Impact of Exploratory Biomarkers on the Treatment Effect of Bevacizumab in Metastatic Breast Cancer

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Abstract

Purpose: The addition of bevacizumab to cytotoxic chemotherapy has demonstrated a progression-free survival (PFS) benefit in the first-line and second-line treatment of advanced or metastatic breast cancer (MBC). However, the addition of bevacizumab to capecitabine in heavily pretreated MBC patients did not show a PFS benefit (AVF2119g phase III trial). The aim of this study was to evaluate the expression of novel putative biomarkers as predictors of benefit from bevacizumab in retrospective subset analyses of the AVF2119g trial.

Experimental Design: In the AVF2119g trial, 462 patients with MBC were randomly assigned to receive capecitabine or capecitabine plus bevacizumab. Primary tumor tissue and outcome data were available for 223 patients. Biomarker expression was assessed by in situ hybridization (VEGF-A, VEGF-B, thrombospondin-2 and Flt4) or immunohistochemistry (VEGF-C, PDGF-C, neuropilin-1, delta-like ligand (Dll) 4, Bv8, p53 and thymidine phosphorylase) on formalin-fixed, paraffin-embedded tissue. PFS was associated with these variables in retrospective subset analyses.

Results: Patients with low scores for Dll4, VEGF-C, and neuropilin-1 showed trends toward improvement in PFS associated with the addition of bevacizumab to capecitabine (P values = 0.01, 0.05, and 0.07, respectively). These observations were not statistically significant following correction for multiple hypothesis testing.

Conclusion: These retrospective subset analyses suggest that expression of Dll4, VEGF-C, and neuropilin-1 may predict benefit from bevacizumab. Such observations are not conclusive but warrant additional testing.

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Introduction

Angiogenesis is necessary for the growth of tumors (1), and is predominantly mediated by proangiogenic factors [e.g., vascular endothelial growth factor A (VEGF); ref. 2]. The expression of VEGF in cancer is controlled by both oncogenic signaling [for instance, epidermal growth factor receptor (EGFR) signaling; ref. 3] and hypoxia (4). Although there is redundancy among proangiogenic factors in advanced cancer (5), many early cancers (6, 7) and in vivo models (8) are VEGF dependent. This observation led to the evaluation of an anti-VEGF monoclonal antibody (bevacizumab) with first-line cytotoxic chemotherapy in advanced/metastatic breast cancer (MBC), which prolonged the median progression-free survival (PFS) in 3-phase III randomized controlled trials [E2100 (9), RIBBON1, and AVADO].

Two randomized controlled trials have evaluated bevacizumab in patients progressing after first-line treatment for MBC. The addition of bevacizumab to second-line chemotherapies improved the PFS of patients with MBC by a median 2.1 months (HR = 0.78; P = 0.0072) in the RIBBON2 trial. However, the earlier AVF2119g trial, evaluating the addition of bevacizumab to capecitabine in first-to fifth-line MBC (including patients relapsing on therapy), demonstrated a 10.7% improvement in response rate (P = 0.001) and no PFS benefit (10). Together with research conducted in preclinical models (8), the AVF2119g data suggest the possibility that anti-VEGF therapies may be more effective against relatively less advanced tumors that...
Translational Relevance

The clinical benefit of bevacizumab in metastatic breast cancer is small and an overall survival benefit is not seen. Furthermore, there are no validated biomarkers of the survival benefit afforded by bevacizumab. Objective response is not predictive of the survival benefit afforded by bevacizumab in colorectal cancer, and improved objective response rates are seen in the absence of a survival benefit in breast cancer. Oncologists cannot objectively select patients to start bevacizumab and cannot tell when resistance develops. This analysis assesses biomarkers that have been shown to be functionally involved in resistance to bevacizumab in preclinical studies. The predictive impact of these markers has not been assessed before in terms of survival data from a randomized controlled trial. Our data suggest that these markers may have predictive significance and is a first step in identifying patients who benefit from bevacizumab and resistant patients who may benefit from alternative therapies.

are VEGF dependent. By contrast, relatively more advanced tumors may exploit additional mechanisms to further drive angiogenesis [e.g., platelet-derived growth factors (PDGF); refs. 5, 8] and to establish mature vascular beds that are potentially less sensitive to anti-VEGF therapies.

Preclinical research has provided several new avenues to investigate the impact of bevacizumab on patient survival. Therapies that target VEGF specifically have been observed to selectively prune endothelial cells that are not covered by pericytes (11). Further research suggests that paracrine endothelial/pericyte signaling mediated by members of the PDGF family, may account for the relative resistance of more mature vessels to anti-VEGF therapies (12). Indeed, combined targeting of VEGF and PDGF signaling has additive antiangiogenic effects (13). Using a model of resistance to anti-VEGF therapy, Crawford and colleagues reported that stromal expression of PDGF-C is a significant feature of anti-VEGF resistance (14). Furthermore, di Tomaso and colleagues revealed that overexpression of PDGF-C by glioblastoma xenografts is associated with relative insensitivity to anti-VEGF therapy (15). No one has tested the implications of these observations in human cancer.

Investigating inherent resistance to anti-VEGF therapy in xenografts, Shojaei and colleagues reported that recruitment of mouse CD11b+Gr+ myeloid cells was sufficient to confer relative insensitivity to anti-VEGF treatments (16). Further studies in mouse models suggest that mobilization of CD11b+Gr+ myeloid cells may be regulated by Bv8 (Bombina variegata peptide 8) expression (17). Nevertheless, there is no clinical evidence to suggest that these factors are associated with the efficacy of bevacizumab.

Delta-like ligand 4 (Dll4) is a Notch ligand that is expressed by endothelial cells (18) and may be induced by VEGF (19). Disruption of Dll4 signaling may impair tumor angiogenesis in xenografts (19, 20). Certain xenografts that are refractory to anti-VEGF therapy are reported to be sensitive to anti-Dll4 (19–21) therapy, and combination treatment with anti-VEGF and anti-Dll4 therapies has additive effects on tumor growth (19). In addition, human umbilical vein endothelial cells transfected with Dll4 downregulate VEGF receptors and show reduced proliferative and migratory responses to VEGF (22). Together these data suggest that Dll4 may have a role in mediating resistance to anti-VEGF therapies.

The aim of this study was to evaluate the expression of VEGF pathway components [VEGF-A, VEGF-B, VEGF-C, fms-like tyrosine kinase (fT) 4, and neuropilin-1], thrombospondin-2 (TSP-2, an antiangiogenic gene; ref. 23), p53, thymidine phosphorylase (TP, a putative biomarker of capcitabine efficacy; ref. 24), PDGF-C, Dll4, and Bv8 in available primary cancer tissue as predictors of benefit from bevacizumab in MBC. These hypotheses were tested in exploratory, retrospective subset analyses on the AVF2119g trial of capcitabine with and without bevacizumab in previously treated MBC.

Materials and Methods

Patients and study design

Patient recruitment and trial design have been described in detail elsewhere (21). In brief, patients with MBC were randomized to receive capcitabine (n = 230) or capicitabine with bevacizumab (n = 232). Enrollment took place in the United States only from November 2000 to March 2002. Local institutional review boards approved the protocol. At the time of enrollment, informed consent was obtained from trial participants to permit research on their archived tissue. All analyses were performed according to the REMARK criteria (25).

Tissue samples

Formalin-fixed, paraffin-embedded tissue blocks and corresponding pathology reports were retrospectively obtained for 181 patients from multiple centers. Where blocks could not be obtained, whole sections were available for 43 patients; the total case series comprised 224 primary tumors. Tissue microarrays were assembled as described previously (26). Sample size was constrained by the number of tissues available from the pathology archives where the original patient samples were stored.

In situ hybridization

Riboprobe synthesis, hybridization, developing, and analysis were carried out as described previously (27). Sense and antisense primers are detailed in Supplementary Table 1.

Hybridization of antisense β-actin riboprobes was confirmed in all tissues. Sense riboprobes were employed as negative controls for hybridization specificity. Tissue microarray cores were scored semi-quantitatively on a scale of zero (no expression) to 3 (very strong signal), according to the overall intensity of the hybridization signal in 10% or
greater of the neoplastic cells (VEGF-A and VEGF-B), stroma (thrombospondin-2), or endothelium (Flt4). The highest score among replicate tissue microarray cores was chosen as the score for the patient. Whole sections were scored on an identical scale. Appropriate tissue and/or cell pellet controls were included in each experiment (23, 28, 29). Scoring was performed blind to treatment and outcome.

**Immunohistochemistry**

Immunohistochemistry was performed on freshly cut tissue sections as described previously. The antigen retrieval, primary antibodies, and detection methods used are described in Supplementary Table 2. All antibodies have been previously validated (Supplementary Table 2) except anti-Dll4 and anti-neuropilin-1. The specificity of anti-Dll4 and anti-neuropilin-1 was assessed using human embryonic kidney (HEK)-293 cell pellet controls transfected with full-length human Dll4 and full-length mouse Dll4, or full-length human neuropilin-1 and empty vector, respectively.

To assess expression, tissue microarray cores and whole sections were scored semi-quantitatively on a scale of zero (no expression) to 3 (very strong signal), according to the intensity of chromogen deposition in 10% or greater of the neoplastic cells (VEGF-C, PDGF-C, neuropilin-1, p53, and TP), endothelium (Dll4 and neuropilin-1), or inflammatory cells (Bv8). Whole sections were scored on an identical scale. The highest score among replicate tissue microarray cores was chosen as the score for the patient. Appropriate tissue and/or cell pellet controls were included in each experiment (Supplementary Figs. 1 and 2). Substitution of the primary antibody with an isotype control antibody confirmed specificity of immunoreactivity. Scoring was performed blind to treatment and outcome.

**Statistical analyses**

To compensate for the large number of statistical hypotheses being tested, a stronger level of evidence is required to determine whether an individual hypothesis is significant. The false discovery rate (FDR) controlling procedure by Benjamini and Hochberg (30) was used to adjust the nominal P values. After adjustment, P values < 0.05 were considered significant. Cutoffs were chosen to discriminate between wholly negative cases (score = 0) and the remainder, or weakly and strongly positive (cutoff score > 1) depending on subgroup size (to ensure a balanced distribution between high and low groups), so that the statistical power can be maximized. Different cutoffs were also used as a sensitivity association. Associations between biomarkers were assessed using Pearson’s χ² test after adjustment for the FDR. Associations between biomarkers and PFS were assessed using survival analyses. All survival analyses refer to PFS from the time of randomization to death or disease progression, whichever comes first [defined by an independent review facility (IRF), investigator’s assessment was used for 3 patients who did not have postbaseline IRF assessments]. Patients without an event were censored at the time of the last tumor assessment. Median survival times were estimated from Kaplan–Meier curves, and their

95% CIs were based on the sign test (31). HRs and 95% CIs were determined from a Cox regression model with terms of the treatment group, the marker (dichotomized to high and low group), the interaction between the treatment and the marker, and the stratification factors (ECOG performance status (dichotomized to 0 and ≥1), prior metastatic disease chemotherapy (yes or no)). P values for the HRs were constructed on the basis of Wald tests and then adjusted for the FDR. Sensitivity analyses were performed from a Cox regression model leaving out the stratification factors terms and a Cox regression model stratified by the stratification factors.

**Results**

**Study group characteristics**

Outcome data were available for 223 of the 224 patients assessed for biomarker expression (99.6%). The cohort of patients in the biomarker population had demographic and pathologic characteristics that were very similar to those in the overall trial population (Table 1). In addition, the estimated PFS treatment effect was not significantly different in the subset of 223 patients compared with the entire study population; the HR was 0.90 (95% CIs, 0.65–1.24) in the subset compared with 0.98 (0.77–1.25) in the overall study population. Analyses of tumors from 223 patients yielded informative data on 102 to 183 patients (Table 2). Results were not available for the remaining cases due to limited amounts of tissue.

**Biomarker expression**

Expression of tumor VEGF-A (146/183, 80%) and stromal TSP-2 (114/157, 73%) were consistent with detailed descriptions elsewhere (23, Supplementary Fig. 3). Hybridization of the antisense riboprobe for VEGF-B was observed over the tumor cells of 42 of 102 (41%) cases (Supplementary Fig. 3). Hybridization of the antisense riboprobe for Flt4 was observed over endothelial cells in 27 of 103 (26%) of tumors (Supplementary Fig. 3).

Cytoplasmic immunoreactivity for VEGF-C (88/161, 55%) and PDGF-C (90/157, 57%) was predominantly observed in tumor cells (Fig. 1). Membranous and/or cytoplasmic neuropilin-1 was observed in tumor-associated endothelial cells from 144 of 162 (89%) patient samples and weakly in the tumor cells of 15 of 162 (9%) samples (Fig. 1 and Supplementary Fig. 2). Similarly, membranous and/or cytoplasmic expression of Dll4 was observed in tumor-associated endothelial cells from 127 of 160 (79%) cancers and in a variable proportion of tumor cells in whole sections from approximately 5% of samples (Fig. 2 and Supplementary Fig. 2). Bv8 (25/161, 15%) was specifically expressed in the cytoplasm of infiltrating inflammatory cells, consistent with previous reports (Fig. 2; ref. 32). Nuclear expression of p53 (33; 82/174, 47%) and expression of TP (24; 127/174, 73%) by tumor cells were consistent with detailed descriptions elsewhere.

Frequency data for in situ hybridization and immunohistochemistry scoring including cutoffs, are detailed in...
Table 2. There were no statistically significant differences in frequency data for tissue microarrays (total \(n = 181\)) and whole sections (total \(n = 43\)). After correction for multiple testing, only positive associations between TSP-2 and Flt4, PDGF-C and VEGF-C, endothelial Dll4 and endothelial neuropilin-1, and endothelial Dll4 and Bv8 were statistically significant (Supplementary Table 3).

### Biomarker association with progression-free survival

Of the biomarkers, only VEGF-A expression by tumor cells showed prognostic significance (though not after FDR correction), a finding previously reported for other tumor types (Supplementary Table 4; ref. 34). VEGF-A, VEGF-B, Flt4, TSP-2, PDGF-C, Bv8, and p53 showed no association with treatment outcome (Table 3). However, subgroups with low expression of endothelial neuropilin-1, TP, VEGF-C, or endothelial Dll4 showed trends toward benefit from the addition of bevacizumab to capecitabine in terms of PFS, but after correcting for multiple hypothesis testing, none of them were significant (Table 3 and Fig. 3). When endothelial Dll4 subgroups were defined using a cutoff score of 0 or 1 versus 2 or 3 (as opposed to 0 vs. 1, 2, or 3), Dll4 did not show statistical significance (data not shown). Expression of neuropilin-1 by tumor cells was not a significant predictive factor (data not shown). Expression of Dll4 by tumor cells was too infrequent to permit meaningful PFS analyses in this series.

### Discussion

Treatment regimens that include bevacizumab have demonstrated broad clinical activity (9, 35-39). However, identification of patient subsets that receive the most clinical benefit would enable more specific treatment...
administration of bevacizumab and allow patients unlikely to benefit the opportunity to seek other treatment modalities. Unfortunately, despite efforts to identify patient subsets with a differential benefit from bevacizumab, no validated biomarkers have been defined. Research in this area may be complicated by the multiple putative mechanisms of action for bevacizumab and competing biomarkers for sensitivity to coadministered therapy. This study interrogated primary breast cancer tissue collected from a phase III trial of capecitabine with and without bevacizumab, for expression of biomarkers associated with angiogenesis. None of the biomarkers showed a definitive association with improved PFS. Nevertheless, several trends were identified for expression of VEGF-C and TP, neuropilin-1 and Dll4.

Retrospective subset analyses on randomized controlled trials have thus far failed to identify patients with a clinically significant differential benefit from bevacizumab. Published data on colorectal cancer have found no effect of p53 mutations (40), K-ras/B-raf mutations (40), VEGF-A expression (23), microvascular density (23), TSP-2 expression (23), or circulating VEGF-A levels (41) on the treatment effect of bevacizumab. Exploratory studies of specific VEGF polymorphisms have been reported to

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<th>Score 2</th>
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<td>35 22</td>
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<td>44 25</td>
<td>10 6</td>
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<td>127 73</td>
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Table 2. Frequency data for in situ hybridization and immunohistochemical scoring

Figure 1. Representative immunohistochemistry for VEGF-C (A, tumor 2+), PDGF-C (B, tumor 2+), and neuropilin-1 (C, endothelium 2+; D, endothelium 2+, tumor +). Brown staining with diaminobenzidine indicates immunoreactivity.
predict overall survival in patients treated with bevacizu-
mab and paclitaxel in MBC in 1 study (42), but have not
been observed in other large randomized studies of bev-
acizumab-based treatment. High levels of circulating ICAM
(intercellular adhesion molecule) were associated with a
prolonged overall survival benefit from the addition of
bevacizumab to first-line chemotherapy in nonsquamous,
non–small cell lung cancer (43). However, the low ICAM
group also derived a small benefit in response rate and
overall survival from the addition of bevacizumab, suggest-
ing that ICAM levels have prognostic value and should not
be used to select patients (43). Additional studies have
shown changes in angiogenesis-related biomarkers in
patients who have received bevacizumab that are suggestive
of activity (44, 45). The authors have yet to relate these
observations to survival, making it difficult to evaluate their
significance. Circulating endothelial cells (CEC) have been
proposed as biomarkers of bevacizumab efficacy (46).
However, to date, there are no data that distinguish the
predictive efficacy of CECs as regards bevacizumab, from
their more general prognostic value (47) in patients not
receiving bevacizumab. Preliminary data from Formica
and colleagues suggest that circulating CA19.9 may serve as
a valid biomarker of survival benefit, though this will need
to be confirmed in a trial population (48).

This study aimed to evaluate several putative biomar-
kers of anti-VEGF efficacy identified by preclinical
research. There is growing evidence from model systems
that Dll4 expression may define tumors that are resistant
to anti-VEGF therapies and inhibition of Dll4 may over-
come resistance to bevacizumab (21, 22). Herein, tumors
with low endothelial Dll4 expression showed a trend
toward a benefit in PFS from the addition of bevacizumab
to capecitabine (median = 4.53 month improvement, \(P = 0.01\)). No benefit was observed in tumors with high
endothelial Dll4 expression. In addition, patients from
the low Dll4 subset who were treated with capecitabine
showed a marginally worse PFS than patients from the
high Dll4 subset, though this was not statistically signifi-
cant. The hypothesis follows that tumors with high
endothelial Dll4 expression have more mature vascula-
ture, but tumors with low endothelial Dll4 expression
have immature, poorly functional vasculature, leading to
hypoxia that is an adverse prognostic factor. When bevac-
izumab is combined with capecitabine, there is an antiangiogenic effect with normalization of the vascular bed in
tumors with immature vessels expressing low levels of Dll4.
The effect of bevacizumab is, perhaps, more restricted in
tumors with mature, functional vasculature expressing high
levels of Dll4. However, the number of patients and events
in these subgroups is very small that leaves these analyses
open to bias from imbalances in patient characteristics.
Although there is a biological rationale to support these
observations, the data are exploratory and independent
validation should be sought.

Several other biomarkers showed trends toward benefit
from bevacizumab including low expression of VEGF-C,
endothelial neuropilin-1, and TP. Redundancy among
VEGF ligands may provide a mechanism for tumor escape
from anti-VEGF agents. VEGF-C and its receptor Flt4 have
a physiologic role in lymphangiogenesis (49), but a
pathologic role in angiogenesis of breast cancer (50).
Expression of VEGF-C by tumor cells and Flt4 by
tumor-associated endothelium may invoke resistance to

Figure 2. Representative
immunohistochemistry for Dll4 (A,
endothelium score 2; B,
endothelium 3+, tumor –), Bv8 (C,
inflammation 3+), and TP (D,
tumor 1+). Brown staining with
diaminobenzidine indicates
immunoreactivity.
Table 3. Cox regression analysis of time to progression-free survival for each biomarker subgroup, after adjustment for stratification factors and interaction between treatment and the biomarker variable

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anti-VEGF-A therapies by providing an alternative pathway for angiogenesis. In addition, VEGF-C is believed to be capable of signaling through VEGF receptor 2 (51). Neuropilin-1 is a cell surface protein that can directly interact with VEGF-A and VEGF receptor 2 and may be expressed by both tumor and endothelial cells (52). Data from Pan et al. suggest that combined inhibition of neuropilin-1 and VEGF-A has additive antiangiogenic effects, prohibiting the organization of endothelium and pericytes to form mature vessels (52). Therefore, it is possible that endothelial neuropilin-1 expression may identify a group of patients amenable to combination anti-neuropilin-1 and anti-VEGF treatment.

High expression of TP, an enzyme involved with the metabolism of capcitabine to 5-fluorouracil, has been reported to predict benefit from capcitabine (24). Herein, the median PFS in the capcitabine treatment arm was 1.97 months longer in the subgroup with high versus low TP expression. Median PFS was similar for low and high expressing TP subgroups in the capcitabine plus bevacitumb treatment arm. Hence, the perceived benefit from bevacitumb in tumors expressing low levels of TP may be an artifactual reflection of the prognostic effect of high TP expression in the control arm.

Due to the limited availability of tissue from metastases, these analyses have used primary tumor samples to predict the behavior of metastatic disease. One may expect differences between primary cancers and their metastases, especially in terms of tumor-stromal interactions and angiogenesis, depending on the host tissue. Further clinical research is needed to investigate the impact of these differences on the observations made herein and elsewhere in biomarker studies.

The lack of an observed predictive effect of VEGF-A and p53 expression is consistent with published predictive studies on the survival benefit of bevacitumb (23, 40, 42). Despite preclinical evidence to suggest a predictive effect (14, 17), neither Bv8 nor PDGF-C expression were significant biomarkers of benefit from the addition of bevacitumb to capcitabine. However, this may be related to dynamic regulation of tumor expression of these factors. Given the potentially long time period between primary breast tumor collection and second- or third-line treatment with chemotherapy for MBC on this study, it is conceivable that tumor expression of these factors had changed substantially. In addition, it is noted that very few of these archival primary breast tumor samples tested expressed Bv8 by immunohistochemistry, preventing an adequate comparison of treatment outcome for patients with high versus low Bv8 expression.

These exploratory analyses suggest that several biomarker subsets show trends toward improved PFS from the combination of bevacitumb with capcitabine in patients with previously treated MBC. However, the observations are not statistically significant after correction for multiple hypothesis testing. Validation and more fundamental research on the appropriate clinical material are required to understand the complexity of angiogenesis and response to antiangiogenic therapy.

Disclosure of Potential Conflicts of Interest

Adrian M. Jubb, Kathy D. Miller, Melody A. Cobleigh, and Hope S. Rugo have received honoraria from Genentech, Inc.; Kathy D. Miller and Melody A. Cobleigh have worked as consultants to Genentech, Inc.; Kathy D. Miller, Melody A. Cobleigh, and Hope S. Rugo have received research funding from Genentech, Inc.; Adrian M. Jubb, Dafeng Chen, James D. Reimann, Maike Schmidt, Virginia K. Langmuir, Kenneth J. Hillan, Daniel S. Chen, and Hartmut Koeppen are employees of Genentech, Inc.; Adrian L. Harris has received research funding from and has acted as an advisor to Roche.

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