Original Article

Morphometric assessment of microvessel density in head and neck squamous cell carcinoma using immunomarker CD105 and its correlation with clinicopathological parameters

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ABSTRACT

Background: Blood vessel counts using CD105 staining are more informative marker of prognosis as compared with staining by other endothelial markers. We conducted a study to compare intratumoral (IT) and peritumoral (PT) microvessel density (MVD) in head and neck squamous cell carcinoma (HNSCC) using endothelial marker CD105 and its correlation with lymph node metastasis, histological grading, and other clinicopathological parameters. **Materials and Methods:** Fifty cases of HNSCC with modified radical neck dissection specimens were included in the study group. Representative blocks were prepared from tumor, PT tissue, tumor margins, and all the lymph nodes. Histopathological diagnosis and other parameters were established on the routine hematoxylin and eosin stain. Immunohistochemical profile of blood vessels in IT and PT tissues was assessed by subjecting one section each from a representative block of the tumor and PT tissue to CD105 immunostain. To determine MVD, four fields with the highest MVD (hotspots) were identified. The mean values were calculated by taking an average of all the measurements. **Results:** No significant association was seen between MVD, IT-MVD, and PT-MVD and different age groups, male/female patients, risk factors, site of tumor, size of tumor, presence/absence of inflammation, pushing/infiltrating margin, and different stages of tumors. When compared in node positive and negative groups, a significantly higher MVD, IT-MVD, and PT-MVD was seen in association with lymph node metastasis. The comparison of MVD between PT and IT area revealed significantly higher IT-MVD ($P = 0.001$). **Conclusion:** In the study, we found a significant association of IT-MVD with lymph node metastasis and also observed CD105 as a highly specific marker for IT microvessels while PT vessels were not stained or weakly stained.

Key words: Angiogenesis, CD105 immunostain, head and neck squamous cell carcinoma, intratumoral, microvessel density, peritumoral

INTRODUCTION

Head and neck malignancies account for around 20% of the entire cancer burden in India. Head and neck squamous cell carcinoma (HNSCC) is the most common type of head and neck cancer (HNC), which has a distinct geographical distribution.[1] HNSCC ranks 6th among all the malignancies worldwide and represents over 6% of the global cancer burden. HNSCC arises from the mucosal epithelium of the upper aerodigestive tract, which includes (1) the nasal

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cavity and paranasal sinuses, (2) the nasopharynx, (3) the hypopharynx, larynx and trachea, and (4) the oral cavity and oropharynx. The major cause of oral and oropharyngeal SCC in the India is tobacco chewing. HNSCC usually presents with locally advanced disease which requires a multidisciplinary team approach to surgery, chemotherapy, and radiation.[2]

The major cause of malignancy-related deaths is metastatic spread of tumor cells. Multiple pathways of tumor dissemination are known including hematogenous and lymphatic spread. Tumor cells can metastasize from the primary site via the vessels which are already present in the tumor. Alternatively, neoangiogenesis or lymphangiogenesis into the tumor could promote the growth of new vessels which provide a new escape route for tumor cells.[3] The relative importance of these escape routes of tumor cells via the established vessels versus new blood and lymphatic vessels is still unclear.[3,4]

Quantification of angiogenesis is done through the staining of blood vessels with different endothelial markers including CD31, CD34, CD105, and factor VIII. Cells showing CD34 expression are normally found in the umbilical cord and bone marrow as hematopoietic cells, as well as endothelial cells of blood vessels but not in lymphatics endothelium (except pleural lymphatics).[5]

CD105 (endoglin) is an 180 kDa homodimeric transmembrane protein. CD105 shows relatively increased expression on the cell surface of proliferating endothelial cells as compared to other endothelial markers. It has protective effect on the endothelial cells from hypoxia-induced apoptosis. CD105 molecule occurs in two isoforms (L and S) which result from the alternate splicing of the transcript. These isoforms have different amino acid composition in their cytoplasmic tails. The two isoforms also differ in the extent of phosphorylation within the tissue, which in turn leads to different functions of these molecules. CD105 is also weakly expressed by other cell types which include fibroblasts, smooth muscle cells, macrophages, histiocytes, activated monocytes, follicular dendritic cells, melanocytes, heart mesenchymal cells, mesangial cells, and leukemic cells of pre-B and myelomonocytic origin but has strong expression on syncytiotrophoblasts of term placenta.[6-10]

Association of CD105 with tumor angiogenesis is ascertained from the fact that it is strongly upregulated in the tumor endothelium as compared to the normal endothelium. CD105 staining is a better prognostic marker for assessment of microvascular density (MVD) as compared with staining by other pan-endothelial markers. Several studies also indicated that blood vessel count by CD105 staining is more informative marker of prognosis as compared with staining by other pan-endothelial markers. However, various studies in HNSCC on MVD assessment with CD105 have shown data with conflicting results. Overall review of data on angiogenesis with preclinical and clinical evidence appears promising, and the implicit role of angiogenesis in HNC metastases needs further substantiation.[6-11]

MATERIALS AND METHODS

Patients

Fifty cases of HNSCC with modified radical neck dissection (MRND) specimens were included in the study group. The specimen was examined grossly for tumor size, consistency, cut surface, and margin. In MRND specimens, lymph nodes at different levels were processed for staging.[12] Specimen was fixed and processed by routine histological technique for paraffin embedding. Representative blocks were prepared from tumor, peritumoral (PT) tissue, tumor margin, and all the lymph nodes. Histopathological diagnosis was established on the routine hematoxylin and eosin stain,[13] and all the histological prognostic parameters including histologic grade,[14] tumor necrosis, tumor inflammation, margins, and lymph node metastasis were assessed.

Immunohistochemistry

Immunohistochemical profile[15] of microvessels in intratumoral (IT) and PT tissue was assessed by subjecting one section each from a representative block of the tumor and PT tissue to CD105 immunostain. Paraffin sections measuring 3–5 μm in thickness on slides coated with suitable tissue adhesive were deparaffinization and hydrated. Endogenous peroxidase was inactivated with 3% hydrogen peroxidase for 20 min, and the sections underwent antigen retrieval with microwave oven heating for 30 min using citrate or tris ethylenediaminetetraacetic acid.

Sections then incubated with the monoclonal antibody CD105 (prediluted) (DAKO) overnight at 4°C. Then, sections were rinsed with tris-buffered saline solution. This was followed by incubation with the secondary antibodies The reaction was visualized using 3,3′-diaminobenzidine, and nuclei were lightly counterstained with hematoxylin. Positive and negative controls were run with each batch of immunohistochemical stain. Positive controls for CD105 were sections from tonsillar tissue. Negative controls were obtained by substituting the primary antibody with an antibody of irrelevant specificity.

Interpretation of results

MVD were quantified in IT and PT area. Endothelium of blood vessels revealed brown, membranous positivity with

CD105. To determine MVD, four fields with the highest MVD (hotspots) were identified at low magnification (×40) 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 (×400). Microvessel was defined as any highlighted endothelial cell or endothelial cell cluster clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements. Vessel lumen was not considered to be necessary for a structure to be defined as microvessel. The mean values were calculated by taking an average of all the measurements.[16]

Statistical analysis

Data obtained were correlated with other clinicopathological parameters including site, size, grade, margin of tumor, presence of necrosis, inflammatory infiltrate, and lymph nodes metastasis. All the data obtained were analyzed statistically usin IBM SPSS Statistics for Windows, Version 20.0. (IBM Corp., Armonk, NY). A value of $P < 0.05$ was considered statistically significant.

RESULTS

In the study, the patient's age ranged from 30 to 82 years. Mean age was 53.9 years (standard deviation ± 12.5) and majority of the patients (60%) belonged to the age group of 41–60 years. Seventy-two percent cases were males while 28% were females. Maximum number of the cases (74%) belonged to oral cavity followed by 8% each in oropharynx and larynx. Sixty percent of our patients had positive history of smoking. History of tobacco chewing and alcohol intake was present in 22% and 30% of cases, respectively.

All the cases were divided into four categories, T1, T2, T3, and T4 depending on the size of tumor (≤2 cm, ≥2.1–4 cm, ≥4.1–6 cm, and >6 cm, respectively). Majority (44%) of cases were in category T2 followed by T3 in 30% of cases. Microscopic examination of tumors revealed moderately differentiated squamous cell carcinoma in 60% of cases followed by well-differentiated squamous cell carcinoma in 32% cases. Inflammation was present in 44% of cases; however, it was mainly mild to moderate. 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. All the cases were categorized into two groups on the basis of presence/absence of lymph node involvement irrespective of level of node involvement. Twenty-three cases were lymph nodes positive while 27 cases were negative. Depending on tumor size (T), lymph node status (N), and distant metastasis (M), all the cases were divided into four stages according to the American Joint Committee on Cancer staging. Majority of the cases (56%) were in Stage III and IV.

When compared, no significant association was seen between MVD and patient's age, gender, site of tumor, and various risk factors including cigarette smoking, tobacco chewing, and alcohol consumption. In relation to various histopathological parameters, higher MVD was correlated with lymph node metastasis ($P = 0.018$) and advanced clinical stage ($P = 0.04$) while no significant association of MVD was seen with size of tumor, grade of tumor, presence/absence of inflammation, necrosis, and pushing/infiltrating margin [Tables 1 and 2].

The comparison of vessel densities between PT and IT areas revealed significantly higher IT MVD ($P = 0.001$) [Figures 1 and 2]. In the sections, CD105 did not stain lymphatic endothelial cells [Figure 3]. PT-MVD and IT-MVD were compared further with various clinicopathological parameters. No significant association was seen with age, gender, risk factors, histological parameters, tumor inflammation, margin, tumor necrosis, and tumor grade and stage. IT-MVD had significant association with tumors in oral cavity ($P = 0.033$) and lymph node metastasis ($P = 0.008$).

DISCUSSION

Angiogenesis is essential for the metastasis of solid tumors. In 1971, Folkman proposed that tumor growth and metastasis are angiogenesis-dependent, and hence, blocking angiogenesis could be a strategy to arrest tumor growth. In 1976, Gullino showed that cells in precancerous tissue acquire angiogenic capacity on their way to becoming cancerous. He proposed that this concept can be used to design strategies to prevent cancer, a hypothesis later confirmed by genetic approaches.[17] Without adequate vascularization, tumors larger than 1 mm^3 may undergo necrosis and cannot grow beyond a critical size or metastasize to another organ. Similarly, without an efficient blood supply, it is difficult to deliver anti-cancer drugs to all regions of a tumor in effective quantities.[11,17]

Microvascular density (MVD) is by far the most commonly used and reliable predictor for metastasis. However, some investigators have failed to find such observation. This is due to the differences in the various techniques used in different studies. IT vascularization is necessary for growth as it provides nutrients for tumoral cells. PT vasculature is essential for invasion and metastasis. CD105 associated with tumor angiogenesis is ascertained from the fact that it was strongly upregulated in the endothelium of various tumor tissues compared with that in normal tissues. MVD assessment with CD105 shows negative correlation with overall patients survival, disease-free survival, and presence of tumor metastasis in various tumors including breast, cervical, endometrial, gastric, renal, colorectal,

Table 1: Comparison of microvessel density with various clinical parameters

Clinical parameters	Groups	Number of cases	MVD		PT-MVD		IT-MVD	
			Mean density±SD	*P*	Mean density±SD	*P*	Mean density±SD	*P*
Age group[a]	0-10	0	-	0.888	-	0.968	-	0.798
	11-20	0	-		-		-	
	21-30	3	61.1±10.8		42.0±6.1		80.1±21.4	
	31-40	6	43.7±16.8		35.3±9.8		52.1±26.7	
	41-50	15	53.0±16.5		37.6±15.2		68.5±23.9	
	51-60	15	54.2±28.1		43.3±39.2		65.2±26.7	
	61-70	6	50.5±15.6		41.7±30.5		59.4±8.5	
	71-80	4	60.4±23.4		45.5±21.1		75.3±43.5	
	81-90	1	58.3		61.5		55.2	
Site[a]	Oral cavity	37	50.5±20.3	0.503	38.6±26.7	0.440	62.5±24.1	0.033
	Oropharynx	4	52.8±18.3		60.7±31.1		44.8±9.9	
	Hypopharynx	1	45.3		31.3		59.4	
	Mandible	2	73.7±7.7		36.5±3.1		110.9±18.4	
	Larynx	4	53.2±28.8		34.4±11.8		92.1±54.8	
	Salivary gland	2	65.1±9.6		65.1±5.2		65.1±14.0	
Gender[b]	Male	36	49.8±17.5	0.065	37.1±17.3	0.218	62.7±28.3	0.273
	Female	14	61.7±25.3		50.9±39.1		72.4±27.1	
Smoking[b]	Present	30	48.8±18.2	0.065	36.3±18.1	0.125	61.3±29.2	0.206
	Absent	20	59.6±22.2		47.7±33.3		71.6±25.6	
Tobacco chewing[b]	Present	11	57.3±24.3	0.450	47.4±42.1	0.341	67.1±13.9	0.734
	Absent	39	52.0±19.3		39.0±19.1		64.9±31.0	
Alcohol[b]	Present	15	47.3±21.3	0.188	32.0±13.3	0.111	62.6±34.8	0.646
	Absent	35	55.6±19.8		44.7±28.7		66.6±25.1	

[a]ANOVA test, [b]Independent *t*-test. MVD: Microvessel density, PT-MVD: Peritumoral microvessel density, IT-MVD: Intratumoral microvessel density, SD: Standard deviation

Table 2: Comparison of microvessel density with various histological parameters

Histological parameters	Groups	Number of cases	MVD		PT-MVD		IT-MVD	
			Mean density±SD	*P*	Mean density±SD	*P*	Mean density±SD	*P*
Tumor size[a]	T1	10	66.8±26.5	0.108	58.1±41.0	0.099	75.4±36.7	0.523
	T2	22	47.9±17.3		35.0±21.0		60.7±24.5	
	T3	15	52.1±18.0		36.8±14.5		67.5±29.9	
	T4	3	51.3±18.0		46.5±20.2		56.1±16.4	
Tumor grade[a]	WDSCC	16	53.5±16.0	0.940	36.4±12.4	0.550	70.7±23.0	0.345
	MDSCC	30	53.4±22.8		41.84±30.5		64.94±60.8	
	PDSCC	4	49.6±22.2		51.55±26.9		47.70±21.8	
Tumor inflammation[a]	Absent	3	33.6±14.6	0.370	33.0±28.0	0.542	34.3±7.4	0.089
	Mild	21	54.2±23.0		47.1±34.8		61.2±22.6	
	Moderate	15	52.7±18.1		36.9±17.1		68.6±29.2	
	Severe	11	57.0±18.5		36.6±17.1		77.5±33.7	
Tumor margin[b]	Pushing	22	48.7±14.5	0.156	34.3±14.2	0.107	63.2±20.7	0.630
	Infiltrating	28	56.6±23.7		46.1±31.2		67.1±33.0	
Tumor necrosis[b]	Present	22	53.7±23.5	0.856	44.5±33.2	0.379	63.0±25.1	0.592
	Absent	28	52.7±18.0		38.0±17.8		67.3±29.9	
Lymph node positivity[b]	Present	23	60.5±16.7	0.018	43.8±13.5	0.464	77.1±32.9	0.008
	Absent	27	46.9±21.4		38.4±32.7		55.4±18.6	
Stage of tumor[a]	I	7	64.7±28.1	0.048	64.9±48.3	0.022	64.5±19.8	0.188
	II	15	41.9±14.0		29.6±21.9		54.2±13.8	
	III	25	56.9±19.4		40.2±13.9		73.5±16.4	
	IV	3	51.3±18.0		46.5±26.2		56.1±16.4	

[a]ANOVA test, [b]Independent *t*-test. MVD: Microvessel density, PT-MVD: Peritumoral microvessel density, IT-MVD: Intratumoral microvessel density, MDSCC: Moderately differentiated squamous cell carcinoma, WDSCC: Well-differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, SD: Standard deviation

nonseminomatous testicular germ cell tumors, nonsmall cell lung cancer, and prostate cancer.[6-11]

There have been a number of studies revealing the expression and prognostic significance of microvessel density in HNSCC with conflicting and contradictory results.[18-23] Many of these studies have used pan-endothelial markers, namely, CD31, CD34, and von-Willebrand factor. These conflicting results may reflect the low specificity of pan-endothelial markers which stain vessels in tumor and normal tissue with equal intensity and are of low sensitivity for IT vessels while endoglin (CD105), being a highly sensitive marker, stains IT vessels intensively whereas vessels in nonneoplastic tissue are not or are weakly stained.[24]

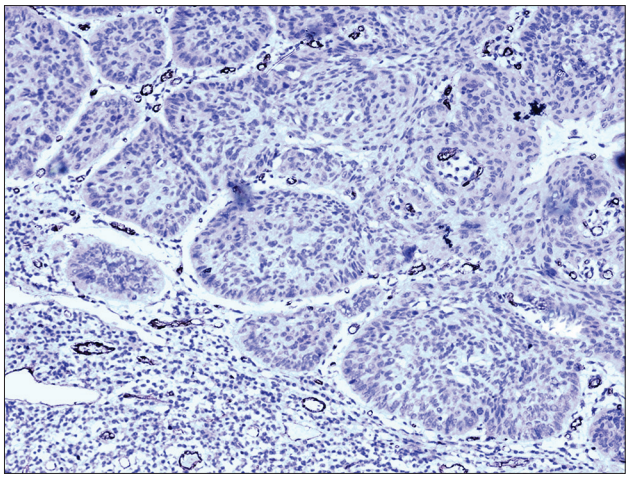

Figure 1: High expression of CD105 in intratumoral microvessel with lower expression of peritumoral microvessel (IHC-CD105, ×100)

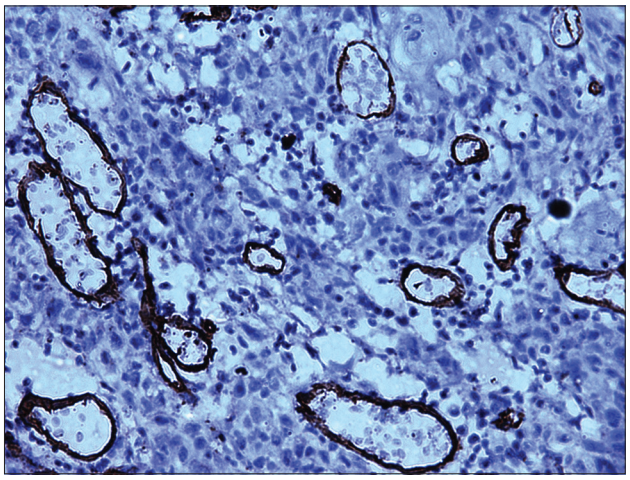

Figure 2: Abundant intratumoral microvessel expression of CD105 (IHC-CD105, ×400)

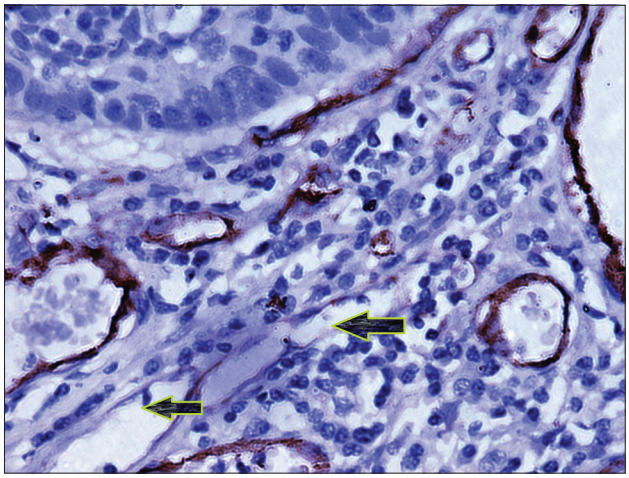

Figure 3: CD105-stained microvessel. Lymphatic vessels (arrow) were not stained (IHC-CD105, ×400)

On comparison, no significant association was seen between MVD and patient's age, gender, site of tumor and various risk factors in our study. Similar observations were also made in various other studies.[18-20] In relation to various histopathological parameters, higher MVD was correlated with lymph node metastasis (P = 0.018) and advanced clinical stage (P = 0.04) while no significant association of MVD was seen with size of tumor, grade of tumor, presence/absence of inflammation, necrosis, and pushing/infiltrating margin. Kyzas *et al*.[21] reported significant association of MVD with advanced clinical stage ($P \leq 0.001$) and lymph node metastasis at the time of diagnosis ($P \leq 0.001$). In study by Miyahara *et al*.[20] on 110 cases of oral SCC, higher MVD values correlated with lymph node involvement and lymphovascular invasion ($P \leq 0.001$) but not with age, sex, tumor size, and grade.

The comparison of vascular densities between PT and IT areas revealed significantly higher IT MVD. Similar observations were also made by Margaritescu *et al*.[22] In study by Xuan *et al*.,[25] PT-MVD was higher than IT-MVD using CD34 as marker of blood vessels while Ohno *et al*.[26] observed higher blood vessel density in both PT and IT area in 50 cases of oral cancer using CD34 as marker of MVD. Higher IT-MVD values were seen with increasing inflammation, infiltrating margin, and tumor necrosis; however, these values were not statistically significant in our study. IT-MVD had significant association with tumors in oral cavity (P = 0.033) and lymph node metastasis (P = 0.008). Similar results were shown by Schimming and Marmé.[18]

Although the use of CD105 offers a feasible solution to the problem of selection of the optimal endothelial marker, there are other equally important difficulties. Measuring MVD by examining small sections of archival tissue at a single point in time does not necessarily represent the angiogenic status of the tumor. There is also significant inter-observer variability for the identification and selection of the "hotspots." Differences between immunohistochemical protocols, selection of paraffin block, sections within the block, and counting procedure are difficult steps for reliable and reproducible assessment of MVD.[21]

The use of diagnostic modalities for the assessment of tumor metastasis based on CD105 may have an important role in clinical management including diagnosis, follow-up, prediction of response to treatment, and prognostic determination. CD105 represents an ideal target for antiangiogenic therapy. However, whether prevention of angiogenesis with anti-CD105 therapy can be an effective treatment or not is still unclear and more studies on MVD are necessary.

CONCLUSION

In the study, we found a significant association of IT microvessel density with lymph node metastasis and clinical

stage in HNSCC and also observed CD105 as a highly specific marker for IT microvessels while PT vessels were not stained or weakly stained. Hence, our results further confirm the previous observations made in the literature that MVD evaluation by CD105 is a promising prognostic factor in patients with HNSCC. The main limitation of our study was short-term follow-up of the patients, so we could not correlate MVD with survival.

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Conflicts of interest
There are no conflicts of interest.

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