Evaluation of the Correlation between Colour Power Doppler Flow Imaging and Vascular Endothelial Growth Factor in Breast Cancer

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This study was designed to evaluate the correlation between blood flow, using colour power Doppler flow imaging [CPDI], and protein levels of vascular endothelial growth factor (VEGF), as measured by optical density (OD), in breast tumours. Breast cancer patients were observed pre-operatively using CPDI and VEGF protein levels were quantified by immunohistochemistry, and the correlation between the two was studied. The relationship between tumour angiogenesis and axillary lymph node (LN) metastasis was also analysed. Blood-flow grade was higher in the LN+ group than in the LN− group; grade II – III blood flow was 88.9% in the LN+ group. The VEGF protein levels in the LN+ group were also higher than in the LN− group. A significant positive correlation was observed between blood-flow grade and OD value for VEGF protein. Breast tumour angiogenesis was closely correlated with axillary LN metastasis. Higher blood flow was related to elevated VEGF protein levels and an increased risk of axillary node metastasis. CPDI could, therefore, indirectly demonstrate tumour angiogenesis before surgery, enabling planning of treatment and assessment of the prognosis.

KEY WORDS: BREAST NEOPLASM; ULTRASONOGRAPHY; COLOUR POWER DOPPLER FLOW IMAGING (CPDI); VASCULAR ENDOTHELIAL GROWTH FACTOR; ANGIOGENESIS; IMMUNOHISTOCHEMISTRY

Introduction

Breast cancer angiogenesis has a substantial role in tumour progression and metastasis, and is closely correlated with prognosis.¹ The close relationship between angiogenesis and tumourigenesis, and the biological behaviour of tumours has been confirmed.² Vascular endothelial growth factor (VEGF) is a type of ω-glycoprotein with a relative molecular weight of 45 kDa. It can influence the growth and angiogenesis of tumours through autocrine and paracrine mechanisms.³ VEGF has an important role in promoting the mitosis of vascular endothelial cells, directly inducing tumour angiogenesis and increasing the...
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permeability of new vessels.4 These biological effects are carried out through the combination of VEGF and its specific receptor in endothelial and tumour cells. Levels of VEGF protein can, therefore, indicate the level of tumour vascularization and is the strongest, most specific factor promoting angiogenesis in tumours.5–7

In the present study, colour power Doppler flow imaging (CPDI) and immunohistochemistry were applied to evaluate vascularization in breast cancer. The relationship between breast cancer angiogenesis and metastasis was also analysed.

Patients and methods

PATIENTS

Patients with breast carcinoma who were referred for surgical treatment at the Sixth People’s Hospital Affiliated to Shanghai Jiao Tong University (Shanghai, China) between October 2008 and August 2009 were enrolled sequentially in this study. The study was approved by the Institutional Ethics Committee of the Sixth People’s Hospital Affiliated to Shanghai Jiao Tong University and written informed consent was obtained from all study subjects.

INSTRUMENTS AND METHODS

A colour Doppler diasonography HDI 5000 (Philips Medical Systems, Bothell, WA, USA) with a linear array transducer (5 – 12 MHz) was used. The breast was initially scanned with grey-scale ultrasonography in order to visualize the target lesion and measure its maximum diameter. Then, CPDI was performed in order to evaluate vascularization within the lesion. Imaging settings (output power, gain and pulse repetition frequency [range 700 – 1500 Hz]) were adjusted for each patient to optimize flow visualization. Blood flow in the breast lesion was classified into one of four grades according to the method established by Adler et al.:8 grade 0, no blood flow; grade I, small amounts of flow, one or two punctiforms or short rod-shapes of colour flow signal; grade II, medium amounts of flow, one main vessel or a few small vessels could be found as a long strip of a curve; and grade III: rich flow, more than four vessels can be found with different diameters and twists or helical colour flow signals. The axillary area was examined to determine whether lymph nodes were swollen and this was compared with histopathological examination after surgery.

IMMUNOHISTOCHEMICAL STAINING

Tumour specimens were fixed in 10% formalin and subject to routine paraffin embedding, and 5-µm serial sections were prepared. Antigen retrieval was carried out by incubating in 0.1 mM citrate buffer, pH 6.0, at 98 °C for 20 min, followed by 10% goat serum at 37 °C for 30 min. Sections were then incubated with a rabbit antihuman VEGF polyclonal primary antibody (BA2090, 1:100; Boster Biological Technology Co, Wuhan, China) for 12 h at 4 °C and then washed three times for 15 min in 0.01 mM phosphate-buffered saline (PBS), pH 7.3. Sections were then incubated with a biotinylated goat antirabbit antibody (1:200; ZhongShan Biological Technology Co., Beijing, China) for 30 min at 37 °C and washed three times, each time for 5 min (total of 15 min) with 0.01 mM PBS. Immunoreactivity for VEGF was visualized using diaminobenzidine (Fuzhou Maixin Biotechnology Development Co., Ltd, Fuzhou, China). The negative control used 0.01 mM PBS as a replacement for the primary antibody.

Tumour cells that were ‘buffy-coated’ or
had dark brown particles distributed in the cytoplasm were defined as being positive for VEGF protein. The VEGF protein levels were quantified by measuring the optical density (OD) at a wavelength of 255 nm, using a digital medical image analysis system (Motic 6.0 imaging software system; Xiamen Motic Instrument Co. Ltd, Xiamen, China), excluding haemorrhagic regions and reacting regions in the verge. Immunohistochemical analyses were carried out by a pathologist with 10 years’ diagnostic experience. The mean OD value was calculated based on 10 vision fields.

STATISTICAL ANALYSIS
Statistical analyses were performed with SPSS® statistical package, version 12.0 (SPSS Inc., Chicago, IL, USA) for Windows®. The relationship between VEGF protein levels and axillary lymph node metastasis was examined using the $\chi^2$-test. The correlation between the OD values of VEGF-positive cells and blood-flow grade was determined by Pearson’s correlation coefficient. A $P$-value < 0.05 was considered to be statistically significant.

Results
The study included 42 patients (mean ± SD age 52.2 ± 10.9 years; range 33 – 80 years). These included 38 cases of infiltrating ductal carcinoma, two of infiltrating lobular carcinoma, one of inflammatory breast cancer and one of ductal carcinoma in situ. Pathological analyses revealed that 27 cases had axillary lymph nodule metastasis (LN+) and 15 did not (LN−) (Table 1).

The CPDI detection of tumour blood-flow signals was 97.6% overall (100% in the LN+ group and 93.3% in the LN− group; a non-significant statistical difference). There was a statistically significant difference between the two groups in the percentage of tumours with grade II – III blood flow (88.9% in the LN+ group and 53.3% in the LN− group, $P < 0.05$). A representative image of grade III blood flow signals in a breast cancer tumour from a patient in the LN+ group is shown in Fig. 1.

The VEGF protein was detected in the cytoplasm and cell membrane of tumour cells, and was visible as widespread or sporadic ‘buffy-coated’ particles (Fig. 2). Of the 42 breast tumours examined, 35 (83.3%) were positive for VEGF immunoreactivity. Endothelial cells of the tumour interstitium and ambient tumour tissue were either negative or weakly positive for VEGF protein. Both the percentage of cases positive for VEGF immunoreactivity and the mean OD were significantly higher in the LN+ group compared with the LN− group ($P < 0.001$; Table 1). There was a notable positive correlation between blood-flow grade and OD ($r = 0.61885$, $P < 0.05$; Fig. 3).

### TABLE 1:
Relationship between the presence of vascular endothelial growth factor (VEGF) protein in breast cancer tumour cells and lymph node metastasis in 42 breast cancer patients

<table>
<thead>
<tr>
<th>Metastasis</th>
<th>Total No. of patients</th>
<th>VEGF protein positive $n$ (%)</th>
<th>Optical density (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN+</td>
<td>27</td>
<td>25 (92.6)</td>
<td>0.6424 ± 0.1540</td>
</tr>
<tr>
<td>LN−</td>
<td>15</td>
<td>10 (66.7)***</td>
<td>0.2896 ± 0.1277***</td>
</tr>
</tbody>
</table>

***$P < 0.001$ compared to the LN+ group.
LN+, lymph node metastasis positive; LN−, lymph node metastasis negative.
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FIGURE 1: Representative colour power Doppler flow image of grade III blood flow signals in a breast cancer tumour from the lymph node positive (LN⁺) group. There was a significant difference between the LN⁺ group and the lymph node negative (LN⁻) group in tumours with grade II – III blood flow.

FIGURE 2: Representative immunohistochemical staining from the lymph node positive (LN⁺) group showing vascular endothelial growth factor protein (staining) present in the cytoplasm and cell membrane of breast cancer tumour cells (scale bar 50 µm)
Discussion

Angiogenesis is a critical process for the growth, invasion and metastasis of tumours.\textsuperscript{9 – 11} Folkman\textsuperscript{12} classified tumour vascularization in the vascular prophase and angiogenesis stage. In the former the tumour grows slowly, with linear growth and a size less than a few millimetres, whereas in the angiogenic stage, the tumour shows potential growth and visible metastatic potency. Malignant breast lesions typically show increased Doppler signals due to tumour neovascularization.\textsuperscript{13 – 15}

It is known that VEGF is a type of D-glycoprotein, with a relative molecular weight of 45 kDa, that can influence the growth and angiogenesis of tumours through autocrine and paracrine mechanisms.\textsuperscript{3} VEGF has an important role in promoting the mitosis of vascular endothelial cells, directly inducing tumour angiogenesis and increasing the permeability of new vessels.\textsuperscript{4} These biological effects are mediated through the combination of VEGF and its specific receptor in endothelial and tumour cells. VEGF expression can, therefore, indicate the level of tumour vascularization and is the strongest, most specific factor promoting angiogenesis in tumours.\textsuperscript{5 – 7} VEGF levels can reflect the degree of vascularization in tumours but only after resection of the primary tumour, since VEGF expression can only be examined via immunohistochemistry. Studies have, therefore, focused on obtaining information regarding angiogenesis before surgical excision.\textsuperscript{16}

The technique of CPDI is based on an integrated power spectrum; it is angle-independent and is not subject to aliasing.

\footnotesize{FIGURE 3: Correlation between the optical density of vascular endothelial growth factor (VEGF)-positive cells and blood-flow grades (according to Adler \textit{et al.}\textsuperscript{8}), as assessed by immunohistochemistry and colour power Doppler flow imaging, respectively, in breast cancer tumours (Pearson’s correlation coefficient, \( r = 0.61885, P < 0.05 \))}

CPDI could more accurately determine tumour perfusion, but quantification is difficult due to lack of a suitable index. Cosgrove et al. observed vessel distribution in tumours using a quantitative method, analysing the colour flow signal by computer; the vessel number per cm² and the percentage of colour pixels per total area were calculated, however this method proved tedious and time-consuming.

In the present study, a more convenient semi-quantitative method (as proposed by Adler et al.) was adopted to estimate the degree of blood flow in breast tumours, and immunohistochemistry was used to detect the presence of VEGF protein. The digital medical image analysis system objectively quantified VEGF protein and reduced subjective influences in microvessel counting by human operators. The positive correlation between blood-flow grade and OD value for VEGF protein observed in tumours in the present study demonstrated a consistency between microscopic vascularization activity and macroscopic blood-flow signals. Vascularization and blood-flow were positively correlated with axillary node metastasis, since the LN⁺ group showed both higher blood-flow grades and VEGF expression. It has been suggested that breast cancer angiogenesis is regulated by vascular factors secreted by tumour cells and has a close relationship with axillary nodule metastasis.

In conclusion, in the present study, the degree of blood flow determined by CPDI was found to correlate with the levels of VEGF protein, as assessed by immunohistochemistry. Blood flow within tumours and axillary node metastasis could, therefore, be demonstrated more accurately through a combination of these two methods. In patients with breast cancer, the increased blood flow shown by CPDI indicated increased VEGF levels, together with a higher risk of axillary node metastasis. Pre-operative CPDI examination in patients with breast carcinoma could, therefore, be used to evaluate tumour angiogenesis indirectly and to provide a useful reference regarding treatment selection and prognosis.

**Conflicts of interest**
The authors had no conflicts of interest to declare in relation to this article.

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**References**


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