Prognostic Value of Vascular Endothelial Growth Factor in Breast Cancer

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ABSTRACT

Angiogenesis, the process leading to the formation of new blood vessels from a preexisting vascular network, is necessary for tumor growth, invasion, and metastasis. Data from experimental and clinical studies indicate that breast carcinoma is an angiogenesis-dependent tumor.

Most retrospective studies evaluating the prognostic value of determination of intratumoral microvessel density (IMD) at the vascular “hot spot” (a surrogate marker of angiogenesis) found that IMD is a significant and independent prognostic indicator in patients with both node-negative and node-positive breast cancers. More recently, the expression of certain endothelial growth factors has been tested. Among these, vascular endothelial growth factor (VEGF), the most potent endothelial cell mitogen and also a regulator of vascular permeability, is emerging as a powerful new prognostic tool. Eight of the nine published retrospective studies reported that VEGF is significantly associated with relapse-free survival, overall survival, or both. Patients with early stage breast cancer who have tumors with elevated levels of VEGF have a higher likelihood of recurrence or death than patients with low-angiogenic tumors, even if treated with conventional adjuvant therapy.

High levels of VEGF can differentiate the subgroups of patients with breast cancer with poor prognosis who benefit minimally from conventional adjuvant therapy but who may benefit from validated anti-VEGF treatments. The Oncologist 2000;5(suppl 1):37-44

INTRODUCTION

Angiogenesis is the term coined by Folkman [1] to identify the complex process leading to the formation of new blood vessels from the preexisting vascular network. Growth, invasion, and metastasis of many cancers depend on angiogenesis; solid tumors require neovascularization to grow beyond ~1 mm³ [2-6]. The molecular mechanisms leading to the angiogenic switch of tumor cells are not completely known, but the modifications over time of the net balance between pro- and antiangiogenic factors appear to be responsible for the angiogenic activity of tumors [7-9]. Several proangiogenic factors as well as naturally occurring inhibitors of angiogenesis have been identified and sequenced [10]. Results from experimental studies suggest that tumor progression and metastasis in breast cancer are angiogenesis dependent [11-16].

In the clinical setting, the seminal study by Weidner et al. [17] reported a significant correlation between the degree of intratumoral microvessel density (IMD) at the vascular “hot spot” with the probability of metastasis in a series of 49 patients with different stages of invasive breast cancer. Subsequently, two independent studies by Horak et al. [18] and Weidner et al. [19] found that IMD is a significant and independent prognostic indicator in human invasive breast cancer. To date, 43 retrospective studies have been published on the prognostic significance of IMD, with more than 6,800 tumors evaluated [20]. Since these studies have been done in series of patients having different pathological and clinical characteristics, a proper meta-analysis is not possible. Nevertheless, more than 75% of the authors reported positive results on the association of IMD with clinical outcome of the patients [20-22], and more than 85% of the 27 studies, including those with multivariate analysis, found that IMD is an independent prognostic variable [20]. However, determination of IMD is quite a crude surrogate
marker of angiogenesis and cannot supply functional information on the molecular pathways involved in the angiogenic activity of each single tumor [23, 24].

Methods have been developed to assess the expression or concentrations of certain angiogenic factors. Among these angiogenic factors, vascular endothelial growth factor (VEGF) has been studied most extensively and is probably the most essential factor for differentiation and development of the vascular system. VEGF is a highly specific and selective mitogen for vascular endothelial cells [25]. It induces proliferation and migration of endothelial cells in vitro [26, 27] while inhibiting apoptosis [28]. In vivo, VEGF is necessary for vasculogenesis [28-30], promotes angiogenesis, and enhances vascular permeability [27, 31-35]. In several different experimental conditions, overexpression of VEGF was accompanied by marked tumor growth and neovascularization [36, 37]. On the other hand, therapeutic blockade of VEGF has been shown to inhibit primary and metastatic tumor growth in animal models [36, 38-48].

Expression of VEGF may be induced by several factors: hypoxia [49-52], altered tumor suppressor genes (e.g., mutated p53) [53], certain cytokines such as interleukin 1b (IL-1b) [54] and IL-6 [55], oncogenes such as v-Raf [56] and v-Src [57], growth factors such as insulin growth factor-1 [58] and transforming growth factor-β [59], or hormones such as progestins [60] under specific experimental conditions.

Five isoforms of VEGF have been identified, the soluble ones being biologically more active in promoting neovascularization [27, 61]. VEGF-soluble proteins bind to two specific tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed preferentially in endothelial cells [62-64].

A recent study reported that VEGF protein is expressed in human breast cancer, with both VEGF-B (preferentially expressed in endothelial cells) and VEGF-C (associated with development of lymphatic vessels) also being expressed at lower levels [65]. In a study that evaluated 46 breast biopsies from ductal carcinoma in situ lesions [66], significant association was found between VEGF mRNA expression and the degree of angiogenesis. The same group of investigators [67] found strong tumor cell expression of VEGF in half of the ductal breast carcinomas in situ and strong or moderate VEGF expression in most of the invasive carcinomas examined. Strong intratumoral endothelial cell expression of VEGF receptors was also observed. Therefore, VEGF could be an important marker of angiogenic activity for prognostic purposes as well as for targeting inhibition of angiogenesis as a novel therapeutic strategy against cancer.

**Methods**

**VEGF Assays**

Toi and colleagues [68] were among the first to assess the expression of VEGF in primary invasive breast cancer in a series of 103 patients. They evaluated the expression of VEGF and factor VIII-related antigen (RA) as a panendothelial marker, with microvessel density (MVD) determined from frozen sections by indirect immunoperoxidase techniques. The anti-VEGF monoclonal antibody and recombinant human VEGF used were developed by Genentech, Inc. (South San Francisco, CA). Immunoreactivities were evaluated with a semiquantitative method and graded as negative, moderate (+), or high (++) immunostaining. Tumors with grades + or ++ were classified as VEGF-positive (rich) cancers. Microvessel density was evaluated by immunostaining with the factor VIII-RA, following Weidner’s method [19]. A heterogenous expression of VEGF was observed in most of the tumors evaluated, and 29 (28%) of 103 tumors were classified as VEGF-rich cancers. There was a statistically significant association between high microvessel density and VEGF expression ($p < 0.01$).

Subsequently, the same group of investigators [69] used the above methods to evaluate the expression of VEGF in 230 patients with either node-negative or node-positive disease. Using a similar immunohistochemical method, Anan and colleagues [70] also recognized the expression of VEGF in human breast cancer and found a strict correlation between VEGF and metastatic potential.

In 1996, Toi and colleagues [71] published a new quantitative method of determination of VEGF protein levels in frozen primary breast cancer tissues. This method was based on an original colorimetric enzymatic immunoassay using a polyclonal human anti-VEGF antibody that reacts with the soluble isoforms VEGF$_{121}$ and VEGF$_{165}$ of the angiogenic peptide. The enzyme reaction used Lumi-phos 530 as substrate, and the minimal detectable level was 1 pg/ml. The study showed a significant association between VEGF concentrations and intratumoral vascularization. Subsequently, similar methods of detecting VEGF levels in cytosolic samples were modified for prognostic purposes in large series of patients [72, 73]. A similar immunometric assay for VEGF was used by Obermaier and colleagues [74].

Relf and colleagues [75] evaluated the expression of multiple angiogenic peptides including VEGF by a quantitative RNase protection assay in 64 invasive breast cancers. Two probes were used for VEGF, the first to protect the smallest isoform, VEGF$_{121}$, and the second to protect the largest soluble fragment, VEGF$_{393}$. They found that the predominant isoform was VEGF$_{121}$, which comprised 50% to 90% of the total VEGF mRNA detected. In 1998,
Eppenberger and colleagues [76] evaluated VEGF protein concentrations on cryopreserved samples of 305 invasive breast cancers using a chemiluminescence immunosorbent assay in the cytosol. This assay recognizes both the secreted isoforms of VEGF (VEGF121 and VEGF165). In addition to VEGF, basic fibroblast growth factor (bFGF), angiogenin, and the proteolysis markers urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type-1 (PAI-1) were assessed by enzyme-linked immunosorbent assays (ELISA). Finally, Linderholm and colleagues published two studies [77, 78] on the determination of VEGF165, using a commercial quantitative immunoassay kit (Quantikine; R&D Systems; Minneapolis, MN). The optimal density for VEGF detection was 450 nm absorbance. The authors reported that the VEGF165 level was significantly inversely associated with estrogen receptor (ER) expression and positively associated with tumor size and histological grade [77]. In the most recent study [78], a positive significant association with mutated p53 protein was found.

All these methods are now considered investigational. Before they are used in routine clinical practice, a prospective, quality-controlled standardization of each technique in different laboratories is needed for proper validation and identification of the method of choice.

Currently no information is available on the prognostic relevance of determining circulating VEGF levels by quantitative ELISA methods in patients who have had surgery for breast cancer. In a recent study [79], researchers found a correlation between circulating levels of VEGF and the stage of disease or tumor burden. However, the cellular source and biological significance of circulating levels of VEGF are not known. Studies have highlighted important methodological pitfalls of these assessment techniques (e.g., activated platelets may transport and release high levels of VEGF) [80, 81].

**Prognostic Results**

The main results from studies on the prognostic value of VEGF tumor levels in human breast cancer are summarized in Table 1 [68, 69, 73-78, 82, 83] and are discussed briefly here. Toi and colleagues [68] evaluated the prognostic value of VEGF expression in 103 patients with breast cancer. The study enrolled 38 node-negative and 65 node-positive patients, followed for a median period of 51 months. The authors reported that the relapse-free survival (RFS) rate of the patients with VEGF-rich tumors was significantly worse than that of patients with VEGF-negative tumors (p < 0.01). In multivariate analysis for RFS, VEGF (p = 0.039), vascularity (p = 0.026), and nodal status (p = 0.010) retained an independent prognostic significance [68]. In a subsequent study [69], the same group of investigators confirmed the results of the preliminary study using the same method in 230 patients with node-negative and node-positive invasive breast cancer, with a median follow-up of 56 months. VEGF was reported to have prognostic significance for RFS in univariate analysis (p < 0.01), but not in multivariate analysis.

In 1997, four studies testing the prognostic value of VEGF were published. Relf and colleagues [75] assessed the expression of VEGF mRNA in 64 breast cancers of patients with node-negative (n = 35) or node-positive (n = 29) disease. The authors used the median value of expression of VEGF to distinguish patients with high (above the median) and low (below the median) VEGF levels. Univariate analysis for RFS showed that patients with high values of VEGF had significantly higher risk of relapse than those with tumors with low levels of VEGF (p = 0.03). Among the other factors tested, ER and epidermal growth factor receptor levels were also significantly associated with prognosis [75]. A second study, conducted by Gasparini and colleagues [73], determined the cytosolic concentrations in a series of 260 node-negative patients with early stage invasive breast carcinoma. The patient population consisted mostly of postmenopausal women (66%), median age 60 years, with ductal invasive histological type disease (80.5%) not treated with adjuvant therapy. The median value of VEGF levels was 126.25 pg/mg of protein (range, 5.0-6,523). The authors found that VEGF, evaluated as a continuous variable, was not associated with any other variable tested and that VEGF was of prognostic value for both RFS and overall survival (OS) in univariate and multivariate analyses based on a median follow-up period of 66 months [73].

The same group of investigators performed another study [72] in the same series of patients by adding other important biological variables besides VEGF. These included thymidine phosphorylase (TP), also known as platelet-derived endothelial cell growth factor), cathepsin D, and p53 protein. VEGF was weakly associated with TP, while TP correlated significantly with p53. In univariate analysis for RFS, tumor size (p = 0.052), VEGF (p < 0.0001), and p53 (p = 0.030) were significantly prognostic for the risk of relapse. VEGF and p53 also retained significance for OS (p < 0.0001 and p = 0.012, respectively). Multivariate Cox regression analysis for RFS indicated that VEGF (p < 0.001), TP (p = 0.050), and their first order interaction terms (i.e., the joint variable VEGF/TP [p = 0.027]), but not p53, were significant and independent prognostic variables [72]. In the fourth study, conducted by Obermair and colleagues [74], 89 patients with both node-positive and node-negative invasive breast cancer were followed for a mean period of 32 months (between year 3 and 5, patients were examined every six months, and yearly thereafter). The authors evaluated only RFS in univariate analysis and reported that VEGF was not of prognostic value. However, taking into account the small and heterogeneous...
series of patients examined and the relatively short follow-up period, the negative results of this preliminary study are to be considered with caution.

In 1998, results from two independent, large-scale studies on the prognostic value of VEGF were reported. Eppenberger and colleagues [76] evaluated VEGF in frozen biopsies obtained from primary breast tumors. The primary study included 305 cases of both node-negative and node-positive patients with a median follow-up of 37 months. A separate validation study with a long median follow-up period of 76 months included 190 node-negative primary tumor samples from patients not treated with adjuvant therapy. In the primary study, VEGF was of prognostic value for RFS in both univariate and multivariate analyses ($p < 0.001$ and $p < 0.01$, respectively). The other variables that retained an independent prognostic value in multivariate analysis were nodal status ($p < 0.0001$), histological grading ($p = 0.04$), uPA ($p < 0.05$), and ER, which was of borderline statistical significance ($p = 0.06$). In the node-negative patient subset, tumor size ($p < 0.001$), VEGF ($p = 0.04$), and uPA ($p = 0.03$) were significant and independent variables for RFS in multivariate analysis. Codetermination of VEGF and uPA added prognostic information in the validation series of 190 node-negative tumors [76].

Linderholm and colleagues [77] evaluated cytosolic levels of VEGF$\text{165}$ in a large, homogeneous population of patients with node-negative breast cancer ($n = 525$) with a median follow-up of 46 months. The median level of VEGF$\text{165}$ was $2.40 \text{ pg/\mu g}$ of DNA (range, 0.11-144.79) and was positively associated with tumor size and histological grade and inversely correlated with ER levels. Elevated levels of VEGF$\text{165}$ were associated with shorter OS. In multivariate analysis for OS, both VEGF and histological grade ($p = 0.019$ and $p = 0.047$, respectively) were independent prognostic variables, while the ER level approached significance ($p = 0.09$) [77].

More recently, Gasparini and colleagues [82] evaluated the prognostic significance of VEGF and TP in a series of node-positive patients treated with either adjuvant chemotherapy (cyclophosphamide, methotrexate, and 5-fluorouracil [CMF] schedule, $n = 137$) or hormone therapy (tamoxifen, $n = 164$), both with a median follow-up of 72 months. The clinicopathological characteristics of the two groups of patients differed with respect to the median age and ER levels (both higher in the group treated with tamoxifen). The distribution of the other variables, including VEGF and TP, were similar in the two groups. In the CMF group, VEGF and TP were not significantly correlated with each other, and VEGF was not associated with any other variable. In univariate analysis, high levels of VEGF were associated with significantly worse RFS and OS. In contrast, elevated levels of TP were associated with better outcome, suggesting that in such a setting TP may be more important as a target enzyme for the antimetabolites and antiproliferative agents of the CMF schedule than as an angiogenic factor. In multivariate analysis, age, VEGF, TP, and their first order interaction terms were significant and independent prognostic variables. In the tamoxifen group, VEGF and TP again did not correlate with each other. In multivariate analysis, only VEGF and the number of positive nodes retained independent prognostic value for RFS.

<table>
<thead>
<tr>
<th>Author [reference]</th>
<th>$n$ of patients</th>
<th>Nodal status</th>
<th>Method</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
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<tbody>
<tr>
<td>Toi [68]</td>
<td>103</td>
<td>N/N*</td>
<td>ICA</td>
<td>&lt;0.01 ND</td>
<td>0.039 ND</td>
</tr>
<tr>
<td>Toi [69]</td>
<td>230</td>
<td>N/N*</td>
<td>ICA</td>
<td>&lt;0.01 ND</td>
<td>ND</td>
</tr>
<tr>
<td>Obermair [74]</td>
<td>89</td>
<td>N/N*</td>
<td>IMA</td>
<td>NS ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Gasparini [73]</td>
<td>260</td>
<td>N*</td>
<td>IMA</td>
<td>&lt;0.001 &lt;0.001</td>
<td>&lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Relf [75]</td>
<td>64</td>
<td>N/N*</td>
<td>RNase</td>
<td>0.03 ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Eppenberger [76]</td>
<td>305</td>
<td>N/N*</td>
<td>ICMA</td>
<td>&lt;0.001 ND</td>
<td>&lt;0.01 ND</td>
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<tr>
<td></td>
<td>190</td>
<td>N</td>
<td>ICMA</td>
<td>0.02 ND</td>
<td>0.04 ND</td>
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<tr>
<td>Linderholm [77]</td>
<td>525</td>
<td>N*</td>
<td>ELISA</td>
<td>ND 0.001 ND</td>
<td>ND 0.02</td>
</tr>
<tr>
<td>Gasparini [82]</td>
<td>301</td>
<td>N*</td>
<td>IMA</td>
<td>&lt;0.01 &lt;0.05</td>
<td>&lt;0.05 &lt;0.05</td>
</tr>
<tr>
<td>Linderholm [78]</td>
<td>833</td>
<td>N/N*</td>
<td>ELISA</td>
<td>ND 0.0001 ND</td>
<td>ND &lt;0.05</td>
</tr>
</tbody>
</table>

Abbreviations: N = nodal status (-, negative; +, positive); ND = not done; NS = not significant; IMA = immunometric assay; ICA = immunohistochemical assay; RNase = RNase protection analysis; RFS = relapse-free survival; OS = overall survival; ICMA = chemiluminescence immunoassay.

Modified with permission [83].
Similarly, Linderholm and colleagues [78] reported that mutated p53 protein and VEGF were significantly correlated and were both predictive of outcome in 833 patients with primary invasive breast cancer, after a median follow-up time of 56 months. This large-scale study enrolled 485 node-negative and 348 node-positive patients. The node-negative patients were not treated with adjuvant therapy, with the exception of 25 high-risk patients who received chemotherapy (CMF schedule). Adjuvant treatments were administered to all node-positive patients. Premenopausal ER-positive patients were randomly selected to CMF or ovarian irradiation, and ER-negative patients were randomly treated with CMF or fluorouracil, epirubicin, and cyclophosphamide (FEC). Postmenopausal patients were randomized to receive tamoxifen for two or five years. In the overall group of 833 cases, both VEGF and p53 were significant prognostic indicators for RFS and OS in univariate analysis.

In the subgroup of node-negative patients, VEGF was of prognostic value for both RFS and OS \( (p = 0.005 \text{ and } p = 0.001, \text{ respectively}) \) in univariate analysis. In multivariate analysis for OS, VEGF \( (p = 0.0075) \) and p53 status \( (p = 0.01) \) were the only independent variables; however, neither VEGF nor p53 were statistically significant indicators for RFS. Similar results were observed in the cohort of node-positive patients. In univariate analysis, both VEGF and p53 were statistically significant prognostic factors for RFS and OS. In multivariate analysis for OS in this subgroup, ER \( (p < 0.0001) \), the number of positive nodes \( (p = 0.0001) \), and VEGF \( (p = 0.005) \) retained an independent prognostic value. For RFS, ER status \( (p < 0.0001) \), the number of positive nodes \( (p = 0.0004) \), and tumor size \( (p = 0.02) \) were independent predictors of the risk of recurrence while VEGF approached statistical significance \( (p = 0.08) \). A further subanalysis by adjuvant treatment was not performed, so it was not possible to identify selective predictive indicators of outcome in relation to the type of treatment.

Because the data are from various retrospective studies with differing methods of VEGF detection, patient characteristics, lengths of follow-up, and diverse treatments administered to the subgroups of patients, a true meta-analysis is not feasible. Nevertheless, determination of VEGF level was reported to be of prognostic value in eight of the nine studies published. Thus, this marker may serve as an important novel prognostic indicator for human breast cancer that would warrant validation in large, prospective clinical studies.

**Conclusions and Future Research**

The retrospective studies reporting the prognostic value of VEGF to date support the notion that human breast cancer is an angiogenic-dependent tumor [83-86]. Although the known angiogenic peptides have not been systematically compared, these retrospective studies suggest that VEGF plays a relevant biological role in the progression of breast cancer. VEGF has been detected using different techniques, thus the identification of the method of choice for prognostic or predictive purposes will need prospective comparative studies, with standardization among different laboratories using quality control programs. Therefore, at present, determination of VEGF can be considered a promising, but investigative, prognostic tool not yet validated for routine clinical use.

In most of the studies described above, VEGF is not correlated with the conventional prognostic factors. Also, a controversial relationship has been reported in two studies [72, 78] regarding the association of VEGF with mutated p53 protein. In general, VEGF levels have been found to be of prognostic value in heterogeneous patient populations as well as in node-positive and node-negative subgroups, regardless of the type of adjuvant therapy administered. These data suggest that patients who have tumors with high levels of VEGF have a biologically aggressive disease and that conventional adjuvant therapy may not be able to reduce the risk of recurrence and death in such patient subgroups.

This information, if validated in homogeneous, large-scale, prospective studies, may be clinically relevant in terms of: A) avoiding unnecessary chemotherapy or hormone therapy in high-risk patients with high tumoral levels of VEGF, and B) using VEGF as both a target for therapy and a potentially useful predictive marker for novel therapeutic strategies (e.g., anti-VEGF compounds) based on inhibition of angiogenesis. Agents that neutralize VEGF protein, such as the humanized monoclonal antibodies (Genentech, Inc., and PLD, Inc.; Fremont, CA), and compounds that block the specific VEGF signaling pathway, such as SU5416 (SUGEN, Inc.; South San Francisco, CA), are currently under early clinical evaluation. It has been suggested that for proper clinical validation of the anticancer activity of angiogenesis inhibitors, the identification of specific surrogate markers of angiogenic activity should be an integral part of the study design for phase II/III clinical trials [87-89]. VEGF seems to be an ideal candidate as a surrogate marker of angiogenesis for prognostic purposes in human breast cancer and for evaluation of the biological activity of antiangiogenic agents in early clinical studies [90].

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