

乳腺疾病病理学及基础研究专题 • 综述 •

An analysis of vascularity and neovascularization in surgical pathology materials of breast carcinoma and its clinical significance

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Breast cancer is one of the most common malignancies among women and its morbidity has recently increased in many parts of the world^[1]. Numerous factors have been reported to be associated with development of breast cancer including angiogenesis. Angiogenesis or the formation of new blood vessel networks, not only plays a pivotal role in human normal development, but also in pathophysiological conditions such as inflammatory diseases and neoplasms^[2], which consists of multiple coordinated, sequential and interdependent steps^[3]. The angiogenic program requires the degeneration of the basement membrane, endothelial cells migration and invasion of the extracellular matrix, with endothelial cell proliferation and capillary lumen formation before maturation and stabilization of the new vasculature^[3]. Results of previous studies demonstrated that normal breast adjacent to malignant breast induced angiogenesis twice as frequently as did tissues from non-neoplastic breast, suggesting that the angiogenic switch occurs before morphological changes are discernible^[3-4]. In addition, angiogenesis precedes, and is considered to be required for tumor progression and metastasis^[5]. When a new tumor reaches the size of 1—2 mm, its growth requires the induction of new blood vessels, which may consequently lead to the development of metastases, via the penetration of malignant cells into the circulation^[5]. The term “angiogenic switch” describes a hallmark of malignancy, in which the tumor initiates recruitment of its own blood supply by shifting the balance between stimulatory and inhibitory factors of angiogenesis^[6-7]. Numerous studies have been reported on the mechanisms of control or regulation

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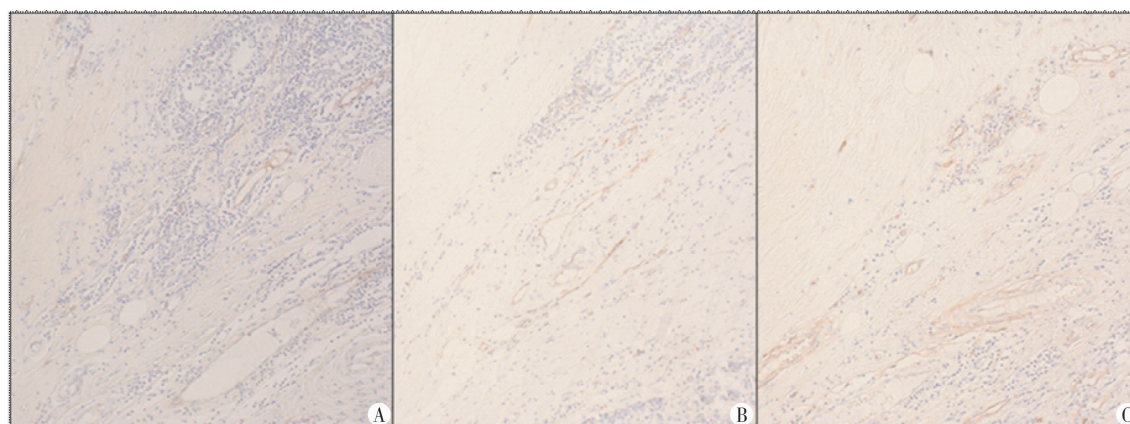
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of angiogenesis since the discovery of endothelial growth factor (VEGF) and angiopoietin family proteins^[7]. Other molecules involved in this process of angiogenesis, including pigment epithelium derived factor (PEDF), platelet factor 4, angiostatin and endostatin, have been proposed as angiogenesis inhibitors^[8]. In addition, the protein called vasohibin has been very recently demonstrated, one of the first established negative feedback regulators of angiogenesis^[2,7-8]. As for antiangiogenic therapy, antiangiogenic trials are under investigation in the adjuvant setting and antiangiogenic agents may be incorporated into the standard therapy of breast cancer treatment in the near future. In that case, it should become very important to evaluate the status of vascularity or neovascularization in the patients with breast carcinoma. This brief review article summarizes the analysis of vascularity and neovascularization in surgical pathology materials of breast carcinoma and its clinical significance.

1 Histopathological evaluation of angiogenesis in surgical pathology specimens of human breast cancer: immunohistochemical analysis of vessel-related protein

Histopathologic studies of angiogenesis in breast carcinoma have examined the possible correlation of tumor vascularity with known prognostic markers and/or clinical outcome of the patients, itself in order to evaluate its clinicopathologic significance^[9]. Immunohistochemical evaluation of various markers of vascular endothelial cells have been employed to identify the vessels. These markers include factor VIII (anti-human von Willebrand factor), CD34 and CD31 (Figure 1)^[9-10], and occasionally with integrin $\alpha_v\beta_3$, CD105 and type IV collagen^[11]. However, results of studies using these markers of vascular endothelial cells have not necessarily provided consistent findings in terms of determining the vascularity of breast cancer tissues and the optimal or appropriate methods evaluating angiogenesis in surgical pathology specimens have been desired in breast carcinoma^[9]. Results of previous studies demonstrated that CD31 was the most sensitive marker and consistently stained more vessels than did either factor VIII or CD34, and is most abundantly expressed in endothelial cells^[9-10]. However, results of several other studies did demonstrate that

anti-CD34 and anti-VWF were associated with better efficiency in terms of detecting the vessels than anti-CD31, although the staining results with different antibodies were generally comparable^[9,12].



A: factor VIII; B: CD31; C: CD34

Figure 1 Representative illustrations of immunohistochemical findings of breast carcinoma case (HE staining $\times 200$)

2 Histopathological technique to quantify intratumoral angiogenesis

Manual methods including microvascular density (MVD), Chalkley count and multiparametric computed image analysis system (CIAS) has been the most frequently used histopathological technique to quantify intratumoral angiogenesis in breast cancer specimens^[11,13-18]. In the analysis of MVD, “microvessels” are identified based on the architecture, i. e., morphologically identifiable lumen lined by endothelial cells, complemented by immunopositivity of the endothelial cells with vascular markers after scanning the immunostained section at low magnification ($\times 40$ and $\times 100$)^[13]. Individual microvessels are subsequently counted at a higher power ($\times 200$ field) in an adequate area (0.74 mm^2 per field using $\times 20$ objective lens and $\times 10$ ocular)^[13]. Any stained endothelial cells or clusters separate from adjacent vessels are counted as a single microvessel, even in the absence of vessel lumen^[13]. Each single count is expressed as the highest number of microvessels identified at these hot spot^[13]. Results of several previous studies demonstrated that MVD is correlated with aggressive tumor behavior, such as

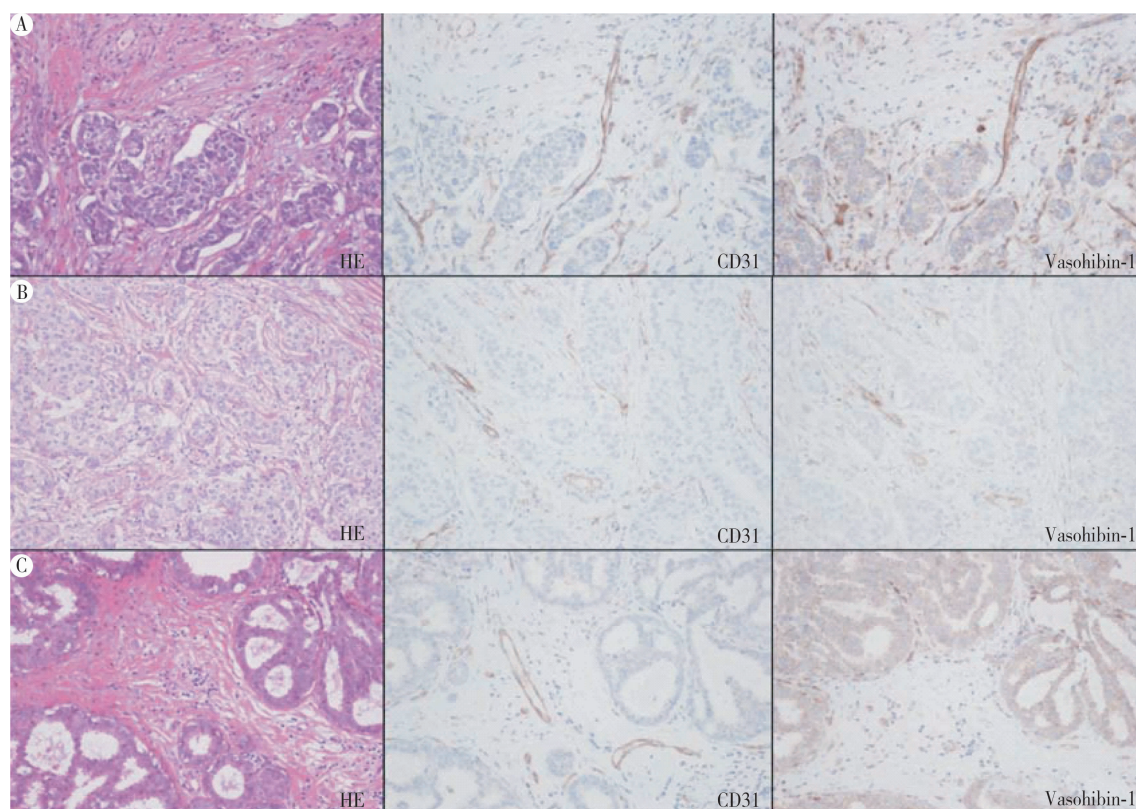
higher stage at presentation, greater incidence of metastasis and decreased patient survival^[13-14]. The Chalkley counting method is another method of evaluating vascularity in surgical pathology specimens of human breast carcinoma and its reproducibility has been evaluated, and the prognostic value confirmed in a large population in an extensive manner^[15-17]. Briefly, the three most vascular areas (hot spots) with the highest number of microvessel profiles are selected in the tumor section in a relatively subjective manner^[16-17]. A 25 point Chalkley eyepiece graticule is then applied to each hot spot area at $\times 250$ magnification with a corresponding Chalkley grid area of 0.196 mm^2 ^[15-17]. The graticule is orientated to permit hitting on the maximum number of points, or within the areas of immunohistochemically highlighted microvessel profiles^[15-17]. The Chalkley count for an individual tumor is taken as the mean value of the three graticule counts^[15-17]. This estimate is categorized according to predefined cut off points at 5 and 7, and used for further prognostic analysis^[15-17]. It takes approximately 5 min for individual pathologists to assess each tumor^[17]. In addition, CIAS could allow for a more objective and reproducible evaluation of tumor angiogenesis and may be used not only to count the number of microvessels within the hot spots, but also to measure other morphologic parameters related to vascularization that may be useful to add further biological and prognostic information^[18]. In addition, CIAS method may be of help to assist a non- experienced pathologist in the evaluation of tumor angiogenesis^[18]. However, this method usually required expensive equipments or instruments compared to previous two methodologies.

3 Newly identified biomarker of angiogenesis

Vasohibin-1 is a recently identified negative feedback inhibitor or suppressor of angiogenesis induced by VEGF^[2,7,19]. Vasohibin-1 was subsequently demonstrated to be specifically expressed in endothelial cells (EC) in response to angiogenic stimulators such as VEGF and basic fibroblastic growth factor (bFGF)^[2,7,19]. Vasohibin-1 is also abundantly

present in human placenta and fetus in which angiogenic event markedly occurred in vivo. VEGF-A is the most potent factor for angiogenesis among known VEGF family members, stimulating protease synthesis, migration and proliferation of EC^[2,7,19]. In addition, the great majority of VEGF-A mediated signals are trasduced *via* VEGF-A receptor 2 (Flk-1) and protein kinase C δ (PKC δ), one of the signals located in important downstream intrasignaling pathway of Flk-1, and they also induced vasohibin-1 expression markedly^[2,7,19]. Our previous study is the first study demonstrating the status of vasohibin-1 in human breast lesions, which indicated that vasohibin-1 is associated with neovascularization and may especially play important roles in the regulation of intratumoral angiogenesis in breast cancer^[2,20]. Vasohibin-1 immunodensity (Figure 2) tended to be consistent with MVD in human breast tissues but they were not always parallel^[2]. The vasohibin-1 immunodensity was significantly higher in IDC than in DCIS but no significant differences of MVD were detected between these two lesions^[2]. In addition, results of double immunostaining analysis which could simultaneously demonstrate two different proteins in the same cells, demonstrated the significant positive correlation between Ki-67 positive proliferating vascular endothelial cells, which may represent neovascular formation and vasohibin-1 positive endothelial cells^[2]. Indeed, the Ki-67 labeling index among vasohibin-1 positive endothelial cells was significantly higher than that in all CD31 positive endothelial cells^[2]. These results clearly demonstrated that vasohibin-1 is considered a more appropriate biomarker for intratumoral neovascularization compared to CD31, which may detect all the vasculartures including both resting and proliferating endothelial cells^[2]. Result of this study also demonstrated that the positive correlation between vasohibin-1 and VEGF-A or bFGF in carcinoma cells or Flk-1 in intratumoral endothelial cells, which also suggest that the vasohibin-1 in vasculature in human breast carcinoma is induced by VEGF-A, bFGF/Flk-1 signaling pathway^[2]. Results of this study demonstrated that the cases with a higher number of vasohibin-1 positive vessels tended to

be associated with worse and statistically significant OS of the patients (Figure 3A)^[2]. In addition, a statistically negative or inverse correlation was detected between vasohibin-1 immunodensity and DFS of the patients (Figure 3B)^[2]. These results all suggest that an evaluation of the number of vasohibin-1 positive vessels may become one of the prognostic markers for metastasis and prognosis but it awaits further investigations to establish this approach as a surrogate marker such as MVD^[2]. In addition, result of our another study demonstrated that increased vasohibin-1 expression was associated with both increased cell proliferation of carcinoma cells and higher nuclear and histological grade^[20]. Therefore, these results clearly indicated that vasohibin-1 expression in DCIS could become one of the appropriate biomarker of the potential of subsequent stromal invasion of carcinoma cells^[20].



A and B: two invasive ductal carcinoma (IDC) cases stained positively for CD31 and vasohibin-1;

C: a ductal carcinoma in situ (DCIS) case stained positive only for CD31 and not for vasohibin-1

Figure 2 Representative illustrations of histological and immunohistochemical findings of breast carcinoma cases examined (HE staining $\times 200$) (Cancer Sci 2009)

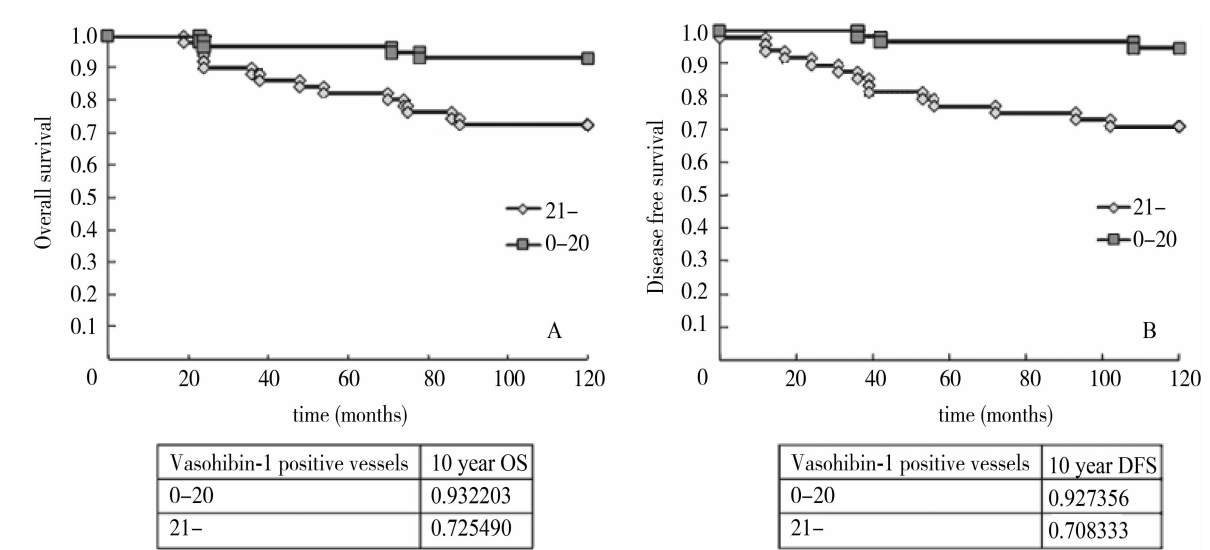


Figure 3 Analysis of (A) overall survival and (B) disease free survival in relation to the status of vasohibin-1 expression (Cancer Sci 2009)

4 Anti-angiogenic therapy

It has been suggested that inhibition of blood vessel formation could be a therapeutic target in solid tumors because many breast cancer cases require neovascularization^[21]. VEGF is one of the most potential promoters of angiogenesis involved in endothelial cells proliferation and motility and blood vessel permeability^[22]. Over-expression of VEGF has been detected in a variety of malignancies including breast cancer, and is associated with a worse relaps-free and overall survival compared with non-over expressing cancers^[23-24]. Bevacizumab is a humanized monoclonal antibody to VEGF that recognized all isoforms of VEGF-A^[25]. Preclinical models have demonstrated regression of solid tumor growth and angiogenesis with anti-VEGF monoclonal antibodies alone or in combination with chemotherapy^[25-26].

Adjuvant therapy trials are being carried out with bevacizumab and numerous other anti-VEGF agents are now being tested in the patients with breast cancer in various settings. It was demonstrated in the 11th St Gallen (Switzerland) expert consensus meeting on the primary treatment of early breast cancer that drugs that target angiogenesis might, in the long run, induce angiogenesis as a rebound phenomenon and have been demonstrated in preclinical studies to induce tumor progression and metastasis^[27]. A possible mechanism for this tumor progression may be the release of

increasing numbers of circulating endothelial cells following some types of chemotherapy^[27]. Importantly, this effect is not seen with metronomic chemotherapy^[28]. Long-term treatment with antiangiogenic drugs together with metronomic chemotherapy was associated with dramatic and profound reduction of VEGF and substantial clinical response in metastatic breast cancer^[27]. The type of cancer vascularization and the extent of VEGF targeting might be a crucial strategic issue in the treatment of malignancies^[27]. In addition, it was also demonstrated in American Society of Clinical Oncology (ASCO) 2009 educational book, that the combination of anti-HER2 and antiangiogenic agents, as well as those agents that target the downstream HER-2 signaling pathway, are areas of active research that may prove to be effective in improving targeted treatment in breast cancer^[28]. Antiangiogenic trials are under investigation in the adjuvant setting and antiangiogenic drugs will be added to the standard therapy for breast cancer treatment in the very near future.

【Key words】 Breast neoplasms; Vascularity; Neovascularization; Surgical pathology; Materials

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