IHC) was performed for E-cadherin and cyclin D1 expression. Statistical analysis (Student's t-test and Chi-square test) was conducted to identify significant association between various histopathological parameters (Worst Pattern of Invasion (WPOI), tumour budding, Tumour-Infiltrating Lymphocytes (TIL)'s, tumour differentiation, T-stage, Lymphovascular Invasion (LVI) Perineural Invasion (PNI) and IHC markers (E-cadherin and cyclin D) in predicting occult nodal metastasis in early stage OSCC. Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS) statistics software windows version 22.0 released 2013.

Results: Among the 50 patients, 24 were Lymph Node (LN)-positive, while 26 were LN-negative. Significant predictors of nodal metastasis included pT2 stage, Depth Of Invasion (DOI) exceeding 5 mm, WPOI grade 5, high tumour budding score, tumour differentiation, TIL's and LVI (p<0.05). Furthermore, LN-positive cases showed a higher prevalence of molecular markers such as E-cadherin loss (p=0.001) and cyclin D1 overexpression (p=0.04), emphasising their role as potential predictive biomarkers.

Conclusion: Identifying histopathological and immunohistochemical predictors of occult LN metastasis can optimise surgical decisions, especially in the context of end for early stage OSCC, leading to more tailored treatment strategies.

INTRODUCTION

The OSCC ranks as the 16th most common cancer worldwide, with over 377,000 new cases annually, 70% of which occur in males [1]. Lip and oral cavity cancers are highly prevalent in South Central Asia (e.g., India, Sri Lanka, and Pakistan) and Melanesia [2]. The incidence of tongue cancer in individuals under 45 years of age has been rising globally, though the cause remains unclear. While tobacco use is the primary cause of OSCC, in some regions, areca nut consumption is the major carcinogen [3].

According to the National Comprehensive Cancer Network (NCCN) Head and Neck Cancer guidelines, treatment for OSCC varies from resection of the primary tumour, with or without neck dissection. For advanced stages, such as T3 and T4, neck dissection is strongly recommended. However, the optimal approach for early stage OSCC (T1-2, N0) remains a subject of debate. END in early stage cases can reduce morbidity by addressing potential nodal metastases. On the other hand, primary tumour resection without neck dissection may improve overall survival in some patients compared to the END group [4-7]. Therefore, it is crucial to accurately detect LN metastasis, especially in early stage OSCC.

DOI is regarded as the most reliable predictor of occult metastatic disease. Other histological factors, such as the WPOI, tumour budding, tumour differentiation, T-stage, LVI are also under investigation as predictors of occult nodal metastasis. These factors play a crucial role in the decision-making process for elective nodal dissection and contribute to reducing the incidence of treatment failure [8,9].

Over the past two decades, molecular studies have revealed that Head and Neck Squamous Cell Carcinoma (HNSCC) progression

involves a multi-step process of genetic alterations in oncogenes, tumour suppressor genes, and cell adhesion molecules, leading to heterogeneous cell populations with varied invasive and metastatic potential [10]. This has spurred interest in identifying molecular markers to assess cancer progression and to serve as reliable prognostic or predictive tools [11].

E-cadherin (120kDa), a calcium-dependent trans-membrane glycoprotein, encoded by the CDH1 tumour suppressor gene located on chromosome 16q21 [11,12]. Serving as a key cell adhesion molecule, it mediates the binding of epithelial cells through homotypic interactions. When these interactions are disrupted, cancer cells can detach from their original site, initiating the process of tumour invasion. Decreased or abnormal expression of E-cadherin has been associated with factors such as increased tumour aggressiveness, greater invasive capacity, and enhanced metastatic potential in various cancers, including OSCC [12]. The cyclin D1 gene (11q13) encodes cyclin D1, a nuclear regulator of CDK-4/6, which drives the G1-S transition through RB inactivation [13,14]. cyclin D1 is overexpressed in 35-40% of HNSCC cases. Consequently, there is a significant need for reliable and consistent prognostic markers to predict occult nodal metastasis and guide treatment decisions.

The current study's objectives were to assess histopathological parameters (WPOI, tumour budding, TIL's, tumour differentiation, T-stage, LVI, PNI) and the role of Immunohistochemical markers-E-cadherin and cyclin D1 in predicting occult nodal metastasis in early stage (T1/T2, N0) OSCC.

MATERIALS AND METHODS

The cross-sectional study was conducted in the Department of Oncopathology at Mahatma Gandhi Medical College and Hospital, Jaipur, Rajasthan, India, from June 2023 to May 2024. The study included fifty patients of Early stage OSCC. Ethical clearance was obtained from the Institutional Ethics Committee (IEC No. MGMC&H/IEC/JPR/2024/3069).

Inclusion criteria: Biopsy-confirmed, early stage (T1/T2), and clinically (N0) OSCC patients.

Exclusion criteria: Incomplete records of OSCC patients, prior neoadjuvant therapy, recurrence, multiple tumours

Study Procedure

Clinical data were recorded, specimens fixed in 10% formalin, processed in an automated system, paraffin-embedded, and sectioned (3-5 μm). Haematoxylin and Eosin (H&E) stained slides were examined per CAP and AJCC 8th edition guidelines [15].

Histopathological analysis included tumour size, grade (well/moderate/poor), DOI, WPOI, LN metastasis, LVI, PNI, and TNM staging. DOI (mm) was measured from the normal basement membrane to the deepest invasion point [16]. WPOI was classified as 1-4 (non-aggressive) and 5. WPOI 1-4 included pushing borders (WPOI 1), finger-like tumour borders (WPOI 2), large islands >15 cells <1 mm from the main tumour (WPOI 3), small islands <15 cells <1 mm (WPOI 4), while WPOI 5 had dispersed satellites >1 mm away [Table/Fig-1] [17].

The extent of peritumoural lymphoplasmacytic infiltration was divided into three grades according to Brandwein-Gensler M et al., [18]. Grade 1 is characterised by a continuous dense layer of lymphoplasmacytic infiltration among tumour and healthy tissue. Grade 2 shows a discontinuous patchy pattern of lymphoplasmacytic infiltration. Grade 3 shows only minimal or no lymphoplasmacytic infiltration [19].

Tumour buds were assessed according to Almangush A et al., scoring criteria [20]. A tumour bud is defined as a single tumour cell or a small cluster of fewer than five cells at the invasive front. Slides were initially scanned using a 4X microscope objective to identify areas with the highest tumour budding, followed by counting the
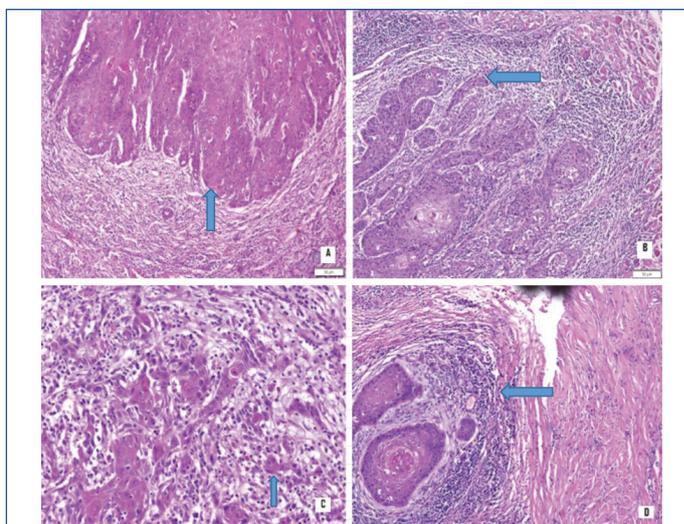
[Table/Fig-1]: a) Tumour with finger-like tumour borders (WPOI 2) of infiltration (H&E stain, 40X); b) Tumour with large islands of >15 tumour cells/island close to <1 mm to the main tumour, WPOI 3(H&E stain, 40X); c) Tumour with small islands of <15 tumour cells/island close to <1 mm to the main tumour, WPOI 4 (H&E stain, 40X); d) Dispersed tumour satellites. >1 mm away from the main tumour WPOI 5(H&E stain, 40X);

tumour buds at a higher magnification. Tumour budding was classified as Low Intensity (LI) when there were fewer than five buds per field (magnification 20X) and High Intensity (HI) when there were five or more buds per field (magnification 20X).

E-cadherin loss (tumour suppressor gene) and cyclin-D1 over-expression (proto-oncogene) were analysed via non-biotin polymer-based HRP IHC. Tumour sections (3-4 μm) were cut onto polylysine-coated slides. E-cadherin detection used a mouse monoclonal antibody (clone 36, Biogenix, USA; 30-min incubation), and cyclin D1 used a rabbit polyclonal antibody (clone EP12, PathnSitu Biotechnologies, Livermore, CA, USA; 30-min incubation). Tonsil sections served as controls. Scoring of E-cadherin loss (tumour suppressor) and cyclin D1 were done using Binary classification by Shergill K et al., [Table/Fig-2-4] [13]. Histopathology and IHC were evaluated as predictive markers of nodal metastasis in early stage OSCC.

| Grading	E-cadherin expression, intensity of membranous immunostaining	Cyclin D1 expression, nuclear staining positive cell (%)
0	Negative[a]	No cells[a]
1+	Weak[a]	1-25[a]
2+	Moderate[b]	26-50[a]
3+	Strong[b]	51-75[b]
4+	Very Strong	76-100[b]

[Table/Fig-2]: Scoring of E-cadherin and cyclin D1 immunostaining
Binary classification: a-negative; b- positive

STATISTICAL ANALYSIS

For statistical analysis, SPSS v22.0 (IBM Corp., Armonk, NY) was used. Descriptive statistics included frequency and proportions for categorical variables, and mean±Standard Deviation (SD) for continuous variables. Inferential statistics included the Mann-Whitney test for comparing mean age, the independent student's t-test for mean tumour size, and the Chi-square test for tumour characteristics, histopathological findings, and the expression of cyclin D1 and E-cadherin between LN-positive and LN-negative groups. Statistical significance was set at $p < 0.05$. All the parameters (Histopathological and cyclin D1 and E-cadherin IHC) related to poor prognosis are indirectly associated with higher stage of patients. However, as the follow-up of patients was not available so the survival data cannot be analysed.

RESULTS

The study involved 50 patients diagnosed with early stage OSCC with clinically negative neck nodes (N0). The patients were categorised
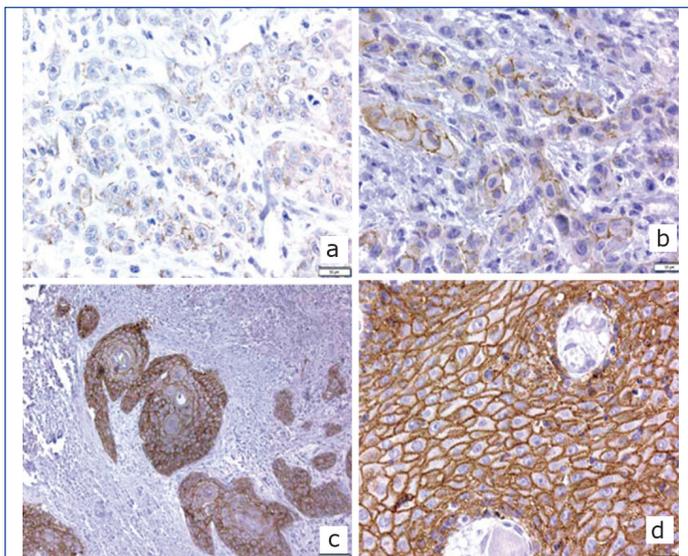
[Table/Fig-3]: Scoring of E-cadherin membranous staining in head and neck squamous cell carcinoma. a) 100 X (score, 1+); b) 100 X (score, 2+); c) 40 X (score, 3+); d) 100 X (score, 3+).
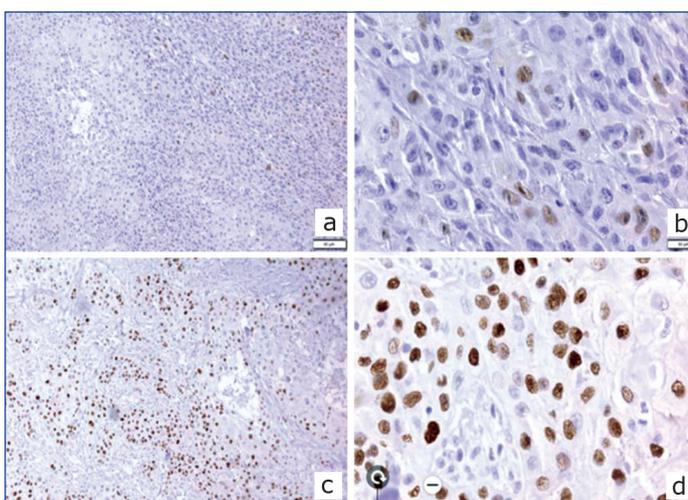
[Table/Fig-4]: Scoring of cyclin D1 staining in head and neck squamous cell carcinoma: a) 40 X (score, 1+); b) X100 X (score, 1+); c) 40 X (score, 4+); d) 100 X (score, 4+).

into two groups based on LN metastasis: LN-positive (n=24) and LN-negative (n=26). The mean age difference between the LN-positive group (50.7±21.12 years) and the LN-negative group (55.12±26.56 years) was not statistically significant (p=0.18). The sex distribution was similar in both groups, with a predominance of males (75% in LN-positive vs. 76.9% in LN-negative, p=0.87, Chi-square Test). The tumour site distribution also showed no significant differences, with the buccal mucosa being the most common site (45.8% in LN-positive vs. 57.7% in LN-negative) [Table/Fig-5].

Tumour staging showed a significant correlation with nodal metastasis. pT2 cases were more frequent in LN-positive 22 (91.7%) than LN-negative 16 (61.5%) groups (p=0.01). Well-differentiated tumours were more common in LN-negative 18 (69.2%) than LN-positive 9 (37.5%) groups (p=0.04), while moderate/poor differentiation prevailed in LN-positive cases. DOI >5 mm was observed more frequently in LN-positive 22 (91.7%) than LN-negative 16 (61.5%) cases (p=0.01). WPOI 5 was higher in LN-positive 7 (29.2%) than LN-negative 2 (7.7%) groups (p=0.04) [Table/Fig-5].

Grade-1 TILs were more common in LN-negative cases 13 (50.0%) cases compared to LN-positive cases 5 (20.8%) cases. In contrast, Grade-2 TILs were predominant in the LN-positive group 14 (58.3%) cases compared to the LN-negative group 7 (26.9%) (p=0.04). High tumour budding (≥5 buds/field) was observed more frequently in LN-positive cases 12 (50.0%) cases than in LN-negative cases 6 (23.1%) cases (p=0.04). LVI was present exclusively in LN-positive

cases 3 (12.5%) (p=0.04). No significant difference was found in PNI between LN-positive 6 (25.0%) and LN-negative 6 (23.1%) cases (p=0.87) [Table/Fig-5]. E-cadherin loss was higher in LN-positive 19 (79.2%) than LN-negative 8 (30.8%) cases (p=0.001). cyclin D1 over-expression was more frequent in LN-positive 16 (66.7%) than LN-negative 10 (38.5%) cases (p=0.04) [Table/Fig-5].

Parameters	Variables	LN Positive (n=24)	LN Negative (n=26)	p-value
Age in years	Mean age ±2SD	50.7±21.12	55.12±26.56	0.18
Sex	Males n (%)	18(75%)	20 (76.9%)	0.87
	Females n (%)	6 (25%)	6 (23.1%)	
Tumour site n (%)	Buccal mucosa	11 (45.8%)	15 (57.7%)	0.81
	Lower alveolus	2 (8.3%)	2 (7.7%)	
	Lower GBS	2 (8.3%)	1 (3.8%)	
	Tongue	6 (25.0%)	7(26.9%)	
	Floor of mouth	2 (8.3%)	1(3.8%)	
	Lower lip	1 (4.2%)	0(0.0%)	
Tumour stage	pT1	2 (8.3%)	10 (38.5%)	0.01*
	pT2	22 (91.7%)	16 (61.5%)	
Tumour grade	Well differentiated	9 (37.5%)	18 (69.2%)	0.04*
	Moderately differentiated	14 (58.3%)	8 (30.8%)	
	Poorly differentiated	1 (4.2%)	0 (0.0%)	
Worst Pattern of Invasion (WPOI)	1-4 WPOI	17 (70.8%)	24 (92.3%)	0.04*
	5 WPOI	7 (29.2%)	2 (7.7%)	
Depth of Invasion (mm)	≤5 mm	2 (8.3%)	10 (38.5%)	0.01*
	> 5 mm	22 (91.7%)	16 (61.5%)	
Tumour-infiltrating Lymphocyte (TIL) n (%)	Grade 1	5 (20.8%)	13 (50.0%)	0.04*
	Grade 2	14 (58.3%)	7 (26.9%)	
	Grade 3	5 (20.8%)	6 (23.1%)	
Tumour budding n (%)	Low score	12 (50.0%)	20 (76.9%)	0.04*
	High score	12 (50.0%)	6 (23.1%)	
LVI	Seen	3 (12.5%)	0 (0.0%)	0.04*
	Not Seen	21 (87.5%)	26 (100.0%)	
PNI	Seen	6 (25.0%)	6 (23.1%)	0.87
	Not Seen	18 (75.0%)	20(76.9%)	
Necrosis	Seen	3 (12.5%)	0 (0.0%)	0.04*
	Not Seen	21 (87.5%)	26 (100.0%)	
Cyclin D1	Positive	16 (66.7%)	10 (38.5%)	0.04*
	Negative	8 (33.3%)	16(61.5%)	
E-cadherin	Positive	5 (20.8%)	18 (69.2%)	0.001*
	Negative	19 (79.2)	8 (30.8%)	

[Table/Fig-5]: Comparison of various parameters in early stage oral cavity tumours between lymph node positive and lymph node negative groups.
LN: Lymph node; SD: Standard deviation; LVI: Lymphovascular invasion; PNI: Perineural invasion; *-Statistically Significant

DISCUSSION

The present study highlights several histomorphological features linked to an increased risk of occult LN metastasis in early stage OSCC. Notably, advanced tumour stage (pT2), poor differentiation, greater DOI (DOI>5 mm), aggressive invasion patterns (WPOI 5), Grade-2 TILs, high tumour budding, LVI, and necrosis were all associated with a heightened risk of metastasis.

In addition, molecular markers such as E-cadherin and cyclin D1 appear to play crucial roles in tumour progression and metastasis. The loss of E-cadherin, a key cell adhesion molecule, may promote tumour cell detachment and dissemination, while cyclin D1 over-expression, a regulator of cell cycle progression, could contribute to unchecked cellular proliferation.

The current study revealed a notable association between tumour differentiation grades and cervical LN metastasis. The LN Positive

group showed a higher occurrence of moderately and poorly differentiated tumours, with 15 (62.5%) cases. Conversely, the LN Negative group demonstrated a greater prevalence of well-differentiated tumours, with 18(69.2%) cases. This finding aligns with the observations of Suresh TN et al., Singh P et al., and Chatterjee D et al., who also reported a significant correlation between histological grades and occult nodal metastasis [21-23]. In the present study, DOI (p=0.01) was statistically significant predictors of regional LN metastasis consistent with the conclusions of Kane SV et al., (p=0.046), Fukano H et al., (p=0.0003), Hubert Low TH et al., (p=0.03), and Ebrahimi A et al., (p=0.001) [24-27]. Furthermore, the present study confirms a strong association between early stage OSCC, WPOI 5, and LN metastasis, corroborating the findings of Arora A et al., (p=0.001), Verma R et al., (p=0.02), and Chatterjee D et al., (p<=0.0001) [10,17,23]. Tumour budding, widely recognised as a marker of poor prognosis in various epithelial tumours, including head and neck squamous cell carcinoma, was also found high score tumour budding significantly associated with nodal metastasis. This observation is in agreement with prior research by Chatterjee D et al., Lu W et al., and Togni L et al., [23,28,29].

Several previous studies [10,23,30] have also reported a significant positive association between LVI and LN metastasis. A recent large meta-analysis of 18 studies by Huang S et al., concluded that LVI was associated with LN metastasis in OSCC [31]. The present study supports this conclusion. The present study found no statistical significance between LN metastasis and occult nodal metastasis in early stage OSCC (p-value =0.87). These findings are consistent with the observations reported by Verma R et al., and Suresh TN et al., [17,21]. Shergill K et al., (p=0.001) and Suresh TN et al., (p=0.001) found that E-cadherin loss was significantly linked to nodal metastasis and higher TNM stage aligning with the present study findings [13,21]. Many studies have investigated the role of cyclin D1 in the carcinogenesis of HNSCCs, including oral cancers. These have shown that amplification or overexpression of cyclin D1 may be a valuable biologic marker of poor prognosis, tumour aggressiveness, and recurrence of these malignancies [13,32-34]. The present study has also indicated that cyclin-D1, is significantly associated with an invasive phenotype and pathological LN status, and that these genetic alterations are a reliable prognostic predictor in OSCCs. Identifying reliable predictors of occult LN metastasis can refine surgical decisions, particularly regarding END in early stage OSCC, enabling more personalised treatment approaches.

The present study has small sample size so the generalisability of results cannot be done. Hence future studies are required to get elaborated datas.

CONCLUSION(S)

This study highlights the importance of specific histomorphological parameters (Tumour grade, DOI, WPOI, LVI, TIL's, Tumour budding) and molecular markers (Loss of E-cadherin and overexpression of cyclin D1) in predicting occult LN metastasis in early stage OSCC. The integration of these factors into clinical evaluation could enhance the accuracy of metastasis prediction and inform more tailored therapeutic approaches, ultimately improving patient management and prognosis.

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