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ORIGINAL ARTICLE

Relationship between peritumoral lymphatic microvessel density and the clinical and pathological characteristics of invasive breast cancer

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Abstract

Objective The aim of the study was to determine the morphological characteristics of lymphatic microvessels and the relationship between lymphatic microvessel density (LMVD) and clinical and pathological characteristics of invasive breast cancer.

Methods Tissue specimens and clinical pathological data of 51 cases of female breast cancer were collected in the General Hospital of Shenyang Military Region (Shenyang, China) from January 2007 to October 2011. Another 20 breast fibroadenoma tissue samples were used as controls. All specimens were cut into 4-μm slices, and immunohistochemically stained using streptomycin-resistant avidin peroxidase antibody D2-40. SPSS 17.0 for Windows was used to perform all analyses.

Results A total of 38 breast cancer tissue specimens showed varied staining with monoclonal antibody D2-40. The rate of positive staining was in these tissues was 74.5% (38/51), which is significantly higher than that observed in breast fibroadenoma tissues (chi-square = 35.197, $P = 0.000$). The average LMVD in 38 cases of breast cancer was (26.46 ± 10.06) microvessels/100× magnification field, which was higher than that in the control group ($t = 10.74$, $P = 0.000$). Microvessels in peritumoral tissues were abundant, with an average LMVD of (38.42 ± 11.38) microvessels/100× magnification field. Based on layered analysis, the expression level of peritumoral LMVD was correlated with metastasis of lymph nodes, tumor size, and the expression of C-erbB-2 ($P < 0.05$); however, there was no correlation with age or expression of estrogen receptors or progesterone receptors ($P > 0.05$).

Conclusion Lymphatic microvessels detected using D2-40 antibody are mainly present in the peritumoral region of breast cancer tissues, and LMVD showed a correlation with lymph node metastasis and the expression of C-erbB-2. Positive lymphatic vessels, especially in the peritumoral region, may provide a path for lymphatic metastasis in breast cancer. Peritumoral LMVD may be used to estimate the prognosis of patients with breast cancer and may aid in research on treatment methods.

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Breast cancer is a malignant disease with complicated and systemic features [1]. When the tumor diameter exceeds 0.2–0.3 cm, new vessels will be generated to provide essential oxygen and nutrition [1–2]. New vessels to promote tumor growth and metastasis are under the control of many positive and negative factors. It is reported that the occurrence, growth, metastasis, and prognosis of breast cancer mostly depends on sustained and uncontrolled angiogenesis and lymphangiogenesis [2–4].

There has been little research on lymphangiogenesis. The lack of specific lymphatic epithelial markers made it difficult to distinguish blood vessels and lymphatic vessels based on morphology, and only hematoxylin-eosin (HE) staining technique has been useful [5].

In this study, the morphologic characteristics of

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microlymphatic vessels were examined in 51 cases of breast cancer. In addition, the relationship between lymphatic microvessel density (LMVD) and the clinical and pathological characteristics of breast cancer were examined.

Materials and methods

Patients and specimens

Tissue specimens and clinical pathologic data of 51 cases of female breast cancer were collected in the General Hospital of Shenyang Military Region (Shenyang, China) from January 2007 to October 2011. All patients underwent surgery. The median age of all patients was 55 (22–75) years; the patients had no family history of breast cancer or other malignancy. Preoperative examination included tumor markers, B-mode ultrasound, computed tomography or positron emission tomography-computed tomography to exclude a second cancer. Based on the World Health Organization Breast Cancer Histopathological Classification (2003), 51 cases were diagnosed with invasive ductal carcinoma. Based on the sixth edition handbook of cancer TNM staging by the American Joint Committee on Cancer (AJCC), 11 cases of stage I, 12 cases of stage II, 28 cases of stage III, and 0 cases of stage IV were diagnosed; 14 cases had more than 3 lymph node metastases, 15 had 1–3 lymph node metastasis, and 22 had no lymph node metastases. Another 20 cases of breast fibroadenoma tissue were used as benign controls. All specimens were cut into 4-μm slices and archived for HE staining.

Main reagents and kits

Instant-applied mouse anti-human D2-40 monoclonal antibodies were purchased from the Zhongshan Jinqiao Biotechnology Company, China. Instant-applied Ultra™ SP kit for immunohistochemistry and DAB-0031 kit for DAB coloring were purchased from Maixin Biological Ltd., Fuzhou, China.

Immunohistochemistry

A total of 51 breast cancer tissue samples and 20 control breast fibroadenoma tissue samples were detected by immunohistochemical technique using streptomycin-resistance avidin peroxidase (S-P) with antibody on D2-40. The specific steps were as follows:

Paraffin sections were dewaxed, and incubated in 3% H_2O_2 for 10 min at room temperature. Antigen retrieval was performed by microwaving for 3 min in citrate buffer at pH 6.0. Nonspecific sites were blocked with 3.5%–10% normal goat serum. The sections were incubated for 10 min at room temperature. Serum was poured off. All sections were exposed to the first D2-40 antibody and incubated overnight at 37 °C. The second biotin-labeled antibody was added. The sections were incubated in wet box for 20 min, followed by horseradish peroxidase-labeled avidin. The sections were rinsed (3 × 5 min) in 0.01 MPBS (pH 7.4), stained with 3,3-diaminobenzidin (DAB), kept at room temperature, washed with distilled water, and hematoxylin stained. Specimens of known colorectal cancer provided by the Zhongshan Jinqiao Company (China) served as positive controls; phosphate buffered saline was used instead of the first antibody as a negative control.

Detection and determination of MLVD

Brown-yellow grains that appeared in the cytoplasm or cell membrane were considered positive stain for D2-40, and represented microtubules, single endothelial cells, or a cell plexus.

The method for MLVD counting was as follows: select two areas with the highest number of positive microvessels at low magnification (× 100), namely the "hot spot"; observe five fields at each hot spot at high magnification (× 400), count the number of microvessels, and calculate the mean number of microvessels in two hot spots and five fields. At same time, count intratumoral LMVD (located at the center of the tumor) and peritumoral LMVD (located in the peripheral tissue within 2 mm of the tumor) [6].

Statistical analysis

Quantitative parameters were expressed as mean ± SD. Qualitative variables were presented as values and percentages. Paired-samples *T*-test and one-way analysis of variance with least significant difference testing were used to compare quantitative parameters. Pearson's chi-square test was used to compare qualitative parameters. A P-value < 0.05 was considered to be statistically significant. The Statistical Package for Social Sciences (SPSS) version 17.0 for Windows was used to complete all analyses.

Results

Morphologic characteristics of lymphatic microvessels labeled with D2-40 antibody

This research included 38 breast cancer tissue specimens with different degrees of immune reactivity to monoclonal antibody D2-40. The positive rate was 74.5% (38/51). Brown-yellow grains appeared as positive cytoplasm or cell membranes. The positive microvessel rate labeled with D2-40 monoclonal antibody was only 20% (4/20) in 20 cases of controls, significantly lower than that in breast cancer tissue (chi-square = 35.197, P = 0.000; Fig. 1).

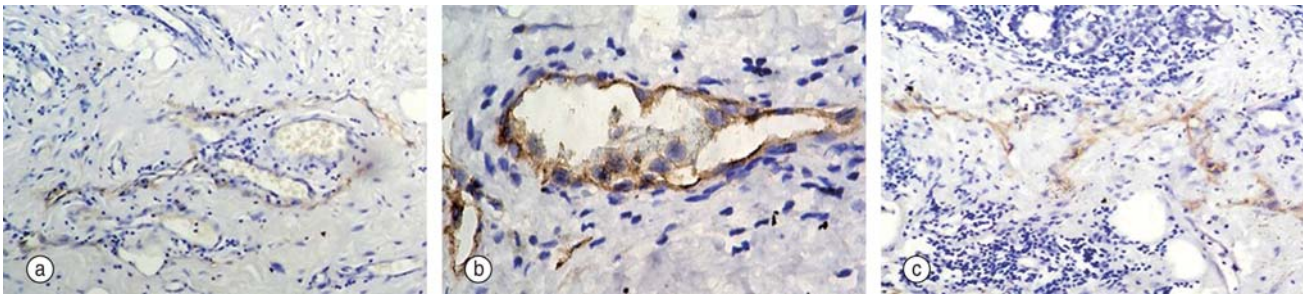

Fig. 1 (a) Positive lymphatic microvessels labeled with D2-40 (original magnification ×100); (b) tumor thrombus appears in lymphatic vessel cavity (original magnification ×400); (c) positive intratumoral lymphatic microvessel, with narrowed lumen, even funicular vessels (original magnification ×100)

Relationship between peritumoral LMVD and clinical and pathological characteristics

The average LMVD in 38 cases of breast cancer was (26.46 ± 10.06) microvessels/100× magnification field, and the average LMVD in the control group was (6.65 ± 1.72) microvessels/100× magnification field (t = 10.74, P = 0.000).

In intratumoral tissues, microvessels marked with D2-40 were sparse, with average LMVD of (7.69 ± 2.30) microvessels/100× magnification field; microvessels in peritumoral tissue were abundant, with average LMVD of (38.42 ± 11.38) microvessels/100× magnification field. Statistical differences between the two regions of breast cancer tissue were obvious (t = –16.31, P = 0.000).

Based on layered analysis, the expression level of peritumoral LMVD in 38 D2-40-positive cases was correlated with metastasis of lymph nodes and tumor size (P < 0.05), but showed no correlation with age (P > 0.05; Table 1).

Table 1 Relationship between LMVD with clinical and pathological features (microvessels/100× magnification field)

Features	No.	LMVD (M, $\bar{\chi} \pm s$)	P value	T or F
age (years)	38		0.49	0.69
< 60	13	41.45, 40.22 ± 11.28		
> 60	25	37.56, 37.48 ± 11.55		
Tumor size (cm)	38		0.02	3.30
< 5 cm	10	32.95, 29.34 ± 7.23		
> 5 cm	28	40.83, 41.66 ± 10.9		
ER state	38		0.211	1.274
–	14	36.12, 35.35 ± 12.63		
+	24	39.06, 40.20 ± 10.46		
PR state	38		0.923	0.098
–	18	38.81, 38.24 ± 8.80		
+	20	39.05, 39.61 ± 13.98		
Lymph node metastasis	38		0.000	40.722
0	15	26.78, 27.80 ± 7.25		
1–3	10	39.06, 39.71 ± 1.83		
> 3	139	51.08, 49.67 ± 4.69		
C-erbB-2	38		0.000	16.577
–	14	30.11, 29.73 ± 9.85		
2+	11	39.05, 38.67 ± 7.57		
3++	13	45.67, 49.18 ± 7.17		

LMVD labeled with D2-40 in the peritumoral region was significantly correlated with the expression of C-erbB-2 in invasive ductal carcinoma (P < 0.05), but showed no correlation with the expression of estrogen receptors and progesterone receptors (P > 0.05). With an increase of C-erbB-2 expression, LMVD also gradually increased.

Discussion

Research on lymphangiogenesis has been limited because of a lack of specific lymphatic endothelial markers available to distinguish between blood vessels and lymphatic vessels. Recent studies have identified some molecules that label lymphatic endothelia. D2-40 is a monoclonal antibody reported by Marks in 1999. It reacts with a 40-kD oncofetal antigen, better known as M2A antigen that is expressed in fetal testis and germ cells. D2-40 reportedly detects lymphatic vessels selectively in the tissues of gastric cancer, prostate cancer, and malignant melanoma by reacting selectively with the fixed antigen on lymphatic endothelium, without reacting with mature lymphatic vessels or capillary vessel endothelium [4, 7]. However, it has seldom been studied in breast cancer.

In our study, the rate of detection of lymphatic microvessels using D2-40 was 74.5%, and the average LMVD was (26.46 ± 10.06) microvessels/100× magnification field, which was significantly higher than that in benign breast tumors (P = 0.000). D2-40 is expected to become a new marker to selectively distinguish lymphatic endothelium.

In our research, immunohistochemical staining showed that positive lymphatic microvessels labeled by D2-40 were heterogeneous. The shape of vessels, size of the cavity, and LMVD in different areas were inconsistent. In the peritumoral tissue, there was an abundance of lymphatic microvessels with thin walls, more wrinkles, and a flat lumen; however, there were only a few vessels with poor lymphatic structure in

the intratumoral tissues. LMVD in two regions showed statistical significance (P = 0.000), in accordance with Roma's research [8]. Prior research suggested the possible reason. New functional lymphatic vessels surrounding stroma were formed when the tumor size was very small. However, owing to the continuous outward expansion of the tumor, these lymphatic microvessels were wrapped around intratumoral tissues. Cancer cells with excessive growth caused high hydrostatic pressure in the center of the tumor. Thus, the lymphatic lumen was compressed and the lymph vessels became non-functional [8].

It was found that LMVD was closely related to tumor invasive ability, metastasis, and prognosis [8–9]. The main cause of death in breast cancer is wide dissemination from the original site. Metastasis of axillary lymph nodes is often the first step to widespread metastasis. Therefore, lymph node metastasis has become the standard for evaluation of prognosis in breast cancer patients and directs the choice of treatment.

Recent research confirmed that when lymph node metastasis occurs, the tumor cells must penetrate the basement membrane and invade the blood vessels or lymphatic vessels before entering the circulation [10]. Stratification analysis shows that lymphatic microvessels labeled by D2-40 are correlated with lymph node metastasis in breast cancer. If the number of metastatic lymph nodes is more than three, the LMVD in interstitial tissue significantly increases, with a significant difference compared to that in lymph node-negative cases ($P < 0.01$).

The results of this research are consistent with those of a recent study [8, 11]. If breast cancer cells are exposed to an environment with more lymphatic microvessels, metastasis of lymph nodes and distant spread are more likely to occur. At the same time, lymphatic microvessels with enlarged lumens in peritumoral tissues have thin walls and discontinuous basement membranes, making it difficult to block tumor cells from entering the lymphatics. These lymphatic microvessels were the main channels for lymphatic metastasis in breast cancer.

The lymph nodes and lymphatic metastasis of tumors are associated with lymphangiogenesis. Lymphatic microvessels around the tumor provide necessary conditions for tumor growth and metastasis, and are expected to be a new target for antitumor therapy.

More than 20% of human tumor tissues express C-erbB-2 [12]. The excessive expression in human breast cancer may cause poor overall survival, with recurrence and metastasis. This study showed that the expression of lymphatic microvessels labeled by D2-40 was related to the expression of C-erbB-2 in breast cancer. We surmise that C-erbB-2 may play an important role in the growth of lymphatic microvessels. Further studies would be needed to validate these findings.

In conclusion, lymphatic microvessels labeled by D2-40 are often expressed in the peritumoral region of breast cancer, and LMVD shows a correlation with lymph node metastasis and the expression of C-erbB-2. The positive lymphatic vessels, especially in the peritumoral region, may provide a path for lymphatic metastasis in breast cancer.

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