Comparison of Microvessel Density with Prognostic Factors in Invasive Ductal Carcinomas of the Breast

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ABSTRACT

Objective: Angiogenesis plays a key role in tumor growth and metastasis. Determination of microvessel density is the most common technique used to evaluate the amount of the intratumoral angiogenesis in breast cancer. We have aimed to investigate the relationship with tumor angiogenesis and prognostic parameters in breast invasive ductal carcinomas.

Material and Method: In this study, a total of 100 invasive ductal carcinoma patients, who were diagnosed at the Department of Pathology, Ataturk University Faculty of Medicine between the years 2003-2008, were re-evaluated. Patient characteristics and clinicopathological findings were obtained from archival records. In the present study, microvessel density was determined by immunohistochemical staining by using anti-CD34 monoclonal antibody in the paraffin blocks. First, the most vascular area was selected in the tumor under a low magnification (40x) by a light microscope and then microvessels were counted under a higher magnification (200x). Patients were classified as low and high microvessel density depending on their microvessel counts. Chi-square test and multivariate linear regression analysis were used for statistical analysis (p≤0.05).

Results: We have determined that microvessel density increases as tumor size increases (p=0.001). Microvessel density was higher in patients with at least 10 lymph node metastases compared to those with no metastasis (p=0.05). However, there was no statistically significant difference between microvessel density and other prognostic factors such as histological grade, nuclear grade, patient age, vascular invasion, estrogen, progesterone receptor status, HER2/neu expression.

Conclusion: In our study, we have found that microvessel density is associated with tumor size and lymph node metastasis in patients with invasive ductal carcinoma.

Key Words: Breast cancer, Invasive ductal carcinoma, Angiogenesis

INTRODUCTION

Breast cancer is the most common malignant tumor among women in Turkey and the world. It accounts for approximately 30% of all cancers in women. According to the 2013 data of Ministry of Health, breast cancer constitutes 24.6% of all cancers among women in our country and the most common type is invasive ductal carcinoma (1). Therefore, the etiologic and prognostic studies on breast cancer are still important.

Angiogenesis is the process of new capillary vessel formation and it is observed in physiological events such as embryonic development, wound healing and organ hypertrophy. However, uncontrolled angiogenesis is held responsible for the progression and etiopathogenesis of many neoplastic formations, especially growth and metastasis of solid tumors (2). The numerical value of tumor angiogenesis is defined as microvessel density (MVD). MVD is measured by counting small and tortuous vessels in the tumor tissue by immunohistochemical staining using antibodies such as CD31, CD34, CD105 and Von-Willebrand factor (Factor VIII) that are specific for vessel endothelium. In the earlier studies, MVD is reported to be associated with advanced pathologic stage and poor prognosis of disease in breast, lung, colon, stomach, prostate and bladder cancers, and malignant melanoma (3-5).

In this study, we aimed to determine the angiogenesis in invasive ductal carcinoma, which is the most common breast cancer type, by using microvessel counting and the relationship between MVD and known prognostic parameters such as patient’s age, tumor size, lymph node metastasis, vascular invasion, estrogen-progesterone receptor status, human epidermal growth factor (HER2/neu) expression.

MATERIAL and METHOD

In this study, a total of 113 patients, who were diagnosed with invasive ductal carcinoma, not otherwise specified (NOS) in the Department of Pathology, Ataturk University School of
Medicine between the years 2003-2008 and did not receive any neo-adjuvant treatment, underwent modified radical mastectomy and axillary lymph node dissection were re-evaluated. However, 13 patients were excluded from the study since we could not access their paraffin blocks from the pathology department archive. Hematoxylin and eosin (H&E) preparations of these patients were re-evaluated and the best formalin fixed-paraffin embedded (FFPE) block representing the tumor for each patient was selected. MVD was determined by immunohistochemical staining with this tissue. Results were compared with clinicopathologic parameters such as patient’s age, tumor size, histological grade, lymph node involvement, the presence of vascular invasion, estrogen-progesterone receptor status, human epidermal growth factor (HER2/neu) expression. The data about clinicopathologic features were obtained from the pathology reports. These features were also used to evaluate mastectomy materials during routine pathology practice. The study was approved by the Ethics Committee of Erzurum Ataturk University, School of Medicine.

The Nottingham modification of Bloom-Richardson system was used for histological grading (6). When 1% and higher nuclear staining was present in the tumor cells at any density, hormone receptor status was accepted as positive. According to immunostaining results, HER2/neu expression was considered as negative (0), equivocal (2+) and positive (3+) (7). Tumor diameter and lymph node status were grouped according to the TNM system.

**Immunohistochemistry**

The 5μ thick samples taken from FFPE blocks of each patient were put on pol-L-lysine coated microscope slides. These samples were washed in phosphate buffered saline (PBS) after deparaffinization with xylene and rehydration process with alcohol. In order to eliminate the endogenous peroxidase activity, they were incubated in 3% hydrogen peroxide solution for 15 minutes. They were washed again in PBS. Then, anti-CD34 primer antibody (Monoclonal Mouse Anti-human CD 34 class II Clone QBend-10) (Dako code No. M 7165), which was diluted at a ratio of 1:50, was dropped onto tissues and waited for 60 minutes. Tissues were re-washed in PBS. Biotinylated-link was treated for 30 minutes as the secondary antibody. It was re-washed in PBS. Treated with streptavidin peroxidase for 30 minutes and washed in PBS. Tissues were incubated for 6 minutes after dropping chromogenic DAB (3,3’-Diaminobenzidine tetrahydrochloride). The samples washed with distilled water were counterstained with Mayer’s hematoxylin and then closed by immunohistochemistry sealing solution after being washed off with distilled water again.

**Microvessel Density (MVD) Calculation**

MVD was evaluated by counting anti-CD34 positive microvessels and calculated by the counting method developed by Weidner using a light microscope (8). Accordingly, after scanning the whole tumoral section with a light microscope under a low magnification (x40), the area with highest number of microvessels was identified as ‘hot-spot’ (Figure 1) and microvessels were counted under a higher magnification (200x) in this area. Any brown-stained single endothelial cell or endothelial cell clusters separated from surrounding tumor cells and connective tissue elements were considered to be a microvessel regardless of whether they had a lumen or not. No erythrocyte was necessarily required in the lumen. Branching vessel structures were counted as a single vessel. Vascularity was not considered in the areas of necrosis within the tumor. After determining microvessel counts of all patients, the average MVD was found as 89.3 (SD±28.74). This value was regarded as the cut-off value. Patients with microvessel counts below this cut-off value were classified as ‘low MVD’ (Figure 2), and patients with microvessel counts above this cut-off value were classified as ‘high MVD’ (Figure 3) (9, 10). Olympus BX51 (Tokyo, Japan) light microscope was used for counting microvessels. Microscopic photographs were captured by Olympus DP70 (Tokyo, Japan) camera.

**Statistical Method**

SPSS 20.0 for Windows (SPSS Inc. Chicago, IL, USA) software package was used to investigate whether there is a significant relationship between all the findings.

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**Figure 1:** Immunohistochemical staining of an invasive ductal carcinoma, NOS with anti-CD34 antibody. The hot-spot with higher density of microvessels was identified and microvessels were counted in this area (CD34; x100).
Chi-square test was used to examine the relationship between MVD and other prognostic parameters. Those with a p-value smaller than 0.25 (tumor size, lymph node involvement, progesterone receptor status, lymphovascular invasion) in the univariate analysis were re-examined using the multivariate linear regression analysis model.

The error value was set as 0.05. p-values either higher or equal to 0.05 were considered to be statistically significant.

RESULTS

All of the patients included in the study were women with an average age of 51.8 (SD=±11.9 years; age range, 26-80). Tumor size ranged from 0.7 cm to 10 cm and the average tumor size was 4.15 (SD=±2.03) cm. No lymph node metastasis was observed in 28% of the patients, while 1-4 lymph node metastases were observed in 34% of the patients, 4-9 lymph node metastases were observed in 24% of the patients and ≥10 lymph node metastases was observed in 14% of the patients, respectively. 2% of the patients were graded as grade 1, 75% of them were graded as grade 2, and the remaining 23% were graded as grade 3, respectively.

When MVD was calculated by anti-CD34 antibody, at least 31 and up to 185 microvessels were counted. High MVD was observed in 48% of the patients and low MVD was observed in the remaining 52%, respectively. In our study, MVD was found to increase as tumor diameter increases (p<0.001). MVD was higher in patients with at least 10 lymph node metastases compared to those with no metastasis (p=0.05). The relationship between lymph node status and MVD is shown in Figure 4.
No significant relationship was found between MVD and clinicopathologic parameters such as patient’s age, histological grade, the presence of vascular invasion, estrogen-progesterone receptor status, human epidermal growth factor (HER2/neu) overexpression. The relationship between MVD and clinicopathological parameters is summarized in Table I.

The clinicopathological parameters with a p-value smaller than 0.25 in the univariate analysis were examined using the multivariate linear regression analysis model. Similar to the univariate analysis, the multivariate linear regression analysis showed a statistically significant relationship between MVD and tumor size and lymph node involvement. The results are summarised in Table II.

Table I: The relationship between clinicopathological parameters and MVD in patients with breast cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>AMC ± SD</th>
<th>MVD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>High (%)</td>
<td>Low (%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 years of age</td>
<td>45</td>
<td>86.33 ± 25.74</td>
<td>23 (51.1)</td>
<td>22 (48.9)</td>
</tr>
<tr>
<td>&gt;50 years of age</td>
<td>55</td>
<td>88.49 ± 31.12</td>
<td>25 (45.5)</td>
<td>30 (54.5)</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 cm</td>
<td>15</td>
<td>71 ± 16.56</td>
<td>2 (13.3)</td>
<td>13 (86.7)</td>
</tr>
<tr>
<td>2–5 cm</td>
<td>60</td>
<td>85.25 ± 27.84</td>
<td>28 (46.7)</td>
<td>32 (53.3)</td>
</tr>
<tr>
<td>5 cm</td>
<td>25</td>
<td>102.88 ± 30.06</td>
<td>18 (72)</td>
<td>7 (28)</td>
</tr>
<tr>
<td><strong>Lymph node involvement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No lymph node involvement</td>
<td>28</td>
<td>80.64 ± 24.64</td>
<td>11 (39.3)</td>
<td>17 (60.7)</td>
</tr>
<tr>
<td>≥10 lymph node involvement</td>
<td>14</td>
<td>106.36 ± 33.08</td>
<td>10 (71.4)</td>
<td>4 (28.6)</td>
</tr>
<tr>
<td><strong>Estrogen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>46</td>
<td>87.41 ± 32.21</td>
<td>23 (50)</td>
<td>23 (50)</td>
</tr>
<tr>
<td>Negative</td>
<td>54</td>
<td>87.61 ± 25.66</td>
<td>25 (46.2)</td>
<td>29 (53.8)</td>
</tr>
<tr>
<td><strong>Progesterone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>58</td>
<td>83.29 ± 26.53</td>
<td>25 (43.1)</td>
<td>33 (56.9)</td>
</tr>
<tr>
<td>Negative</td>
<td>42</td>
<td>93.36 ± 30.84</td>
<td>23 (54.8)</td>
<td>19 (45.2)</td>
</tr>
<tr>
<td><strong>HER2/neu</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>47</td>
<td>85.04 ± 25.84</td>
<td>20 (42.6)</td>
<td>27 (57.4)</td>
</tr>
<tr>
<td>Equivocal</td>
<td>15</td>
<td>92.80 ± 35.43</td>
<td>9 (60)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>88.50 ± 29.66</td>
<td>19 (50)</td>
<td>19 (50)</td>
</tr>
<tr>
<td><strong>Lymphovascular invasion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>82</td>
<td>88.34 ± 28.73</td>
<td>42 (51.2)</td>
<td>40 (48.8)</td>
</tr>
<tr>
<td>No</td>
<td>18</td>
<td>83.78 ± 29.09</td>
<td>6 (66.7)</td>
<td>12 (33.3)</td>
</tr>
</tbody>
</table>

AMC: Average number of microvessel count, SD: Standard deviation, MVD: Microvessel density

Table II: The relationship between clinicopathological parameters and MVD by multivariate linear regression analysis

<table>
<thead>
<tr>
<th>Model</th>
<th>B</th>
<th>SD</th>
<th>Beta</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>46.738</td>
<td>11.352</td>
<td>4.117</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>15.195</td>
<td>4.145</td>
<td>0.332</td>
<td>3.666</td>
<td>0.000</td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td>8.91</td>
<td>2.739</td>
<td>0.315</td>
<td>3.253</td>
<td>0.002</td>
</tr>
<tr>
<td>Progesterone</td>
<td>-7.477</td>
<td>5.227</td>
<td>-0.129</td>
<td>-1.43</td>
<td>0.156</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td>-8.333</td>
<td>7.057</td>
<td>-0.114</td>
<td>-1.181</td>
<td>0.241</td>
</tr>
</tbody>
</table>

R: 0.484, R²: 0.234, F: 7.269
DISCUSSION

The most important clinicopathologic factors influencing the biological behavior and treatment of the disease of breast cancer are patient’s age, tumor size, tumor type, axillary lymph node involvement, the presence of vascular invasion, estrogen-progesterone receptor status and human epidermal growth factor (Her2/neu) overexpression (11-13). Prognostic factors can be useful to identify poor clinical outcomes and select patients who will receive adjuvant therapy (14).

As known, angiogenesis plays a key role in tumor growth, invasion and metastasis. In recent years, the growing importance of targeted approaches in treating cancer highlights the target treatment options that inhibit angiogenesis in cancer treatment (15-17). Angiogenesis inhibitors slow and inhibit tumor growth and metastasis via different mechanisms. For example, anti-angiogenic drugs target directly pro-angiogenic molecules, while some of them inhibit angiogenic receptors, signal pathways or angiogenic external factors. Using anti-angiogenic drugs together or combining them with chemotherapeutic agents are more effective in treating breast cancer (18).

In this study, we have determined angiogenesis in invasive ductal carcinoma by counting microvessels using anti-CD34 antibody and compared MVD that we have obtained from each patient with prognostic factors. We have found a statistically significant relationship between increasing tumor size and MVD (p=0.001). There are consistent studies (19-21) with our results in the literature as well as some studies (16, 22) found no significant relationship between MVD and tumor size and some studies found an inverse correlation between them (23).

When we compared MVD with lymph node involvement we have found that MVD was higher in patients with at least 10 lymph node metastasis compared to those with no metastasis (p=0.05). Similar to our results, there some studies found that high MVD is correlated with axillary lymph node metastases (21, 24, 25). However, there are also some other studies found no relationship between MVD and axillary lymph node metastases (16, 26).

In our study, we have found no relationship between MVD and prognostic parameters such as patient’s age, tumor size, histological grade, vascular invasion, estrogen-progesterone receptor status, human epidermal growth factor (HER2/neu) overexpression. In the literature, some studies have reported that there is no relationship between MVD and prognostic parameters such as patient’s age (25, 27), histological grade (28), lymphovascular invasion (9), estrogen and progesterone receptor status (27), HER2/neu overexpression (27). On the other hand, there are also some other studies that found a significant relationship between high MVD and patient’s age (29), high histologic grade (16, 25, 27), presence of lymphovascular invasion (30), estrogen (16, 22) and progesterone receptor negativity (22) and HER2/neu overexpression (31).

As it can be seen, there are different results in the literature regarding the relationship between MVD and prognostic parameters. One of the reason of this may be using different antibodies such as CD34, CD31, Factor VIII and CD105 to highlight the microvessels (24, 32, 33). In the literature, some studies reported that the anti-CD34 monoclonal antibody is more sensitive than the anti-CD31 antibody and anti-factor VIII-related antigens in the calculation of MVD in breast cancer (31, 32). Therefore, we used the anti-CD34 monoclonal antibody to calculate the MVD. Since we did not use any other antibodies in the calculation of MVD in breast cancer, we do not know whether our results were affected by this selection.

One another reason for having different results may be the calculation method of MVD. Weidner et al. have identified the hot-spot area with the largest number of microvessels at low magnification (x40 and x100) to determine MVD and counted microvessels in this area under a magnification of x200 (34). This method used by Weidner is used in many studies conducted on microvessel count (16, 28, 35). Some authors have counted a single area under x200 or x250 magnification, while some other counted a single area under x400 magnification (36-38). In this study, we have used the microvessel counting method used by Weidner.

In the tumoral area, heterogeneity of microvessel distribution may be another reason for the different results (39). Bosari et al. have shown that the number of microvessels counted in a single area is 20% more than the average number of microvessels counted in three areas (9). Heterogeneity of MVD is thought to be reduced with increasing number of areas counted (23). Examining each tumor tissue blocks and applying immunohistochemistry for all tumor tissue blocks may be useful in order to overcome the problem of heterogeneity. However, this is an expensive and time consuming process and it is difficult to maintain its sustainability in routine practice. In our study, after scanning the whole tumor sections, we have counted the microvessels in appropriate tumor tissue by immunostaining with anti-CD34 antibody. And also, all tissue samples that we have used to count microvessels were resected materials. Due to tumor heterogeneity, MVD should be determined in the resection materials and it should be avoided to determine MVD from biopsy specimens.
Another reason for the different results between studies may be different cut-off values used to classify patients depending on their MVD. Some studies identify the cut-off value as the average number of microvessels (9, 27), but in other studies, the cut-off value is the median number of microvessels (10, 40). There are also some other studies accepted absolute values as the cut-off value (36, 41, 42). In this study, we have accepted the average number of microvessels as the cut-off value. All these different cut-off values may be the main cause of different results by affecting the p value. However, we found similar results when we re-analyzed the data by accepting the median value as the cut-off value.

In conclusion, we have found MVD in invasive ductal carcinomas associated with tumor size and lymph node metastasis. However, there are different results regarding the relationship between MVD and prognostic parameters in the literature. These differences may be due to different microvessel counting methods and antibodies used to count microvessels. Since the exact identification of MVD may be helpful in estimating the impact of the anti-angiogenic drugs used in the treatment of breast cancer and the selection of high-risk patients who will receive adjuvant therapy, the microvessel counting method and antibodies used to count the microvessels should be standardized.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**REFERENCES**


