HER2/neu expression correlates with vascular endothelial growth factor-C and lymphangiogenesis in lymph node-positive breast cancer

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Background: Vascular endothelial growth factor-C (VEGF-C) is the main inducer of lymphangiogenesis. VEGF-C overexpression is associated with lymphovascular tumor cell invasion, an increased rate of lymph node metastasis and adverse prognosis in various human cancers. However, little is known about the upstream inducers of VEGF-C expression. Recent studies have shown that human epidermal growth factor receptor 2 (HER2/neu) overexpression is associated with high VEGF-C levels in human breast cancer cells. In addition to blocking of HER2/neu, tyrosine kinase significantly decreased VEGF-C expression in vitro.

Patients and methods: VEGF-C expression, lymphatic microvessel density (LMVD), lymphovascular invasion (LVI) and HER2/neu expression were evaluated with immunohistochemical/FISH methods in a collective of 150 lymph node-positive human breast cancers with long-term follow-up.

Results: Cases with 3+ HER2/neu protein expression showed a significantly stronger VEGF-C expression than all other cases (P = 0.006). In addition, we found a significant correlation between VEGF-C expression and LMVD (P = 0.012) and a strong positive association between LMVD and LVI (P < 0.001).

Conclusion: Our data provide evidence for a clinically relevant association between HER2/neu and VEGF-C expression in human breast cancer. Inhibiting HER2/neu may reduce tumor progression by blocking VEGF-C-mediated tumor cell proliferation and lymphogenic metastasis.

Key words: breast cancer, HER2/neu, lymphangiogenesis, lymph node metastases, VEGF-C

Introduction

The discovery of the human epidermal growth factor receptor 2 (HER2/neu gene) and the subsequent development of targeted therapies against its protein product can be considered as one of the major recent advances in the therapy of breast cancer [1, 2].

The HER2/neu gene encodes for a transmembrane protein, which is involved in the regulation of cellular proliferation, differentiation and survival [3]. HER2/neu gene amplification occurs in 15%–30% of breast cancers and is associated with an aggressive tumor phenotype and poor prognosis [3]. Although overexpression of HER2/neu increases the metastatic potential of cancer cells, the underlying mechanisms remain mostly unknown [4].

Due to the discovery of specific lymphatic endothelial cell markers and the lymphatic endothelial growth factor vascular endothelial growth factor-C (VEGF-C), lymphangiogenesis, the formation of new lymphatic vessels, has become one of the expanding fields of tumor research over the past years [5].

Vascular endothelial growth factor-C (VEGF-C) overexpression, a subsequent increase in lymphangiogenesis and a higher rate of lymphovascular invasion (LVI), has been shown to worsen breast cancer prognosis [6, 7].

Recently, HER2/neu kinase stimulation by heregulin-β1 was shown to up-regulate VEGF-C expression in the human breast cancer cell line MCF-7, following activation of p38 MAP kinase and nuclear factor ‘kappa-light-chain-enhancer’ of activated B cells. This VEGF-C overexpression was significantly inhibited by the HER2/neu tyrosine kinase inhibitor PD153035 [8].

These data indicate a possible key role of HER2/neu in the regulation of VEGF-C-modulated tumor lymphangiogenesis.

Aim of this study was to explore the impact and clinical relevance of HER2/neu expression and gene amplification on VEGF-C expression, lymphangiogenesis and LVI in a large cohort of lymph node-positive breast cancers.

Patients and methods

Patients and tissues

The study population consisted of 150 randomly collected cases with stage II node-positive invasive breast cancer according to International Union
Against Cancer (UICC) that participated in one of five prospective, randomized, multicenter trials conducted by the Austrian Breast and Colorectal Cancer Study Group. All cases included in this study were from one center [9]. Special care was taken to include only sections with sufficient amounts of normal breast tissue directly adjacent to the invasive tumor. Due to a lack of sufficient material, anti-podoplanin immunostaining was only carried out in 112 cases.

All patients underwent breast surgery and dissection of axillary lymph nodes, containing at least 10 nodes.

immunohistochemistry

Immunohistochemistry was carried out on paraffin-embedded tumor specimens fixed in 4% buffered formalin. Four-micrometer-thick histological slides were deparaffinized in xylol and heated in 0.01 M citrate buffer for 16 min in a microwave oven. After cooling for 20 min and washing in phosphate-buffered saline (PBS), endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 min, followed by incubation with PBS containing 10% normal goat serum for 30 min.

For immunostaining of podoplanin, specimens were incubated at 20°C with the polyclonal rabbit antibody in a dilution of 1 : 200 for 1 h. Rabbit anti-human podoplanin IgG was raised against the recombinant human homologue of the rat 43-kDa glycoprotein podoplanin as described previously [10].

For immunohistochemical detection of VEGF-C protein expression, a polyclonal anti-VEGF-C antibody (Zymed Laboratories, South San Francisco, CA) was incubated at 20°C in a dilution of 1 : 800 for 1 h.

Positive staining reaction was illuminated using biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), for 30 min at 20°C followed by a streptavidin–peroxidase complex, according to the manufacturer’s instructions. As chromogens diaminobenzidine (DAB; SERVA, Heidelberg, Germany) or 3-amino-9-ethylcarbazole (BioGenex, San Ramon, CA) were used, respectively. Slides were counterstained with hematoxylin.

For negative control, a slide was prepared from the same tissue block and a preimmune serum or PBS was used instead of the primary antibodies. A block of cervical cancer used in a previous study served as positive control for VEGF-C and podoplanin [11].

Immunohistochemistry for HER2/neu protein expression was carried out using the HercepTest® (Dako, Glostrup, Denmark), according to the manufacturer’s instructions. Briefly, after deparaffinization and rehydration, tissue sections were immersed in epitope retrieval solution for 40 min at 95°C. After cooling for 20 min at room temperature, endogenous peroxidase activity was blocked by incubation with the provided reagent for 5 min. The primary polyclonal antibody against rabbit anti-human c-erbB-2 oncprotein (A0485; DAKO) or negative control reagent was applied followed by incubation for 30 min at room temperature. After washing, the specimens were incubated with visualization reagent (provided) and applied with DAB substrate chromogen. Counterstaining was carried out with hematoxylin.

morphometry

Determination of lymphangiogenesis quantified as lymphatic microvessel density (LMVD) assessed by immunostaining for podoplanin was carried out as indicated by Weidner [12]. In brief, after scanning the immunostained section at low magnification (×40), the area of tissue with the greatest number of distinctly highlighted microvessels (hot spot) was selected. LMVD was then determined by counting all immunostained vessels at a total magnification of ×200 corresponding to an examination area of 0.7386 mm². Determination of the staining reaction was strictly confined to the hot spots. LVI was considered evident if at least one tumor cell cluster was clearly visible inside the podoplanin-stained vascular space.

Intensity of immunostaining of VEGF-C in cancer cells was graded semiquantitatively as strong, medium or weak-to-absent expression.

The expression of HER2/neu was classified as negative, negative (1+), weakly positive (2+) or positive (3+) depending on the immunostaining pattern of the cellular membrane, following the diagnostic criteria of HercepTest®.

Estrogen receptor density was determined using the dextran–charcoal method from snap-frozen tumor samples as described previously. For definition of estrogen receptor positivity, cut-off values of >10 fmol/l were used [13, 14].

Two independent observers (SFS and PB) carried out the analysis of immunohistochemistry. The mean values of results from both observers were used for all further calculations. If differences of >30% between observers occurred (evident in <10% of cases), these slides were reinvestigated by both investigators on a multileveled microscope.

FISH

HER2/neu gene amplification in all 3+ and 2+ cases at HercepTest®, as well as in 79 randomly selected 0 or 1+ cases, was investigated using the PathVysion Gene Detection System (Vysis Inc., Downers Grove, IL) as described previously [15].

Slides were analysed using 4'-6-diamidino-2-phenylindole (DAPI), single (orange and green) bandpass and triple bandpass filters with an Olympus BX 60 fluorescence microscope. Slides were scanned at low power (×10 objective) using the DAPI filter to identify areas with optimal tissue digestion and areas with tumors. At least 100 and up to 200 cells were counted from all tumor areas in accordance with the standardized counting guide included in the assay kit. All cases with more than a mean number of four fluorescence signals per two signals of the centromere of chromosome 17 were considered amplified [16]. Specimens were considered negative when <10% of tumor cells showed amplification of HER2/neu, paralleling the situation in immunohistochemistry.

statistics

Spearman’s coefficient of correlation, regression analysis, Kruskal–Wallis U-test and Mann–Whitney U test were used as appropriate.

Overall survival (OS) was defined as the interval between the day of surgery and the patient’s death. Data on patients who had survived until the end of the observation period were censored at their last follow-up visit.

Death from another cause than breast cancer or survival until the end of the observation period was considered censoring events. Disease-free survival (DFS) was defined from the end of primary therapy until first evidence of progression of disease.

Univariate analysis of OS and DFS was carried out as outlined by Kaplan and Meier [17]. The Cox proportional hazards model was used for multivariate analysis of OS and DFS. VEGF-C expression, patient’s age at time of diagnosis, histological grading and tumor stage were included into the analysis.

For all tests, a P value of 0.05 was considered as significant. All P values given are results of two-sided tests.

results

clinical data

The mean patient’s age at time of surgery was 51.3±10.27 years (median 50 years, range 27–70 years). Eighty-one patients (54%) were premenopausal, 65 (43%) were postmenopausal, and in 4 patients (2.7%), the status was not known. Mean estrogen receptor density was 70.5±99.6 fmol/l. One hundred and twenty-six (84%) patients were estrogen receptor positive and 20 (13.3%) were staged as receptor negative. In four
patients, the receptor status was not determined due to lack of material. As surgical treatment, breast conservation (usually wide excision) was carried out in 27 patients (18%) and mastectomy in 123 (82%). After breast conservation, the majority of patients were treated with adjuvant radiotherapy, except for a small subgroup of patients with minimal risk. Following surgery, 11 patients (7.3%) received no adjuvant therapy. In 64 of the patients (42.7%), tamoxifen was administered for 5 years at a dose of 20 mg/day. Seventy-three patients (48.7%) received a combined adjuvant chemotherapy (6×CMF i.v. for 6 cycles, days 1 and 8, recycled on day 28, at the given doses: cyclophosphamide 600 mg per square meter, methotrexate 40 mg/m² and 5-fluorouracil 600 mg per square meter plus tamoxifen).

Documentation and follow-up is complete, comprehensive and consistent as all patients were treated within prospective clinical trials.

All 150 patients were staged lymph node positive. Ten tumors (6.7%) were graded G1, 64 (42%) G2 and 76 (50.7%) G3 according to Elston [18]. Eighty-five tumors (56.7%) were staged pT1 and 61 tumors (40.7%) pT2 according to UICC stages. One hundred and twenty-five patients (83.3%) had invasive ductal not otherwise specified carcinomas and 25 patients (16.7%) had tumors histologically typed as invasive lobular carcinomas (also see Table 1).

**morphometry**

VEGF-C expression was graded as strong in 37 (24.7%) and as medium in 36 (24%) cases. Seventy-seven (51.3%) showed a weak or absent VEGF-C protein expression (Figure 1A).

Median LMVD was 8.5 microvessels/field (range 1–18 microvessels) (Figure 1B). LVI was observed in 36.6% of cases, mainly seen in open lymphatic vessels and absent in narrow or collapsed lymphatic spaces as described previously [6] (Figure 1C). HER2/neu expression was rated 0/+ in 113 patients (75.3%), 2+ in 16 patients (10.7%) and 3+ in 21 patients (14%) (Figure 1D and E). FISH analysis was carried out in 98 cases; HER2/neu gene amplification was found in 18 specimens, which were all graded 3+ at HercepTest®.

LMVD was significantly higher in patients with LVI compared with those without LVI (11.3 ± 3.7 microvessels/field versus 7.6 ± 3.9 microvessels/field, P < 0.001, Mann–Whitney U test) (Figure 2A). LMVD correlated significantly with VEGF-C expression (8.1 ± 4 microvessels/field for cases with weak-to-absent VEGF-C expression versus 10.1 ± 4.4 microvessels/field for cases with moderate or strong VEGF-C expression, P = 0.012, Mann–Whitney U test) (Figure 2B).

Cases with 3+ HER2/neu protein expression had a significantly stronger VEGF-C expression than all others cases (median: medium versus absent/weak expression; P = 0.006, Mann–Whitney U test).

When comparing the LMVD between cases with and without HER2/neu gene amplification assessed by FISH, a clear trend toward higher LMVD in amplified cases was observed, albeit

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**Table 1.** Clinical data

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<tbody>
<tr>
<td>Age (years)</td>
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<tr>
<td>Tumor stage (UICC), n (%)</td>
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<tr>
<td>pT1</td>
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<tr>
<td>pT2</td>
<td>63 (42)</td>
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<tr>
<td>Grading, n (%)</td>
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<td>G1</td>
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<tr>
<td>G2</td>
<td>64 (42.7)</td>
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<tr>
<td>G3</td>
<td>76 (50.7)</td>
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<td>Histological type, n (%)</td>
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<td>Ductal NOS</td>
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<tr>
<td>Lobular</td>
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<tr>
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<td>16 (10.7)</td>
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<tr>
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<tr>
<td>LVI−</td>
<td>71 (63.4)</td>
</tr>
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</table>

UICC, International Union Against Cancer; NOS, not otherwise specified; EgR, estrogen receptor; LVI, lymphovascular invasion.
missing significance ($P = 0.083$, Mann–Whitney $U$ test) (Figure 2D). A similar trend toward higher LMVD was also seen in cases with 3+ HER2/neu expression in immunohistochemistry (Figure 2C), which also missed significance ($P > 0.05$).

A strong association between LVI and LMVD was established ($P < 0.001$, Mann–Whitney $U$ test).

Regression analysis for LVI including HER2/neu expression, LMVD and VEGF-C expression showed that LMVD was the only significant variable ($P < 0.001$).

Histological grading was significantly lower in tumors with HER2/neu amplification (median 3 versus 2, $P = 0.006$, Mann–Whitney $U$ test), but no association between histological grading, VEGF-C expression and LMVD was found ($P > 0.05$, Kruskal–Wallis test).

There was no correlation between LVI, estrogen receptor density, tumor stage and HER2 expression ($P > 0.05$).

**survival analysis**

The mean observation time was 110 months (range 1–170 months). During this observation time, 56 patients (47.1%) developed recurrent disease and 43 (36.2%) died from their cancer.

Although clear trends toward diminished survival in patients with strong expression of VEGF-C were seen, no significant influence on OS and DFS was found.

In Cox regression for OS and DFS including patients’ age, histological grading, HER2/neu expression, tumor stage and VEGF-C expression, only grading remained as independent prognostic factor (data not shown).

**discussion**

VEGF-C plays a dual role in promoting breast cancer progression: On the one hand, it stimulates lymphangiogenesis and subsequent lymphatic spread and metastasis, and on the other hand, VEGF-C has a stimulating cellular migratory function and increases proliferation rate by direct and/or autocrine action on cancer cells [11, 19–21].

In human breast cancer, VEGF-C expression is directly correlated with a high risk of lymph node metastasis and worse
clinical outcome [22, 23]. However, in contrast to tumor-induced blood vessel growth, in which several antiangiogenic substances have already been introduced into standard therapeutic regimens, no specific antilymphangiogenic cancer therapy is currently available [24, 25].

Recent data now indicate a direct association between HER2/neu and lymphangiogenesis by showing that VEGF-C expression is mediated by transactivation of HER2/neu via the Src kinase pathway in non-small-cell lung cancer [26].

Further experimental studies revealed that the treatment of VEGF-C-overexpressing breast tumor cells with inhibitors of Src, epidermal growth factor receptor or Her2/neu and p38 MAP kinases, decreases VEGF-C production and inhibits cellular migration [27]. Specifically, VEGF-C production in MDA-MB-231 breast cancers cells was significantly reduced by the HER2/neu kinase inhibitor PD153035 [28].

Here, we investigated the correlation of HER2/neu and VEGF-C expression and its impact on lymphangiogenesis and LVI in patients with advanced breast cancer. In our collective, high HER2/neu expression in tumors was significantly associated with a high levels of VEGF-C expression. Consequently, tumors overexpressing VEGF-C showed higher amounts of lymphangiogenesis, assessed by microvesSEL density. In these cases with a strong lymphangiogenic reaction, LVI was significantly more often evident.

Although a clear trend was seen toward increased lymphangiogenesis in HER2/neu-overexpressing cases, the analysis missed statistical significance.

These findings are in good concordance to previous data showing that expression of VEGF-A, -C and -D was positively correlated with HER2/neu expression in human breast carcinomas [29].

Due to the significant role HER2/neu plays in breast cancer pathogenesis and the accessibility of the extracellular portion of the receptor, it was recognized as a potential candidate for targeted therapies. The humanized anti-HR2 antibody trastuzumab (Herceptin) was approved by the Food and Drug Administration in 1998 for use in metastatic breast cancer and has subsequently shown clinical benefit when used in combination with cytotoxic chemotherapy, as first-line and adjuvant therapy [30, 31]. Although the mechanisms for response to trastuzumab in breast cancer are not completely understood, clinical benefit is attributed to interference with signal transduction pathways, impairment of extracellular domain cleavage, inhibition of DNA repair, as well as induction of cell cycle arrest and antibody-mediated cellular cytotoxicity [32].

Studies in an animal model of HER2-overexpressing breast cancer also indicate that blood vessel angiogenesis may be inhibited by trastuzumab [33, 34].

For lymphangiogenesis, only few in vitro data exist, in which VEGF-C messenger RNA and protein expression decreased significantly in breast cancer cells after interference with trastuzumab [8, 35]. Today, various therapeutic substances (antibodies, tyrosine kinase inhibitors and small molecules) targeting HER2/neu are under development or in early phase clinical trials.

Our data provide further evidence for a clinically relevant association between HER2/neu and VEGF-C expression. Therefore, it seems likely that HER2/neu protein expression could influence cell migration and proliferation as well as lymphangiogenic tumor spread via VEGF-C up-regulation. Blocking HER2/neu may on one hand reduce direct proliferative effects of VEGF-C on tumor cells and on the other hand diminish lymphangiogenesis and lymphogenic metastasis.

**References**