Original Article

Prognostic Significance of Angiogenesis in invasive ductal carcinoma

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ABSTRACT:

Background: *Angiogenesis is essential for tumor growth and metastasis. Axillary lymphnode status has been the most important prognostic factor in operable breast carcinoma, but it does not fully account for the varied disease outcome. More accurate prognostic indicators would help in selection of patients at high risk for disease recurrence and death who are candidates for systemic adjuvant therapy. Microvessel density in invasive ductal carcinoma (measures of tumor angiogenesis) is associated with metastasis and thus may be a prognostic indicator.*

Objective: *To correlate intratumoral and peritumoral angiogenic microvessel density with lymphnode metastasis in invasive ductal carcinoma.*

Material and Methods: *It is a cross sectional observational study, carried out at the department of Pathology, BSMMU from January 2016 to December 2017. A total 48 mastectomy samples with axillary nodes from histologically confirmed invasive ductal carcinoma were included in this study. Weidner method was used for calculating micro vessels density. Sections examined to evaluate the density of angiogenic vessels by immuohistological stain with vWF expression in invasive breast cancer. Correlation between angiogenic vessels density with or without lymph-node metastasis was taken.*

Results: *In this study angiogenic vessel count is more in the intratumoral area than peritumoural area. There was a positive significant correlation between lymphnode metastasis with micro vessel density in both peritumoral and intratumoral areas in Weidner method in vWF stain.*

Key Words:
Angiogenesis, invasive breast carcinoma, vWF, Weidner method.

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Introduction

Angiogenesis is critical process for tumour growth, invasion, and metastasis.[1] Thus, measurement of vascular growth may be clinically important in breast cancer specimens. Breast cancer is the most common cancer in women, comprising almost one third of all malignancies in female. It is the most frequently diagnosed cancer among women in 140 of 184 countries worldwide.[2] Breast cancer is one of the major malignant disease burdens in Bangladesh, with an estimated incidence of about 22.5 per 1, 00,000 in females.[3] Breast cancers are notorious for their invasive and metastasizing potential. The axillary lymph node involvement, tumor size, nuclear grade, hormone receptor status, patient's age are well recognized prognostic factors for patients with operable invasive breast cancer. The lymph node involvement predicts the choice of adjuvant chemotherapy and radiotherapy after surgery for primary breast cancer.[4] The markers used to detect blood vessel invasion include elastic van Gieson stain, factor VIII-related antigen (vWF), CD34, CD31. vWF is useful marker of vascular endothelium. vWF is a trasmembrane glycoprotein in platelet edothelial adhesion molecule-1in the immunoglobin superfamily. It is expressed on monocyte, platelet, selected T cell subsets and endothelial cell and is found more commonly on blood vascular endothelial cell than lymphatic endothelial cell [5].

vWF is useful markers for vascular density (VD). It can be used in routinely preserved breast cancer tissue. Thus vascular density can be assessed and correlated with lymph node metastasis to find out the relationship and its role as potential indicators in breast cancer cases. The common pathologic approach to assess angiogenesis involves microscopic estimation of vascular density or microvessel density using endothelial markers by immunohistochemistry [6].

Studies of various tumors have shown the potential clinical significance of angiogenesis, suggesting that vascular microvessel density correlate with tumor growth and metastasis.[7] The two principal approaches in this regard are direct microscopic immunohistochemistry and semiautomated image cytometry. Direct immunohistochemical analysis of microvessel density is relatively inexpensive and widely available in diagnostic pathology departments [8].

Therefore, this study was aimed to assess intratumoral and peritumoral angiogenic microvessel density and to find out the possible relationships between intratumoral and peritumoral microvessel density and lymphnode metastasis in invasive breast cancer.

Materials and 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. After the approval of the Institutional Review Board (IRB) of BSMMU, Dhaka, the specimens were selected following the inclusion and exclusion criteria. Surgically resected formalin fixed total mastectomy specimen including axillary dissection were collected from BSMMU and all the relevant information were recorded. In this study, sections of normal vermiform appendix were taken as positive control.

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 areas and two slides were made. Tissues were processed according to routine histopathological processing protocol of BSMMU. The slides were routinely stained with H&E method from the cases histologically diagnosed as invasive ductul carcinoma. All samples were selected for vWF stain on the basis of suspicion of vascular invasion in H&E slides. Histopathological categorization of tumor and grading (Nottingham modification of the Bloom– Richardson Grading System) of all the cases were done. Vascular invasions were recorded. Each lymph node was histologically examined to determine metastasis.

Immunohistochemistry study

Immunohistochemistry of all cases were performed using Dako Autostainer Plus at the immunohistochemistry laboratory of the department of Pathology, BSMMU. Polyclonal Rabbit Anti-Human Von Willebrand factor, Ready to use (link) (code, IR527) was used for endothelial cells. From paraffin-embedded, 5-micrometer thick sections were cut, deparaffinized with xylene and rehydrated through a graded series of alcohol. Antigen

retrieval was done by water bath. The sections were stained with vWF according to protocol followed at the department of Pathology, BSMMU. The numbers of blood vessels were counted by Weidner's 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 0.74mm2(20X objective, 10X ocular; equivalent to 0.7386 mm2 per 200X field.[10] First three hot spots were chosen in intratumoral and peritumoral area. In each hot spot, microvessel count was performed at power X20 and finally microvessel density was calculated as the mean of the total number of microvessels in those three hot spots.

Result and Observation

Ages of the 48 study subjects ranged form 22 to 85 years and subjects were grouped on the basis of decades (table-I). 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	Percentage
≤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 of the study sample ranged from 1-9 cm and were grouped on the basis of tumor size (table-II). It was 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	Percentage
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. it was observed that 21(43.8%) sample 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	Percentage
Grade 1	8	16.6
Grade 2	21	43.8
Grade 3	19	39.6

Total 25 cases had lymphode metastasis.The number of lymph node involved ranged from 0-17 in the case. The cases 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	Percentage
N0	23	47.9
N1	5	10.4
N2	17	35.4
N3	3	6.3
Mean±SEM	3.2±0.6	

N0=0, N1=(1-3) lymphnode, N2=(4-9) lymphnode
N3=(≥10) lymphnode

The blood vascular invasion was observed in vWF stain in study sample in (table-V). It was observed that 15(31.3%) was positive and 33(68.7%) negative.

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

BVI	Number of the sample	Percentage
Positive	15	31.3
Negative	33	68.7

Micro vessel density in peritumoral area (PT) and intertumoral area (IT) in Weidner method in vWF stain ranged from 1-92 and intratumoral area was ranged from 0-95. Table VI shows the Mean±SEM MVD in PT was 36.81±2.90 and Mean±SEM MVD in IT was 42.68±2.88.

Table VI: Distribution of the study sample by micro vessel density in peritumoral area (PT) and intertumoral area (IT) in Weidner method (vWF stain) (n=48)

Micro vessel density	Mean±SEM	Range (min-max)
MVD in PT	36.81±2.90	1-92
MVD in IT	42.68±2.88	0-95

The table-X shows the Mean±SEM MVD in PT was 25.80±1.87 and Mean±SEM MVD in IT was 24.75±1.99.

Vascular 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-I).

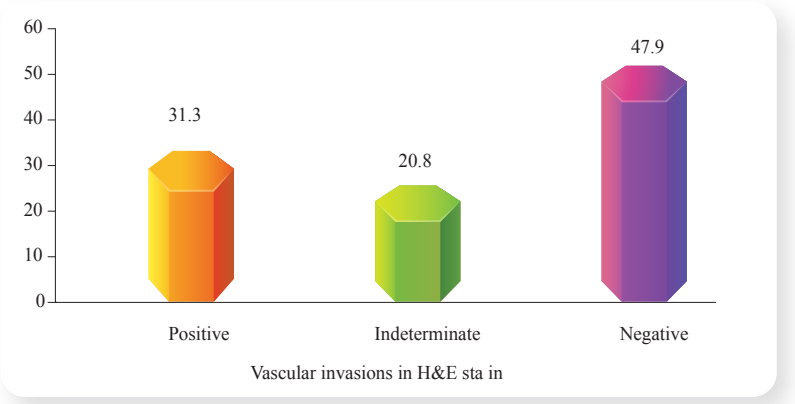

Figure I: Bar diagram showing vascular invasions in H&E stain of the study samples

.Lymphnode metastases of 48 cases were expressed the number and micro vessel density in peritumoral area (PT) in Weidner method in vWFstain was expressed in number/mm2. A positive correlation was found between microvessel density in PT and lymphnode metastases (Figure-II).

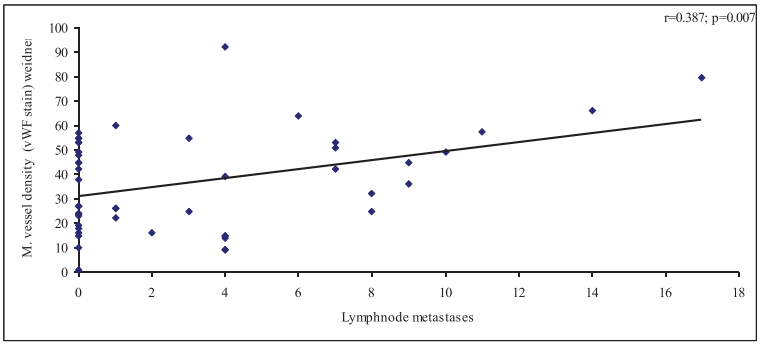

Figure II: Scatter diagram showing Pearson's positive significant correlation (r=0.387; p=0.007) between micro vessel density in peritumoral area in Weidner method in vWF stain and lymph node metastases.

The lymph node metastases of 48 cases were expressed in number and micro vessel density in intratumoral area (IT) in Weidner method in vWF stain was expressed in number/mm2. A positive correlation was found between MVD in IT and lymphnode metastases (Figure-III).

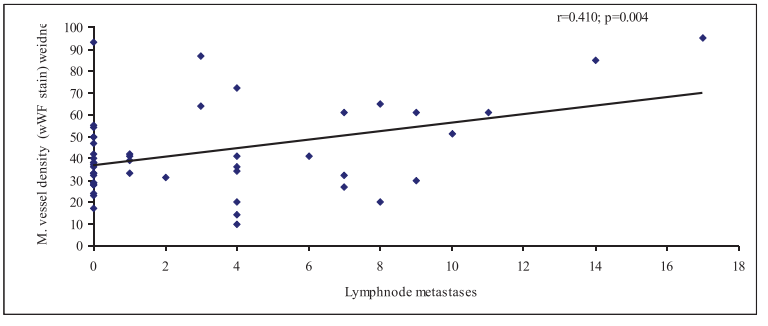

Figure-III: Scatter diagram showing Pearson's positive significant correlation (r=0.410; p=0.004) between micro vessel density in intratumoral area in Weidner method in vWFstain and lymphnode metastases.

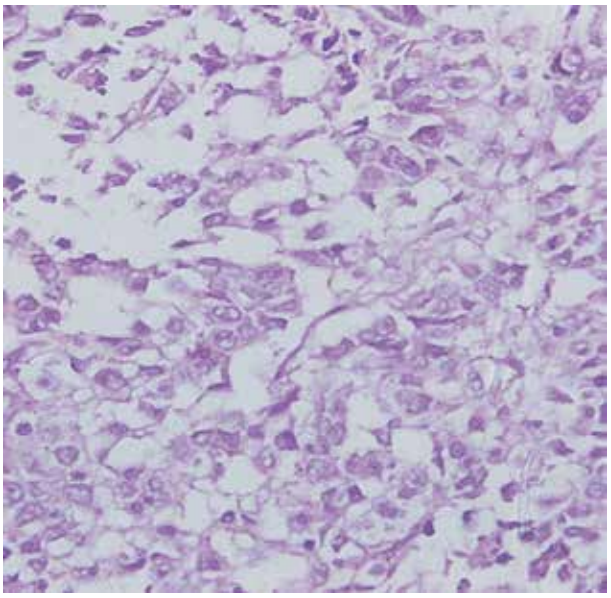

Fig.IV: photo micrograph shows invasive ductal carcinoma grade II (H&E stain x200)

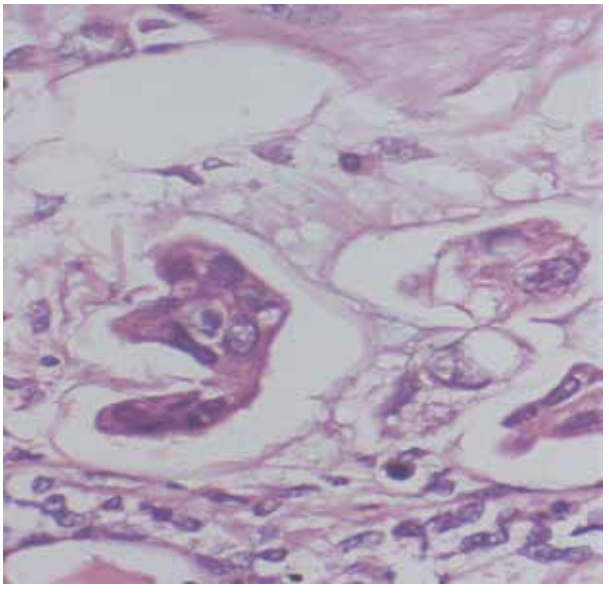

Fig.V: Photo micro graph shows lymph vascular invasion (H&E x200)

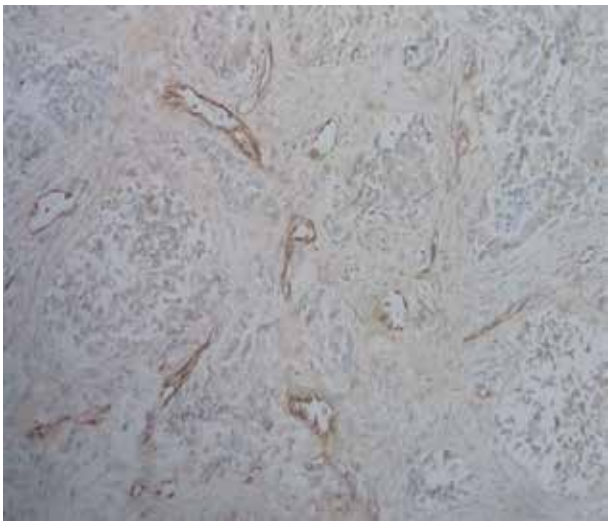

Fig.VI: Photo micrograph shows blood vessel proliferation in intratumoral area (vWFstain x200)

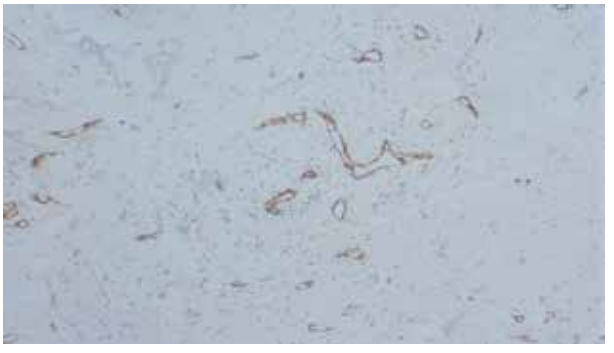

Fig.VII: Photo micrograph shows blood vessel proliferation in peritumoral area (vWF stain x200)

Discussion

Angiogenesis plays a key role in tumour growth invasion and metastasis. Tumour angiogenesis has long been claimed as an important factor for tumour spread. To see this, blood vessels proliferation was estimated in intratumoral and peritumoral areas. There were correlation with lymphnode metastasis. Immunohistochemically using vWF were empioyed to identify blood vessels. This study also estimated the density of expression of Von willebrand factor in angiogenic vessel in invasive breast cancer with or without lymphnode metastasis.

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% 4cm, which is comparable with the current study.

In this present study, it was observed that 43.8% sample had ivasive 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 vari¬ability 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 preva¬lence 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 Micro vessel density in peritumoral area (PT) and intertumoral area (IT) in Weidner method in vWF stain ranged from 1-92 and intratumoral area was ranged from 0-95.So 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.

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.[18-19] El-Gohary et al.[5] and Choi et al.[18] reported that the associations between peritumoral LVD and tumor grade, tumor stage, lymphatic invasion, LNM, and overall survival in breast cancer. However, the relationship between intratumoral LVD and clinicopathological behavior is still uncertain.

In this study, there was a positive significant Pearson's correlation (r=0.387; p=0.007) was found between LN Metastases with micro vessel density in peritumoral area (PT) 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 micro vessel density in intratumoral area (IT) in Weidner method (vWF stain). El-Gohary et al.[5] 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.Peritumoral LMD was statistically significantly higher than intratumoral LMD. Both correlated significantly with CD31-detected MD, and all correlated significantly with lymph node metastasis, nuclear grade, histologic grade, clinical stage, and vascular inva-sion detected by CD 31. [20]

Present study was done to observe the microvessel density using Weidner method. In Weidner method it was observed that there was significant positive correlation between micro vessel (blood vessel) density and lymph-node metastasis. Therefore, further analysis is needed for evaluation.

Conclusion

It can be said that both peritumoral and intratumoral angiogenic vessel count (density) stained by anti vWF antibody correlated with lymph node metastasis. angiogenic vessel count is more in the intratumoral area. Weidner method was used for calculating microvessel(blood vessel) density. In this study Weidner method was found simple and gave significant result . The specific blood vessel marker vWF proved to be a valuable tool in highlighting vascular density and vascular invasion, and therefore a reliable predictor of lymph node metastasis.

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