

# Immunohistochemical analysis of stromal fibrocytes and myofibroblasts to envision the invasion and lymph node metastasis in oral squamous cell carcinoma

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## Abstract

**Background:** Tumor cells work in close coordination with stromal elements from its stage of emergence to metastasis. The study was designed to assess the presence and distribution pattern of stromal fibrocytes and myofibroblasts in oral squamous cell carcinoma (OSCC). Possibility of using these stromal cells as a marker for invasion and lymphnode metastasis was evaluated.

**Materials and Methods:** A total of 40 cases of OSCC consisting twenty cases of each lymph node positive (pN+) and lymph node negative (pN0) samples and ten normal oral mucosa (NOM) tissues were subjected to double immunostaining using CD34 and alpha-smooth muscle actin ( $\alpha$ -SMA) antibodies. Stained sections were evaluated semiquantitatively.

**Results:** CD34 fibrocytes were seen in 70% of NOM and none of OSCC samples.  $\alpha$ -SMA myofibroblasts were seen in 80% of OSCC and none of NOM samples. A statistically significant difference was found in fibrocyte values ( $P < 0.001$ ) and myofibroblast values ( $P < 0.001$ ) between NOM and OSCC study samples. No statistical significance in myofibroblast values between pN0 and pN+ study groups; however, their distribution pattern appreciably varied.

**Conclusions:** This study suggested that fibrocytes could be used as one of the markers for early invasion. Abrupt loss of fibrocytes at the transition zone toward carcinoma and statistical significance in their values supported this inference. Heterogeneity in the distribution pattern of myofibroblasts in tumor stroma indicates that this variability may predict the tumor behavior toward nodal metastasis rather than their mere presence or absence.

**Keywords:** Alpha-smooth muscle actin, CD34, fibrocytes, myofibroblasts, oral squamous cell carcinoma, tumor stroma

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## INTRODUCTION

Tumor, “the wound that does not heal,” is a highly complex tissue where tumor cells coexist with active

stroma composed of various stromal cells and extracellular matrix (ECM).<sup>[1]</sup> These tumor cells and stromal elements work in concert for tumor evolution toward metastasis.<sup>[2]</sup>

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Oral squamous cell carcinoma (OSCC) is one of the top ten cancers in the world,<sup>[2]</sup> ranks first among males and third among females in India.<sup>[3]</sup> Regardless of advanced diagnosis and treatment modalities, it carries potentially poor prognosis with an estimated survival rate of only 56%.<sup>[4]</sup> Prognosis of OSCC is intimately related to nodal metastasis. In general, a single ipsilateral lymph node metastasis reduces the survival rate by 50%.<sup>[5]</sup> There is a need for biomarkers that would predict the invasion and nodal metastasis in early stages from the biological samples collected with minimally invasive techniques to aid in prompt treatment planning.

Recent researches have shown loss of fibrocyte, a blood borne potent antigen presenting cell in carcinomatous stroma with concurrent appearance of smooth muscle like fibroblast called myofibroblast.<sup>[6]</sup> This phenomenon has been observed in the stroma of invasive carcinoma of cervix,<sup>[6]</sup> skin, breast, larynx, oral cavity and gastrointestinal tract.<sup>[7]</sup> Myofibroblasts are thought to contribute significantly for tumor growth, differentiation, progression and metastasis in numerous ways.<sup>[8]</sup>

In the present study, we assessed the presence and distribution pattern of fibrocytes and myofibroblasts in normal oral mucosa (NOM) and OSCC tissues with and without nodal metastasis. Possibility of using fibrocytes and myofibroblasts as a marker for invasion and nodal metastasis was assessed. In addition, attempt was also made to identify the cells with coexpression of CD34 and alpha-smooth muscle actin ( $\alpha$ -SMA) to test the hypothesis of fibrocytes being the origin of myofibroblasts in the tumor stroma.

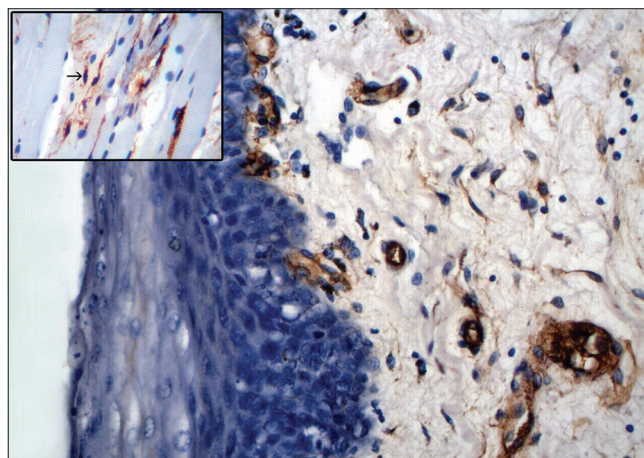
## MATERIALS AND METHODS

A total of 40 cases of OSCC (excision with radical neck dissection), twenty of each pN + and pN0 were obtained as study samples. The majority of the samples were from buccal mucosa (34), some were from gingiva (03) and floor of the mouth (03). Recurrent cases were excluded. NOM tissue sections were obtained during disimpaction procedures. Institutional ethical committee clearance was obtained for the study. Double immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections using CD34 and  $\alpha$ -SMA (1:50 dilution, mouse monoclonal, clone QBEnd/10 and  $\alpha$ -sm1, Vector Labs, CA, USA) antibodies, R.T.U Vectastain Elite ABC immunoperoxidase detection kit and Vector VIP purple and ImmPACT DAB Chromogens (Vector Labs, CA, USA). Trypsin digestion (0.1%) was used for antigen retrieval. Counterstaining was done using Harris hematoxylin.

Endothelial cells and smooth muscle cells of blood vessels acted as internal control for CD34 and  $\alpha$ -SMA, respectively. Membranous brown staining in the cells with bipolar processes and flattened oval nuclei is considered as fibrocytes. Cytoplasmic purple staining in spindle to stellate stromal cells with multiple cytoplasmic extensions and flat nuclei is considered as myofibroblasts. Sections were evaluated semiquantitatively by two observers independently and were graded as 0 (no staining), 1 (focal positivity) and 2 (multifocal/diffuse positivity). Semiquantitative method was adopted as the cells of interest were in syncytium with their cytoplasmic processes intermingled with each other. The values were subjected to Kruskal–Wallis ANOVA ranking and Mann–Whitney U-test. Blinded observation was done to assess the distribution pattern of myofibroblasts in pN0 and pN+ subgroups of OSCC. Distribution pattern was graded as 0 (no myofibroblasts), 1 = loose arrangement with focal (LAF) distribution, 2 = thick syncytium with focal distribution, 3 = loose arrangement with multifocal distribution and 4 = thick syncytium with multifocal distribution (TSMF).

## RESULTS

In the lamina propria of the NOM, fibrocytes were evident (70%) as evenly scattered dendritic cells [Figure 1]. They were more densely arranged around submucosal structures such as adipocytes, blood vessels and minor salivary glands compared to superficial lamina propria. Around deeper structures such as skeletal muscles, they were seen forming reticular network [Figure 1 inset]. None of the OSCC samples showed the presence of fibrocytes ( $P < 0.001$ ) [Tables 1 and 2].

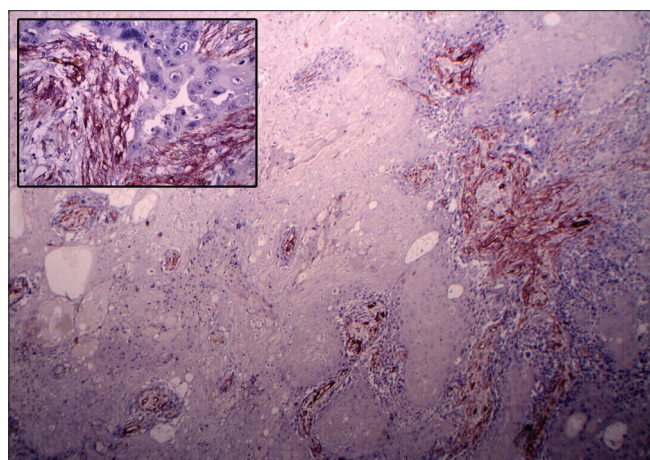


**Figure 1:** Section from normal oral epithelium shows scattered dendritic cells with bipolar thin cytoplasmic processes and flat nuclei in connective tissue (double immunostaining, DAB and VIP purple chromogen, CD34 and alpha-smooth muscle actin monoclonal antibody, original magnification,  $\times 40$ ). Inset shows reticular network of fibrocytes around muscle fibers (original magnification,  $\times 100$ )

Myofibroblasts were completely missing in NOM. Most of the OSCC samples (80%) showed the presence of myofibroblasts in the stroma ( $P < 0.001$ ) [Tables 3 and 4]. Between pN0 and pN+ groups, no significant difference was observed ( $P = 0.66$ ) [Table 4]. Myofibroblasts were heterogeneous in distribution pattern within a single section, abundant in one area while nearly absent in others [Figure 2]. Myofibroblasts were distributed as loose focal [Figure 3a] to diffuse syncytial [Figure 3b] patterns. Nine out of sixteen positively stained pN0 of OSCC had predominantly LAF (56.25%) in contrast to pN+ where 9 out of 16 positively stained cases had predominantly TSMF (56.25%). When myofibroblasts formed thick syncytium near tumor island, less inflammatory cells are seen [Figure 4]. In most cases, myofibroblast network was associated with new capillaries. There was no cell that coexpressed CD34 and  $\alpha$ -SMA. Most of the sections showed myofibroblasts in the stroma between tumor islands [Figure 2], less consistently at invasive front [Figure 5].

## DISCUSSION

Our study revealed the presence and characteristic distribution pattern of fibrocytes in NOM and myofibroblasts in OSCC. Fibrocytes were characteristically more around deeper submucosal structures compared to lamina propria in the oral cavity. Reason behind such diversity is unclear. In the dermal tissues, fibrocytes are thought to nurture skin adnexal structures which contain presumed follicular stem cells.<sup>[9]</sup> Similarly, in the submucosal structures of the oral cavity, they can be postulated to have supportive function in differentiation and proliferation of



**Figure 2:** Section from oral squamous cell carcinoma shows thick syncytium of myofibroblasts in between the compact tumor cells (double immunostaining, DAB and VIP purple chromogen, CD34 and alpha-smooth muscle actin monoclonal antibody, original magnification,  $\times 10$ ). Inset shows myofibroblasts (original magnification,  $\times 100$ )

immature cells.<sup>[10]</sup> Fibrocytes in lamina propria also act as an antigen presenting cell. This fact is supported by their expression of major histocompatibility complex Class I

**Table 1: Kruskal–Wallis ANOVA ranks for fibrocyte values show significant difference between each study groups ( $P < 0.001$ )**

Groups	Means	SD	Median	H	P
n	1.0000	0.8165	1.00	59.0138	<0.001
SCC	0.0000	0.0000	0.00		

SD: Standard deviation, SCC: Squamous cell carcinoma

**Table 2: Pair-wise comparison of the study groups for fibrocytes values by Mann–Whitney U-test ( $P < 0.001$ )**

Groups	Means	SD	Median	Z	P
n	1.0000	0.8165	1.0000	3.3955	0.0007
SCC	0.0000	0.0000	0.0000		

SD: Standard deviation, SCC: Squamous cell carcinoma

**Table 3: Kruskal–Wallis ANOVA ranks for myofibroblast values show significant difference between each study group ( $P < 0.001$ )**

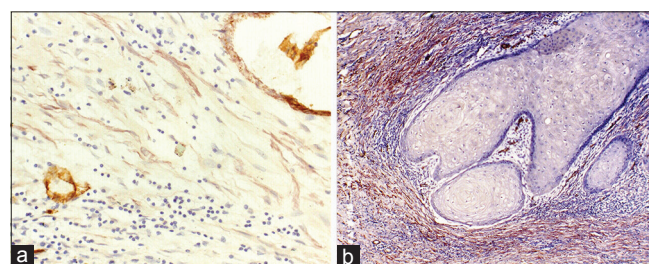
Groups	Means	SD	Median	H	P
n	0.0000	0.0000	0.00	43.4681	<0.001
SCC	1.3000	0.7910	1.50		
pN0	1.2500	0.7864	1.00		
pN+	1.3500	0.8127	2.00		

SD: Standard deviation, SCC: Squamous cell carcinoma

**Table 4: Pair-wise comparison of the study groups for myofibroblast values by Mann–Whitney U-test**

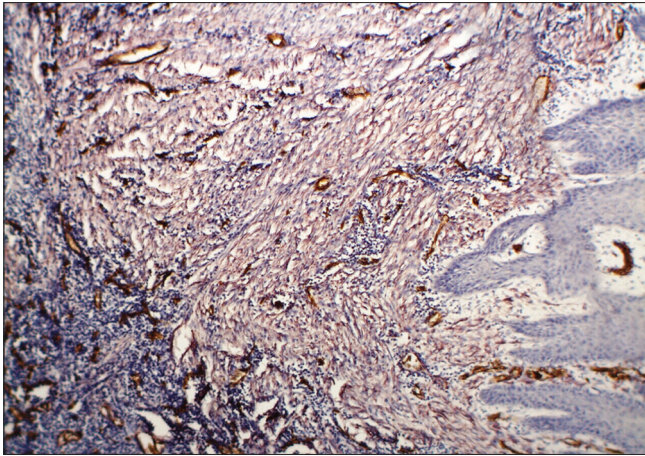
Groups	Means	SD	Median	Z	P
n	0.0000	0.0000	0.0000		
SCC	1.3000	0.7910	1.5000	3.8806	0.0001
pN0	1.2500	0.7864	1.0000	3.5195	0.0004
pN+	1.3500	0.8127	2.0000	3.5195	0.0004
pN0	1.2500	0.7864	1.0000		
pN+	1.3500	0.8127	2.0000	0.4328	0.6652

Significant amount of myofibroblasts were seen in OSCC compared to NOM ( $P < 0.001$ ), both pN0 and pN+ ( $P < 0.001$ ). However, values between pN0 and pN+ groups are insignificant ( $P = 0.66$ ). SD: Standard deviation, SCC: Squamous cell carcinoma, NOM: Normal oral mucosa, OSCC: Oral squamous cell carcinoma



**Figure 3:** (a) Section from oral squamous cell carcinoma shows loosely arranged myofibroblasts in the tumor stroma (double immunostaining, DAB and VIP purple chromogen, CD34 and alpha-smooth muscle actin monoclonal antibody, original magnification,  $\times 40$ ). (b) Section from OSCC shows diffuse syncytial band of myofibroblasts around the tumor islands (double immunostaining, DAB and VIP purple chromogen, CD34 and alpha-smooth muscle actin monoclonal antibody, original magnification,  $\times 40$ )



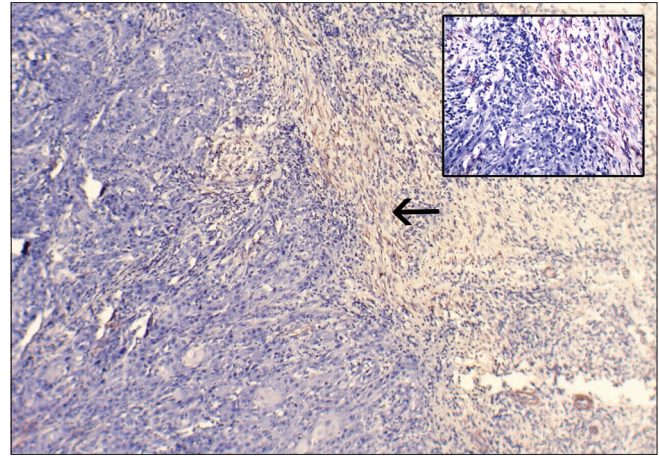


**Figure 4:** Section from oral squamous cell carcinoma shows band of myofibroblasts between tumor islands and inflammatory cell infiltrate (double immunostaining, DAB and VIP purple chromogen, CD34 and alpha-smooth muscle actin monoclonal antibody, original magnification,  $\times 40$ )

and II, costimulatory molecules CD80 and CD86,<sup>[11]</sup> thus suggesting their role in host immune surveillance. According to Fumio *et al.*,<sup>[12]</sup> fibrocytes could be stromal reserve cells with capability of differentiation toward variety of mesenchymal cells such as myofibroblasts, adipocytes, fibroblasts and factor XIIIa-positive dendrocytes. Perivascular location of fibrocytes may represent their recent homing into the tissues after differentiating from their precursors from peripheral blood. In 30% of our normal study samples (3 out of 10), fibrocytes were lacking. This may be due to downregulation of CD34 expression over time. *In vitro* studies of long time fibrocytes culture have supported this view.<sup>[13]</sup>

In OSCC stroma, fibrocytes were totally absent. Abrupt loss of antigen-presenting cells such as fibrocytes at tumor-margin intersection suggests that the tumor has escaped from host immune surveillance.<sup>[14]</sup> Barth *et al.*<sup>[6]</sup> has also hypothesized that mediators released from tumor cells may induce apoptosis of these antigen-presenting cells. However, the mediator and the mechanisms still remain unclear. Statistical significance and fibrocytes loss at tumor-margin interphase suggest that loss of fibrocytes could be sensitive marker for invasion. However, this phenomenon has been also documented in the areas of lymphocytic infiltration.<sup>[7]</sup> Hence, it cannot be considered as specific for invasion.

In the oral cavity, contractile matrix-modifying myofibroblasts are mostly seen in periodontal ligament where tissue undergoes continual remodeling in response to stress.<sup>[15]</sup> Since NOM consisted of buccal mucosa tissues, no myofibroblasts were found. In 80% (32 out of 40 cases) of OSCC samples, myofibroblasts were evident. Presuming



**Figure 5:** Section from invasive front zone of oral squamous cell carcinoma shows loosely arranged myofibroblasts (double immunostaining, DAB and VIP purple chromogen, CD34 and alpha-smooth muscle actin monoclonal antibody, original magnification,  $\times 10$ ). Inset shows myofibroblasts (original magnification,  $\times 40$ )

tumor as a nonhealing wound,<sup>[16]</sup> myofibroblasts are likely to be seen in the tumor stroma. Studies on murine model of bleomycin-induced lung fibrosis have shown that myofibroblasts escape Fas ligand (FasL)-mediated killing, allowing their uninterrupted accumulation. The same might be true in tumor stroma where myofibroblast emerges as an immune-privileged cell phenotype and resists their apoptosis.<sup>[17]</sup> However, further studies are necessary to prove this phenomenon in the case of tumor stroma.

In the tumor stroma, myofibroblast density varied from thick syncytium to loose individual cells. ECM stress is one of the key factors for differentiation of myofibroblasts.<sup>[18]</sup> According to Sarntinoranont *et al.*,<sup>[19]</sup> in solid tumors, neoplastic cells are rapidly dividing and expanding leading to a constant compressive stress on ECM. This may lead to thick syncytium myofibroblast network between compact tumor islands. Myofibroblasts which were located close to small tumor cell clusters mostly presented as loose individual cells and displayed much larger size. Less compressive stress on ECM and less contractile activity could be responsible for such finding. This view is also supported by *in vivo* experiments of collagen stiffness and myofibroblasts morphology.<sup>[18]</sup>

Distribution pattern of myofibroblasts varied from diffuse/multifocal thick syncytium occupying most of the tumor stroma to focal/loose arrangements. Thode *et al.*<sup>[20]</sup> have described two distinct patterns of myofibroblast appearance, one as “diffuse network” pattern and the other focally adjacent to few tumor islands as “spindle” pattern. In our study, pN+ predominantly showed multifocal/diffuse syncytium and pN0 showed focal/loose arrangement, suggesting that variation in the distribution

pattern and their abundance may have impact on tumor behavior since myofibroblasts in tumor stroma act as factories of various mediators.<sup>[21]</sup> However, distribution within a single section was highly variable with abundance of cells in one area to nearly nil in other. This is consistent with Vered *et al.*,<sup>[22]</sup> who found heterogeneous distribution pattern of myofibroblasts in tumor stroma. Although there is no statistical significance in myofibroblasts values between pN0 and pN+, their distribution pattern varied considerably. Kellermann *et al.*<sup>[23]</sup> reported that abundance of myofibroblasts in tumor stroma is associated with highly aggressive behavior but also is well correlated with nodal metastasis.

Myofibroblasts were seen in both well and poorly differentiated tumor stroma. This observation is consistent with literature where authors have suggested that myofibroblasts appear in tumor stroma irrespective of its degree of differentiation.<sup>[24]</sup> However, in well-differentiated tumors, myofibroblasts formed thicker syncytium around tumor islands, and in poorly differentiated tumors, they were loosely arranged throughout stroma. According to the literature, myofibroblasts differentiation occurs in early stages of invasion<sup>[15,24]</sup> suggesting that just a break in basement membrane and invasion of the cells into stroma is sufficient to provoke their establishment. To test this, we stained and examined few early invasion OSCC lesions (five samples). However, there were no myofibroblasts seen around early invasive tumor islands. Larger sample assessment is necessary before concluding on the findings.

Our study samples showed the presence of myofibroblasts mostly in between the tumor cell islands and less frequently at invasive front. However, literature documents their presence mostly at invasive front.<sup>[25]</sup> Myofibroblasts in between tumor islands presented in thick syncytium form whereas at invasive front loose bands. This observation is supported by Kawashiri *et al.*<sup>[26]</sup> studied collagen distribution pattern in the tumor stroma and concluded that there is very less desmoplasia at the invasive front compared to tumor stroma. Enhanced myofibroblasts expression of plasmin,<sup>[25]</sup> stromalysin-1, N-cadherin, platelet-derived growth factor-1, epitaxin, transforming growth factor-beta), stem cell factor and other cytokines<sup>[27]</sup> contribute to tumor cell proliferation, migration and aggressive behavior.

Myofibroblasts showed diverse arrangement pattern and inverse distribution relationship with inflammatory cell infiltration. Thick band of myofibroblasts in between tumor islands and inflammatory cells gave an impression of myofibroblasts being a barrier. However, when myofibroblasts are arranged in loose forms, inflammatory

cells were seen amidst of them. De Wever and Mareel<sup>[25]</sup> suggested that myofibroblasts in thick syncytium prevent the inflammatory cell infiltration toward the tumor islands thus forming a physical barrier, mask the tumor cells from host immune surveillance and prevents tumor cell killing. Wallach-Dayana *et al.*<sup>[17]</sup> based on *in vitro* studies showed that myofibroblasts cause FasL-mediated apoptosis of lymphocytes. In the tumor stroma, inflammatory cells are thought to play both pro- and anti-tumorigenic roles.<sup>[28]</sup> It can be hypothesized that tumor-enslaved inflammatory cells along with myofibroblasts and derived cytokines may act synergistically to enhance the tumor invasion.

As mentioned earlier, we found nonuniform distribution pattern of myofibroblasts in the tumor stroma with their heterogeneity varying from abundant in one area to near absent in other within the same sample. This observation suggests that when there is less stroma available for evaluation in a given sample, there is a high possibility of having false negative results. This may be considered as a reason for the absence of myofibroblasts in 20% of (8 out of 40 cases) study samples.

The earlier studies on myofibroblasts correlation with nodal status have used tongue carcinomas as major sample size.<sup>[23,26]</sup> Possibility of having site factor influence on lymph node metastasis in these studies cannot be ruled out and also has not been discussed. Our study has analyzed the majority of buccal mucosal samples. Other sites include gingiva and floor of the mouth. The number of nodes evaluated to determine the lymphnode status in these earlier studies is unclear.<sup>[29]</sup> We have evaluated an average of 13.4 nodes in each case before concluding on their nodal status. There are very few studies in literature which have analyzed fibrocytes and myofibroblasts correlation in OSCC lesions.<sup>[7,29]</sup> However, those studies have smaller sample sizes, and site details are also unavailable.

## CONCLUSIONS

The study elicited the presence and distribution pattern of fibrocytes and myofibroblasts in NOM and OSCC study groups. Instantaneous loss of fibrocytes and appearance of myofibroblasts at transition zone may hint at fibrocytes being the origin of myofibroblasts. However, we could not obtain sufficient support for this hypothesis since the technique used was inefficient in detecting a single cell with dual marker expression. Immunoelectron microscope which provides better resolution could be a solution. Our study showed a diverse distribution pattern of myofibroblasts in tumor stroma; further research aiming at factors responsible for such diversity and its influence on



tumor microenvironment may provide insight into tumor behavior toward metastasis. The study evidenced that loss of fibrocytes is a sensitive marker for invasion, although not specific. However, gain of myofibroblasts is neither sensitive nor specific for invasion. Further investigations are necessary to explain the reason for their varied distribution pattern and its influence on lymph node metastasis.

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### Conflicts of interest

There are no conflicts of interest.

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