

Prognostic and Predictive Value of Tumor Vascular Endothelial Growth Factor Gene Amplification in Metastatic Breast Cancer Treated with Paclitaxel with and without Bevacizumab; Results from ECOG 2100 Trial

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Abstract

Purpose: Clinically validated biomarkers for anti-angiogenesis agents are not available. We have previously reported associations between candidate *VEGFA* single-nucleotide polymorphisms (SNP) and overall survival (OS) in E2100. The associations between tumor *VEGFA* amplification and outcome are evaluated here.

Experimental Design: E2100 was a phase III trial comparing paclitaxel with or without bevacizumab for patients with metastatic breast cancer. FISH to assess gene amplification status for *VEGFA* was conducted on paraffin-embedded tumors from 363 patients in E2100. Evaluation for association between amplification status and outcomes was conducted.

Results: Estrogen receptor (ER)+ or progesterone receptor (PR)+ tumors were less likely to have *VEGFA* amplification than ER/PR– tumors ($P = 0.020$). *VEGFA* amplification was associated with worse OS (20.2 vs. 25.3 months; $P = 0.013$) in univariate analysis with a trend for worse OS in multivariate analysis ($P = 0.08$). There was a significant interaction between *VEGFA* amplification, hormone receptor status, and study arm. Patients with *VEGFA* amplification and triple-negative breast cancers (TNBC) or *HER2* amplification had inferior OS ($P = 0.047$); amplification did not affect OS for those who were ER+ or PR+ and *HER2*–. Those who received bevacizumab with *VEGFA* amplification had inferior progression-free survival (PFS; $P = 0.010$) and OS ($P = 0.042$); no association was seen in the control arm. Test for interaction between study arm and *VEGFA* amplification with OS was not significant.

Conclusion: *VEGFA* amplification in univariate analysis was associated with poor outcomes; this was particularly prominent in *HER2*+ or TNBCs. Additional studies are necessary to confirm the trend for poor OS seen on multivariate analysis for patients treated with bevacizumab. *Clin Cancer Res*; 19(5); 1281–9. ©2012 AACR.

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Introduction

E2100 was a randomized, phase III trial in the first-line setting for patients with metastatic breast cancer (1). This trial showed a significant improvement in progression-free survival (PFS) for the addition of bevacizumab to paclitaxel when compared with paclitaxel alone. These data led to the accelerated approval by the U.S. Food and Drug Administration (FDA) for bevacizumab in this setting. Although subsequent first-line trials such as AVADO and RIBBON-1 also met their primary endpoints for showing significantly improved PFS, the absolute improvements in median PFS were much more modest than in the original E2100 trial, and none of the trials showed an improvement in overall survival (OS; refs. 2, 3). On the basis of these more modest benefits in combination with a unique toxicity profile, the U.S. FDA subsequently recommended rescinding approval for bevacizumab in this setting (4).

Translational Relevance

The use of bevacizumab for breast cancer has been a highly controversial topic. This agent, based on nonspecific implementation in the clinical trial E2100, received accelerated approval by the U.S. Food and Drug Administration (FDA). On the basis of the less impressive improvements in progression-free survival in subsequent trials (AVADO and RIBBON-I) and the lack of overall survival in all trials coupled with a unique toxicity profile, the approval was rescinded. Interestingly, despite the same data, the European Union has maintained approval. Despite the controversy, most would agree that identification of a successful biomarker for bevacizumab to establish which subgroup might obtain the greatest benefit would be of highest importance. Prior studies in breast cancer have shown that amplification of the therapeutics' target gene can serve as an excellent predictive marker (i.e., *HER2* and trastuzumab). In this study, we set out to evaluate the role of *VEGFA* amplification as a biomarker for bevacizumab in E2100.

One major drawback for the clinical use of bevacizumab (as well as other anti-angiogenic therapies) is the lack of a validated biomarker to predict which patients might be expected to gain the most benefit (5, 6). Our group previously reported single-nucleotide polymorphisms (SNP) that predicted a genetic subgroup that derived substantial benefit in OS for those who received bevacizumab in E2100 (7). These SNPs have been tested in other settings and have been validated in some but not all studies (8–10). Somatic aberrancy also has great potential to serve as a prognostic or predictive marker (11). Gene amplification and deletion are common aberrancies and are the basis for one of the most successful predictive and prognostic biomarkers to date for breast cancer (i.e., *HER2* amplification; ref. 12). Those with *HER2*-amplified tumors gain substantial benefit from therapies that target the *HER2* protein, including trastuzumab (13–16), lapatinib (17), pertuzumab (18), and T-DM1 (19). In this correlative study of E2100, we evaluate the ability for tumor amplification of the target gene of bevacizumab, *VEGFA*, to predict outcome.

Patients and Methods

Samples

In the E2100 parent trial, there were 671 eligible patients with 641 disease progression events and 544 deaths as of May 1, 2009 (1). Patients were randomized to paclitaxel with bevacizumab (arm A) or paclitaxel alone (arm B). The results from the parent trial have previously been reported. Paraffin-embedded tumor blocks were available from 367 for assessment of *VEGFA* amplification by FISH. In all cases, these blocks were derived from the patient's primary tumor. Median follow-up for surviving patients was 59 months at the time of this analysis. All specimens were provided to the investigators of this trial in a de-identified manner. For

VEGFA FISH, 178 samples were available from arm A and 189 from arm B. This retrospective correlative trial was approved by the Institutional Review Board at Indiana University (Indianapolis, IN) and The North American Breast Cancer Group Correlative Sciences Committee.

VEGFA FISH

A *VEGFA*/centromere enumeration-6 (CEN-6) probe set was previously created and validated (20). The validation included a test of the DNA clones by restriction enzyme fragment measurements. The final product contained a bacterial artificial chromosome (BAC) probe, RP11-710-L16, covering 183 kb including the *VEGFA* gene and flanking regions with a start position of 43,633,251 and an end position of 43,817,196 according to UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly. Upstream the probe overlaps the entire *MRPS18* gene (human mitochondrial ribosomal protein *S18-2*) and 20% of the *RSPH9* gene; downstream there is overlap with approximately 67% of the *AK097853* gene. The CEN-6 probe was labeled with fluorescein isothiocyanate (FITC)-labeled peptide nucleic acid (PNA) oligonucleotides and the *VEGFA* probe labeled with Texas Red. Both the *VEGFA* and CEN-6 probes were tested on metaphase spreads to localize the signals to chromosome 6 and exclude cross-hybridization to other chromosomes. The concentrations of Texas Red *VEGFA* and FITC CEN-6 were fine-tuned to give well-balanced red and green signals when hybridized on human breast cancer tissue.

A tissue microarray with 93 human primary breast tumor core specimens was then evaluated by FISH for the presence of *VEGFA* gene amplification and deletion on formalin-fixed, paraffin-embedded tumors using a protocol similar to the manufacturer's protocol for *TOP2A* FISH pharmDx Kit (21). Results were interpreted using a fluorescence microscope equipped with appropriate filters for the fluorophors. Cancer cells were located and then scored for total number of *VEGFA*/CEN-6 signals. A ratio was calculated from the average number of signals for each probe. Normal cells in the analyzed tissue section served as an internal positive control of pretreatment and hybridization efficiency. On the basis of this validation array, a ratio <0.8 was considered deleted; a ratio ≥ 1.5 but <2 was considered borderline-amplified; and a ratio ≥ 2 was considered amplified.

All samples were scored by an experienced technologist using the *TOP2A* FISH scoring guidelines (21). The signals were preferably scored in 3 distinct tumor areas and totaled. The signals were scored in nonoverlapping nuclei where bright and point-shaped signals of balanced size could be identified. Nuclei were scored until 60 red *VEGFA* signals were reached and then the green signals were scored in the same nuclei (22). A minimum of 6 nuclei were scored and a total of 60 nuclei were scored in samples at or near the cutoff (1.80–2.20 for amplification and 0.70–0.90 for deletion) or near the 1.5 ratio for borderline amplification. Reproducibility was tested in 17 samples with interobserver concordance 88.2%; these were re-scored by a second evaluator who counted nuclei from 3 more tumor areas until 60 red

signals were reached. The final ratio for each specimen was based on all scoring.

Statistical design

Comparison of VEGFA amplification with PFS and OS.

To optimize power, we combined those patients who had a tumor that was amplified and borderline-amplified (this group will be referred to as amp/BA). Those with amp/BA status were compared with all other groups (normal + deleted). We evaluated the PFS and OS for those with amp/BA status compared with those who did not in univariate analysis. Univariate analysis was conducted using log-rank test. We also conducted multivariate analysis with Cox proportional hazard model using significant covariates from backward elimination stepwise regression. VEGFA amplification tests were formally evaluated using Kaplan–Meier curves. These were also conducted on subgroups based on estrogen receptor/progesterone receptor (ER/PR) status and arm of study. A formal test for interaction between arm of study and amplification status was also conducted using the Cox regression model. Cox regression analyses and Kaplan–Meier curves were conducted in R. $P < 5\%$ was considered statistically significant.

Results

Performance and frequency of VEGFA gene amplification in E2100

Of the 367 cases available for assessment of amplification for VEGFA by FISH, 324 had successful hybridization (success rate: 88.3%). Twenty-one cases showed VEGFA amplification, 31 had borderline amplification, 251 were VEGFA normal, and 21 had deletion; (Table 1). The characteristics and outcome of the subgroup of patients studied in this subgroup fared similarly to the parent trial (Supplementary Tables S1 and S2).

VEGFA amplification by arm of E2100 and by ER/PR and HER2 status

VEGFA amplification status is summarized in Table 1. A total of 52 patients (16%) had amp/BA status. Amp/BA status was well-balanced across arms in E2100 comprising 15.3% and 16.8% in arms A and B, respectively. There was less amplification in those who had ER- and/or PR-positive

tumors (12.4%, $n = 26$ of 210) than in those who were ER/PR-negative (23.0%, $n = 23$ of 100; $P = 0.020$ by Fisher exact test). E2100 was predominately designed for HER2-negative patients, and thus only 6 patients in this correlative study had HER2 amplification; however 50.0% ($n = 3$ of 6) of these patients showed VEGFA amp/BA.

Association of VEGFA amplification with efficacy

On univariate analysis in the entire study population including both treatment arms, patients with tumor VEGFA amp/BA had significantly worse median PFS (7.8 vs. 8.3 months; $P = 0.040$) and median OS (20.2 vs. 25.3 months; $P = 0.013$) than those whose tumors did not exhibit amplification (Fig. 1). Multivariate analysis was conducted and included covariates that significantly impacted PFS (arm of study, ER/PR status, use of hormonal therapy) and OS (ER/PR status and use of hormonal therapy). On multivariate analysis, amp/BA status had no statistically significant impact on PFS ($P = 0.178$) or OS ($P = 0.08$). Because hormonal sensitivity and the arm of study nullified the significant results seen on univariate analysis, further evaluation was conducted to see whether there was an interaction between arm of study, ER/PR status, and amplification status.

In addition to having a lower likelihood of having VEGFA amp/BA, those with ER- or PR-positive tumors did not experience inferior outcome (PFS; $P = 0.418$ and OS; $P = 0.321$) whether amplified or not (Fig. 2). Patients with HER2-positive tumors were not analyzed separately for association with outcome due to insufficient numbers but were combined with those with triple-negative breast cancers (TNBC) as prior data have shown higher expression of VEGFA in both subtypes than in those who have hormone receptor-positive tumors. When combining the subtypes that displayed a higher frequency of VEGFA amp/BA (HER2-positive and triple-negative populations), those with amp/BA had a trend for inferior PFS with the curves separating late ($P = 0.092$) and a statistically significantly inferior OS ($P = 0.047$; Fig. 2). The improvement in OS was no longer statistically significant when excluding those patients with HER2+ tumors ($P = 0.143$).

When considering treatment arm, patients in arm A (bevacizumab and paclitaxel) of E2100 had a statistically

Table 1. Amplification by arm of trial and ER/PR/HER2 status

	<i>n</i>	Amplified + borderline-amplified, <i>n</i> (%)	Amplified (ratio ≥ 2)	Borderline-amplified (ratio ≥ 1.5 but < 2)	Normal	Deleted (ratio < 0.8)
All patients	324	52 (16.0)	21	31	251	21
Arm A	157	24 (15.3)	6	18	120	13
Arm B	167	28 (16.8)	15	13	131	8
ER+ or PR+/HER2–	210	26 (12.4)	6	20	172	12
TNBC	100	23 (23.0)	13	10	69	8
HER2+	6	3 (50.0)	2	1	2	1

NOTE: Ratio = VEGFA/CEN-6 signal ratio; arm A = paclitaxel with bevacizumab; arm B = paclitaxel alone; TNBC = triple negative breast cancer.

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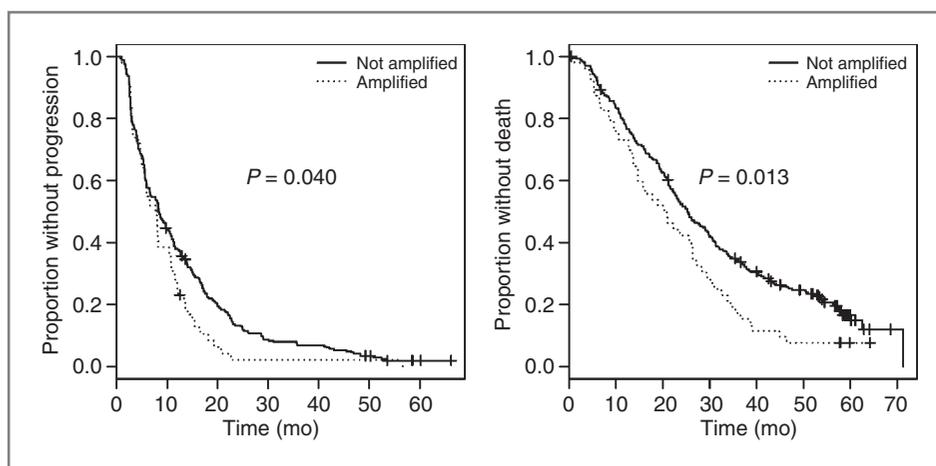


Figure 1. PFS (left) and OS (right) for all patients comparing those with VEGFA amplification/borderline amplification versus those without.

significant inferior median PFS for those with amp/BA status (10.5 months) compared with those who do not (11.3 months; $P = 0.010$; Fig. 3). These patients also had inferior median OS for those with amp/BA status (21.0 months) than those without (25.6 months; $P = 0.042$). Those in arm B (paclitaxel alone) did not have differential PFS ($P = 0.676$) or OS ($P = 0.123$) based on amplification

status (Fig. 3). All patients in arm B, however, fared worse than those on arm A with a median PFS of 5.6 months and a median OS of 23.7 months.

As stated above, patients with amp/BA status do worse than those who do not have amp/BA status. When specifically considering those patients in this poor prognosis group (amp/BA), there is no difference in median PFS

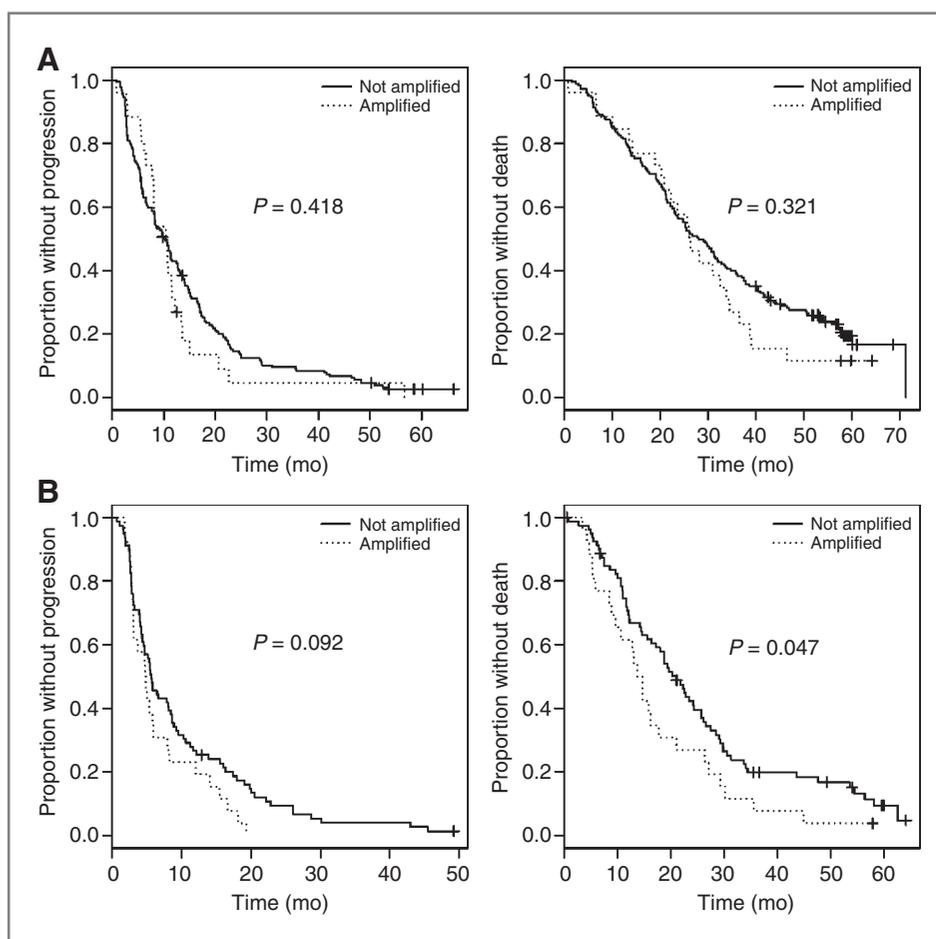
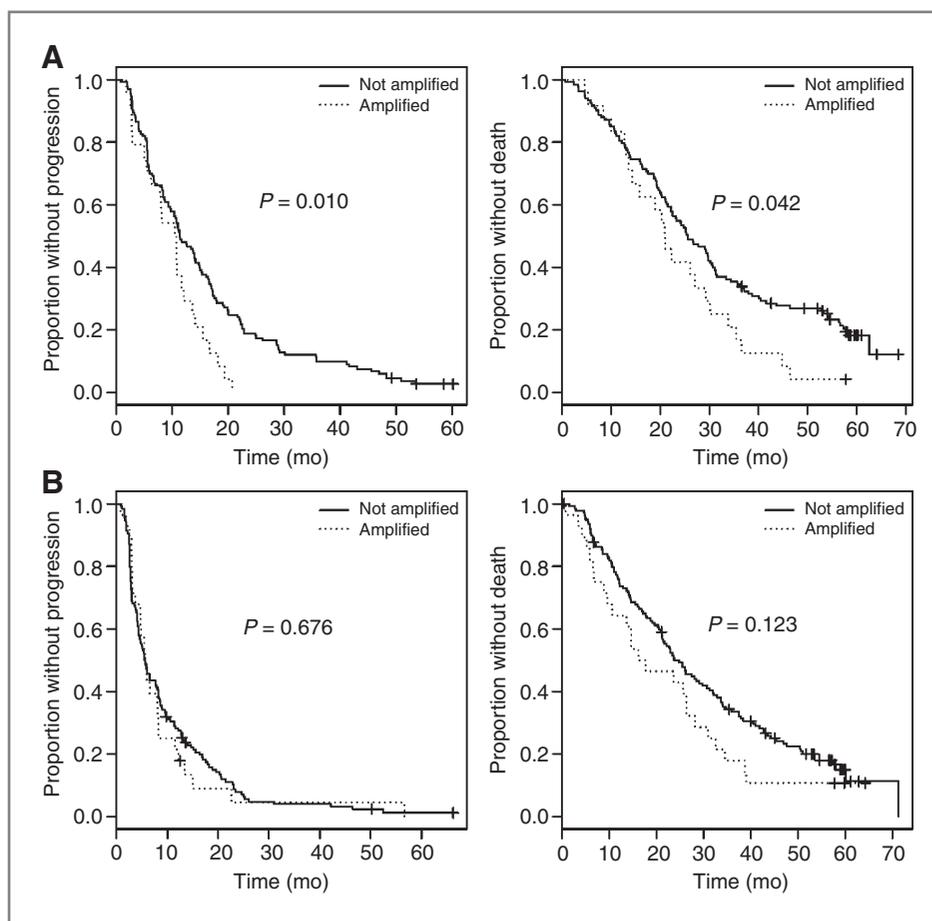


Figure 2. PFS (left) and OS (right) for (A) ER- or PR-positive and (B) TNBCs and HER2-positive patients. Comparison of those with VEGFA amplification/borderline amplification versus those without.

Figure 3. PFS (left) and OS (right) for patients in (A) arm A and (B) arm B of E2100. Comparison of those with VEGFA amplification/borderline amplification versus those without. Arm A, paclitaxel and bevacizumab; arm B, paclitaxel alone.



(10.5 vs. 5.7 months; $P = 0.438$) or median OS (21.0 vs. 16.9 months; $P = 0.973$) for those receiving bevacizumab (arm A) compared with placebo (arm B) suggesting bevacizumab has no effect in this subgroup (Fig. 4). For those patients who are not amplified, however, there is a significant improvement in median PFS for those who received bevacizumab (arm A) compared with those who did not in arm B (11.3 vs. 5.5 months; $P = 6.29 \times 10^{-5}$). This improvement in PFS did not translate into an improvement in median OS (25.6 vs. 24.8 months; $P = 0.472$). When considering both the arm of study and amplification status simultaneously, those patients who did not have amp/BA status and who received bevacizumab had the best PFS ($P = 8.55 \times 10^{-6}$; Fig. 5). There was no corresponding OS improvement, however, seen for those who were not amp/BA and received bevacizumab and paclitaxel ($P = 0.136$). A formal test for interaction between amplification and arm of the trial was not significant for PFS ($P = 0.220$) or OS ($P = 0.690$).

Discussion

In E2100, paclitaxel and bevacizumab showed an improvement over paclitaxel alone in measurement of PFS but not OS (1). Subsequent first-line trials testing bevacizumab in breast cancer, including a meta-analysis of all

trials, also showed significantly improved PFS but not median OS (2, 3, 23). Despite consistent improvement in PFS observed when bevacizumab is added to chemotherapy, the absolute improvement varied with the type of chemotherapy it was partnered with, with the greatest benefit when partnered with paclitaxel (24). Upon the basis of the results of the AVADO and RIBBON-1 trials, the accelerated approval initially granted by the U.S. FDA was rescinded (25), whereas the European Medicine Agency (EMA) granted approval in combination with paclitaxel as first-line therapy. Bevacizumab therefore remains approved for the treatment of metastatic breast in multiple countries throughout the world, based largely on the results of the E2100 trial. In addition, anti-VEGF therapy with bevacizumab and other agents remains important for other tumor types (26–31). Predictive biomarkers that identify which patients derive benefit and toxicity from anti-angiogenic agents are needed (32).

Amplification of *HER2* is a well-studied prognostic and predictive biomarker for patients with breast cancer and represents one of the gold standards of clinical applicability (12). Here, we tested for amplification of the target gene for bevacizumab, *VEGFA*, using FISH. The samples from E2100 were derived from paraffin-embedded tissue from the primary tumor. In this study, we show that testing archived

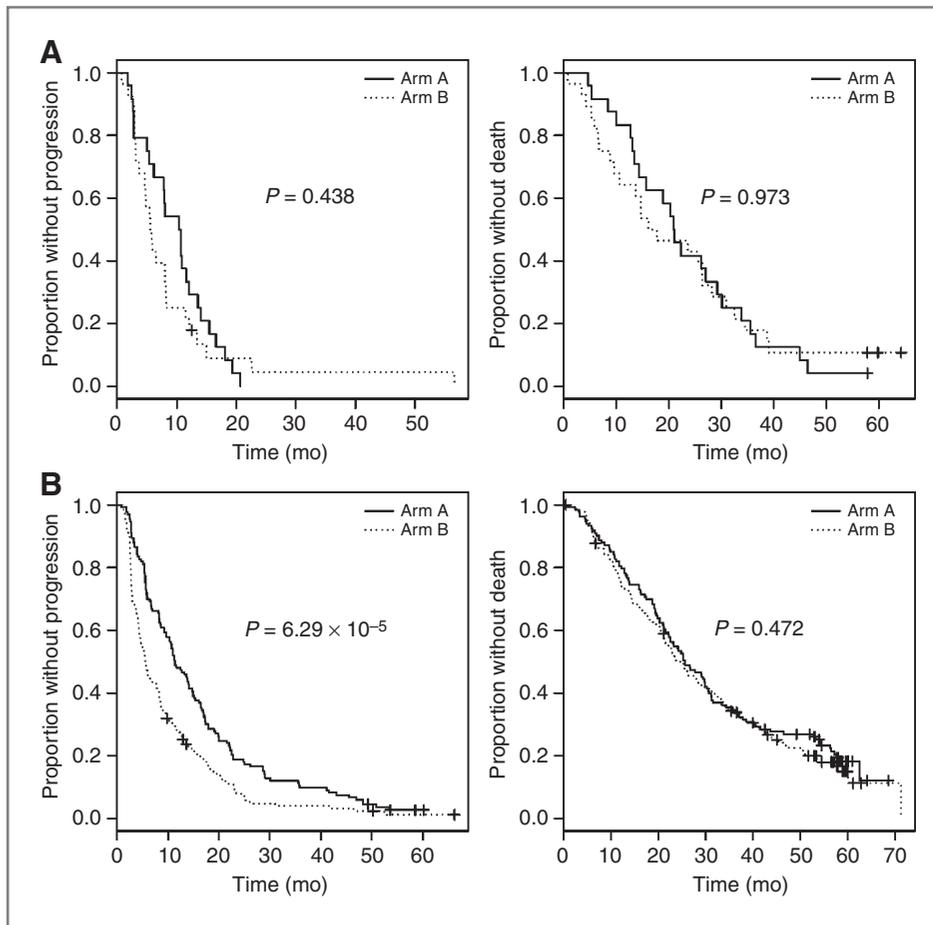


Figure 4. PFS (left) and OS (right) by arm of E2100 for those with (A) amplified/borderline-amplified and (B) not amplified/borderline-amplified tumor status.

tumor blocks for *VEGFA* amplification by FISH is feasible. Previously, we showed that VEGF expression (determined by immunohistochemistry) did not correlate with outcome in E2100 (7). From a technical standpoint, amplification is easier to quantify than expression (7, 33). From a biologic standpoint, it is likely that genomic amplification is less dynamic over time in response to changes in the microen-

vironment than protein expression. For example, VEGF expression is highly variable in response to hypoxia and thus may represent a less reliable surrogate to describe the potential range of *VEGFA* influence at various time points in the life of a metastatic tumor (34, 35). However, it is critical to remember that bevacizumab targets the VEGFA protein. The variable nature of protein expression is a problem

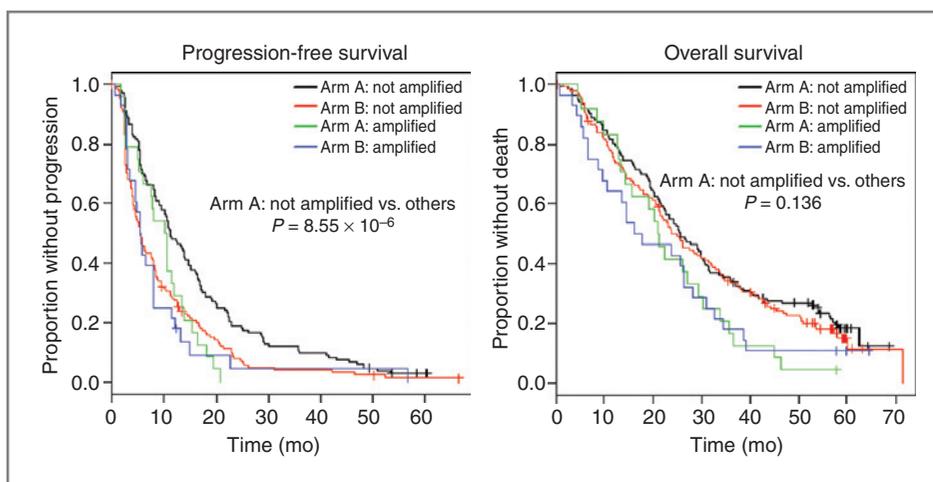


Figure 5. PFS (left) and OS (right) by *VEGFA* amplification status and arm of therapy.

exacerbated in a metastatic trial (like E2100) where the primary tumor being assessed is often far removed in time from the initiation of treatment in the metastatic setting. This issue may also be relevant to the *VEGFA* gene but data are not available.

While the majority of tumors have similar *HER2* amplification status when comparing the primary tumor with a metachronous metastasis, several studies have shown that a clinically relevant fraction (as high as 15%) does change (36–38). As all samples evaluated in this correlative study included the primary tumor, any biologic change that took place at the time of metastasis cannot be accounted for here. Another inherent limitation of this correlative study, like many others, is that samples are not available from all cases of the parent trial. This limits statistical power and can also introduce an unintended confounder if the subgroup evaluated does not reflect the characteristics of the population and outcome in the parent trial. This latter concern is somewhat dampened as this subgroup closely mirrors the parent trial in terms of important characteristics and outcome.

Amplification of the *VEGFA* gene has previously been shown to serve as a poor prognostic marker in patients with colorectal cancer and osteosarcoma (39, 40). As far as we are aware, the use for *VEGFA* amplification as a predictive biomarker for anti-VEGF therapy has not been previously reported in a phase III trial. In this correlative study from E2100, we show those patients with *VEGFA* amplification and borderline amplification (together as a group) do worse than those without amplification in univariate analysis and thus this may represent a prognostic marker as seen in other tumor types. The significant inferiority seen in those with amplification was no longer significant in multivariate analysis which included ER positivity as a significant variable. There appears, then, to be an important biologic interaction with *VEGFA* amplification and ER expression. Tumors that were hormone-sensitive were almost 2 times less likely than those who were ER-negative to have *VEGFA* amplification. ER-positive patients also had lower frequency of *VEGFA* amplification than *HER2*-positive patients, but the number in the latter subgroup was too small to formally compare. Higher *VEGFA* expression has previously been seen in *HER2*-positive and TNBCs than ER-positive tumors; further validating this biologic correlation (41–45). In addition to lower frequency of *VEGFA* amplification, the implication of this amplification in the ER-positive subgroup appears to be different as well. In the ER-positive population, prognosis does not appear to be adversely impacted by amplification. Those with TNBCs or *HER2* positivity, however, do appear to have outcome adversely impacted by amplification. While the effect of *VEGFA* amplification on *HER2*-positive tumors (as a unique group) was not evaluated here due to small numbers, the biology would suggest it to be a provocative area of future investigation.

In this study, those patients who had *VEGFA* amplification did worse in univariate analysis and appeared to have no incremental benefit from bevacizumab. While the path-

ophysiology is not elucidated, this may simply represent a scenario where the amplification "overwhelms" the ability to successfully block *VEGFA*. Conversely, a significant benefit for bevacizumab in PFS was maintained in those who did not have amplification. Similar to the results of the parent trial, this effect did not carry over to a benefit in OS. The test for interaction between treatment arm and amplification was not statistically significant and thus *VEGFA* amplification cannot be formally considered a predictive marker for bevacizumab based on these data. However, the difference in PFS was statistically and clinically substantial when comparing those who did not have amplification but did receive bevacizumab against those who were either amplified or did not receive the anti-*VEGFA* blockade. It does beg the question as to whether the PFS could have been more robust in other data sets (e.g., AVADO and RIBBON-1) if the subgroups with *VEGFA* amplification were excluded. Furthermore, would the enrichment of this population also translate into improved OS with greater numbers and more statistical power (e.g., the first-line meta-analysis) or does the biology of this drug dictate that an improvement in OS is simply not possible for this disease type and setting?

We have previously shown that host-derived genetic variation (i.e., SNPs) correlated with improved OS for patients who received bevacizumab in E2100 (7). In that study, 2 genotypes (*VEGFA* -2578AA and -1154AA) had superior OS in the bevacizumab-containing arm. Combining germ line (SNPs) and somatic (tumor-specific) variability to create a predictive signature is a potentially complex approach but may be necessary to uncover the most accurate biomarker. Specifically, is there a genetic subgroup (based on SNPs) that is able to overcome the poor prognostic effect seen in those with *VEGFA* amplified tumors while receiving bevacizumab? Alternatively, are those who do not have tumor *VEGFA* amplification and who have the good SNP profile destined to experience a meaningful improvement in OS? Recently, a prospective trial has begun enrolment (the MERIDIAN trial) with the goal of prospectively evaluating outcome using a biomarker-guided approach with baseline plasma *VEGFA* levels. The result from this study may further inform biomarker-driven trials such as MERIDIAN. Furthermore, the interaction between the host (i.e., SNPs) and the tumor represents the entire picture of variability and this interaction warrants further evaluation in larger data sets.

Disclosure of Potential Conflicts of Interest

B.P. Schneider, M.N. Dickler, and M.A. Cobleigh are on the advisory board of Genentech (compensated). K.D. Miller has received honoraria and research funding from Genentech. J. Galow has received Commercial Research Grant from Amgen, Novartis, Genentech, and Roche. E.A. Perez has received Commercial Research Support from Genentech and GlaxoSmithKline and is in the advisory board of Genentech, Merrimack, Eisai, and Celgene. T.N. Shenkier is on the advisory board of Amgen. J.A. Sparano has received consulting fees from Genentech. S. Müller holds a team leader position at Dako-Denmark. No potential conflicts of interest were disclosed by the other authors. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

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