Original Article

Preoperative platelet-lymphocyte ratio and neutrophil-lymphocyte ratio as predictors of occult lymph node metastasis detected using Desmoglein 3 and Cytokeratin in Indian population

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Abstract

Aim: This study aims to assess whether preoperative platelet-lymphocyte ratio (PLR) and neutrophil-lymphocyte ratio (NLR) can predict occult metastasis in oral squamous cell carcinoma (OSCC).

Materials and Methods: Thirty-five OSCC cases were analyzed for clinicopathological and hematological data. Cases without metastasis (pN0) were checked for micrometastasis immunohistochemically using Desmoglein 3 (DSG3) and Cytokeratin (CK). Mean PLR and NLR were compared and analyzed between the study groups.

Results: Metastatic deposits were detected in 9 out of 26 pN0 cases (34.6%) accounting for 11 out of 62 (17%) lymph nodes subjected to immunohistochemistry. The mean PLR was higher in OSCC cases with or without occult metastasis in comparison to controls ($P < 0.001$). No significant difference was found in the mean PLR and NLR between OSCC cases with and without occult metastasis. Furthermore, we found DSG3+ sinus histiocytes within the lymph nodes in majority of cases which is least reported in literature.

Conclusion: A significant percentage of cases showed occult metastasis in this study which led to upstaging of tumor. Although PLR was elevated in OSCC cases, it did not have a positive correlation with the presence of occult metastasis but was able to successfully distinguish OSCC patients from healthy individuals.

Keywords: Occult metastasis, oral squamous cell carcinoma, platelet lymphocyte ratio

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies which occurs in the head and neck region, especially in the developing countries.[1] Delay in diagnosis of OSCC leads to increased morbidity and mortality due to disease progression towards advanced stages, development of lymph node metastasis (LNM) and distant metastasis.[2] Recently, systemic inflammatory markers have emerged as significant prognostic determinants and

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have a proven role in disease progression and development of LNM in various cancers.[3,4] Preoperative hematological ratios such as neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) have been investigated in a variety of malignancies in which higher values were associated with increased LNM and worse prognosis.[5] Some authors have even concluded that preoperative PLR and NLR act as potential predictors of mortality and recurrence. It has been suggested that the stratification of patients based on NLR or PLR measurements is as effective as tumor–node–metastasis (TNM) staging in predicting survival.[6,7]

Occult LNM or micrometastasis is an early metastasis in the form of small tumor cell clusters and individual tumor cells (ITCs) to the lymph nodes which are clinically or histopathologically undetectable. Patients without clinical signs of lymph node involvement (N0) have a significant incidence of occult LNM, ranging from 10% to 50%.[8,9] This is a major drawback of clinical TNM staging as due to this limitation, patients with clinically negative nodes often undergo elective neck resection or radiation, which is associated with consequent morbidity and poor quality of life. As occult LNM easily goes undetected on routine histopathologic sections stained with hematoxylin and eosin, often, immunohistochemistry (IHC) is employed for its detection in biopsy specimens using epithelial cell-specific markers such as cytokeratin (CK).[10] Other epithelial markers which have been used previously as biomarkers include epithelial membrane antigen, pemphigus vulgaris antigen/Desmoglein-3 (DSG3) and Desmoglein-1 (DSG1).

Many recent molecular and IHC studies have confirmed that there is upregulation of various desmosomal components including DSG3 in cancer and the increase in expression can be reliably used as a diagnostic and prognostic marker in a variety of cancers, including head and neck, lung, skin and esophagus.[11-13] Its expression can act as a biomarker for the detection of micrometastasis in lymph nodes of OSCC patients with 100% accuracy.[14-16] DSG3 was found to show highly specific expression in cancerous and noncancerous epithelial cells.[15]

In the stages of early metastasis, the dissemination of tumor cells to the lymph nodes and distant organs is via systemic routes. The induction of cytokines and recruited leukocytes due to inflammation have an essential role in epithelial-mesenchymal transition and re-adhesion of the cancer cells.[17] This led us to ponder whether systemic inflammation can have a role in early micrometastasis to cervical lymph nodes and whether systemic inflammatory markers will be able to predict the same.

Hence, the current study was undertaken to assess whether preoperative PLR and NLR were able to predict occult LNM detected using DSG3 and CK in OSCC cases.

MATERIALS AND METHODS

Patient cohort

Our patient cohort consisted of 35 histopathologically confirmed cases of OSCC who were treated with surgical excision along with neck dissection between September 2016 and April 2018. The selection of the cases was strictly based on inclusion and exclusion criteria. Patients were excluded from the study if they were not willing to participate, had undergone preoperative chemo-radiotherapy, or had evidence of systemic infection or inflammation that would acutely or chronically evoke an inflammatory response. In addition, patients with the previous history of hematological disease or malignancy, distant metastasis or those who were taking immunomodulatory drugs such as corticosteroids or β-agonists were excluded from the study. The baseline data for comparison was derived as part of a previous study using 100 healthy subjects without any systemic disease or drug history. This study was approved by the Institutional Ethical committee.

Clinicopathological data, including age, gender, tumor location, extension, type of lesion, tumor size and TNM staging were recorded as per 7th Edition American Joint Committee on Cancer classification. Histopathological grading was performed following Anneroth's grading system. Complete hemogram was obtained preoperatively, 1 day to 1 week prior to the surgery using automatic hematological analyzer (Sysmex, XT 2000i, Japan). Hematological values such as total leukocyte count, platelet count (PC) and differential leukocyte count were recorded. PLR and NLR values were calculated. PLR is absolute PC divided by absolute lymphocyte count. NLR is absolute neutrophil count divided by absolute lymphocyte count.

Histopathological evaluation

Formalin-fixed paraffin-embedded blocks of the lymph nodes were serial sectioned at 2 μm thickness. After staining with hematoxylin and eosin, the slides were examined by three independent pathologists for metastatic tumor deposits. The subjects in which lymph nodes showed tumor cells were termed histopathologically positive for metastasis (pN+). The subjects in which lymph nodes did not show any tumor cells were assessed as histopathologically negative for metastasis (pN0).

Immunohistochemistry

In pN0 cases, all the level I and II cervical lymph nodes having diameter >1 cm were selected for IHC. If none was >1 cm, then the lymph node having the largest diameter were selected and subjected to IHC using DSG3 primary antibody (Dilution-1:90, Mouse monoclonal antibody, 5G11 clone, Santa Cruz, USA) incubated overnight at 4°C. Antigen retrieval was performed by pressure method (Decloaking chamber, Biocare Medical, USA) at 100°C for 10 min. Master Polymer Plus Detection System, HRP (Master diagnostics, Dako, Denmark) was used for detection and visualisation. Standard IHC protocol provided by the manufacturer was followed. The presence of immunoreactive cells, morphologically consistent with cancer cells within the substance of the lymph nodes was evaluated. In the cases where the immunoreactivity to DSG3 was doubtful, the H and E sections were reassessed. As further clarity was needed, all the sections were stained with pancytokeratin immunohistochemical primary antibody (Ready to use, mouse monoclonal antibody, AE1/AE3, Leica Biosystems, Germany) for confirmation of occult metastasis.

The metastatic tumor deposits were categorized according to the following criteria:

A. Individual tumor cells (ITC) - Tumor deposits <0.2 mm
B. Micrometastasis (MM) - Tumor cell aggregates of size between 0.2 and 2 mm
C. Macrometastasis - Tumor deposits >2 mm.

Positive controls (normal oral epithelium) and negative controls (by omitting primary antibody) were run parallelly. All sections were examined independently by three oral pathologists. Interobserver variability was overcome by discussion and agreement to provide a consensus.

RESULTS

All the 35 cases which underwent surgical resection and neck dissection were assessed for pathological parameters such as pathological tumor size, pathological nodal status, pathological TNM (pTNM) and involvement of surgical margins. Out of 35 cases, 8 cases (22.85%) were T4a, and 9 cases (25.7%) were T3 on clinical examination, but pathologically only 5 cases (14.28%) were T4a and 6 cases (17.14%) were T3 [Table 1]. Hence, a total of 6 cases showed a down-staging of the tumor size. Upon clinical and radiographic examination, 18 cases (51.42%) were N0 while on pathological examination 26 cases (74.28%) were N0 [Table 2]. Clinically, 13 cases (37.14%) were stage III and 11 cases (31.42%) were Stage IVa, while pathologically, 9 cases (25.7%) were Stage III and 8 cases (22.85%) were Stage IVa [Table 3].

Among the nine cases in which cervical nodal metastasis was present on routine H and E sections, level I and II were involved. Out of these cases, level IB and level IIA were involved in 5 cases (14.28%) and 2 cases (5.7%), respectively. One case each involved two nodal levels on the ipsilateral side simultaneously, i.e., level IA + IB and level IB + IIA [Table 4]. The surgical margins were found to be involved in 34.28% of cases, whereas the remaining cases had tumor-free surgical margins.

Upon examination, metastatic deposits were detected in 9 out of 26 cases (34.6%) using IHC. This accounted for 11 out of 62 (17%) lymph nodes showing occult metastasis. These were further classified according to size into ITC, micrometastasis and macrometastasis. ITC was observed in 6 out of 9 cases, among which 3 cases were positive only on DSG3, 1 case was positive only on CK

Table 1: Comparison of clinical tumor size and pathological tumour size in oral squamous cell carcinoma cases which underwent resection (*n*=35)

Tumour size	cT, *n* (%)	pT, *n* (%)
T1	3 (8.57)	9 (25.7)
T2	15 (42.85)	15 (42.85)
T3	9 (25.7)	6 (17.14)
T4a	8 (22.85)	5 (14.28)

cT: Clinical tumor, pT: Pathological tumour

Table 2: Comparison of clinical nodal stage and pathological nodal stage in oral squamous cell carcinoma cases which underwent resection (*n*=35)

Nodal size	cN, *n* (%)	pN, *n* (%)
N0	18 (51.42)	26 (74.28)
N+	17 (48.57)	9 (25.7)

cN: Clinical nodal, pN: Pathological nodal

Table 3: Comparison of clinical stage and pathological stage in oral squamous cell carcinoma cases which underwent resection (*n*=35)

TNM stage	cTNM, *n* (%)	pTNM, *n* (%)
I	1 (2.8)	8 (22.85)
II	10 (28.57)	10 (28.57)
III	13 (37.14)	9 (25.7)
IV	11 (31.42)	8 (22.85)

TNM: Tumor-node-metastasis, cTNM: Clinical TNM, pTNM: Pathological TNM

Table 4: Various levels of lymph nodes involved in pathologically node positive oral squamous cell carcinoma cases which underwent resection (*n*=35)

Level of nodes involved on H&E	
	n (%)
Level IB	5 (14.28)
Level IIA	2 (5.7)
Level IA+IB	1 (2.8)
Level IB+IIA	1 (2.8)

H and E: Hematoxylin and eosin

and 2 cases were positive on both DSG3 and CK. Out of the two cases showing micrometastasis, one case was detected on immunostaining with DSG3 and the other one with CK. Overall 3 cases showed macrometastasis, all of which were positive for both DSG3 and CK [Figure 1]. Hence, micrometastasis and ITC together accounted for 23% (6/26) of the pN0 cases and 12.9% (8/62) of the lymph nodes stained with IHC.

Mean PLR and NLR were compared between the histopathologically N0 OSCC cases with and without occult LNM upon IHC. The mean PLR was higher in OSCC cases with or without occult LNM in comparison to controls and the difference was highly significant ($P < 0.001$). There was no significant difference in the mean PLR and NLR between OSCC cases with and without occult metastasis [Table 5].

DISCUSSION

Over the years, evidence has suggested that inflammation may initiate or follow tumor development in various cancers. Exposure to dietary and environmental carcinogens can induce chronic inflammation which might have a tumor-promoting effect by activating the prointerleukins. Another mechanism often suggested is that many tumors initiate an inflammatory response, which creates a favorable microenvironment for tumor growth and spread.[3,4] Also, certain oncogenes such as RAS and MYC induce remodeling of the tumor microenvironment through increased expression and activation of chemokines and cytokines, induction of neoangiogenesis and recruitment of leukocytes leading to enhanced invasive potential. This provides the autonomously proliferating cancer cells with additional growth factors generated by newly recruited immune cells.[18] Thus clearly, systemic inflammation has a role in promoting LNM. LNM is the most important prognostic indicator and thus it is essential to accurately assess the same.

Table 5: Comparison of mean platelet-lymphocyte ratio between pathologically N0 oral squamous cell carcinoma cases with or without occult metastasis upon immunohistochemistry and controls using one-way ANOVA (*n*=26)

Occult metastasis	Mean	SD	*P*
PLR			
Group I	101.92	36.99	0.001*
Group II: Occult metastasis absent	143.62	40.64	
Group II: Occult metastasis present	173.89	67.98	
NLR			
Group I	2.71	7.13	0.591
Group II: Occult metastasis absent	4.01	2.49	
Group II: Occult metastasis present	4.50	2.87	

$P < 0.001$ – highly significant SD: Standard deviation, PLR: Platelet-lymphocyte ratio, NLR: Neutrophil-lymphocyte ratio

In the current study, 17% of 35 cases demonstrated a downstaging of tumor size, which could be attributed to inflammatory cell infiltration and fibrosis surrounding the tumor, resulting in erythema, edema and induration. As a result, the apparent tumor size may appear larger clinically although the pathological extent is smaller. Usually, the clinical and radiological staging is assessed on the size of the enlarged node and those <10 mm in size are generally not considered to be harboring any metastasis, which might not be always true. Furthermore, reactive nodes may become clinically palpable due to inflammatory reaction in response to the tumor and may even appear enlarged on ultrasonography, magnetic resonance imaging and computed tomography.[9] Hence, down-staging of tumor size and less number of cases showing LNM on H and E in our study have contributed to the overall pTNM down-staging.

In the present study, the lymph node levels I and II were involved pathologically. This was consistent with the findings of Clark *et al*.[8] who reported nodal metastases on neck dissection in 34 out of 93 patients. Among these 34 patients, ipsilateral level I nodes were involved in 14 (15%) cases and level II in 16 (18%) cases. In addition, contralateral level I nodes were involved in 4 cases. Although OSCC generally has a predictable pattern of metastasis to level I and II cervical nodes, occasionally skip metastasis may also occur. There was no evidence of metastasis to a higher level of lymph nodes or skip metastasis on pathological examination in our study. Byers *et al*.[19] reported that 16% of cases of SCC involving the

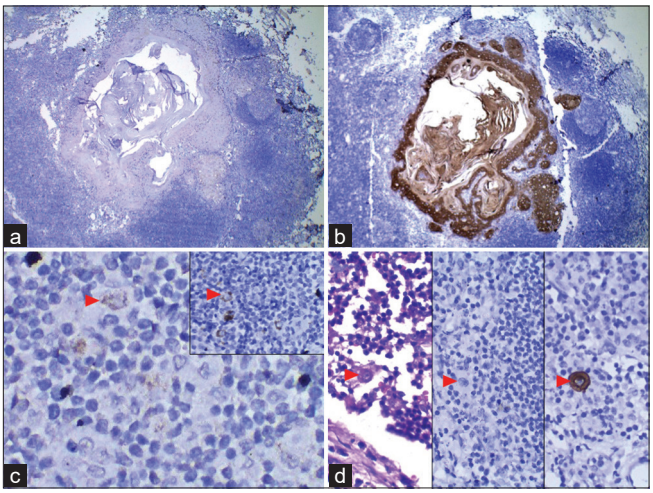

Figure 1: Photomicrograph showing immunopositivity in macrometastatic deposit on staining with (a) DSG3 (IHC, 10x) and, (b) CK (IHC, 10x), (c) Photomicrograph of another case showing presence of ITC on immunostaining with DSG3 (IHC, 40x). Inset showing positivity with CK within the same cells (IHC, 40x), (d) Photomicrograph showing presence of ITC (Left, HE, 40x). Positive immunostaining noted with CK (Right, IHC, 40x), but negative staining was noted with DSG3 (Middle, IHC, 40x)

tongue showed skip metastasis in their series, suggesting that, it is a frequent finding in OSCC involving the tongue.

Kazakydasan *et al*.[20] reported the prevalence of micrometastasis in tongue OSCC cases as 23.5% and in buccal mucosa OSCC cases as 17.6%. Other studies have found a prevalence rate from 10% to 40%.[10,21-23] The variation in results obtained with DSG3 and CK staining could be a result of the different planes of section employed for IHC staining. Being very small in size the ITC which stained positively with one antibody may not have been present in consecutive sections thus giving negative result for the other antibody. However, this difficulty was not encountered in case of macrometastasis due to larger size of tumor cell islands [Figure 1].

The DSG3 staining pattern is primarily membranous, but cytoplasmic and perinuclear staining was also noticed in tumor cells. This could be attributed to the synthesized desmosomal components present intracellularly. Using the same dilution of antibody, the DSG3 staining was not as intense in tumour cells as observed in normal oral epithelium [Figures 1 and 2]. This could be ascribed to the fact that DSG aids in the maintenance of the cell junctions in oral epithelium. Therefore, the alteration of desmosomal components is required to some extent for the cells to metastasise.[24] Further, its expression may vary with the degree of differentiation of the epithelial cells.

It was observed that ITCs were very difficult to identify with DSG3 due to low intensity of staining and nonspecific staining observed in sinus histiocytes and endothelial cells. We only accepted the cells morphologically consistent with epithelial cells with membranous and cytoplasmic or perinuclear staining as ITCs. Nagvekar *et al*.[25] also reported the presence of DSG3+ histiocytes in lymph node tissue and they confirmed the origin of these cells by immunostaining with CD 68 (pan macrophage marker) and CD163 (marker for M2 phenotype). The current study also found substantial number of macrophages in subcapsular sinuses, interfollicular areas, medullary sinuses as well as within the lymphoid follicles which stained positively with DSG3, which is a less reported but interesting finding [Figure 3]. These cells on detailed microscopic examination were identified to be slightly smaller in size having a smaller densely staining nucleus when compared to epithelial cells. A characteristic granular pattern of cytoplasmic staining was noted. Patel *et al*.[15] also used DSG3 as a marker for detection of occult LNM, but, they have not reported this finding.

Though a greater number of ITCs were detected with DSG3, the DSG3+ macrophages posed difficulty in the identification of ITCs and resulted in reduced specificity of the marker. In addition, case 1 showed cytoplasmic staining in epithelioid granuloma cells on staining with DSG3 which further adds up to the nonspecificity of DSG3. CK generally exhibited higher intensity of staining and less amount of nonspecific staining. Although CK has been consistently used as an IHC marker for detection of LNM, it may also show cross-reactivity with the dendritic cells as reported by Atula *et al*.[21] Similar staining of dendritic cells with CK was observed in the current study. According to Nagvekar *et al*.,[25] these macrophages show positivity for both DSG3 and CK and this may be attributed to the presence of ingested processed antigens within these cells, before migration to lymph nodes. Thus, micrometastatic and macrometastatic deposits were easier to identify in comparison to ITC on staining with DSG3.

The rate of immunohistochemical detection of micrometastasis was 23% in the current study which is clinically relevant as these micrometastases may lead to further recurrence in such cases. Also, the detection rate of ITC was found to be higher with DSG3, although it proved to be a relatively nonspecific marker due to extensive

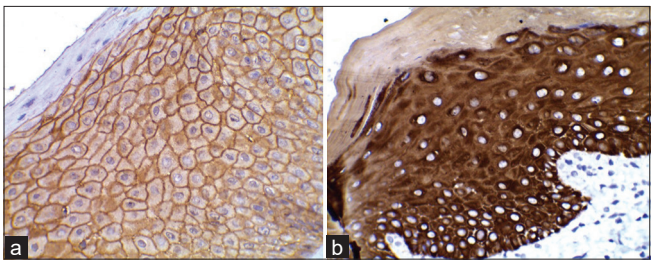

Figure 2: Photomicrograph showing normal oral epithelium stained with, (a) DSG3 (IHC, 40x), (b) CK (IHC, 40x)

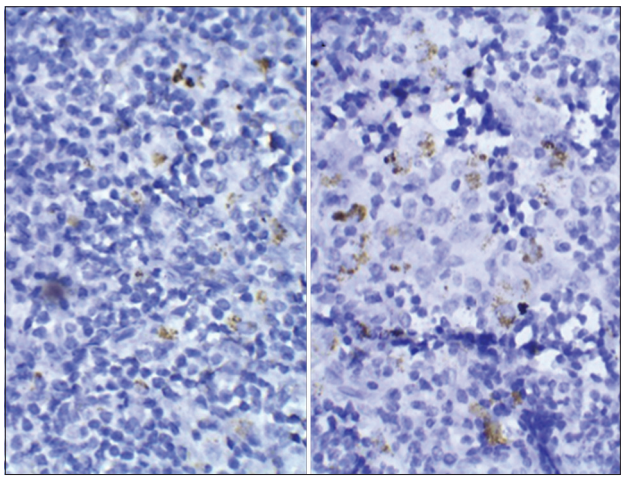

Figure 3: Photomicrograph showing granular positivity in sinus histiocytes on immunostaining with DSG3 (Left, IHC, 40x) and CK (Right, IHC, 40x)

staining of sinus histiocytes. However, it could be used as an adjunct to other markers such as cytokeratin for the detection of micrometastasis and macrometastasis.

Many studies over the years have revealed that various indices using SIR, such as PLR and NLR, can be used as prognosticators in OSCC and other carcinomas.[6,7,26-33] One of the studies in Indian population has also proved that PLR can be used as a predictive marker for LNM in OSCC.[7] As far as we know this is the foremost study attempting to correlate occult LNM with preoperative PLR and NLR. The mean PLR was found to be significantly higher in pN0 OSCC cases with or without occult metastasis in comparison to controls ($P < 0.001$). However, there was no statistically significant difference in the mean PLR and NLR between the cases showing occult metastasis and those without occult metastasis, although these were higher in the former group. The nonsignificant difference could be due to the smaller sample size in the present study. Hence, further multicenter prospective studies with a larger cohort of patients might be able to elucidate significant results. However, the current study has highlighted that preoperative PLR may prove be helpful in distinguishing the OSCC cases from healthy controls. Hence, it could be utilized as a biomarker for early detection of OSCC effectively.

Interestingly, we were able to find occult metastasis in significant percentage of OSCC cases using IHC indicating that the rate of metastasis is actually higher than what is detected through routine histopathologically. This finding supports elective neck dissection in cases negative for metastasis on imaging alone as the treatment strategy rather than the watchful waiting approach as it will reduce the risk of disease recurrence. However, more ITC were immunopositive for DSG3, although it proved to be a less specific marker as it also stained a significant number of histiocytes and few endothelial cells along with tumor cells leading to confusion.

CONCLUSION

CK had better staining characteristics and lesser nonspecific staining in comparison to DSG3 for detection of occult metastasis.

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Conflicts of interest
There are no conflicts of interest.

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