Clinicopathological Significance of Tumor Lymphatic Vessel Density in Head and Neck Squamous Cell Carcinoma

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Abstract Various studies have demonstrated that the lymphatic system is the additional route for solid tumor metastasis. Lymph nodes metastasis in Head and neck squamous cell carcinoma (HNSCC) is a major prognostic indicator for disease progression and a guide for therapeutic strategies. We conducted a study to compare intratumoral (IT) and peritumoral (PT) lymphatic vessel density (LVD) in HNSCC using lymphatic marker D2-40 and its correlation with lymph node metastasis, histological grading and other clinicopathological parameters. Fifty specimen of HNSCC with modified radical neck dissection tissue were included in the study group. Tissue from tumor, peritumoral tissue, tumor margin and all the lymph nodes were processed for paraffin wax blocks and histopathological diagnosis. Immunohistochemical profile of

lymphatic vessels in intratumoral and peritumoral tissue was assessed by subjecting one section each from the tumor and peritumoral tissue to D2-40 immunostain. To determine LVD, four fields with the highest LVD (hot spots) were identified. The mean values were calculated by taking an average of all the measurements. The comparison of LVD between peritumoral and intratumoral area revealed significantly higher PT-LVD ($P = 0.001$). No significant association was seen between LVD, IT-LVD and PT-LVD and different age groups, gender, site of tumor, risk factors, size of tumor, tumor inflammation, pushing/infiltrating margin and stage of tumors. Significantly higher LVD, IT-LVD and PT-LVD was seen in association with lymph node metastasis. Both high intratumoral and peritumoral LVD were found significantly associated with the presence of lymph node metastasis, however lymphatic vessels were found to be significantly more numerous and larger in peritumoral areas as compared to intratumoral lymphatics. The specificity of D2-40 as a lymphatic endothelial marker was also confirmed. The results of our study support the possibility of using the determination of tumor lymphangiogenesis to identify patients of HNSCC who are at risk of developing the lymph node metastasis.

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Introduction

Head and neck malignancies are one among the 10 most common cancers worldwide [1]. According to the Indian Council of Medical Research (ICMR) Atlas, each year

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number of new patients diagnosed with head and neck cancer (HNC) are approximately 0.2 to 0.25 million which account for around 20% cancer burden in India [2]. Head and neck squamous cell carcinoma (HNSCC), is the most common type of HNC [1]. Use of tobacco products is the major cause for HNSCC especially for oral cancer. Alcohol Consumption acts as a synergistic risk factor. Depending upon the clinical extent of the disease and the sub-site of origin, five-year survival varies from 20 to 90% [3].

Hematogenous and lymphatic spread are the major pathways of tumor dissemination. Various theories for the tumor cells dissemination via lymphatic system have been proposed. It is considered that the entry of tumor cells into the lymphatic vessels may be easier due to their thin and discontinuous basement membrane which does not provide a significant barrier to tumor cells. Another theory suggest that neoangiogenesis or lymphangiogenesis which is formation of new blood or lymphatic vessels provides an alternative exit route for tumor cells [4]. For the initial metastatic spread of tumor cells, invasion of tumor cells in the established vessels versus new blood and lymphatic vessels is still unclear [4, 5]. Conventionally, it was assumed that the tumor cells are passively taken up by lymphatic vessels along with the protein-rich interstitial fluid, which is facilitated by the higher permeability of lymphatic vessels due to absence of the regular basement membrane barrier [6].

Lymph node status is a predictor of patient survival and guides the clinical management strategy. Various methods are available to differentiate normal lymph nodes from metastatic lymph nodes, but their sensitivity to detect micrometastasis is low. Therefore, there is a pressing need of more reliable markers which can predict metastasis in the lymph node in early stages of cancers to modify therapeutic decision [7–10].

Neoangiogenesis has been studied extensively till now. Although quantification of tumor-induced lymphangiogenesis is a valuable method for predicting lymph node metastasis, it is not clear whether detection of lymphangiogenesis might be used in the early detection of metastasis in lymph nodes or elsewhere. After the identification of the first lymphangiogenic factor, vascular endothelial growth factor C (VEGF-C) as well as suitable markers that distinguish blood from lymphatic vascular endothelium, there is increased interest in the study of lymphangiogenesis in tumors [6]. Novel lymphatic markers (LYVE-1, podoplanin, β -chemokine receptor D6, macrophage mannose receptor, desmoplakin, and D2-40) which can accurately differentiate lymphatic from blood vessels in histopathological sections have yielded new insights into mechanisms of metastasis.

Lymphatic markers have been commonly used in various studies to assess lymphatic vessel density/lymphatic

area as a prognostic variable for patient survival and as an important tool for predicting lymph node status in malignancy [11, 12]. Immunoreactivity by D2-40 antibody is useful in detecting lymphatic vessels in sections from formalin-fixed and paraffin-embedded blocks. In a direct comparison of the D2-40 antibody and an antibody against podoplanin on paraffin sections in a series of HNSCC, both the antibodies showed extremely high specificity and sensitivity for lymphatic endothelium. Role of lymphatic endothelial marker D2-40 in various solid malignancies has been studied with conflicting results including papillary thyroid carcinoma, non small cell lung cancer, malignant melanoma, epithelial ovarian cancer, prostate cancer, breast cancer, rectal cancer, bladder transitional cell carcinoma, SCC of the cervix, colon cancer, colorectal cancer, endometrial carcinoma, gastric carcinoma and renal cell carcinoma [8].

Several novel lymphangiogenesis parameters might be more sensitive prognostic indicators of metastasis to lymph nodes and disease progression than current methods. These include lymphatic vessel density at tumor sites and in tumor draining lymph nodes, expression of lymphangiogenic growth factors, and invasion of cancer cells into lymphatic vessels and altered lymph flow and volume in tumor draining lymph nodes. Weidner et al. in 1991 defined a method to assess tumor-associated lymphatic vessel density (LVD) by counting the number of immunostained vessels in paraffin tissue section [13]. LVD is determined in areas which contain numerous microvessels termed as vascular ‘hot spots’. Areas with an elevated vascular density have been used for the assessment of LVD with an inherent assumption that in these ‘hot spots’, there is an associated functional increase in the number of lymphatic vessels. The significance of counting lymphatic vessels in ‘hot spots’ vs an overall lymphatic vessel count is questionable as the data on the association of lymphangiogenesis with hypoxia is still contradictory as compared to neoangiogenesis [8].

So far, studies on the importance of lymphangiogenesis for tumor growth have yielded inconsistent conclusion and this is mostly due to differences in the applied methodology and the lack of standardization. A proposition for the standardization of the immunohistochemical method for lymphangiogenesis assessment is given. There is a prodigious volume of data on lymphangiogenesis that has shown promising results. However, unfortunately at the head and neck front, we are yet to gather substantial evidence to use these novel approaches into mainstream clinical practice. Majority of these have been retrospective studies that have been underpowered to reach any meaningful conclusion and further quantification of this may prove valuable as a prognostic indicator. Hence, we plan this study to assess the role of lymphatic vessel density as a prognostic

indicator in HNSCC and their correlation with various clinical and pathological parameters.

Materials and Methods

Patients

Fifty specimen of HNSCC with modified radical neck dissection tissue were included in the study group. Tissue from tumor, peritumoral tissue, tumor margin and all the lymph nodes were processed for paraffin wax blocks and histopathological diagnosis on routine hematoxylin and eosin stain [14]. Histological prognostic parameters including histologic grade [15], tumor necrosis, tumor inflammation and margins were also assessed. Lymph nodes at different levels in MRND specimen were processed [16].

Immunohistochemistry

Immunohistochemical profile [17] of lymphatic vessels in intratumoral and peritumoral tissue was assessed with D2-40 immunostain. Paraffin sections measuring 3–5 μm in thickness on slides coated with tissue adhesive were deparaffinization, hydrated and incubated with 3% hydrogen peroxidase for 20 min to inactivate endogenous peroxidase. Antigen retrieval was done with microwave oven heating for 30 min with citrate or tris EDTA.

Sections incubated with D2-40 (prediluted) (DAKO), the monoclonal antibody, overnight at 4 °C and then rinsed with tris buffer solution (TBS) solution and followed by incubation with the secondary antibodies. The reaction was visualized using DAB (3,3'-Diaminobenzidine), and nuclei were lightly counterstained with hematoxylin. Tonsillar tissue was used as positive controls for D2-40.

Interpretation of Results and Statistical Analysis

Lymphatic endothelium was stained with D2-40 and lymphatic vessel density (LVD) was quantified in intratumoral (IT) and peritumoral (PT) area. Endothelium of lymphatic vessels revealed brown membranous positivity with D2-40. To determine LVD, four fields with the highest lymphatic vessel density (hot spots) were identified at low magnification (40X) within the tumor mass and within an area of 500 μm from the tumor border, and vessels were counted using a computer aided image analysis system under higher magnification (400X). Any highlighted endothelial cell or endothelial cell cluster clearly separated from adjacent microvessels, tumor cells and other connective tissue

elements were defined as microvessel even with absence of lumen. The mean values were calculated by taking an average of all the measurements [13]. All the data obtained were analyzed statistically using SPSS statistical software and correlated with other clinicopathological parameters (site, size, grades, margin of tumor, presence of necrosis, inflammatory infiltrate and lymph nodes metastasis). A value of $P < 0.05$ was considered statistically significant.

Results

In the study, the patient's age ranged from 30 to 82 years with mean age of 53.9 years ($\text{SD} \pm 12.5$). Seventy-two percents cases were males. Maximum number of the cases (74%) belonged to oral cavity. Positive history of smoking, tobacco chewing and alcohol intake was present in 60, 22 and 30% of cases respectively. Depending on the size of tumor, cases were divided into four categories as T1 to T4 (≤ 2 cm, ≥ 2.1 –4 cm, ≥ 4.1 –6 cm and > 6 cm). Majority (44%) of cases were in category T2. Most common tumor differentiation was moderately in 60% of cases. Mild to moderate inflammation was present in 44% of cases. Fifty-six percent of the tumors had infiltrating margin, while pushing margin was present in 44% of the cases. Necrosis in tumor tissue was seen in 44% of the cases. Twenty-three cases were positive for tumor metastasis. Majority of the cases (56%) were in stage III and IV according to AJCC cancer staging.

Lymphatic vessels were observed both within the tumor mass and in the peritumoral area (Figs. 1 and 2). Peritumoral lymphatics were found to have more dilated, open lumina than intratumoral lymphatics (Fig. 3). Few lymphatic vessels contained lymphocytes, whereas red blood cells were not seen within the positively stained vessels. No significant association was seen between LVD and different age groups, male/female patients, site of tumor and various risk factors (cigarette smoking, tobacco chewing and alcohol intake) (Table 1).

LVD was compared with different histopathological parameters and no significant association was found in relation to size of tumor, presence/absence of inflammation, pushing/infiltrating margin and different stage of tumors. When compared in node positive and negative groups, a significantly higher LVD was seen in association with lymph node metastasis ($P = 0.014$) (Table 2). The comparison of LVD between peritumoral and intratumoral area revealed significantly higher PT-LVD ($P = 0.001$). PT-LVD and IT-LVD were compared further with various clinico-pathological parameters. No significant association was seen with age, gender, risk factors, histological parameters, tumor inflammation, margin, tumor necrosis,

Fig. 1 Peritumoral and intratumoral expression of D2-40 in squamous cell carcinoma

Fig. 2 Intratumoral lymphatic vessel with compressed lumina

tumor grade and stage. Mean of PT-LVD and IT-LVD were significantly associated with lymph node metastasis ($P = 0.031$ and 0.023 respectively). In the study, D2-40 staining was seen in tumor cells in 72% of the cases, however this D2-40 positivity in tumor cells did not correlate with any clinicopathological parameters (Fig. 4).

Discussion

Head and neck squamous cell carcinoma (HNSCC) accounts for significant global cancer burden which usually presents with locally advanced disease which requires a multidisciplinary team approach [18]. Lymph nodes

Fig. 3 Peritumoral lymphatic vessel with open lumina

metastasis in HNSCC is a major prognostic indicator and a guide for therapeutic strategies. Methods of lymphangiogenesis quantification in solid tumors rely upon the use of markers that allow an accurate discrimination between lymphatic vessels and blood vessels in histological tissue sections. At present the most reliable marker is likely to be podoplanin, which is recognised by the monoclonal D2-40 antibody with a high specificity and sensitivity. Recently, standardization of the immunohistochemical method for lymphangiogenesis assessment is given which recommended that multiple immunohistochemical stains on serial sections of tumor in random subgroups of cases are used to confirm the actual staining of lymphatic vessels [8]. Manipulation of lymphangiogenesis pathway offers the opportunity for therapeutic strategies designed to inhibit or stimulate growth of lymphatic vessels in conditions such as lymphedema, cancer and infectious diseases.

No significant association was seen between LVD and different clinicopathological parameters except lymph node status in our study. When compared in node positive and negative groups, a significantly higher LVD was seen in association with lymph node metastasis ($P = 0.014$).

Miyahara et al. [19] observed that higher LVD values correlated significantly with higher scores of T classification ($P < 0.001$), lymph node involvement ($P < 0.001$), and mode of invasion ($P < 0.001$) but not with others clinicopathological parameters in oral SCC including tongue and other region. The comparison of LVD between peritumoral and intratumoral area revealed significantly

higher PT-LVD ($P = 0.001$). Similar observation was made in various other studies [20–22] while a few reported higher IT-LVD as compared to PT-LVD [23, 24].

When compared further with various clinicopathological parameters, no significant association was seen between IT-LVD and PT-LVD with age, gender and risk factors. In our study, presence/absence of inflammation, margin (pushing or infiltrative) and tumor necrosis did not show significant association with IT-LVD and PT-LVD. Similar observations were made in different other studies [25, 26]. In study by Beasley et al. [27], there was a significant association between an infiltrating tumor margin and IT-LVD when all three subgroups (oral cavity, oropharynx and larynx) of HNSCC were considered together ($P = 0.004$). When seen individually, this was found only in the oropharyngeal carcinoma subgroup ($P = 0.046$).

Significant association was not found between IT-LVD, PT-LVD and different tumor grade in our study. Similar observations were made in different other studies [25, 27], but higher IT-LVD correlated with a more aggressive tumor phenotype, including poor histological differentiation in few studies [26, 28]. In study by Audit et al. [26], a significant association was found between IT-LVD and poorly differentiated tumors in the laryngeal carcinoma group ($P = 0.02$). This association was not present in the oropharyngeal or hypopharyngeal groups. Higher IT-LVD and PT-LVD correlated with the poor histological differentiation ($P = 0.023$) in study by Baradaranfar et al. [24].

Table 1 Comparison of lymphatic vessel density (LVD) with various clinical parameters

Clinical parameters	Groups	No. of cases	LVD		PT-LVD		IT-LVD	
			Mean density $\pm$ SD	<i>P</i> value	Mean density $\pm$ SD	<i>P</i> value	Mean density $\pm$ SD	<i>P</i> value
Age group ^a	0–10	0	–	0.821	–	0.763	–	0.814
	11–20	0	–		–		–	
	21–30	3	30.4 $\pm$ 34.2		42.9 $\pm$ 49.9		17.9 $\pm$ 19.0	
	31–40	6	23.4 $\pm$ 14.1		29.2 $\pm$ 19.3		17.6 $\pm$ 10.8	
	41–50	15	19.3 $\pm$ 8.8		24.9 $\pm$ 10.5		13.7 $\pm$ 10.2	
	51–60	15	23.5 $\pm$ 10.5		29.9 $\pm$ 13.9		17.1 $\pm$ 09.1	
	61–70	6	18.5 $\pm$ 9.6		24.0 $\pm$ 14.3		13.0 $\pm$ 6.6	
	71–80	4	21.2 $\pm$ 8.2		31.7 $\pm$ 19.8		10.8 $\pm$ 6.3	
Site ^a	81–90	1	23.1		24.7		21.6	
	Oral cavity	37	20.7 $\pm$ 12.6	0.753	26.8 $\pm$ 17.3	0.640	14.7 $\pm$ 9.7	0.663
	Oropharynx	4	28.1 $\pm$ 14.8		39.7 $\pm$ 22.1		16.5 $\pm$ 15.2	
	Hypopharynx	1	33.5		47.6		19.5	
	Mandible	2	18.4 $\pm$ 6.6		25.7 $\pm$ 13.3		11.2 $\pm$ 0.0	
	Larynx	4	22.6 $\pm$ 8.7		30.2 $\pm$ 16.2		14.9 $\pm$ 5.2	
	Salivary gland	2	26.0 $\pm$ 4.1		25.7 $\pm$ 1.5		26.3 $\pm$ 6.6	
Gender ^b	Male	36	22.4 $\pm$ 12.3	0.603	29.7 $\pm$ 18.3	0.423	15.2 $\pm$ 9.2	0.908
	Female	14	20.4 $\pm$ 11.4		25.30 $\pm$ 13.4		15.53 $\pm$ 11.0	
Smoking ^b	Present	30	21.1 $\pm$ 9.4	0.571	27.9 $\pm$ 13.3	0.775	14.3 $\pm$ 08.1	0.363
	Absent	20	23.0 $\pm$ 15.3		29.3 $\pm$ 21.8		16.8 $\pm$ 11.7	
Tobacco chewing ^b	Present	11	22.9 $\pm$ 17.3	0.756	30.7 $\pm$ 24.2	0.628	15.1 $\pm$ 11.8	0.937
	Absent	39	21.6 $\pm$ 10.3		27.8 $\pm$ 14.7		15.3 $\pm$ 9.1	
Alcohol ^b	Present	15	20.1 $\pm$ 8.6	0.513	25.6 $\pm$ 10.2	0.445	14.7 $\pm$ 9.3	0.646
	Absent	35	22.6 $\pm$ 13.2		29.7 $\pm$ 19.2		15.5 $\pm$ 9.9	

LVD lymphatic vessel density, PT-LVD peripheral lymphatic vessel density, IT-LVD intratumoral lymphatic vessel density

^aANOVA test

^bIndependent t test

In our study, size of the tumor and staging also did not show significant association with IT-LVD and PT-LVD. This is supported by different studies including Beasley et al. [27] and Franchi et al. [25], but higher IT-LVD correlated with higher clinical stage ($P < 0.001$) in study by Kyzas et al. [28].

Both PT-LVD and IT-LVD were significantly associated with lymph node metastasis ($P = 0.031$ and 0.023 respectively). Beasley et al. [27] observed that IT-LVD revealed a statistically significant association ($P = 0.027$) with lymph node metastasis in oropharyngeal carcinoma, but not in oral cavity carcinoma or laryngeal carcinoma. They further opined that the formation of intratumoral lymphatic vessels may not facilitate nodal metastasis in all types of HNSCC. Alternatively, other important factors (as yet unidentified) in addition to mere access to lymph vessels may also be required by some tumors to metastasize to lymph nodes. Similar to our study, HNSCCs presenting with lymph node metastasis were found to have

significantly higher LVD, both within the tumor mass and in the peritumoral area ($P = 0.003$ and $P = 0.01$, respectively) in study by Franchi et al. [25].

However, in a study by Audet et al. [26], some node positive tumors were without evidence of intratumoral lymphatics who suggested that the intratumoral lymphatics through which they had spread were too small to be identified and were missed on the tissue sections sampled for analysis, or were destroyed after the metastasis occurred. This highlights some of the potential pitfalls in examining single sections of a tumor taken at a single point of time and the interaction of other biological systems on the process of tumor metastases. In our study we observed higher mean density in both peri and intratumoral area in positive lymph node group thus suggesting that both these lymphatics may be involved in tumor metastasis in lymph node.

Kyzas et al. [28] in their study of 81 cases of HNSCC, found a significant association of both PT and IT-LVD and

Table 2 Comparison of lymphatic vessel density (LVD) with various histological parameters

Histological parameters	Groups	No. of cases	LVD		PT-LVD		IT-LVD	
			Mean density $\pm$ SD	<i>P</i> value	Mean density $\pm$ SD	<i>P</i> value	Mean density $\pm$ SD	<i>P</i> value
Tumor size ^a	T1	10	22.8 $\pm$ 9.9	0.973	30.9 $\pm$ 16.8	0.923	14.6 $\pm$ 8.8	0.888
	T2	22	21.0 $\pm$ 11.8		26.8 $\pm$ 14.9		15.1 $\pm$ 10.7	
	T3	15	22.3 $\pm$ 15.0		29.5 $\pm$ 22.1		15.2 $\pm$ 9.7	
	T4	3	23.1 $\pm$ 5.7		26.6 $\pm$ 3.9		19.6 $\pm$ 10.0	
Tumor grade ^a	WDSCC	16	19.6 $\pm$ 9.5	0.600	24.9 $\pm$ 12.5	0.500	14.4 $\pm$ 9.0	0.901
	MDSCC	30	22.5 $\pm$ 13.7		29.5 $\pm$ 19.1		15.6 $\pm$ 9.9	
	PDSCC	4	25.7 $\pm$ 5.83		35.1 $\pm$ 17.1		16.3 $\pm$ 12.4	
Tumor inflammation ^a	Absent	3	21.4 $\pm$ 9.4	0.401	35.5 $\pm$ 22.3	0.460	7.3 $\pm$ 3.6	0.111
	Mild	21	21.4 $\pm$ 13.8		28.4 $\pm$ 22.7		14.4 $\pm$ 8.4	
	Moderate	15	18.8 $\pm$ 7.5		23.5 $\pm$ 10.3		14.1 $\pm$ 7.3	
	Severe	11	27.0 $\pm$ 13.6		33.3 $\pm$ 15.4		20.7 $\pm$ 13.4	
Tumor margin ^b	Pushing	22	22.6 $\pm$ 14.2	0.690	28.7 $\pm$ 19.7	0.921	16.6 $\pm$ 10.7	0.411
	Infiltrating	28	21.2 $\pm$ 10.2		28.2 $\pm$ 14.9		14.3 $\pm$ 8.8	
Tumor necrosis ^b	Present	22	23.3 $\pm$ 14.5	0.442	29.4 $\pm$ 20.0	0.734	17.3 $\pm$ 10.9	0.186
	Absent	28	20.7 $\pm$ 9.7		27.7 $\pm$ 14.6		13.7 $\pm$ 8.3	
Lymphnode positivity ^b	Present	23	26.3 $\pm$ 13.9	0.014	34.0 $\pm$ 19.8	0.031	18.6 $\pm$ 10.1	0.023
	Absent	27	18.1 $\pm$ 8.7		23.7 $\pm$ 12.8		12.5 $\pm$ 8.4	
Stage of tumor ^a	I	7	21.3 $\pm$ 10.9	0.144	29.3 $\pm$ 18.6	0.170	13.4 $\pm$ 10.2	0.246
	II	15	16.2 $\pm$ 6.6		20.68 $\pm$ 9.5		11.69 $\pm$ 6.6	
	III	25	25.3 $\pm$ 14.3		33.1 $\pm$ 19.8		17.4 $\pm$ 10.6	
	IV	3	23.1 $\pm$ 5.7		26.6 $\pm$ 3.9		19.6 $\pm$ 10.0	

Bold *P* values indicate statistically significant results

LVD lymphatic vessel density, PT-LVD peripheral lymphatic vessel density, IT-LVD intratumoral lymphatic vessel density

^aANOVA test

^bIndependent t test

lymph node metastasis. In the study by Zhao et al. of 86 cases of oral SCC, both higher IT-LVD and PT-LVD were significantly associated with the presence of lymph node metastasis at the time of diagnosis ($P < 0.001$). The correlation between IT-LVD and PT-LVD was also significant statistically ($P < 0.001$) [21]. In the study by Frech et al. significant relationship was identified between the presence of IT-LVD and nodal metastases ($P = 0.049$). There was no significant association between the PT-LVD and lymph node metastasis [22]. While comparing LVD, it was observed peritumoral LVD was significantly higher than intratumoral LVD ($P = 0.002$), indicating that lymphatic present in peritumoral area most likely are more relevant in the process of lymphatic dissemination, since they are well formed functional vessels while intratumoral lymphatic are compressed and may be nonfunctional. Peritumoral lymphatics were found to have more dilated, open lumina than intratumoral lymphatics in our study.

As D2-40 is being used mainly as a lymphatic vessel marker, staining of other cells rather than lymphatic endothelial cells is important to be distinguished to

minimize errors in quantification of LVD (Fig. 4). In our study, D2-40 staining was seen in tumor cells in 72% of the cases, while Franchi et al. [25] reported no staining of tumor cells in their cases. Data similar to our study is also available in the literature showing that D2-40 also stains carcinoma cells [29, 30]. When we further tried to correlate D2-40 positivity in tumor cells with other clinicopathological parameters, no significant association was found. However, Stinga et al. [30] in their study of SCC of oral tongue ($n = 42$), found a significant association of tumor cells positivity with lymph node metastasis and stage of tumor, while Longatto Filho et al. [29] found a significant association with lymphovascular invasion in oral cancers but not with lymph node metastasis. Based on the fact that D2-40 is specific for M2A region of podoplanin, a molecule found to be overexpressed in some tumors cells and positive staining reactions in tumor cells, these authors suggested that podoplanin (D2-40) may actually play a role in promoting the spread of tumor cells through lymphatic vessels. However conflicting results have further demonstrated that lymphatic markers are differentially expressed

Fig. 4 D2-40 positivity in tumor cell of HNSCC

by lymphatic endothelial cells from different organs indicating clear phenotypic heterogeneity depending on the region within tumor tissue [19, 29–31].

Conclusion

In our study both high intratumoral and peritumoral lymphatic vessel density were found significantly associated with the presence of lymph node metastasis, however lymphatic vessel were found to be significantly more numerous and larger in peritumoral areas as compared to intratumoral lymphatics suggesting that lymphatic vessels located around the tumors are the conduits for metastasis as compared to intratumoral lymphatics which may be compressed and non-functional. The specificity of D2-40 as a lymphatic endothelial marker was also confirmed in the study. The main limitation of our study was short term follow up of the patients so we could not correlate LVD with survival.

The results of our study support the possibility of using the determination of tumor lymphangiogenesis to identify patients of HNSCC who are at risk of developing the lymph node metastasis, however this needs further large scale validation and if this possible correlation between lymphangiogenesis and cervical lymph node metastasis is confirmed, these potential markers can be eventually incorporated in future into clinical practice. Further studies are also necessary to decide whether assesment of LVD

could become a criterion to the practice of elective lymph node dissection.

Compliance with Ethical Standards

Conflict of interest All authors state that there are no conflicts of interests.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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