Intratumoral and Peritumoral Angiogenic and Lymphangiogenic Microvessel Density in Invasive Breast Carcinoma and their Correlation with Lymph Node Metastasis

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Abstract

Background: Breast cancer is the most common malignant tumor in females. Although ample efforts have been made in the field of early diagnosis and adjuvant therapy, the incidence and overall mortality of breast cancer continues to increase. Lymph node status is routinely used to check a patients tumor stage prognosis and the modality of treatment. Lymph node and distance metastasis are bad prognostic factor.

Objective: To correlate intratumoral and peritumoral angiogenic and lymphangiogenic microvessel density with lymphnode metastasis in breast carcinoma.

Methods: It is a cross sectional observational study, carried out at the department of Pathology, BSMMU from January 2016 to December 2017. A total of 48 mastectomy samples with axillary lymph nodes from histologically confirmed breast carcinoma were included in this study. Weidner and Chalkley methods were used for calculating both microvessel densities. Sections were examined to evaluate the density of lymphangiogenic and angiogenic vessels by podoplanin and vWF expression respectivelyCorrelation between lymphangiogenic and angiogenic vessels density with or without lymph node metastasis was taken.

Results: Lymphatic vessel count was higher in the peritumoral area whereas angiogenic vessel count was higher in the intratumoral area. There was a positive significant correlation between lymph node metastasis with microvessel density in both peritumoral and intratumoral areas in Weidner method in podoplanin and vWf stain.. But there was no significant correlation between lymph node metastasis with micro vessel density in both peritumoral and intratumoral areas in podoplanin and vWF stain in Chalkley method.

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Keywords: Angiogenesis, lymphangiogenesis, invasive breast carcinoma, podoplanin, vWF.

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Introduction

Breast cancer is the most common cancer in women, comprising almost one third of all malignancies in female.[1] It is also one of the major malignant disease burdens in Bangladesh, with an estimated incidence of about 22.5 per 1, 00,000 in females.[2] Breast cancers are notorious for their invasive and metastasizing potential. Axillary lymph node involvement, tumor size, nuclear grade, hormone receptor status, patient's age are good prognostic factors for patients with invasive breast cancer. The lymph node involvement predicts the choice of adjuvant chemotherapy and radiotherapy after surgery. for Lee et al.[3] emphasized that the lymphatic and blood vessel invasion to regional lymph nodes or distant sites occur early in tumor metastasis. The markers used to detect blood vessel invasion include elastic van Gieson stain, factor VIII-related antigen (vWF), CD34, CD31, and vascular endothelial growth factor receptor-3.[4] The marker for lymphatic invasion include podoplanin/D240, lymphatic vessel endothelial hyaluronan receptor-1, laminin and type IV collagen. The podoplanin and vWF are useful markers of lymphatic and vascular endothelium respectively. vWF is a trasmembrane glycoprotein in platelet edothelial adhesion molecule-1 in the immunoglobin superfamily. It is expressed on monocyte, platelet, selected T cell subsets and endothelial cells and commonly found on vascular endothelial cell than lymphatic endothelial cell.[5] Podoplanin is a monoclonal antibody generated against an oncofetal membrane antigen M2A and has been reported to be specific marker for lymphatic endothelium in normal and neoplastic tissue.[5] However, this finding was not supported by other published studies.[6]

Lymphangiogenesis and angiogenesis are critical processes for tumor growth, invasion and metastasis.[7] The common pathologic approach to assess angiogenesis and lymphangiogenesis involves microscopic estimation of vascular density or microvessel density using endothelial markers by IHC.

Podoplanin and vWF are useful markers for lymph vessel density and vascular density. Studies of various tumors have shown the potential clinical significance of angiogenesis and lymphangiogenesis, suggesting that blood and lymphatic microvessel density correlate with tumor growth and metastasis.[8] The two principal approaches in this regard are direct microscopic IHC and semiautomated image cytometry. Direct IHC analysis of microvessel density is relatively inexpensive and widely available.

Therefore, this study was aimed to assess intratumoral and peritumoral lymphatics and angiogenic density and to find out the possible relationships between intratumoral and peritumoral LVD and LNM in breast cancer.

Methods

A total of 48 histologically diagnosed cases of invasive ductal carcinoma samples were included in this cross sectional observational study which was carried out in the Department of Pathology, (BSMMU) from January 2016 to December 2017. Total mastectomy specimen including axillary dissection were collected and all the relevant information were recorded.

Statistical analysis:

Statistical analyses of the results were obtained by using Microsoft Office Excel version 2007. The results were calculated using relevant statistical formula(Pearson's correlation) and were presented in tables, figures and diagrams.

Histopathological study

5 mm thick consecutive tissue sections were cut from each blocks including intratumoural

and peritumoural and two slides were made. Tissues were processed according to routine histopathology processing protocol of BSMMU. The slides were routinely stained with H&E method. and then selected for podoplanin and vWF stain on the basis of suspicion of LVI in H&E slides. Histopathogical categorization of tumor and grading (Nottingham modification of the Bloom–Richardson Grading System) of all the cases were done. Lymphpovascular invasions were recorded. Each lymph node was histologically examined to determine metastasis.

Immunohistochemistry study

IHC of all cases were performed using Dako Autostainer Plus at the IHC laboratory of the department of Pathology, BSMMU**.** Normal vermiform appendix were taken as positive control. Monoclonal mouse Anti-Human podoplanin, clone D2-40, Ready to use (link), (code, IR072) was used for detecting lymphatic vessel. Polyclonal Rabbit Anti-Human Von Willebrand factor, Ready to use (link) (code, IR527) was used for endothelial cells. The sections were stained with podoplanin and vWF according to protocol followed at the department of Pathology, BSMMU. The numbers of lymphatic and blood vessels were counted by Weidner's and Ckalkley method in peritumoral and intratumoral areas.[9-10]

Microvessel quantification

Microvessel densities were calculated according to Weidner's method by Olympus microscope model BH51. At first the sections were scanned at low power (X100) looking for hot spots. Three hot spots were selected in both intratumoral and peritumoral areas. When the hot spots were detected, microvessel count was performed by counting the individual stained microvessels (at power X20) representing a field size of 0.74 mm^2(20X objective, 10X ocular; equivalent to 0.7386 mm^2 per 200X field.[10] First three hot spots were chosen in both areas. In each hot spot, MVC was performed at power X20 and finally MVD was calculated as the mean of the total number of microvessels in those three hot spots. A 50 point Chalkley eyepiece graticule was applied to same hot spots at a high magnification (X200 magnification) and oriented to permit the maximum number of points to hit on or within the stained angiogenic and lymphangiogenic microvessels by vWF and podoplanin. Chalkley one point means 2% of area covered by the immunostained vessels at hot spot. Finally MVD was calculated as the mean percentage of area covered by immunostained microvessel in these three hot spots.

Results

Ages of the 48 study subjects ranged form 22 to 85 years. These were grouped on the basis of decades (table-I) and ~~it was~~ observed that one third (41.7%) sample belonged to age ≤40 years.

Table I: Distribution of the study sample by age (n=48)

Age (in years)	Number of the sample	%
≤40	20	41.7
41-50	14	29.1
51-60	13	27.1
>60	1	2.1
Mean±SEM	45.38±1.6	

Tumor sizes ranged from 1-9 cm, grouped on the basis of tumor size (Table-II) and observed that (60.4%) sample belonged to tumor size of 2-5 cm.

Table II: Distribution of the study sample by tumor size (n=48)

Tumor size (cm)	Number of the sample	%
0-2	11	22.9
2-5	29	60.4
>5	8	16.7
Mean±SEM	3.63±0.3	

Table III shows histological grading of the ductal carcinoma and observed that 21(43.8%) had invasive ductal carcinoma, grade-II followed by 19(39.6%) grade-III and 8(16.6%) grade-I.

Table III: Distribution of the study sample by histological grade (n=48)

Histological Grade	Number of the sample	%
Grade 1	8	16.6
Grade 2	21	43.8
Grade 3	19	39.6

Total 25 cases had lymph node metastasis. The number of lymph node involved ranged from 0-17, were grouped according to the numbers of lymph node metastases as N0, N1, N2, N3 (Table-IV) and observed that almost half (47.9%) of the sample had no lymph node metastases N0.

Table IV: Distribution of the study sample by number of lymph node metastases (n=48)

Number of Lymphnode metastases	Number of the sample	%
N0	23	47.9
N1	5	10.4
N2	17	35.4
N3	3	6.3
Mean±SEM	3.2±0.6	

LVI was observed in podoplanin stain and BVI in vWF stain in Table-V. It was observed that 24(50.0%) and 15(31.3%) were positive and 24(50.0%) and 33(68.7%) were negative, respectively.

Table V: Distribution of the study sample by lymph vascular invasions in podoplanin stain and blood vascular invasions in VWF stain (n=48)

	Number of the sample	%
LVI		
Positive	24	50.0
Negative	24	50.0
BVI		
Positive	15	31.3
Negative	33	68.7

LVI=Lymph vascular invasion
BVI=Blood vascular invasion

Table VI shows MVD in PT and IT area in Weidner method in podoplanin stain ranged from 0-41and 0-35. In Chalkley method ranged from 0-46 and 0-40.The Mean±SEM MVD in PT were 7.42±1.03 ; 5.56±1 and the Mean±SEM MVD in IT were 4.72±0.61; 2.98±1.04, respectively.

Table VI: Distribution of the study sample by MVD in PT and IT area in Weidner method and Chalkley method in podoplanin stain. (n=48)

	Mean±SEM	Range(min-max)
Weidnermethod		
MVD in PT	7.42±1.03	0-41
MVD in IT	4.72±0.61	0-35
Chalkley method		
MVD in PT	5.56±1.31	0-46
MVD in IT	2.98±1.04	0-40

MVD=Microvessel density
PT=Peritumoral area
IT=Intratumoral area

MVD in PT and IT l area in Weidner method in vWF stain ranged from 1-92 and 0-95.In Chalkley method ranged from 2-50 and 0-62.Table VII shows the Mean±SEM MVD in PT were 36.81±2.90, 27.37±1.87 and

Mean±SEM MVD in IT were 42.68±2.88 and 24.75±1.99, respectively.

Table VII: Distribution of the study sample by MVD in PT and IT area in Weidner and Chalkley method in vWF stain. (n=48)

	Mean±SEM	Range (min-max)
Weidnermethod		
MVD in PT	36.81±2.90	1-92
MVD in IT	42.68±2.88	0-95
Chalkley method		
MVD in PT	27.37±1.87	2-50
MVD in IT	24.75±1.99	0-62

Table VIII shows the value of Pearson's correlation coefficient of Lymph vessel in podoplanin stain in PT and IT were 0.662 and 0.54. The correlation value of blood vessel in vWF stain in PT and IT were 0.498 and 0.533. All of these show positive correlation between Weidner and Chalkley method which was statistically significant (p=0.001).

Table VIII: Correlation between Weidner and Chalkley method

Micro vessel density	Weidner method and Chalkley method	
	r	p value
Lymph vessel in podoplanin stain PT	0.662	0.001[s]
Lymph vessel in podoplanin stain IT	0.54	0.001[s]
Blood vessel in vWF stain PT	0.498	0.001[s]
Blood vessel in vWF stain IT	0.533	0.001[s]

Lymphvascular invasions in H&E stain was detected in 15(31.3%) cases, 10(20.8%) cases were indeterminate and 23(47.9%) cases were negative (Figure-II).

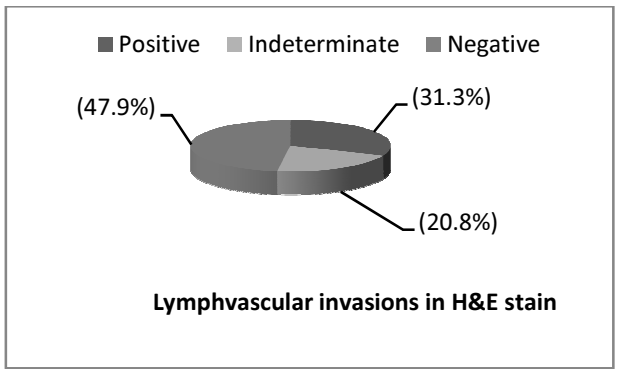

Figure 1. Pie chart showing lymph vascular invasions in H&E stain of the study samples

Lymph node metastases of 48 cases were expressed the number and micro vessel density in peritumoral and intratumoural area in Weidner method in vWF stain were expressed in number/mm^2. A positive correlation was found between MVD in PT and lymph node metastases and also MVD in IT and lymph node metastases. A Pearson's positive significant correlation (r=0.387; p=0.007) and (r=0.410; p=0.004) between micro vessel density in peritumoral and intratumoural area and lymph node metastases respectively (Figure-2).

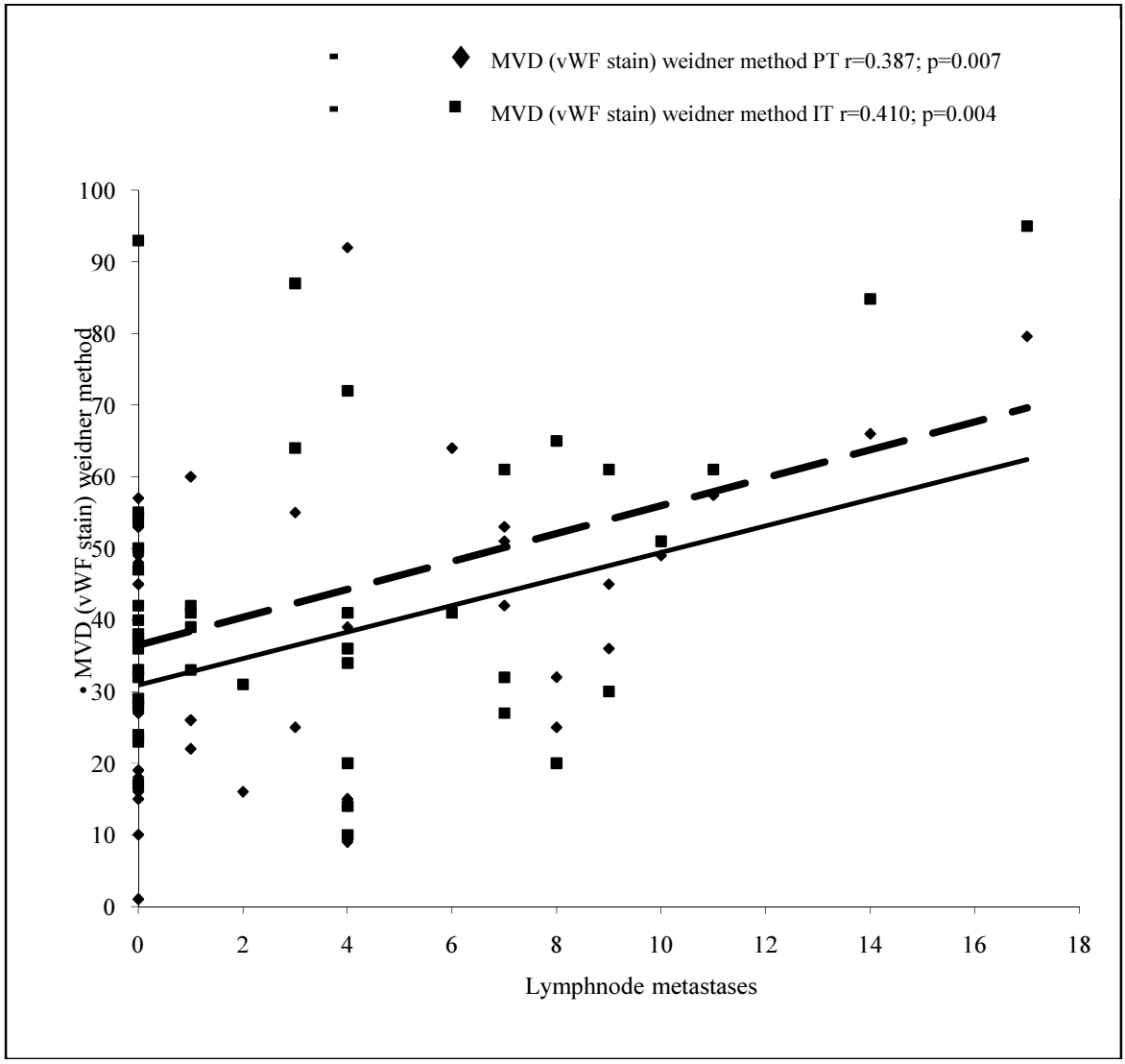

Figure 2. Scattered diagram showing Pearson's positive significant correlation between microvessel density in peritumoral and intratumoural area in vWF stain and lymph node metastases in Weidner method.

Lymph node metastases of 48 cases were expressed in number and micro vessel density in peritumoural and intratumoural area in Weidner method (Podoplanin stain) was expressed in number/mm^2. A positive correlation was found between MVD in PT and lymph node metastases and also MVD in IT and lymphnode metastases (Figure-3).The value of Pearson's correlation coefficient were (r= 0.306); (r= 0.406) and it is significant (p=0.036) and (p=0.004), respectively.

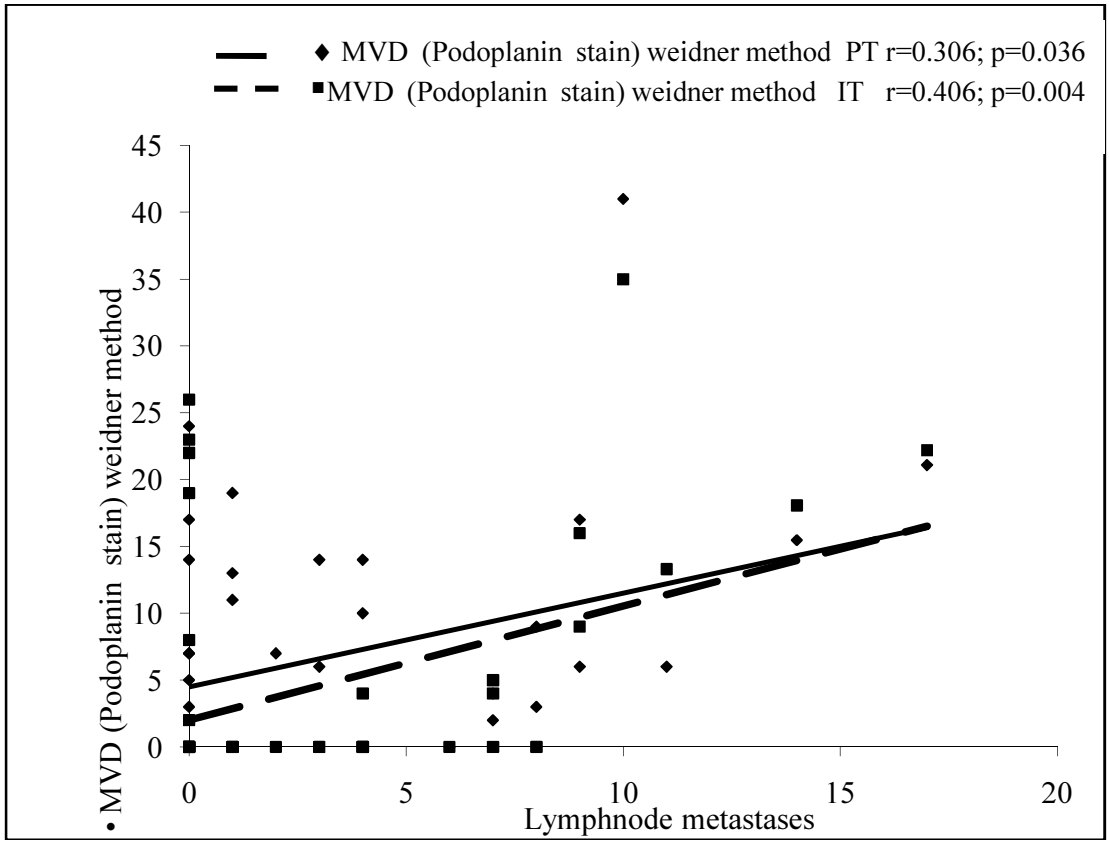

Figure 3. scattered diagram showing Pearson's positive significant correlation between micro vessel density in peritumoral and intratumoural area in podoplain stain and lymph node metastases in Weidner method.

In Chalkley method lymphnode metastases of 48 cases were expressed in number and micro vessel density in peritumoral and intratumoral area in vWF and podoplanin stain were expressed percentage. A positive correlation was found between MVD in PT and lymph node metastases and also MVD in IT and lymphnode metastases in vWF stain. A Pearson's positive correlation (r=0.134; p=0.362) and (r=0.226; p=0.123) between micro vessel density in peritumoral area and intratumoural area respectively but not significant. .

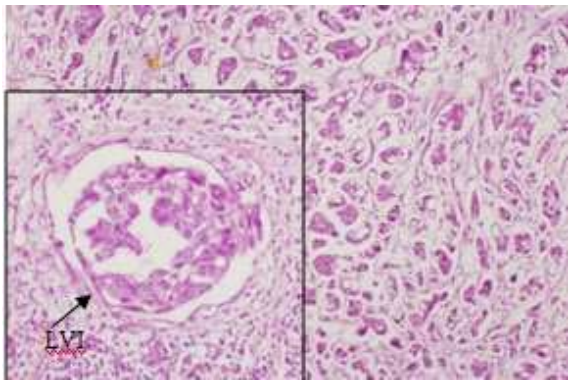

Figure. 4. Photo micrograph shows invasive ductal carcinoma grade II (case no 6. H&E stain x200) and inset figure shows lymph vascular invasion (LVI) (case no 1.H&E x200)

A positive correlation was found between MVD in PT and lymph node metastasis and also MVD in IT and lymphnode metastases in podoplanin stain. A Pearson's positive correlation (r=0.242; p=0.098) and (r=0.235; p=0.083) between micro vessel density in both peritumoral area and intratumoral area and lymphnode metastases but not significant.

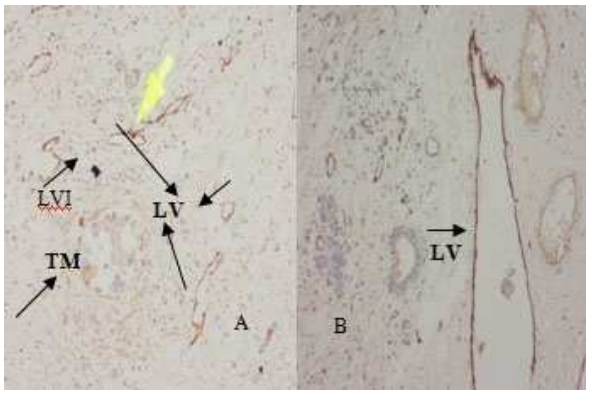

Figure 5. A. Photomicrograph shows (LV) lymph vessel proliferation in intratumoral area. Tumor (TM) is present close to the vessel. B. shows (LV) lymph vessel proliferation in peritumoral area (case no1, podoplanin immunostain x200)

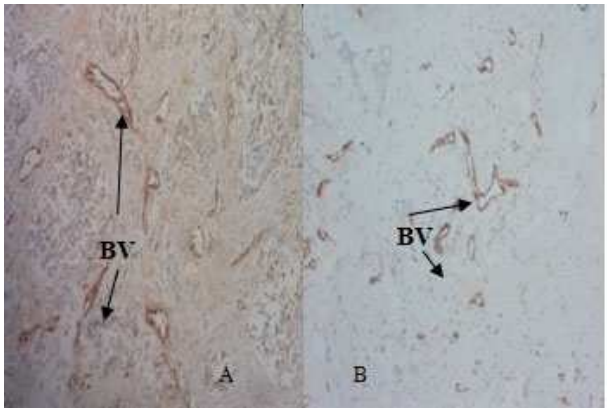

Figure 6. A. Photomicrograph shows (BV) blood vessel proliferation in intratumoral area, B shows (BV) blood vessel proliferation in peritumoral area (case no 6. vWF stain x200)

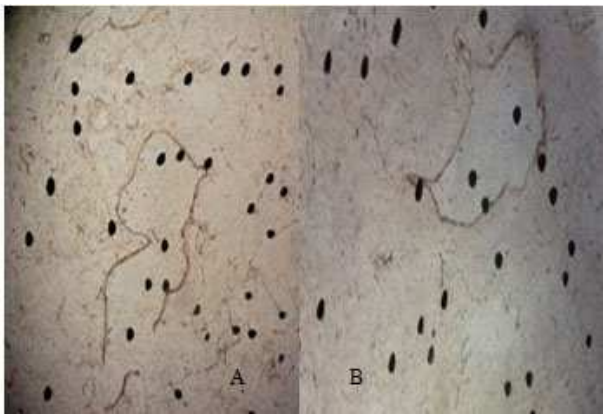

Figure 7. A. Photomicrograph shows lymph vessel in podoplanin stain (Chalkley method x200) B. shows blood vessel in vWF stain (Chalkley method x200)

Discussion

Tumour angiogenesis has long been claimed as an important factor for tumour spread.To see this, lymphvessel and blood vessels proliferation was estimated in intratumoral and peritumoral areas.There were correlation with lymphnode metastasis. Immunohistochemically using podoplanin and vWF were empioyed to identify lymphatic vessels and blood vessels respectively. In the present study, 41.7% sample belonged to age ≤40 years and the mean±SEM age was 45.38±1.6 years ranged from 22 to 85 years. Similarly in Italy Raica et al.[11] found that women having invasive breast carcinoma age varied from 26 – 81 years. Almost similar age ranged also observed by Zhao et al.[5] in China, where they found age varied from 29 – 75 years.

In the current study, tumour size ranged from 1-9cm. 60.4% sample belonged to tumor Size 2-5 cm and only 16.6% had >5 cm tumor size. The mean±SEM of tumor size was 3.63±0.3 cm.. Lee et al.[3] found 51.2% had 1cm, 41.3% 2cm, 5.0% 3cm and 2.5% 4 cm, which is comparable with the current study.

In this present study, it was observed that 43.8% sample had invasive ductal carcioma grade-II followed by 39.6% grade-III.

Lee et al.[3] showed 44.9% LVI positive tumors were histological grade-III. Valencak et al.[12] and Braun et al.[13] also demonstrated similar findings and which can be explained by the speculation that aggressive tumors are more capable of invading lymphatic vessels.

In this study, it was observed that 47.9% sample belonged to LN metastases stage 0, followed by 35.4% stage II. Schoppmann et al.[14] demonstrated that LVI assessed by anti-podoplanin immunostaining has been strongly associated with the presence of lymph-node metastases and unfavorable for overall survival in human breast cancer.

In the current study, regarding the vascular invasions (VI) in H&E stain, it was observed that 31.3% were VI positive, 47.9% negative and 20.8% indeterminate. Previous reports have suggested that vascular invasion (blood vessel invasion and lymphatic vessel invasion) are significant prognostic factors.[15-16]

In our study, it was observed that 50.0% had LVI positive and 50.0% negative. Kato et al.[17] observed that LVI positive had 32.4% in Japanese and 37.0% in British. LVI negative had 67.6% in Japanese and 63.02% British. However, Kato et al.[17] study showed that LVI was not contribute to the Japanese-British disparity in breast cancer and LVI variability which could not explain the survival differences between Japanese and British patients. In another study, Lee et al.[3] demonstrated that LVI was detected by D2-40, podoplanin and H&E stain in 10.0%, 8.8%, and 5.2% tumors respectively.

In the current study, regarding the blood vessel invasions (BVI) it was observed that 31.3% was BVI positive and 68.7% negative. BVI positive had 20.2% in Japanese and 26.1% in British. BVI was negative in 79.8% of Japanese and 73.9% of British, which indicated that the prevalence of BVI in British patients was particularly high as reported by Kato et al.[17] They also evaluated BVI by H&E staining alone and found a rate of 6.5%. By H&E staining alone, it was difficult to detect blood vessels filled with tumor cell emboli, to distinguish between small blood vessel invasion and lymphatic vessel invasion. In another study Lee et al.[3] mentioned that BVI was detected by CD31 stain in 22.5% tumors, which is slightly lower our present study.

In the present study, regarding the micro vessel density in peritumoral area (PT), it was observed that the mean±SEM MVD in Weidner method PT (Podoplanin stain) was 7.42±1.03 with ranged from 0-41 and mean±SEM MVD IT was 4.72±0.61 with ranged from 0-35. So microvessel density was greater in peritumoral area in podoplanin stain and microvessel density was greater in intratumoral area in vWF stain. Weidner et al.[9] showed that the intensity of tumor neovascularization is highly predictive for overall and relapse free survivals in patients with early stage (I or II) invasive breast carcinoma.

In this present study, a positive significant Pearson's correlation (r=0.306; p=0.036) was found between LN metastases with micro vessel density in peritumoral area (PT) in Weidner method (Podoplanin stain). Similarly, there was also a positive significant Pearson's correlation (r=0.406; p=0.004) between LN metastases with micro vessel density in intratumoral area (IT) in Weidner method (Podoplanin stain). El-Gohary et al.[4] also showed a positive correlation of peritumoral and intratumoral D2-40detected LMD with lymph node metastasis. Only D2-40 LMD (peritumoral and intratumoral)

correlated significantly with the presence of LVI detected by D2-40, which were r=0.346 (p=0.016) and r=0.492; (p=0.001) respectively. Choi et al.[18] studied LMD using D2-40 antibody and found that increased LMD correlated with several prognostic factors, including lymph node metastasis. The studies of Mohammed et al.[19] and Tezuka et al.[20] demonstrated that intratumoral lymphatics are detectable. Intratumoral lymphatics were generally detected in 40.07% of breast cancer specimens, and the detection rate of peritumoral lymphatics was 77.09%. Moreover, intratumoral lymphatics are believed to be functional, as tumor cells have been observed to flow within the vessels.[21]

It is well known that blood vessel density, an indicator of tumor angiogenesis, is closely associated with the clinicopathological outcomes of breast cancer.[17] The methods used for assessing angiogenesis are usually used to measure the lymphangiogenesis of breast cancer as well.[22,23]

In this study, there was a positive significant Pearson's correlation (r=0.387; p=0.007) was found between LN Metastases with MVD in Weidner method (vWF stain). Similarly, there was also a positive significant Pearson's correlation (r=0.410; p=0.004) between LN metastases with MVD in IT in Weidner method (vWF stain). El-Gohary et al.[4] reported that CD31-detected MD correlated significantly (r=0.378; P=0.008) with vascular invasion and vascular invasion was detected in 23% by CD31. They also stated that peritumoral lymphatics in 96.0% of invasive carcinoma of the breast. Peritumoral LMD was statistically significantly higher than intratumoral LMD. We observed positive correlation with micro vessel density and lymphnode metastasis in both Weidner method and Chalkley method but positive significant correlation was seen in Weidner method than Chalkley method.

Chalkley method is an internationally accepted method. it is used worldwide for counting microvessel density. In this study Weidner method gave significant result.We also showed separate correlation between two methods but these gave positive significant correlation. It is occurred due to observer variations or using these two methods together. Therefore, further analysis is needed for evaluation.

Conclusion

It can be said that both peritumoral and intratumoral lymphangiogenic and angiogenic vessel count (density) stained by podoplanin and vWF respectively, correlated with lymph node metastasis. Lymphatic vessel count is more in the peritumoral area whereas angiogenic vessel count is more in the intratumoral area. In this study Weidner method was found simple and gave significant result compared to Chalkley method. The specific lymphatic endothelial marker podoplanin and blood vessel marker vWF proved to be a valuable tool in highlighting vascular density and lymphovascular invasion, and therefore a reliable predictor of lymph node metastasis.

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