The Expression of Vascular Endothelial Growth Factor Correlates with Mutant p53 and Poor Prognosis in Human Breast Cancer

Barbro K. Linderholm, Thomas Lindahl, Lars Holmberg, Sigrid Klaar, Johan Lennерstrand, Roger Henriksson, and Jonas Bergh

Department of Oncology, Radiumhemmet, Karolinska Institute and Hospital, SE-181 87 Stockholm, Sweden [B. K. L., T. L., S. K., J. B.]; Department of Cancer Epidemiology, Akademiska Hospital, Uppsala University, SE-751 85 Uppsala, Sweden [E. H.]; Institute of Oncology, Umeå University, SE-901 85 Umeå, Sweden [R. H.]; and Virco United Kingdom, Cambridge, England [J. L.]

ABSTRACT

Wild-type p53 protein has been shown to inhibit angiogenesis through thrombospondin in the preclinical setting. Here, we determined the associations between the expression of the angiogenic factor vascular endothelial growth factor (VEGF) and the p53 status, including different mutation sites and types, in primary breast cancer. Cytosols from 224 primary breast cancer patients were analyzed with an enzyme immunoassay for determination of human VEGF165 protein content. p53 status was determined by cDNA-based sequencing of the entire coding region, by immunohistochemistry (IHC), and by a p53 luminometric immunoassay (LIA) method. Statistically significant associations were found between higher VEGF content and non-wild-type p53 status for all methods; sequence-based data (P = 0.0019), IHC data (P = 0.0068), and the LIA method (P = 0.427; P > 0.001). Highest VEGF values were detected in tumors with p53 insertions, deletions, and stop codon mutations (P = 0.0043). Combining p53 status and VEGF content resulted in additional prognostic information, relapse-free survival (RFS; P = 0.0377), overall survival (OS; P = 0.0319), and breast cancer corrected survival (BCCS; P = 0.0292). In multivariate analysis, the relative hazard increased when the VEGF data were added to the p53 status, with a relative hazard of 1.7 for RFS and 3.0 for BCCS, compared with 1.1 for RFS and 1.4 for BCCS among the patients with either high VEGF content or p53 mutation. Higher VEGF content was statistically significantly correlated with a worse outcome for patients with estrogen receptor-positive tumors receiving adjuvant tamoxifen: RFS (P = 0.0471), OS (P = 0.0134), BCCS (P = 0.0064), as well as in multivariate analysis with point estimates of 3.4 and 2.1 for BCCS and RFS, respectively. VEGF expression is related to p53 status in human breast cancer patients. Combining VEGF with p53 status resulted in better prognostic prediction.

INTRODUCTION

Solid experimental and clinicopathological evidence demonstrates that growth of tumors and the process of metastasis are dependent on angiogenesis (1–3). In breast cancer, several studies have suggested that the degree of vascularization of the primary tumor is a predictor of survival, regardless of the nodal status (4, 5). The induction of tumor vasculization involves the release of angiogenic peptides (6). VEGF3 is a cytokine that selectively induces endothelial cell proliferation and migration, increases the permeability of microvessels, and activates proteolytic enzymes involved in tumor invasiveness (7–9). A higher VEGF expression has been shown to correlate with a worse prognosis for patients with primary breast carcinomas (10–13).

Mutations or more unspecific increased p53 protein levels have been described to be associated with a worse prognosis in primary human breast cancer (14–20). Wild-type p53 protein has been shown, in cell lines, to suppress angiogenesis via regulation of TSP-1 expression (21) and to down-regulate the promoter activity of the angiogenic factor VEGF in a dose-dependent manner (22). Preclinical studies have shown wild-type p53 protein to enhance the expression of TSP-1, an inhibitor of angiogenesis, and that down-regulation of TSP may be observed when alterations of the p53 protein occur (21).

To date, there are few clinical studies published concerning the relationship between p53 status and angiogenesis in human breast cancer. The primary aim of this study was to determine the association between VEGF expression and mutant p53 according to cDNA gene sequence data, overexpression of p53 protein determined by IHC and a LIA method in 224 primary breast cancer patients. Secondary aims were to investigate the clinical relevance of VEGF expression, alone and in combination with p53 status for RFS, BCCS, and OS.

MATERIALS AND METHODS

Study Materials and Patient Data. Three hundred and fifteen operated breast cancer patients from a population-based cohort were included from the time between January 1, 1987 and December 31, 1989 and are described in detail elsewhere (18, 20, 23). The tumors were collected at the Department of Pathology, Akademiska Hospital (Uppsala, Sweden). Of those, 224 patients had sufficient remaining cytosols for measurement of VEGF protein content. The clinical and tumor biological characteristics of the patients are shown in Table 1. Patient records were reviewed blindly for biological markers, with regard to primary adjuvant treatment including radiotherapy, relapse information, relapse treatment, and date and cause of death. Fatal outcome was classified as death attributable to breast cancer or death of unrelated causes.

Therapy and Clinical Follow-Up. All patients were operated. Postoperative radiotherapy was given as part of a randomized study to those operated with sector resection, and after the closure of the study it was recommended routinely to all of those patients operated with sector resection (24). Systemic adjuvant treatment were given routinely to all patients with lymph node-positive disease as outlined elsewhere (18, 20). In general, premenopausal patients received adjuvant chemotherapy with i.v. cyclophosphamide, methotrexate, and 5-fluorouracil. Tamoxifen was offered to postmenopausal patients with node-positive disease and to node-negative patients with T2 tumors for 2 or 5 years within a clinical trial (25). In this patient population, a total of 66 patients received adjuvant endocrine therapy, and 22 patients received adjuvant polychemotherapy.

Follow-Up. All patients treated for breast cancer in Uppsala County were routinely seen on a regular outpatient basis for at least 5 years. The routine follow-up consisted of clinical examination, blood tests, and X-ray procedures performed when indicated.

Tumor Tissue Preparation. Fresh tumor material was sectioned for routine histology, estrogen and progesterone receptor assessment, and DNA analysis and stored for further use in −70°C.

VEGF Analysis. A VEGF assay was performed using a commercial quantitative immunoassay kit for human VEGF165 (Quantikine, human VEGF; R & D Systems, Minneapolis, MN), as earlier described (11, 13). VEGF content was expressed as pg protein/mg of total cytosol protein.

Sequence-based Analysis of p53. RNA isolation, conversion to cDNA, and sequence analysis were performed as described previously (18, 20). The
entire p53 gene was analyzed. The sequence was compared with the wild-type p53 sequence. Every mutation was verified by reamplification and sequencing of the fragment using the cDNA preparation as starting material. The evolutionarily conserved regions were defined as follows: region I, exons 2–4; region II, exon 5; region III, exon 6; region IV, exons 7–8; and region V, exons 9–11.

LIA Analysis of the p53 Protein. Cytosols from the tumor samples were prepared as earlier described (19). p53 protein content in the cytosols was determined using a LIA (LIA-mat p53) from Sangtech Medical AB (Bromma, Sweden), using the monoclonal antibodies DO1 and 1801 (20).

IHC Analysis of p53 Protein. p53 status in tumors was analyzed by immunohistochemistry on paraffin sections using the monoclonal mouse antibody 1801 as earlier described (20).

IHC Analysis of c-erbB-2 Overexpression. c-erbB-2 overexpression in tumors was analyzed by immunohistochemistry on paraffin sections using the monoclonal mouse antibody CB11 as described earlier (26).

Statistical Methods. The Pearson χ² test was used for testing associations between VEGF content, and p53 status obtained by either cDNA sequence data or by IHC was tested by the Spearman’s nonparametric test, used to describe the association between quantitatively measured VEGF and p53 protein according to the LIA method, with the tested factors as continuous variables. Distribution of other established prognostic or predictive factors in different groups according to VEGF expression was tested by the Pearson χ² test. Lymph node status was determined as negative (0), positive (1), and unknown (2). Statistical analysis was performed using the Kaplan-Meier method, and comparison between study groups was performed with the log-rank test. The median value of VEGF content and wild-type p53 versus mutant p53 according to cDNA sequencing results was used in univariate survival analysis. To evaluate the simultaneous effect on different factors on survival, the Cox’s proportional hazard model was used. The variables included were used as above, with the exception of tumor size and age, which were used as continuous variables. Survival time was measured from date of diagnosis to date of first recurrence or to death. In all tests, the significance level was set to 0.05, and all tests were two-sided.

RESULTS

Clinical Outcome. The median age at time for diagnosis was 64.5 years (range, 28–94 years). Sixty-two patients had histopathologically verified lymph node metastasis (27.7%), and 154 patients presented with a node-negative disease. Node status was unknown in 8 patients. The median tumor size was 20 mm (range, 2–65 mm). Steroid receptor status was determined in 220 cases; 17.8% was ER negative, 80.2% ER positive, 13.4% were PgR negative and 84.8% PgR positive (Table 1). Of the 224 patients included in this study, 37 died of breast cancer, and 20 died of unrelated causes. The 5-year OS was 65.6%, and the 5-year BCCS was 71.3%. The patients were followed for a median time of 58 months (range, 51–85 months).

Distribution of VEGF. A wide range of VEGF protein content was found. The median value was 256.4 pg/mg total protein (range, 7.5–9084.2 pg/mg). There was no statistically significant difference between the node-negative group (median, 244.2 pg/mg; range, 13.9–6725.1) and the node-positive group (median, 307.8 pg/mg; range, 7.5–6199.4; P = 0.3122).

Sequence-based Analysis of p53 Status. Alterations in the p53 gene were detected in tumors from 37 of the 224 patients (16.5%). Twenty-two mutations were found in lymph-node negative patients, 14 in lymph-node positive patients, and 1 mutation was found in a patient with unknown lymph node status. p53 mutations were detected throughout the entire coding region. Eighteen mutations (48.7%) were detected within the evolutionarily conserved regions. Twenty-nine point mutations (78.4%) and 8 “severe” mutations, including insertions, deletions, and stop codon mutations, were found.

p53 Status Based on IHC. Positive IHC was found in 39 patients (17.4%), 183 were IHC negative, and 2 patients had unknown IHC p53 status.

Association between p53 Status and VEGF Content. A statistically significant association was found between mutant p53 according to sequence-based data and an increased VEGF expression (P = 0.0019). Twenty-seven patients with mutant p53 (73.0%) had a VEGF content above the median value. A statistically significant association was also found between a high VEGF content and insertions, deletions, and stop codons (insertions, deletions, and stop codon mutations versus point mutations versus wild-type p53; P = 0.0043; Table 2). A significant association was also found between an increased VEGF content and positive p53 IHC (P = 0.0068; Table 2), as well as quantitatively measured p53 protein with the LIA method (P < 0.001, Spearman r = 0.427).

Association between VEGF Content and Other Variables. A statistical significant inverse association was seen between VEGF content and ER status (positive versus negative; P = 0.0261). A significant association was found between VEGF content and ploidy (diploid versus aneuploid; P = 0.0075). No significant correlation was found between VEGF and tumor size, PgR content, S-phase fraction, c-erbB-2 overexpression, or nodal status (Table 3).

VEGF Correlated with Survival. Univariate analysis demonstrated a nonsignificant trend of reduced survival times for patients with VEGF content above the median value, compared with patients with a lower VEGF content (BCCS, P = 0.0725; OS, P = 0.0900). VEGF expression was not correlated with RFS (P = 0.4103). In the group that received adjuvant endocrine therapy (n = 66), a significant difference was seen, with reduced survival times for patients with VEGF content above the median value (RFS, P = 0.0413; BCCS, P = 0.0092; and OS, P = 0.0145). In this group, 8 patients were found to be steroid receptor negative, and 2 patients had unknown
receptor status; the results, when they were excluded, were similar (RFS: $P = 0.0471$; Fig. 1A; BCCS: $P = 0.0064$; Fig. 1B; and OS: $P = 0.0319$). A worse outcome was disclosed for the high-risk group, with a 5-year BCCS of 50.0%; the best survival was seen for patients with wild-type p53 and lower VEGF content, with a 5-year BCCS of 79.4% ($P = 0.0555$; Table 4). Survival analysis of the ER-positive patients that received adjuvant endocrine therapy showed statistically significant differences in survival times: RFS, $P = 0.0488$; OS, $P = 0.0233$; and BCCS, $P = 0.0342$ (Table 4).

**Multivariate Analysis.** Cox proportional hazards models were constructed to analyze the influence of VEGF on BCCS and RFS in the presence of other, classical prognostic factors. Those were: patient age at operation in years, tumor size in mm, presence of axillary metastasis (yes versus no), estrogen and progesterone receptor status (positive versus negative), and S-phase fraction (high versus low).

### Table 3
Associations between VEGF content (≤ median versus > median) and other (established) markers in breast carcinoma

<table>
<thead>
<tr>
<th>Marker</th>
<th>$\chi^2$ test</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size ($n = 224$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20 mm vs. &gt;20 mm</td>
<td>0.1405</td>
<td></td>
</tr>
<tr>
<td>ER ($n = 220$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER+ vs. ER-</td>
<td>0.0261</td>
<td></td>
</tr>
<tr>
<td>PgR+ vs. PgR-</td>
<td>0.4973</td>
<td></td>
</tr>
<tr>
<td>Lymph-node status ($n = 216$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N- vs. N+</td>
<td>0.1972</td>
<td></td>
</tr>
<tr>
<td>Ploidy ($n = 224$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid vs. aneuploid</td>
<td>0.0075</td>
<td></td>
</tr>
<tr>
<td>S-phase fraction ($n = 215$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤7% vs. &gt;7%</td>
<td>0.5392</td>
<td></td>
</tr>
<tr>
<td>c-erbB-2 status ($n = 222$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative vs. positive</td>
<td>0.2738</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion ($n = 202$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative vs. positive</td>
<td>0.2225</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Probability of RFS ($P = 0.0471$; A) and BCCS ($P = 0.0064$; B) by VEGF content in 56 ER-positive patients. The median value of VEGF was used as a cutoff value.

### Table 4
The 5-year BCCS according to VEGF content (≤ median versus > median) in all patients who received adjuvant tamoxifen ($n = 66$) and in the 56 patients that had ER-positive tumors. The 5-year BCCS according to VEGF content and p53 status (wild-type versus mutant) in the total patient population

<table>
<thead>
<tr>
<th>Group</th>
<th>5-year BCCS</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant tamoxifen ($n = 66$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low VEGF</td>
<td>78.9%</td>
<td></td>
</tr>
<tr>
<td>High VEGF</td>
<td>41.7%</td>
<td>0.0120</td>
</tr>
<tr>
<td>ER-positive patients, adjuvant tamoxifen ($n = 56$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low VEGF</td>
<td>77.8%</td>
<td></td>
</tr>
<tr>
<td>High VEGF</td>
<td>35.3%</td>
<td>0.0099</td>
</tr>
<tr>
<td>Total patient population</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low risk (wild-type p53, low VEGF)</td>
<td>79.4%</td>
<td></td>
</tr>
<tr>
<td>Intermediate risk (mutant p53 or high VEGF)</td>
<td>69.4%</td>
<td>0.0555</td>
</tr>
<tr>
<td>High risk (mutant p53 and high VEGF)</td>
<td>50.0%</td>
<td></td>
</tr>
</tbody>
</table>

Combination of p53 and VEGF in Correlation with Survival.

The patients were classified in three groups according to p53 status, determined by cDNA sequencing and VEGF content. These were a low-risk group with wild-type p53 and low VEGF expression, an intermediate group with either p53 mutations or increased VEGF expression, and a high-risk group, consisting of patients with both p53 mutations and a higher VEGF expression. Univariate analysis generated statistically significant different survival times between the groups: RFS ($P = 0.0377$; Fig. 2A), BCCS ($P = 0.0292$; Fig. 2B), and OS ($P = 0.0319$). A worse outcome was disclosed for the high-risk group, with a 5-year BCCS of 50.0%; the best survival was seen for patients with wild-type p53 and lower VEGF content, with a 5-year BCCS of 79.4% ($P = 0.0555$; Table 4). Survival analysis of the ER-positive patients that received adjuvant endocrine therapy showed statistically significant differences in survival times: RFS, $P = 0.0488$; OS, $P = 0.0233$; and BCCS, $P = 0.0342$ (Table 4).

Fig. 2. Probability of RFS ($P = 0.0377$; A) and BCCS ($P = 0.0292$; B) by VEGF and p53 status for patients with primary breast cancer. The patients were divided in three risk groups: low risk with lower VEGF content and wild-type p53, an intermediate group with either higher VEGF or mutant p53, and high risk with higher VEGF and mutant p53.
VEGF added as ≤ versus > the median value did not add to the model fit; for BCCS, the RHs were 1.2–1.5, and for RFS, around 1.1. None of the estimates were statistically significant. When the models were stratified on type of adjuvant treatment (none, radiotherapy only, chemotherapy +/- radiotherapy, or tamoxifen +/- radiotherapy), VEGF was associated with RHs <1.0 for both BCCS (RH, 0.1; 95% CI, 0.003–3.6) and RFS (RH, 0.1; CI, 0.003–1.3) for women treated with chemotherapy. On the contrary, the RHs were above unity in women treated with tamoxifen, with RH of 3.4 (95% CI, 0.9–12.6) for BCCS and 2.1 (95% CI, 0.8–5.5) for RFS. Because of the seemingly different results for women given adjuvant chemotherapy and tamoxifen, respectively, an interaction analysis between VEGF and adjuvant treatment was performed. For both BCCS and RFS, the interaction terms were associated with RHs around 0.4 and 2.2, respectively; however, none of the interactions were statistically significant (interaction term with endocrine treatment; RFS, P = 0.2 and BCCS, P = 0.3).

When the combination of p53 status and VEGF expression in three risk groups were included, an additional predictive effect was obtained. The patients were stratified in the same way, as in the univariate analysis, and the low-risk group (wild-type p53 and low VEGF) was used as a reference group. The results in the total patient population showed an increased RH in the high-risk group, including patients with both p53 mutations and higher VEGF expression (RH, 3.0; CI, 1.30–6.95), compared with the intermediate group (with one factor, p53 mutations or increased VEGF expression; RH, 1.44; CI, 0.73–2.86) for BCCS (Table 5). Similar results were obtained for patients that received adjuvant tamoxifen with RH of 2.9 for BCCS and 2.6 for RFS in the high-risk group compared with RHs of 2.5 and 1.6, respectively, for the intermediate group.

**DISCUSSION**

This study demonstrates a statistically significant association between p53 status and VEGF expression in human breast cancer. This correlation was reported earlier in preclinical studies (22, 27). The most pronounced association was found between an increased VEGF expression and p53 mutations according to cDNA sequence data. Associations were also found between higher VEGF content and increased p53 protein content determined by protein-based methods. This indicated that increased VEGF expression is correlated with wild-type p53 loss and supports that angiogenesis may be regulated, in part, by p53 tumor suppressor gene function. Tumors with insertions, deletions, or nonsense mutations were found to have the highest degree of correlation with increased VEGF expression. Those types of mutations give rise to truncated proteins, which seldom can be detected by protein-based methods (20). In accordance, the few patients in our study (n = 8), with this type of p53 mutation were all p53 immunohistochemistry negative, except one where IHC data were unknown. This might explain that a lower association was found between VEGF expression and the protein-based methods for determination of p53 status compared with sequence-based data.

To our knowledge, investigations comparing the angiogenic activity with p53 status according to complete gene sequence data in primary breast cancer have not been reported before. Two smaller studies have used sequence-based data from part of the p53 gene, i.e., exons 4–10 in 27 patients with non-small cell lung cancer (28) and exons 5–9 in 19 patients with angiosarcomas (29). The small populations might explain the absence of associations between increased angiogenesis and p53 mutations in those studies. Moreover, lack of sequence data from the entire p53 coding region must be considered, because it has been demonstrated that p53 mutations can be detected throughout the entire coding region of the gene (18).

Concerning breast cancer, three studies have, in contrast to our results, reported the absence of a correlation between p53 positivity, determined by IHC, and an increased microvessel count (5, 30, 31). Although a high correlation is reported between vessel density and the cytosolic VEGF content in primary breast tumors (32), the differences in determination of both the angiogenic activity and the p53 status in those studies, compared with ours, might be suggested as explanations. However, our results are supported by another study, which suggests that VEGF levels are associated with p53 expression (33).

Combining p53 status and VEGF content seems to yield additional prognostic information for the patients’ outcomes, both in univariate and multivariate analyses. The best outcome was found for the patients with wild-type p53 and low VEGF content. The shortest RFS, OS, and BCSS times were found for those with p53 mutations and higher VEGF content. Multivariate analysis that included all patients showed an increase of the RH for BCSS from 1.4 in the intermediate group to 3.0 in the high-risk group, including patients with primary tumors with both p53 mutations and higher VEGF expression.

The predictive value of VEGF content was, in this study, restricted to the group of patients that received adjuvant endocrine treatment and also restricted when the receptor-negative patients were excluded from the survival analysis. Patients with higher VEGF content were found to have both significant reduced RFS and BCSS. Multivariate analysis also showed an increased RH for both BCSS and RFS for those patients (3.4 and 2.1, respectively). Tamoxifen has been shown in experimental studies to have an antiangiogenic effect by decreasing transforming growth factor α, which is a stimulator of angiogenesis in ER-positive tumors (34, 35). Our results may indicate that tamoxifen alone is insufficient as adjuvant systemic treatment for patients with a high VEGF expression, despite receptor positivity.

Although the number of patients was limited, a worse outcome was indicated for patients with lower VEGF expression treated with adjuvant chemotherapy. Similarly, in multivariate analysis, the RH was <1.0 for both BCSS and RFS. The results of the Cox proportional models should, however, be interpreted cautiously. The number of events, especially for BCSS, are few in relation to the number of variables tested, so that an irregular pattern could occur by chance.
alone (36). The interaction analysis has low power, and in a study of many different factors that may reflect host characteristics, tumor biology, and treatment indications, it is not self-evident how the mathematical model should be constructed. On the other hand, a possible interaction between different types of treatment and VEGF was preconceived, and a lack of effect of endocrine therapy for women with tumors with high vessel count has been observed previously (30, 37).

In summary, increased VEGF expression seems to correlate with p53 status, especially for p53 mutations determined by p53 gene sequence data. Highest VEGF values were detected in tumors with p53 insertions, deletions, and stop codon mutations. This might indicate that angiogenesis, at least in part, is regulated by p53 function. Combining those two markers yielded additional prognostic information. Further analysis of the expression of angiogenic factors and inhibitors in clinical tumor samples might provide useful information about genetic involvement in the regulation of angiogenesis.

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