Original

Microvascular Density Measurements and Clinical Pathological Analyses of Breast Cancer Tumors

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Abstract: In this study, we measured microvascular densities using two types of vascular endothelial markers, CD34 and nestin, and performed clinical pathological analyses of breast cancer tumors. The subjects included 144 primary breast cancer patients who had undergone surgical resection. Microvascular densities were obtained by randomly selecting three fields of the tumor margins of CD34-and nestin-immunostained samples under high magnification and adding the numbers of positive blood vessels from these fields together. Both CD34 and nestin immunostaining revealed that the microvascular density was higher in patients with lymph node metastases, distant metastases, and lymphatic vessel invasion compared to those without. Although no significant correlation was observed between microvascular density and age, significant correlations were detected between microvascular density and tumor diameter, nuclear grade (NG), and hormone receptor expression. Moreover, a significant decrease in overall survival was observed in cases with high microvascular densities compared to low density cases as measured by CD34 and nestin. The results of this study suggest that microvascular densities in breast cancer tumors may reflect the metastatic growth potential of the tumor or the prognosis of a patient. No obvious differences were observed between CD34 or nestin, the two vascular endothelial markers used to measure microvascular densities in this study.

Key words: breast cancer, neovascularization, microvessel density, CD34, nestin

Introduction

The correlation between tumor proliferation and neovascularization has been studied in various carcinomas ever since it was reported by Folkman1) in 1971. Neovascularization is also considered to be closely associated with the proliferation/metastasis of tumors in breast cancer, and some reports have suggested the possibility of using this as an independent prognostic factor2-12).

As an assessment method that reflects the degree of neovascularization, the microvascular density of a tumor is often measured in tissue sections obtained from immunostained sam-

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Vascular endothelial markers are usually used to clarify blood vessels. Many reports have stated that higher microvascular densities are correlated with higher metastatic rates and poor prognoses in breast cancer patients\(^2\text{-}^{12}\), a situation similar to that found in other carcinomas. In contrast, some studies have reported that there is no correlation between microvascular density and metastasis or prognosis\(^3\text{-}^{11},^{13}\text{-}^{15}\). One of the reasons for this discrepancy is the use of different antibodies for immunostaining, and differences in the sites used to measure the microvascular density. With this in mind, Kato et al\(^16\) measured microvascular densities at three different sites—the tumor margins, tumor centers, and areas where microvessels are gathered—using the Factor VIII vascular endothelial marker, and reported that measurements taken at the tumor margins were useful for evaluating the significance of neovascularization.

In this study, we measured microvascular densities at the tumor margins using the vascular endothelial markers CD34 and nestin in breast cancer tumor resection samples, and assessed the possibility of a correlation between microvascular density and tumor metastasis and patient prognosis.

**Subjects and Methods**

**Subjects**

One hundred and forty-four patients who had undergone surgical resection of primary breast cancer at our hospital during the two-year period from 1998 to 1999 were included in this study (Table 1). Treatments were not administered to any of the patients prior to the operation, and post-operative adjuvant therapy was performed according to the operational method and the stage of the disease. The mean observation period for these patients was 68 months. In April 2005, there were 115 surviving patients, 24 deaths, and 5 unknown cases. Among the deaths, 20 were due to breast cancer, and 4 were associated with other causes.

**Methods**

Thin sections (thickness: 3 \(\mu\)m) were prepared for immunohistochemical studies from formalin-fixed paraffin-embedded blocks containing tumor margins. Immunostaining was performed using the ChemMate EnVision Detection kit (DakoCytomation, Denmark). The anti-human CD34 monoclonal antibody (1:50; QBEnd10, DakoCytomation, Denmark) and anti-human nestin monoclonal antibody (1:50; 10C2, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used as primary antibodies.

Three fields were randomly selected under high magnification from the immunostained tumor margin samples using a light microscope, and microvascular densities were calculated by adding the numbers of CD34- and nestin-positive blood vessels in each field (Fig. 1). These were missing elastic lamina and forming tubular structures. The correlations between microvascular densities and clinical pathological factors, and between microvascular densities and patient prognoses were calculated. Statistical assessments were made using the Mann-Whitney test, in which \(P < 0.05\) was defined as being statistically significant. Relapse-free survival and overall survival included the period following the operations until death or until observations had been completed.

**Results**

*Relationship between microvascular density and clinicopathological factors*
The following clinicopathological factors were assessed to determine whether these correlated with the microvascular density of the tumor: age, tumor diameter, lymph node metastasis, distant metastasis, lymphatic vessel invasion, nuclear grade (NG), estrogen receptor (ER), and progesterone receptor (PgR) (Table 2).

Regarding age, separate assessments were made for patients younger than 50 years of age and for those who were 50 years of age or older. Although the microvascular density was low in the older group of patients (50 years of age or older), no significant differences in microvascular density were observed.
To assess the relationship with tumor diameters, separate assessments were made for tumors with different TNM classifications, and included T1 tumors (< 2 cm), T2 tumors (2–5 cm), and T3 tumors (> 5 cm). Microvascular densities were significantly higher in tumors with large diameters in CD34-stained samples only.

**Table 2. Clinicopathological factors and microvessel density (MVD)**

<table>
<thead>
<tr>
<th>CD34</th>
<th>MVD mean value ± SD</th>
<th>n</th>
<th>Nestin</th>
<th>MVD mean value ± SD</th>
<th>n</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt; 50</td>
<td>(26) 8.2 ± 3.6</td>
<td>(27) 8.0 ± 4.2</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 50</td>
<td>(95) 7.3 ± 3.6</td>
<td>(96) 7.9 ± 4.5</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>(1 (≤ 2 cm)) (48) 7.0 ± 3.6</td>
<td>(48) 7.3 ± 4.4</td>
<td>.047</td>
<td>8.4 ± 4.5</td>
<td>(73)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(2, 3 (&gt; 2 cm)) (71) 8.0 ± 3.5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nodal status</td>
<td>negative (63) 6.0 ± 2.9</td>
<td>(64) 5.7 ± 2.9</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>positive (53) 9.5 ± 3.6</td>
<td>(54) 10.5 ± 4.7</td>
<td>&lt; .001</td>
<td>12.5 ± 5.4</td>
<td>(28)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Distant metastases</td>
<td>negative (94) 6.8 ± 3.3</td>
<td>(95) 6.5 ± 3.0</td>
<td>&lt; .001</td>
<td>12.5 ± 5.4</td>
<td>(28)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>positive (27) 10.1 ± 3.4</td>
<td>(54) 10.5 ± 4.7</td>
<td>&lt; .001</td>
<td>12.5 ± 5.4</td>
<td>(28)</td>
<td>&lt; .001</td>
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| Lymph : lymphatic vessel invasion, ER : estrogen receptor, PgR : progesterone receptor, NS : not significant

Fig. 1. Microvessels were highlighted by CD34 immunostaining
Overall survival was significantly shorter in the high value group in comparison with the low value group with both CD34 and nestin. \(p < 0.05\)

Fig. 2. Overall survival of subgroups (CD34 low / high and nestin low / high)

Microvascular densities were significantly higher in patients with lymph node metastases and distant metastases in both CD34- and nestin-stained samples. Similarly, microvascular densities were also significantly higher in patients with positive lymphatic vessel invasion in both CD34- and nestin-stained samples.

To assess the nuclear grade (NG) of the tumor, separate assessments were made for tumors with different nuclear grades as described in the Breast Cancer Handling Conventions in Japan (Vol. 15), and included NG1 + 2 and NG3 tumors. Microvascular densities were significantly higher in NG3 tumors in both CD34- and nestin-stained samples.

Separate assessments were also made for the estrogen (ER) and progesterone (PgR) hormone receptors. Negative results were defined as those in which the ratio of positively stained cells was less than 10\%, and positive results as those with a ratio of 10\% or higher. Significant differences in microvascular densities were observed in nestin-positive samples expressing ER, and in CD34- and nestin-positive samples expressing PgR.

**Relationship between microvascular density and overall survival**

The mean values of the microvascular densities calculated with CD34 and nestin were 7.51 and 7.91, respectively. When the relationship between these values and overall survival (OS) was studied separately for the low-value group (less than 7) and the high-value group (7 or more), the OS was significantly shorter in the high-value group with both CD34 and nestin (Fig. 2).

**Discussion**

A 1991 study of 49 primary breast cancer cases, Weidner et al\(^2\) reported that microvascular density is related to lymph node metastasis and distant metastasis. Since then, many studies have described the correlation between microvascular density and metastasis and patient prognosis in breast cancer\(^7\)\(^-\)\(^\)\(^12\). However, some reports have found no correlation between microvascular density and cancer progression\(^5\)\(^,\)\(^11\)\(^,\)\(^13\)\(^\)\(^-\)\(^15\). The cause of this discrepancy can possibly be attributed to the lack of a common method for measuring microvascular densities\(^12\). Specifically, this discrepancy may be caused by differences in the areas where the vascular densities are measured and by the vascular endothelial markers used. Kato
et al\(^{16}\) evaluated the different sites used for measuring microvascular densities using three methods: AMC (average microvessel count: the entire margin of the tumor); CMC (central microvessel count: 6 sites at the center of the tumor); and HMC (highest microvessel count: 3 hot spots), and reported that AMCs were useful in the assessment of neovascularization. However, only one vascular endothelial marker, Factor VIII, was used in their study.

In the present study, two vascular endothelial markers were used: CD34 and nestin. CD34 is widely used as a vascular endothelial marker to assess microvascular densities in various carcinomas including breast cancer\(^{5,13,17-19}\). In contrast, nestin is usually used as a marker for stem cells and progenitor cells, which differentiate into various kinds of cells. However, nestin has been gaining attention recently as a useful vascular endothelial marker\(^{20,21}\), and has been used to assess microvascular densities in gastric and colonic cancer\(^{18,19}\).

Based on these previous studies, we measured microvascular densities in tumor margins using CD34 and nestin, and investigated whether there was a correlation between microvascular density and metastasis and patient prognosis. Our results demonstrate that microvascular densities were significantly higher in patients with lymph node metastases and distant metastases compared to those without, based on the results of both CD34 and nestin immunostaining. We believe that this result reaffirms the fact that neovascularization contributes to the metastatic growth of tumors, and supports the usefulness of the AMC microvascular density measurement method reported by Kato et al\(^{7}\). In addition, it appears that it is not necessary to use a specific vascular endothelial marker to measure microvascular densities.

In the present study, the clinicopathological factors that were significantly correlated with microvascular densities were the presence of lymph node metastases and distant metastases, lymphatic duct invasion, tumor diameter, nuclear grade, presence of hormone receptors, and overall survival. These results emphasize that microvascular density measurements are an effective indicator of metastasis and patient prognosis in breast cancer patients, just as they are in other carcinomas. In contrast, the correlation between microvascular densities and hormone receptors is considered to be an aspect specific to breast cancer. Furthermore, neovascularization is induced by various factors associated with cancer proliferation\(^{22}\), and the fact that microvascular densities tended to be lower in elderly patients in this study corresponds to reports stating that neovascularization is suppressed by decreases in estrogen concentrations\(^{23,24}\). Although further studies must be conducted to gain a better understanding of breast cancer and to develop treatment applications for breast cancer in relation to microvascular densities, it will also be necessary to consider the assessment of breast cancer-specific factors that are associated with the induction of neovascularization.

**Conclusion**

A significant correlation was observed between microvascular densities, which were measured using CD34 and nestin immunostaining, and metastasis and disease prognosis in the breast cancer cases treated in our hospital.

**References**

The Evaluation of Neovascularization in Breast Cancer


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