HER-2 Gene Amplification Correlates with Higher Levels of Angiogenesis and Lower Levels of Hypoxia in Primary Breast Tumors

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ABSTRACT

Purpose: This study investigated the connection among HER-2 gene amplification, HER-2 protein expression, and markers of tumor angiogenesis and oxygenation in patients with operable, invasive breast tumors.

Experimental Design: From 1988 to 1995, 425 patients with metastatic breast cancer were enrolled in a study of high-dose chemotherapy with autologous transplant. Primary tumor blocks were obtained and evaluated using immunohistochemistry (IHC) staining of vessels with von Willebrand factor antibody. Mean microvessel densities (MVD) were determined by counting von Willebrand factor stained cells in three separate “vascular hot spots” using image analysis. Tumor samples were also stained for HER-2 by IHC, HER-2 gene amplification by fluorescence in situ hybridization, carbonic anhydrase 9 by IHC, and vascular endothelial growth factor (VEGF) by IHC. Plasma from 36 patients with primary tumor samples had VEGF (R&D Systems, MN) and d-dimer (American Diagnostica, Greenwich, CT) levels determined.

Results: There was a significant positive correlation between HER-2 gene amplification and both maximum and average MVD (Spearman coefficient = 0.51 and 0.50; \(P = 0.03\) and 0.05, respectively). There was an inverse correlation with HER-2 gene amplification and expression of the tumor hypoxia marker CA-9 (\(\chi^2 P = 0.02\)). The level of HER-2 gene amplification correlated with plasma d-dimer levels (Spearman coefficient = 0.43; \(P = 0.021\)). Interestingly, tumors with HER-2 by IHC had decreased amounts of VEGF staining (\(\chi^2 = 5.81; P = 0.01\)). There was no correlation between HER-2 by IHC and MVD or d-dimer. Of all the variables examined, only average \((P = 0.0016)\) and maximum MVD \((P = 0.0128)\) predicted disease-free survival (Cox univariate model).

Conclusions: HER-2-amplified breast cancers have increased amounts of angiogenesis, decreased amounts of hypoxia, and increased markers of fibrin degradation. These findings have prognostic, predictive, and therapeutic implications in breast cancer treatment.

INTRODUCTION

The HER-2 gene encodes a 185-kDa transmembrane phosphoglycoprotein with tyrosine kinase activity and is a member of the human epidermal growth factor receptor gene family (1). Slamon et al. (2) first described the association of HER-2 gene amplification and poor prognosis in breast cancer, and this finding has been confirmed in subsequent studies. Several studies have also shown a correlation between HER-2 overexpression in tissue and serum and response to both chemotherapy and hormonal therapy, suggesting its role as a predictive factor (3–10). Currently, there are a number of breast cancer therapies that inhibit signaling through the HER-2 pathway. These therapies are either Food and Drug Administration approved for the treatment of breast cancer (Herceptin; Genentech, South San Francisco, CA) or being studied in clinical trials.

Clinically, HER-2 overexpression has been correlated with higher histological grade and S-phase fraction, increased tumor size and number of involved lymph nodes, lymphoid infiltration, p53 mutation, negative estrogen receptor, absence of bcl-2, and absence of lobular histology (11–14). HER-2 amplification is one of the most consistent alterations found in human breast adenocarcinomas. Although the biological pathways activated by HER-2 are not completely characterized, the oncogenic potential of HER-2 has been clearly established. Cells transfected with HER-2 acquire a more malignant phenotype (15), with stimulation of cell proliferation, invasion (16), and metastasis (17). In addition, HER-2 amplified cancer cells have increased signaling through the ras/mitogen-activated protein kinase pathway (18, 19). This altered signaling could increase cytokine production thus leading to favorable growth conditions such as increased angiogenesis in the tumor microenvironment (20).

Little information is known about the connection between specific gene amplification and tumor angiogenesis (21). In neuroblastoma cell lines, n-MYC amplification leads to decreased interleukin 6, a potent inhibitor of angiogenesis (22). In NIH 3T3 fibroblasts that have been transfected to overexpress HER-2, vascular endothelial growth factor (VEGF) production
is increased, and this increased production of VEGF is blocked using a monoclonal antibody directed at HER-2 (20). This HER-2-associated increase in VEGF could lead to increased angiogenesis and decreased tumor hypoxia. In breast cancer patients, we know that VEGF production and angiogenesis are important prognostic and predictive factors (23). As markers of angiogenesis, intratumoral microvessel density (MVD) counts have been shown in many human tumor types, including breast cancer, to be predictive for response to therapy and prognostic for patient outcome (24–28).

Prior studies have examined HER-2, CA9, and VEGF independently as prognostic factors (2, 8, 22, 27–31). Preclinical work in both breast cancer cell lines and other cancer cell lines has described a relationship between HER-2 and VEGF (20, 32, 33). This study is the first to examine the relationship among HER-2 amplification, tumor hypoxia, and angiogenesis in human breast cancer. In addition, we examined the relationship between HER-2 and a number of other factors that have been associated with angiogenesis, such as fibrin degradation (d-dimer). These relationships are important, because many novel therapies target angiogenesis, and the relationships we describe suggest that the antiangiogenesis therapeutic approach should be especially promising in HER-2-amplified cancers. Furthermore, we examined these relationships in the context of high dose chemotherapy, which may be useful to determine their relevance as predictive factors.

MATERIALS AND METHODS

Patients and Treatment. Between 1988 and 1995, 425 patients with measurable, metastatic breast cancer were enrolled in a study at Duke University of high-dose alkylating agents with autologous cellular support as consolidation after intensive AFM therapy. To be eligible for the study, patients were required to have histologically demonstrated measurable metastatic breast cancer not involving the brain or bone marrow and good performance status (34). Patients received two to four cycles of doxorubicin (25 mg/m² × 3 days), 5-fluorouracil (500 or 750 mg/m² × 5 days), and methotrexate (250 mg/m²) and were evaluated for best response. Those who achieved a complete remission (n = 113) were randomized to immediate high-dose consolidation versus observation and transplant at the time of relapse. Patients who attained a partial response (n = 202) went on to immediate high-dose therapy. The high-dose regimen consisted of cyclophosphamide (5625 mg/m²), cisplatin (165 mg/m²), and carbustine (600 mg/m²).

A total of 230 tissue blocks could be retrieved out of 330 requested. The remaining cases could not be requested due to missing record. Reasons for lack of retrieval were that the center did not keep the paraffin blocks beyond 5 years (25%), they did not release blocks for research purposes (5%), or inadequate pathology data were available to determine where the original pathology was located (20%). Out of 230 obtained, 207 had adequate tissue for the initial study (34). From that cohort, 86 sections remained for the current analysis. Thirty six of the patients with evaluable tumor sections also had stored plasma within the Duke University bone marrow transplant plasma bank.

Gene Amplification Staining for HER-2. HER-2 gene amplification was measured by fluorescence in situ hybridization (FISH) at Labcorp Inc. using the Ventana INFORM kit, and results were published previously (34). Five-µm sections of formalin-fixed, paraffin-embedded breast cancer tissues were mounted on microscope slides. A H&E-stained section was evaluated for tumor, and the corresponding tumor area was etched on the FISH slide for reference during the scoring process. Sections were deparaffinized, and tissues were digested with proteinase. Target DNA in sections and the biotinylated HER-2 probe were codenatured in a 90°C oven for 12 min. Target and probe sequences were hybridized overnight at 37°C. Sections were washed in 0.5× SSC to remove unhybridized probe. The hybridized probe was detected by incubation with fluorescein-labeled avidin. The nuclei were counterstained with 4',6-diamidino-2-phenylindole. Slides were examined with an epifluorescence microscope equipped with dual and single filters to detect the fluorescein and 4',6-diamidino-2-phenylindole fluorochromes. Two fields of 20 tumor cells were evaluated, and the number of HER-2 signals per nucleus was determined. The HER-2 gene was considered amplified if, on average, there were four or more signals per tumor nucleus.

Immunohistochemical Staining for HER-2, CA9, von Willebrand factor (vWF), and VEGF. Immunohistochemistry (IHC) was carried out using procedures described by Hsu et al. (35). Briefly, paraffin-embedded tissues were sectioned (5 µm), and antigen retrieval was performed using heat in a citrate buffer (Biogenex). Individual tissue sections were treated with primary antibody against HER-2 protein (CB11ab; dilution of 1:100; BioGenex), carbonic anhydrase 9 (1:100; gift of Dr. Egbert Oosterwijk, University Hospital Nijmegen, Nijmegen, Netherlands), vWF VIII (dilution of 1:100; DAKO), or VEGF (dilution of 1:80; Neomarkers). Secondary and tertiary antibodies were provided in a kit (314KLD; Innovex), and the location of the reaction was visualized with 3,3′,6-diamidino-2-phenylindole. Slides were counterstained with hematoxylin and mounted with cover slips. Negative controls for the IHC were treated with human IgG and did not display any background staining. IHC by CB11 was performed on the initial cohort of 207 using published methods (34).

Assignment of HER-2, CA9, vWF, and VEGF staining was conducted with no knowledge of clinical parameters of patient outcome. HER-2 scoring was performed as described previously (34). CA-9 and VEGF quantitation was based on the amount of the tumor sample staining as well as the intensity of staining. Tumors were graded as 2+ if >50% of the tumor showed cytoplasmic staining and 3+ if >75% of the tumor showed cytoplasmic staining. Tumors with a score of 0 or 1+ were considered to not have CA9/VEGF protein overexpression, and tumors with a score of 2+ or 3+ were considered to have CA9/VEGF protein overexpression.

Plasma VEGF and d-Dimer Levels. Plasma VEGF levels were quantified using the Quantikine human VEGF colorimetric sandwich ELISA kit (R&D) according to the manufacturer’s instructions. d-Dimer levels were obtained using the DIMERTTEST immunoassay (American Diagnostica, Greenwich, CT). This commercially available kit recognizes all of the isoforms of VEGF (A, B, C, and D). This commercially available d-dimer monoclonal antibody recognizes an epitope that is a specific product of cross-linked fibrin that has been subsequently degraded by plasmin. Therefore, the plasma d-dimer
efficiency higher maximum MVD and average MVD (Spearman correlation coefficient, 0.51 and 0.50; P = 0.03 and 0.05, respectively; Table 1; Fig. 2). A total of 35 tissue blocks had adequate tumor tissue to determine CA9 expression. In this subset, HER-2 amplification was inversely correlated with CA9 expression (x² P = 0.02; Spearman correlation coefficient = −0.39; P = 0.021; Table 2).

Twenty-nine patients had adequate plasma samples for analysis of plasma VEGF and D-dimer levels. Thirty-three patients had adequate tissue samples to determine VEGF expression by IHC. The mean plasma VEGF value was 130.3 pg/ml (SD = 152.0). The mean plasma D-dimer level was 381.6 ng/ml (SD = 434.4). HER-2 amplification did not correlate with VEGF expression by IHC (24) and modified for use with an image analysis system (Optimas image analysis software). Once the tissue was stained for vWF, tissues were then examined at x10 magnification to identify areas of vascular “hot spots.” At x20 power, vessels were marked on the image, the image was then converted to a threshold image using the image analysis program, and the marked vessels were quantitated. MVD were classified both by the average MVD (mean value of the quantitation of 3 “hot spots”) and the maximum MVD (largest number of vessels within a single “hot spot”). For outcome (event-free survival) analysis, both average MVD and maximum MVD were dichotomized at the mean values (39 counts and 47 counts, respectively), and each tumor was classified as either “low” or “high” for each MVD category.

Statistical Analysis. We estimated overall and disease-free survival according to the Kaplan-Meier product limit method and applied the log-rank test to compare two distributions. We calculated disease-free survival as time from first cycle of chemotherapy to disease progression or death, whichever occurred first. Patients who were alive and disease free were censored at the date of last follow-up visit. Overall survival was calculated from time of first cycle of chemotherapy to death, and patients who were alive were censored at date of last follow-up visit. We used Cox proportional hazards models to determine which variables were significant predictors of overall and disease-free survival. Statistical significance was taken as P < 0.05.

RESULTS

HER-2 Amplification Correlates with Higher Levels of Tumor Angiogenesis, Lower Levels of Tumor Hypoxia, and Higher η-Dimer Levels. A total of 66 tissue blocks had adequate invasive cancer in the archival sections to determine MVD. The average tumor size was 3.2 cm (range, 1.5–8.4 cm), and the average number of involved axillary lymph nodes was 3 (range, 0–17 axillary lymph nodes). The mean average MVD for these 66 tumors was 39 counts (SD = 16.3; range, 6–69), and the mean maximum MVD was 47 counts (SD = 12.2; range, 14–83). HER-2 gene amplification correlated with both higher maximum MVD and average MVD (Spearman coefficient = 0.51 and 0.50; P = 0.03 and 0.05, respectively; Table 1). HER-2-amplified tumors had a mean of 6 more vessel counts in both average MVD and maximum MVD determination than tumors that did not have amplified HER-2. HER-2 overexpression by IHC (2 and 3+) did not correlate with either maximum MVD or average MVD (Fig. 1). A total of 35 tissue blocks had adequate tumor tissue to determine CA9 expression. In this subset, HER-2 amplification was inversely correlated with CA9 expression (x² P = 0.02; Spearman correlation coefficient = −0.39; P = 0.021; Table 2).

Twenty-nine patients had adequate plasma samples for analysis of plasma VEGF and η-dimer levels. Thirty-three patients had adequate tissue samples to determine VEGF expression by IHC. The mean plasma VEGF value was 130.3 pg/ml (SD = 152.0). The mean plasma η-dimer level was 381.6 ng/ml (SD = 434.4). HER-2 amplification did not correlate with tissue VEGF expression by IHC (Table 1). HER-2 expression, however, was inversely correlated with VEGF expression. Eleven of 38 (29%) HER-2 non-overexpressing tumors had tissue VEGF expression 2+ or higher, whereas only 2 of 15 (13%) HER-2 overexpressing tumors had tissue VEGF expression of 2+ or higher (χ² value = 5.81; P = 0.01). HER-2 expression did not correlate with plasma VEGF levels. HER-2 amplification correlated with plasma η-dimer levels (Spearman correlation of 0.65; P = 0.02; Table 1; Fig. 2).

HER-2 Amplification Correlates with HER-2 Protein Expression. Both HER-2 amplification by FISH and HER-2 protein expression by IHC were available in 58 patients. The median number of gene copies by FISH was 2.45, and 17 of 58 (29%) were considered gene amplified (<4 gene copies). A significant correlation existed between HER-2 gene amplification and HER-2 protein expression (2/3+; Table 3; χ² P < 0.01). There was concordance between HER-2 gene

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**Table 2** HER-2 amplification was inversely correlated with CA9 expression (x² P = 0.02)

<table>
<thead>
<tr>
<th>CA9 expression</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
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<tr>
<td>Her-2 negative</td>
<td>5</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Her-2 positive</td>
<td>11</td>
<td>5</td>
<td>16</td>
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**Table 1** HER-2 amplification correlates with MVD* and plasma η-dimer

<table>
<thead>
<tr>
<th></th>
<th>Maximum MVD</th>
<th>Average MVD</th>
<th>Plasma η-dimer</th>
</tr>
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<tr>
<td></td>
<td>n² = 59</td>
<td>n = 59</td>
<td>n = 29</td>
</tr>
<tr>
<td>HER-2</td>
<td>r = 0.51</td>
<td>r = 0.50</td>
<td>r = 0.65</td>
</tr>
<tr>
<td>By FISH</td>
<td>P = 0.03</td>
<td>P = 0.05</td>
<td>P = 0.02</td>
</tr>
</tbody>
</table>

* MVD, microvessel density; VEGF, vascular endothelial growth factor; FISH, fluorescence in situ hybridization.

**Table 2** HER-2 amplification was inversely correlated with CA9 expression (x² P = 0.02)
amplification and HER-2 protein overexpression in 42 of 58 (73%) of the cases.

Higher Levels of Angiogenesis Correlate with Poor Prognosis. Both average MVD (Fig. 3A) and maximum MVD (Fig. 3B) correlated significantly with event-free survival (Cox proportional hazard $P$ of 0.006 and 0.034, respectively). Patients with high average and high maximum MVD values had significantly worse event-free survival. VEGF tissue expression, D-dimer, HER-2 (IHC), and HER-2 (FISH) did not correlate with event-free survival. None of the variables examined (average MVD, maximum MVD, tissue VEGF, plasma VEGF, HER-2 by IHC, HER-2 by FISH, or plasma D-dimer) were significant in predicting overall survival in either univariate or multivariate models.

DISCUSSION

This study is the first to look at the connection between HER-2 amplification and markers of angiogenesis and oxygenation in human breast tumors. The increases in MVD between HER-2-amplified tumors compared with nonamplified tumors were both statistically significant as well as potentially clinically significant. Weidner et al. (24) reported an increase of 10 vessel counts translated into a relative risk of 1.59 for involvement of axillary lymph nodes in a study examining 49 breast tumors. We find a similar magnitude (median 6 vessel count) difference between HER-2-amplified and -nonamplified tumors. The significant increase in angiogenesis seen in HER-2-amplified breast tumors may partially explain why HER-2 is such an important prognostic factor in breast cancer. In addition, this finding confirms in human breast tumors that HER-2 might be an important determinant in angiogenesis, a relationship first reported in cell culture models by Petit et al. (20).

This study also showed a decrease in the hypoxia marker, CA9, in HER-2-amplified tumors. This finding is the first to provide a link between increased tumor angiogenesis and decreased amounts of chronic tumor hypoxia, a factor that is known to play an important role in both radiation and chemotherapy resistance. CA9 has been shown in many tumor types to be an important prognostic marker but has not been examined as a predictive marker for response to therapy (29, 31). Perhaps this connection between HER-2 amplification and improved tumor oxygenation offers some insight into why HER-2 amplification might serve as a positive predictive role for chemotherapy sensitivity. A number of preclinical and clinical models have shown that many chemotherapeutic drugs are less effective in the setting of tumor hypoxia (36–38). Therefore, if HER-2 bestows improved tumor oxygenation via increased angiogenesis, this might alter the sensitivity to many different types of chemotherapy. Additional examination of the effects of im-
proved oxygenation in HER-2-amplified tumors in predicting chemotherapy response is necessary.

This study failed to show a connection between HER-2 protein expression by IHC and markers of angiogenesis and hypoxia. This discrepancy between the correlations seen with HER-2 gene amplification but not seen with HER-2 expression is probably reflective of the fact that gene amplification is a much more precise predictor of increased HER-2 signaling and dependence. This discrepancy is also representative of the fact that this study is limited by a small number of tumor samples, its retrospective design, and that available tumor specimens were 5–10 years old in some cases, which may have affected immunohistochemical assay quality. Nonetheless, correlations between HER-2 amplification and protein overexpression suggest that, at least for these markers, the tissue was adequate for study.

This study also found that HER-2-amplified tumors have lower levels of VEGF expression. This finding differs from that seen in preclinical models where transfection of the HER-2 gene into fibroblasts increased expression of VEGF (20). The higher amounts of angiogenesis seen in the HER-2-amplified tumors might have led to less tumor hypoxia (as indicated by the lower amounts of CA9 staining), and in turn, decreased production of VEGF. Alternatively, this finding may be explained by the complexity of VEGF regulation. Ocharoenrat et al. (39) have demonstrated that HER-2 up-regulation in head and neck carcinoma cells leads to increased HIF-1α signaling. In this study, HER-2 up-regulation led to increases in VEGF isoform A and C levels, no changes in VEGF isoform-B levels, and down-regulation of VEGF isoform-D levels. However, this cell culture model cannot account for complex effects of the in vivo tumor environment. Our observation in human tumors that VEGF as detected by an antibody that binds to all of its isoforms is down-regulated in HER-2-overexpressing tumors warrants additional investigation.

Finally, we found a significant correlation with HER-2 amplification and D-dimer levels. This correlation is not surprising, because the HER-2 amplified tumors had higher amounts of angiogenesis, and D-dimer appears to be associated with other markers of tumor neovascularization (40, 41). Clinically, markers of angiogenesis are needed and, therefore, we decided to look at D-dimer to see if there was any correlation with HER-2 amplification status. In addition, there are reports of HER-2 amplification leading to up-regulation of urokinase plasminogen activator, which would lead to an additional increase in fibrin degradation associated with HER-2-amplified tumors (42). Our findings would confirm that there are higher levels of fibrin turnover occurring in HER-2-amplified tumors, possibly because of higher amounts of tumor angiogenesis, higher urokinase plasminogen activator activity, or both.

In conclusion, this study has significant implications in the therapy of HER-2-positive breast cancer. This study provides possible mechanisms through which HER-2 acts in breast cancer, which result in the HER-2 signaling pathway being such an important prognostic and predictive factor. The finding that HER-2-amplified tumors have increased angiogenesis implies that this tumor phenotype might benefit from the use of antiangiogenic therapies more so than HER-2-negative tumors. Trastuzumab (Herceptin) has been reported to have antiangiogenic properties in preclinical models (43, 33), and this finding is consistent with the findings of our study. HER-2 amplification has also been shown to correlate with increased HIF-1α expression (44) in human breast cancers, and certainly this relationship as far as its therapeutic potential needs additional investigation. Hopefully, additional information regarding the downstream physiological effects of HER-2 amplification, including its up-regulation of angiogenesis, will lead to improved therapeutic combinations for this poor-prognosis subset of breast cancer patients.

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