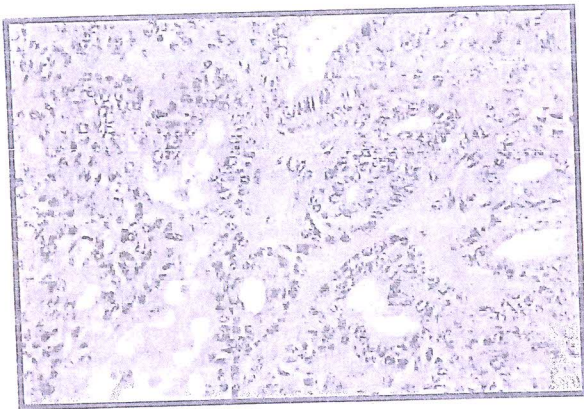


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ORIGINAL ARTICLE

Evaluation of histomorphological and immunohistochemical parameters as biomarkers of cervical lymph node metastasis in squamous cell carcinoma of oral cavity: A retrospective study

Suresh TN, Hemalatha A, Harendra Kumar ML, Azeem Mohiyuddin SM¹

Departments of Pathology and ¹Ear, Nose and Throat, Head and Neck Surgery, Sri Devaraj Urs Medical College, Sri Devaraj Urs Academy of Higher Education and Research Center, Kolar, Karnataka, India

Address for correspondence:

Dr.Suresh TN,
Department of Pathology, Sri Devaraj Urs Medical
College, Sri Devaj Urs Academy of Higher
Education and Research Centre, Kolar - 562 103,
Karnataka, India.
E-mail: sureshstn@rediffmail.com

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ABSTRACT

Introduction: Oral squamous cell carcinoma (OSCC) is seen worldwide but is more common in India. Lymph node (LN) metastasis has been shown to be the strongest prognostic factor in OSCC. Many histopathological and immunohistochemical markers have been studied to predict the LN metastasis. **Aims:** To identify clinicopathological factors and immunohistochemical (IHC) biomarkers which predict cervical metastasis in OSCC patients. **Study and Design:** A total of 105 cases of OSCC were taken up for our study. Histopathological parameters such as tumor thickness, depth, degree of differentiation, pattern of invasion (POI), lymphovascular and neural invasion were assessed. IHC was done on all cases using antibodies against Ki-67, cyclin D, E-cadherin, p53, CD31 and each antibody was assessed according to the standard protocol. **Statistical Analysis:** To calculate the relation between clinical, histopathological parameter, IHC marker and the occurrence of LN metastasis, chi-square test was used. Variables were tested using multivariate logistic regression method to assess the predictive significance. **Results:** Out of 105 cases studied, 29 cases showed LN metastasis. Maximum numbers of cases affected were females with involvement of buccal mucosa. We found significant association of cervical LN metastasis with high grade of differentiation, lack of E-cadherin expression, high Ki-67 and cyclin D1 expression. In our study; tumor depth, thickness, extent of peritumoral lympho-plasmacytic infiltration, presence of eosinophils, tumor nest type, p53 and microvessel density (MVD) showed no significant correlation. **Conclusion:** Significant association of cervical LN metastasis with high grade of differentiation, lack of E-cadherin expression, high Ki-67 and cyclin D1 expression was seen.

Key words: Cyclin D1, E-cadherin, grade of differentiation of OSCC, Ki-67, lymph node metastasis

INTRODUCTION

Oral cancer is the sixth most common cancer worldwide with high prevalence in South Asia.^[1,2] Oral cancers are most

prevalent in Kolar and constitute 29.66% of total cancer incidence in Kolar.^[3] Despite substantial development in both diagnosis and treatment in recent decades, the prognosis of the oral squamous cell carcinoma (OSCC) remains poor. Lymph node (LN) metastasis is shown to be the strongest prognostic indicator in OSCC. The 5- year survival drops considerably from 63 to 86% in patients with no nodal involvement to 20–36% in patients with LN metastasis.^[4,5]

Many methods are used to detect cervical LN metastasis. The sensitivity of preoperative imaging by computed tomography or magnetic resonance imaging and clinical examination is only

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around 70% and there is no imaging study capable of detecting micrometastasis in cervical LN.^[6] Clinical assessment by the Tumor Node Metastasis (TNM) system is widely and routinely used to define the extent of tumor load and thus determines the treatment option for OSCC patients. Although 20–30% of clinically N0 patients will show occult metastasis on elective neck dissection, a considerably large number of patients will remain pathologically N0 and therefore do not benefit from neck dissection.^[7,8] One of the major criticisms of the TNM system is that it ignores individual histological characteristic of tumor.^[9] Therefore many studies proposed histological grading system to predict the biological behavior of LN metastasis. Multiple histological features have been tested over last 2 decades to predict LN metastasis that includes nuclear pleomorphism, mitotic index, lympho-plasmacytic response, pattern of invasion (POI), tumor nest, tumor thickness, tumor depth, lympho-vascular invasion, perineural invasion and grade of tumor. Many studies also used immunohistochemical (IHC) markers like p53, Ki-67, cyclinD1, epidermal growth factor receptor, CD31, cyclooxygenase 2, mucin 1(MUC1), laminin 5γ2, E-cadherin and β-catenin to find out useful IHC marker to predict LN metastasis.^[10–14] Aim of this study was to identify clinicopathological factors and IHC biomarkers in predicting cervical metastasis in OSCC patients.

MATERIALS AND METHODS

Total number of 2,538 cases of malignancies were reported in the Department of Pathology, between January 2006 and December 2011. During study period, 523 patients were diagnosed as OSCC. A total of 115 patients underwent wide excision of primary tumor with simultaneous selective resection of regional LNs. In 105 cases, it was possible to retrieve surgical pathology material for review and to conduct IHC study. Patients with prior radiotherapy/chemotherapy/surgery for malignancy in head and neck region were excluded from the study. Clinically, TNM staging was done. Growth pattern of the tumor on gross examination was recorded. Tumors which were ulcerated, exophytic and cauliflower-like were described as ulceroproliferative. Ulcerated tumor which shows mainly an invasive component was termed as ulceroinfiltrative. Bulky exophytic tumor with superficial invasion and broad front were labeled as verrucous. Paraffin blocks and hematoxylin and eosin slides were retrieved from filing section of Department of Pathology. All the slides were reviewed by two pathologists without the knowledge of patient's clinical details and LN status.

Microscopic tumor thickness was defined as maximum tumor thickness excluding the keratin coat, taking the vertical extent of tumor from the surface to its deepest extent in a perpendicular fashion. Microscopic tumor depth was taken as infiltrative portion of the tumor which extended below the surface of the adjacent mucosa. Cases in which the epithelium was destroyed, it was measured after reconstituting a virtual surface. The starting points differed for these two

measurements, but the deepest point of invasive tumor border was identical for both. In 18 cases, we could not measure the tumor depth, as adjacent normal mucosa was not identified on section.

Grade of differentiation was divided into well, moderate, or poor as described by Martinez-Gimeno *et al.*,^[15] and the extent of peri tumoral lympho-plasmacytic infiltration was divided into three grades according to Brandwein-Gensler *et al.*^[16] Grade 1 is characterized by a continuous dense layer of lympho-plasmacytic infiltration among tumor and healthy tissue. Grade 2 shows a discontinuous patchy pattern of lympho-plasmacytic infiltration. Grade 3 shows only minimal or no lympho-plasmacytic infiltration.

POI types 1–4 are identified as defined by Bryne *et al.*^[17] Type 1 represents tumor invasion in a broad pushing manner with a smooth outline. Type 2 represents tumor invasion with broad pushing fingers or separate large tumor islands with a stellate appearance. Type 3 represents invasive islands of tumor, >15 cells/island. Type 4 represent tumor islands, <15 cells/island. 20% was used as the minimal cut off for incorporating into any particular type. The shape of tumor nest is classified as type A where tumor had oval shape or sheet like nest with a round margin (with >80% tumor area showing these features). Tumors that had asteroid shape tumor nest with a speculated margin or scattered small tumor nest (>20% tumor area showing these features) were classified as type B.^[18]

IHC was performed using non-biotin polymer based HRP detection system. Slides were incubated with p53, Ki-67, CD31, cyclin D1 and E-cadherin primary antibodies (Biogenix, USA) at room temperature according to manufacturer's recommendation. Percentage of p53, Cyclin D1 and Ki-67 positive tumor cells were calculated by counting the number of brown-stained tumor nuclei in hotspots. In each case approximately 1000 tumor cells were examined. According to previous literature, we classified sample as positive if >10, >40 and >30% of tumor nuclei were stained by anti-p53, anti-cyclinD1 and Ki-67 antibody receptors, respectively.^[14,19] Expression of E-cadherin was defined as high when membrane staining of >50% of cells was observed and low when membrane staining ≤50% of the cells stained. Small blood vessels were visualized by staining endothelial cells for CD31 antibodies. Microvessel density (MVD) was calculated as highest number of vessels on high power field (×40) at invasive front of tumor and the patients were then divided into two groups, with a median value as the dividing line. In calculating the MVD, areas of inflammation and adjacent benign tissues were excluded.

Statistical analysis

In order to explore the relation between clinical, histopathological parameters, IHC markers and the frequency

of LN metastasis; chi-square test was used. In second step, all variables were tested in Multivariate logistic regression method to assess the predictive significance of above parameters. All calculations were carried out using statistical Package for Social Sciences (SPSS) version 16. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

A total of 105 patients (15 males and 90 females) with a mean age of 50.9 years (range 25–70 years) were included in this study. The site of primary origin was in buccal mucosa in 89 (84.7%) cases, lower alveolus in 12 (11.4%) cases, anterior two-third of the tongue in two cases and floor of mouth in two cases. Primary tumor was on the left side in 63 cases, on right side in 38 cases and in the midline in four cases. Clinical TNM staging showed six (5.7%) cases in Stage I, 22 (21.0%) cases in stage II, 31 (29.5%) cases in stage III and 46 (43.8%) cases in stage IV. Out of 105 patients, 29 (27.61%) patients had cervical LN metastasis. On statistical analysis, sex and anatomical site showed no significant association with respect to node metastasis [Table 1].

The distribution of histopathological parameters between tumor with and without cervical LN metastasis is summarized in Table 2.

Grade of differentiation and POI showed significant correlation with the occurrence of cervical LN metastasis. Majority of well-differentiated SCC (78.8%) showed no LN metastasis as compared to high occurrence of metastasis in moderate- and poorly-differentiated SCC (55%). Highly infiltrating SCC (POI type 4) was significantly associated with higher likelihood of LN metastasis [Figure 1].

The measurement of tumor thickness and tumor depth in primary tumor revealed an average value of 8.24 and 5.78 mm, respectively, with a range of 1–21 and 0–20 mm, respectively. However, none of the cut off values for tumor thickness or tumor depth achieved statistical significance to predict LN metastasis.

Lymphovascular invasion was found in two patients and both the patients showed LN metastasis. Perineural invasion associated with nodal metastasis was seen in three out of four patients. In our study extent of peritumoral lymphoplasmacytic infiltration, presence of eosinophils and tumor nest type showed no significant correlation.

A total of 904 LN were examined with an average of 8.6 per patient. Out of 282, 65 (23.0%) LNs studied in metastatic group showed metastasis.

Nuclear expression of p53, Ki-67 and cyclinD1 ranged from 0 to 95%, 5 to 70% and 5 to 90%, respectively. E-cadherin expression ranged from 0 to 90%. MVD ranged from

Table 1: Frequency distribution of clinical features with respect to node metastasis

Parameter	No. of patients with metastasis (n=29)	No. of patients without metastasis (n=76)	P value
Sex			
Male	4	11	0.929
Female	25	65	
Anatomic subsite			
Buccal mucosa	26	63	0.641
Alveolus	3	9	
Tongue	0	2	
Floor of mouth	0	2	

Table 2: Distribution and significance of histopathological parameters

Parameter	No. of patients with metastasis (n=29)	No. of patients without metastasis (n=76)	Total number	P value
Grades of differentiation				
G1	18	67	85	0.006
G2	10	9	19	
G3	1	0	1	
Pattern of invasion				
Type 1	1	2	3	0.004
Type 2	7	20	27	
Type 3	12	50	62	
Type 4	9	4	13	
Lymphoplasmacytic infiltration				
Grade1	6	12	18	0.520
Grade2	16	52	68	
Grade3	7	12	19	
Eosinophils	12	42	54	0.203
Tumor thickness, mm				
0 to <5	5	22	27	0.347
5 to <10	11	32	43	
10 to <15	8	11	19	
15 to <20	5	11	16	
Tumor depth, mm				
0 to <5	9	35	44	0.383
5 to <10	7	19	26	
10 to <15	1	7	8	
15 to <20	4	5	9	
Perineural invasion	3	1	4	
Lymphovascular invasion	2	0	2	

2 to 56/HPF with a median of 12/HPF. Decreased expression of E-cadherin and increased Ki-67 and cyclin D1 expression was significantly associated with LN metastasis [Figures 2–4]. However, MVD and p53 expression showed no significant correlation [Table 3].

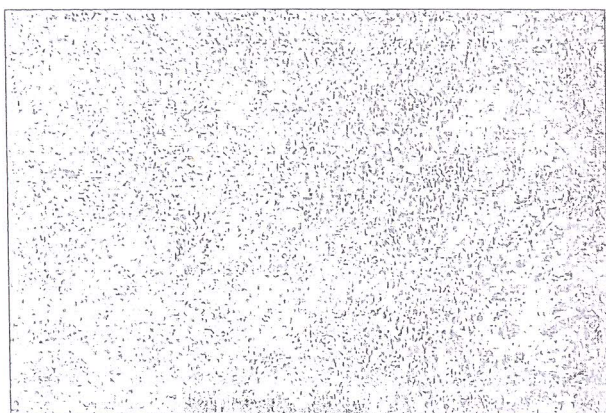


Figure 1: Squamous cell carcinoma showing type 4 pattern of invasion (H&E stain, $\times 100$). H&E = Hematoxylin and eosin

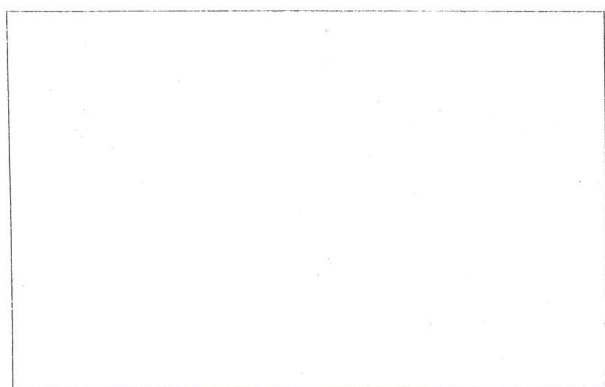


Figure 3: Immunohistochemical staining of Ki-67 in squamous cell carcinoma showing increased mitotic activity (IHC stain, $\times 400$)

Multivariate analysis of clinico-pathological factors and IHC biomarkers showed association of cervical LN metastasis with high grade of differentiation, low E-cadherin expression, high Ki-67 and Cyclin D1 expression ($P = 0.007, 0.001, 0.029$ and 0.020 , respectively).

CONCLUSION

Clinical TNM system is widely used by clinician for the management of OSCC patients. But it has been found that surface tumor size and size of the cervical LN does not correlate with the occurrence of metastasis in OSCC.^[20,21] Studies have shown that LN in excess of 20mm may be histologically reactive hyperplasia without metastasis.^[22] General policy of elective neck dissection based on clinical TNM staging exposes many OSCC patients to neck dissection that may not be necessary. Detailed pathological study of sentinel LN appears to be accurately predicting presence of metastasis and prevent the morbidity associated with unnecessary neck dissection in clinically N0/N1 patients.^[23] However, sentinel LN biopsy in head and neck cancer is not the standard of care and is practiced only in few centers. The complex drainage pattern of head and neck region and

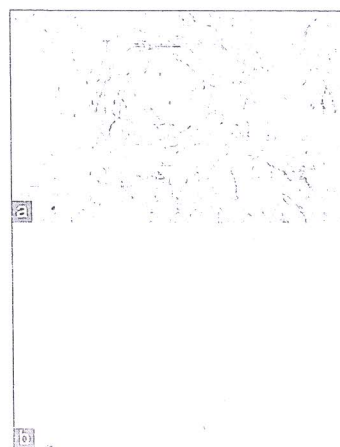


Figure 2: (a) Photomicrograph showing high expression of E-cadherin (IHC stain, $\times 400$). (b) Photomicrograph showing low expression of E-cadherin (IHC stain, $\times 400$)

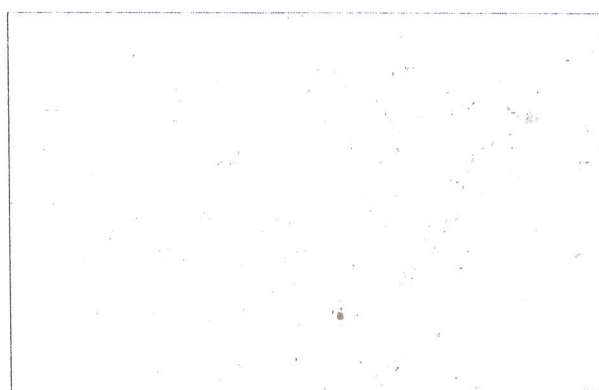


Figure 4: Immunohistochemical staining of cyclin D1 in squamous cell carcinoma showing high expression (IHC stain, $\times 100$)

proximity of LN to injection site raised concerns that sentinel LN may not be accurate in head and neck cancer. Many studies have investigated histopathological parameters, IHC and molecular markers for potential predictive factors. If these predictive factors accurately identify LN metastasis before neck dissection, the treatment would be more selective and overall cost and morbidity would be minimized.

We found significant correlation between grades of differentiation and cervical LN metastasis. Reports by Martinez-Gimeno *et al.*, Byers *et al.*, Sparano *et al.*, Kurokawa *et al.* and Pimenta *et al.*, also found a significant correlation between histological grades and occult nodal metastasis.^[15,24-27] In the study by Chen *et al.*, prevalence of nodal metastasis in elective neck dissection was 32% for well and 75% for poorly-differentiated carcinoma.^[28]

In our study there was a significant correlation between POI and cervical LN metastasis. Only 20 out of 102 (19.6%) patients in low risk group (POI type 1, 2 and 3), but nine out of 13 (69.2%) patients of high risk group (type 4)

Table 3: Immunohistochemical biomarkers and cervical metastasis

Biomarker	No. of patients with metastasis (n=29)	No. of patients without metastasis (n=76)	Total number	P value
E-cadherin (%)				
>50	12	71	83	0.001
<50	17	5	22	
Ki-67 (%)				
>30	20	29	49	0.005
<30	9	47	56	
CyclinD1 (%)				
>40	18	29	47	0.028
<40	11	47	58	
p53 (%)				
>10	29	70	99	0.119
<10	0	6	6	
MVD				
>12/HPF	20	46	66	0.424
<12/HPF	9	30	39	

MVD=Microvesseldensity, HPF=High power field

revealed cervical LN metastasis. Various other studies by Hiratsuka *et al.*, Kurokawa *et al.*, Osaki *et al.*, Nagata *et al.*, Okamoto *et al.*, Goerkem *et al.* and Borges *et al.*, showed POI/ mode of invasion (MOI) as important predictor of cervical LN metastasis.^[20,26,29-33]

Chang *et al.*, proposed invasive pattern grading score (IPGS) which is based on Bryne *et al.*'s criteria.^[10,17] In their study, two most prevalent patterns at the invasive front of OSCC were considered similar to Gleason's score in carcinoma of prostate. Since each invasive score was assigned a number between 1 and 4, total summed score ranged from 2 to 8. Twenty percent was used as minimal cut off for incorporating into grading system. They found statistically significant correlation between IPGS, LN metastasis, distant metastasis and tumor recurrence. Highly significant association between presence of lymphovascular/perineurial invasion and LN metastasis was well established in the studies by Sparano *et al.*, Pimenta *et al.*, Borges *et al.*, Shingaki *et al.*, and Brown *et al.*^[23,27,33-35]

In contradiction to the results published for elective neck dissection, neither tumor thickness nor tumor depth was significantly associated with cervical LN metastasis. Lim *et al.*, Kurokawa *et al.* and Fakih *et al.*, suggested tumor depth more than 4 mm, but Kane *et al.* and Fukano *et al.*, found that tumor depth more than 5 mm carries a high risk for cervical LN metastasis.^[14,26,36-38] In a study by Yuen *et al.*, tumor thickness from 3 to 9 mm was associated with a 50% nodal metastasis rate; whereas, Hoşal *et al.*, found that thickness of ≥ 9 mm was the only variable that predicted occult metastasis in tongue carcinoma.^[39,40] However, in Goerkem *et al.*'s study of 78 patients no significant association between tumor thickness

or tumor depth with cervical LN metastasis was reported.^[32] Most of the above stated studies were done on carcinoma of tongue and floor of mouth. In our study, tongue carcinoma and carcinoma of floor of mouth were seen in only two cases each. Majority of our cases showed buccal carcinoma (83.8%). Carcinoma of buccal mucosa appears to behave differently in relation to metastatic potential as compared to carcinoma of tongue and floor of mouth. Tumor thickness and tumor depth which is widely described as predictor of cervical LN metastasis in tongue carcinoma may not be applicable to carcinoma of buccal mucosa. Hence, large prospective studies are required in buccal carcinoma to establish the significance of tumor thickness and tumor depth for predicting regional node involvement.

Oral carcinogenesis is a multistep process in which 6–10 genetic events leads to the disruption of the normal regulatory pathways that controls basic cellular functions including cell division, differentiation and cell death.^[41] Mutation in the p53 tumor suppresser gene is the most common genetic change in human cancers and is regarded as an early event in carcinogenesis.^[42,43] In our study, 99(94.2%) cases showed positivity to p53 antibodies. However, there was no significant difference between metastatic and non-metastatic group in our study.

E-cadherin is a calcium-dependent intracellular adhesion glycoprotein in epithelial cells. It plays a very important role in cell-cell adhesion, homotypic binding of epithelial tissue and also in its morphogenesis and cancer metastasis.^[44] Several studies reported existence of association between low expression of E-cadherin expression and LN metastasis.^[14,45,46] Wang *et al.*, have found low expression of E-cadherin at the invasive front of the tumor than the central/superficial part in tumors with LN metastasis. It was also shown that low expression inversely correlated with invasive front grading system score, tumor thickness, poor survival rate and tumor size.^[47] In our study we found a statistical significance association of low E-cadherin expression with LN metastasis ($P = 0.001$).

Ki-67 is a proliferation marker. High Ki-67 index is associated with poor prognosis in tongue SCC.^[48] Study by Carlos *et al.*, revealed a correlation between Ki-67 index and histological grades of differentiation in OSCC patients.^[49] In our study, Ki-67 (>30% tumor cells) showed positivity in 49 (46.6%) cases. Ki-67 positivity statistically correlated with cervical LN metastasis ($P = 0.005$).

Cyclin D1 overexpression in carcinoma cells indicates accelerated G1 progression and entry into S phase of cell cycle with lower cell dependence on growth factors for proliferation. Cyclin D1 over expression has been reported in OSCC and is correlated with cytological grade, infiltrative pattern and metastasis.^[41,50-52] Carlos *et al.*'s study showed that cyclin D1 expression was significantly associated with

advanced tumor stage, LN metastasis and high Ki-67 index (>50% tumor positivity).^[49] In our study, 47 (44.7%) cases showed cyclin D1 (>40% tumor cells) positivity and there was significant correlation with cervical LN metastasis ($P = 0.0028$).

In our study, MVD in the primary tumor did not show any significant correlation with metastasis. Few studies have reported that MVD acts as an independent prognostic factor and is also not associated with tumor grade.^[53]

CONCLUSION

In literature there is a lacuna regarding the clinical and radiological factors that predict the cervical LN metastasis, hence there arises a need to evaluate histomorphological and immunohistochemical parameters as biomarkers of cervical LN metastasis. Our study shows that significant association of cervical LN metastasis exists with high grade of differentiation, lack of E-cadherin expression, high Ki-67 and cyclin D1 expression. An assessment of these factors in primary tumor may help us to predict LN metastasis, thereby minimizing the number of neck dissection.

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