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ORIGINAL ARTICLE

Vascular endothelial growth factor and microvessel density for detection and prognostic evaluation of invasive breast cancer*

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Abstract

Objective The purpose of this study was to evaluate the distribution of vascular endothelial growth factor (VEGF) and CD105-microvessel density (MVD) in invasive breast carcinomas. We also aimed to analyze the relationship between VEGF and MVD expression with other standard prognostic parameters associated with invasive breast cancer, such as size, grade, stage of the cancer, metastases, and tumor recurrence.

Methods Immunohistochemistry via the Ultra Sensitive™ S-P method was used to detect VEGF and MVD expression in 128 cases of invasive breast carcinoma. Specimens were evaluated for CD105 expression. Positively stained microvessels were counted in dense vascular foci under 400× magnification. MVD in the peripheral area adjacent to the lesion and in the central area within the lesion in invasive breast carcinomas and benign leisions groups were also assessed. Fifty cases of benign breast disease tissue were selected as the control group.

Results Results showed that 64.1% of invasive breast cancer samples were VEGF-positive, higher than in benign breast disease tissue (22.0%, $P < 0.05$). There was a positive correlation between VEGF overexpression and histological grade, lymph node metastasis, and distant metastasis of invasive breast cancer. VEGF expression was not related to age or size of the tumor ($P > 0.05$). MVD of the peripheral area adjacent to the lesion was significantly higher than those central area within the lesion in both invasive breast cancer and benign breast disease groups ($P < 0.01$ for each group). There were significant differences in the mean CD105-MVD, between invasive breast tumors with a histological grade of I or II and grade III; between tumors with lymph node or distant metastasis; and between patients with or without recurrence ($P < 0.05$). However, there was no difference in the mean MVD between the two age groups (≤ 50 years vs. > 50 years) or the two tumor diameter groups (≤ 2 cm vs. > 2 cm), $P > 0.05$.

Conclusion Overexpression of VEGF and MVD may be important biological markers for invasion and lymph node and distant metastases of invasive breast cancer. Combined detection of the two tumor markers could provide better prognostic monitoring for disease recurrence and metastasis, as well as aid with clinical staging of breast tumors. Prediction of the risk for metastasis and recurrence, as well as recurrence patterns based on VEGF and MVD post-surgery, could aid design of better follow-up regimens and appropriate treatment strategies for breast cancer patients.

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Key words: invasive breast carcinoma; vascular endothelial growth factor; microvessel density; detection; immunohistochemistry

In recent years, the global incidence of breast cancer has risen significantly. There is an increasing focus on breast cancer treatment, improving prognostic accuracy and identifying postoperative recurrences in breast

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cancer patients [1–2]. Early diagnosis, surgical treatment with systematic lymph node dissection, and appropriate chemotherapy have improved the survival of patients with breast cancer. However, even after a curative resection, tumor recurrences are likely to assume a variety of forms in various organs. The prediction of risk for recurrences and recurrence patterns after surgery could help the design of better follow-up programs and appropriate treatment strategies for breast cancer patients. However, despite advances in diagnostic techniques and surgical procedures, the prognosis after resection has remained unsatisfactory due to a high incidence of cancer lymph node metastases and recurrence. The identification of variables in breast tumor biology may lead to a more precise assessment of outcome and response to therapy. The development of prognostic markers that can accurately predict outcome is crucial in identifying patients who could benefit from aggressive therapy. Vascular endothelial growth factor (VEGF) promotes angiogenesis and plays an important role in the establishment, development, metastasis, and recurrence of various tumors. Endoglin (CD105) is an accessory receptor of transforming growth factor. The highest levels of endoglin synthesis and expression have been found in vascular endothelial cells. The involvement of endoglin in angiogenesis and angiogenesis-dependent processes has been observed [3]. In this study, we investigated the prognostic significance of CD105 and microvessel density (MVD) recognized by CD105 based on the number of CD105-positive vessels in various tissues of invasive breast cancer and surrounding epithelium in resected breast tissue and evaluated the relationship between MVD, lymph node metastasis, and post-operation tumor recurrence in breast cancer.

Materials and methods

Patients

We collected tissues from 128 patients with invasive breast cancer during surgical resection at Rizhao People's Hospital from June 2000 to June 2013. The age of the patients ranged from 22 to 79 years, with a mean of 48.7 years. As a control group, 50 patients with benign breast disease, with a mean age of 45.3 years, ranging from 21 to 77 years, were selected. Patients diagnosed with invasive breast cancer had not been treated with hormone endocrine therapy, anti-neoplastic chemotherapy, or radiotherapy during the 6 months prior to the study. In the present study, we investigated the significance of vessels recognized by CD105 as endothelial markers using immunohistochemical staining in 128 cases of curatively resected breast cancer and analyzed the relationship between VEGF and MVD by CD105 and clinical outcomes. In addition, we also correlated the results of CD105 expression with other standard prognostic parameters, such as size, grade, stage of the disease, metastases, and patient survival to identify the clinical and pathological characteristics associated with invasive breast cancer. This will enable the improvement of clinical diagnosis of breast cancer, allow monitoring of the effectiveness of anti-cancer treatment on regression, and improve detection of tumor recurrences.

Methods

Immunohistochemistry was employed to detect of VEGF and CD105 expression. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were deparaffinized and rehydrated using standard procedures. Serial sections (4 μm) were deparaffinized in xylene and hydrated through a graded series of ethanol. The specimens were washed in phosphate-buffered saline (PBS) for five minutes and examined under a binocular dissecting microscope. Immunoreactions were processed using the Ultra Sensitive™ S-P Kit (Maixin-Bio, China) according to the manufacturer's instructions and signals were visualized using 3,3'-diaminobenzidine as a substrate, which stains the target protein yellow. Negative controls were used. The primary antibody was replaced with PBS containing 0.1% bovine serum albumin. Tissues known to express the antigen studied were used as positive controls. VEGF was localized in the cytoplasm and the membrane. Samples were classified as negative when the number of positive cells was <25% and positive when there were visible brown particles and the number of positive cells was ≥ 25%. VEGF, which promotes angiogenesis and proliferation of endothelial cells, also exerts an important effect in the establishment, development, metastasis, and recurrence of various tumors. The CD105-MVD was evaluated under light microscopy according to the procedure described by Kopczyńska *et al* [3]. Briefly, after scanning the sections at low magnification, five tumor areas with the greatest number of distinctly highlighted microvessels were selected. The number of microvessels was counted in the each field at high magnification (400×) and the average counts of the fields were recorded. Each brown-stained endothelial cell or endothelial cell cluster, which was clearly separate from the adjacent microvessels, tumor cells, and connective tissue elements was considered a single, countable microvessel. The pathological categorization was determined according to the current World Health Organization classification system (WHO 2012) [4]. The pathological diagnosis was independently verified using histology by two pathologists blinded to the subject's clinical history and immunohistochemistry results. The pathological reading was determined for each biopsy slide and an overall pathological diagnosis determined for each subject. The tumor grade was determined according to the modified Bloom-Richardson score. The grade was

Table 1 Abnormal expression of VEGF in different tissues

Groups	*n*	–	+	χ^2	*P* value
Invasive breast carcinoma	128	46	82 (64.1%)	0.96	0.03
Benign breast lesions	50	39	11 (22.0%)		

Table 2 Correlation between VEGF and biological parameters of invasive breast cancer

Biological parameters	*n*	–	+	χ^2	*P* value
Age at diagnosis (years)					
≤ 50	60	19	41	0.51	0.48
> 50	68	27	41		
Tumor size (cm)					
≤ 2	46	16	30	0.14	0.71
> 2	82	30	52		
Histological grade					
I + II	96	39	57	15.70	0.00
III	32	7	25		
Lymph node metastasis					
Present	83	14	69	13.02	0.00
Absent	45	32	13		
Distant metastasis					
Present	37	7	30	0.51	0.48
Absent	91	39	52		

obtained by summing the scores for tubule formation, nuclear pleomorphism, and mitotic count, which were scaled as 1, 2, or 3. The final scores ranged between 3 and 9 and were then divided into three grades (I–III). The final grading scores were as follows: total points, 3–5, grade I; 6–7, II; and 8–9, III.

Statistical analysis

SPSS 17.0 statistical software was used to analyze the data. Results are expressed as mean ± SD ($\bar{\chi} \pm s$). Measurement data between groups was compared with a t-test. Enumeration data was compared using a χ^2-test. The *P*-value was considered to be significant if it was smaller than 0.05.

Results

Expression of VEGF and relationship with clinical-pathological parameters

The positive rate of VEGF in invasive breast cancer was 64.1% and was higher than in hyperplasia breast tissues (22.0%), ($\chi^2 = 0.957$, $P = 0.028$), as shown in Table 1.

There was a positive correlation in overexpression of VEGF with the histological grade, lymph node metastasis, and distant metastasis in invasive breast cancer. VEGF expression was not correlated with age and size of tumor ($P > 0.05$), as shown in Table 2.

Table 3 Abnormal expression of MVD recognized by CD105 in different tissues

	n	MVD ($\chi \pm s$)	*t* value	*P* value
Invasive breast carcinoma	128	31.69 ± 8.62	22.17	< 0.01
Benign breast lesions	50	10.04 ± 3.98		

Abnormal expression of MVD and relationship with clinical-pathological parameters

Various tissues from invasive breast cancer, benign breast lesion, and adjacent epithelium were stained by immunohistochemistry for CD105. MVD was assessed based on the number of CD105-positive vessels. Immunohistochemical results were shown in Tables 3 and 4. The MVD based on CD105 expression had statistical significance ($P < 0.01$) in invasive breast cancer (31.69 ± 8.62) and benign breast lesion groups (10.04 ± 3.98) (Table 3).

MVD of the peripheral area adjacent to the lesion was significantly higher than that of the central area within the lesion in every group ($P < 0.01$ for each group), as shown in Table 4.

We also examined clinical-pathological parameters and their relationship with CD105-MVD expression in invasive breast cancer, as shown in Table 5. There were significant differences in the mean abnormal expression of CD105-MVD between estrogen and/or progesterone receptor (ER/PR)-positive and -negative samples from invasive breast cancer patients; samples of invasive breast cancer with a histological grade of (I + II) and grade III; and samples of lymph node metastasis and distant metastasis ($P < 0.05$). However, there was no difference in the mean expression of CD105-MVD between the two age groups at diagnosis (≤ 50 years and > 50 years) and the two groups of tumor size (≤ 2 cm and >2 cm) ($P > 0.05$).

Discussion

Breast cancer is the most common form of cancer disease in women and the second leading cause of death by a malignant disease in females after lung cancer [5]. In recent years, the incidence of breast cancer has risen significantly in both the East and West. Therefore, breast cancer treatment, effective assessment of therapies, precise prognosis, and early detection of postoperative recurrence in patients have recently garnered more attention.

VEGF is capable of promoting angiogenesis and plays an important role in the establishment, development, metastasis, and recurrence of various tumors. In the process of tumorigenesis and development, tumor regenerative capillaries capable of providing nutrients to tumor cells and favorable conditions for distal metastasis are the precondition to induce local growth, infiltration, and distal metastasis of malignant tumors. Inhibition of tumor angiogenesis is currently a hot research topic [6]. VEGF,

Table 4 Abnormal expression of MVD in different areas within the lesions

Groups	*n*	CD105-MVD ($\bar{\chi} \pm s$)		*t* value	*P* value
		Central area within the lesion	Peripheral area adjacent		
Invasive breast carcinoma	128	10.23 ± 6.13	30.36 ± 3.59	47.28	0.00
Benign breast lesions	50	0.00 ± 0.00	10.04 ± 3.98		

Table 5 Correlation between MVD and biological parameters of invasive breast cancer

Biological parameters	*n*	CD105-MVD		
		MVD ($\chi \pm s$)	*t* value	*P* value
Age at diagnosis (years)				
≤ 50	60	29.89 ± 5.56	1.09	0.28
> 50	68	31.28 ± 5.22		
Tumor size (cm)				
≤ 2	46	34.25 ± 7.98	1.82	0.07
> 2	82	37.00 ± 8.36		
Histological grade				
I + II	96	27.77 ± 7.63	8.27	< 0.01
III	32	40.13 ± 6.28		
Lymph node metastasis				
Absent	45	22.94 ± 3.31	15.80	< 0.01
Present	83	39.55 ± 7.87		
Distant metastasis				
Absent	91	27.89 ± 6.62	10.84	< 0.01
Present	37	41.05 ± 5.13		

one of the strongest and most specific factors in promoting tumor angiogenesis, can not only induce proliferation of endothelial cells, but also regulate and participate in angiogenesis. Due to its intimate association with the genesis, development, metastasis, and infiltration of breast cancer, VEGF is an important indicator for metastasis and infiltration of breast cancer in the clinic. VEGF is localized in the cytoplasm and the membrane. In the study, VEGF expression in different pathological stages of patients was analyzed. The results revealed that, with increasing pathological stage, VEGF levels in the invasive breast carcinoma group gradually increased and the statistical significance was remarkably presented compared with grade I or II ($P < 0.01$). There was a significant difference between VEGF expression in the different pathological stages of patients ($P > 0.05$). In the study, VEGF expression in patients with and without lymph node metastasis were compared in the observation group. Results revealed that patients with lymph node metastasis had significantly and markedly higher VEGF expression than those without lymph node metastasis ($P < 0.01$). There was a positive correlation between VEGF overexpression and histological grade, lymph node metastasis, and distant metastasis in breast cancer. VEGF expression was not correlated with age and size of tumor ($P > 0.05$). In this study, VEGF expression was clearly higher in the recurrence group than in the non-recurrence group ($P < 0.01$). This result suggests that detection of VEGF can improve prognostic accuracy and assist in monitoring recurrence and metastasis.

In our study, we investigated the prognostic significance of CD105 and MVD assessed based on the number of CD105-positive vessels in various tissues from invasive breast cancer, benign breast lesions, and adjacent epithelium. Results showed that high expression of CD105 and MVD were significantly associated with poorer prognosis, a histological grade of III, tumor invasion, and lymph node metastasis. Tumor angiogenesis and its clinical significance have been studied in a variety of neoplasms [3, 7–8]. Studies suggest that angiogenesis contributes to the pathogenesis of various cancers, and MVD may improve our ability to predict breast cancer expansion. MVDs are significantly higher in primary tumors of patients with metastatic disease than in those without metastases [3, 7]. In addition, an association between MVD in the peripheral area adjacent to the lesion and in the central area within the lesion has been observed in invasive breast carcinomas and benign lesions groups. It is interesting that MVD in the peripheral tissue adjacent to the lesion is significantly higher than that in the central area within the lesion in breast cancer. CD105 is a proliferation-associated and hypoxia-inducible glycoprotein abundantly expressed in angiogenic endothelial cells and is essential in angiogenesis. The intensity of staining for CD105 is greater in blood vessel endothelia within neoplastic tissues than within normal tissues, indicating that CD105 is a powerful marker of neovascularization in solid malignancies [9]. However, despite advances in diagnostic techniques and surgical procedures, prognosis after resection has remained unsatisfactory due to a high incidence of cancer lymph node metastases and recurrence. The identification of variables in breast tumor biology may lead to a more precise assessment of outcome and response to therapy. Our results support that CD105-MVD is closely relevant to lymph node metastasis and breast cancer recurrence, and may act as a valuable indicator of prognosis. CD105-MVD may be useful as a predictor for the recurrence of breast cancer and have a specific association with the development of locoregional and hematogenous recurrence. In breast cancer, the potential application of CD105-MVD as a tumor angiogenesis marker for cancer diagnostics and clinical application is anticipated. CD105-MVD may also serve as a prognostic marker for breast cancer. A combination of biomarkers may improve the ability to identify cancer patients at high risk of disease [10–12]. Here, we showed for the first time that MVD by

CD105 indicates recurrence in patients with breast cancer. Significantly higher MVD was found in tumors with lymph node metastasis, advanced stage, and tumor recurrence. When tumors were divided into grades I–II and grade III, high MVD was also significantly associated with advanced grade (III). However, there was no difference in the mean CD105 levels and MVD between the two groups of age at diagnosis (≤ 50 years and > 50 years) and tumor size (≤ 2 cm and > 2 cm) ($P > 0.05$). Elevated expression of CD105 and MVD are associated with tumor progression, invasion, and metastasis. However, further studies are needed to understand the exact pathogenic mechanism. In breast carcinoma, abnormal expression of CD105 and MVD is associated with poor differentiation, similar to higher grade lesions and metastatic disease [13].

In summary, our results show that joint detection of VEGF and MVD can complement each other in the diagnosis, treatment, and prognosis of breast cancer, and can be regarded as a crucial reference indicator. VEGF and MVD expression is important in assessing lymph node metastasis, recurrence monitoring, and clinical staging of tumors in clinic. In conclusion, VEGF and CD105-MVD may be useful predictors of tumor metastasis and recurrence, thereby assisting the refinement of therapeutic decisions in breast cancer. Risk prediction for metastasis and recurrence, as well as recurrence patterns based on MVD after surgery, could help the design of better follow-up programs and appropriate treatment strategies for breast cancer patients. Future studies should therefore preferentially explore a broader target set of potential biomarkers.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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