Proteomic Characterization of Breast Cancer Xenografts Identifies Early and Late Bevacizumab-Induced Responses and Predicts Effective Drug Combinations

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Abstract

**Purpose:** Neoangiogenesis is an important feature in tumor growth and progression, and combining chemotherapy and antiangiogenic drugs have shown clinical efficacy. However, as treatment-induced resistance often develops, our goal was to identify pathways indicating response and/or evolving resistance to treatment and inhibit these pathways to optimize the treatment strategies.

**Experimental Design:** To identify markers of response and/or resistance, reverse-phase protein array (RPPA) was used to characterize treatment-induced changes in a bevacizumab-responsive and a non-responsive human breast cancer xenograft. Results were combined with bioinformatic modeling to predict druggable targets for optimization of the treatment.

**Results:** RPPA analysis showed that both tumor models responded to bevacizumab with an early (day 3) upregulation of growth factor receptors and downstream signaling pathways, with persistent mTOR signaling until the end of the *in vivo* experiment. Adding doxorubicin to bevacizumab showed significant and superior growth inhibition of basal-like tumors, whereas no additive effect was seen in the luminal-like model. The combination treatment corresponded to a continuous late attenuation of mTOR signaling in the basal-like model, whereas the inhibition was temporary in the luminal-like model. Integrating the bevacizumab-induced dynamic changes in protein levels with bioinformatic modeling predicted inhibition of phosphoinositide 3-kinase (PI3K) pathway to increase the efficacy of bevacizumab monotherapy. *In vivo* experiments combining bevacizumab and the PI3K/mTOR inhibitor BEZ235 confirmed their significant and additive growth-inhibitory effect in the basal-like model.

**Conclusions:** Treatment with bevacizumab caused compensatory upregulation of several signaling pathways. Targeting such pathways increased the efficacy of antiangiogenic therapy. *Clin Cancer Res; 20(2); 1–9*. ©2013 AACR.

Introduction

Angiogenesis represents a critical step in cancer growth, invasion, and metastasis, with VEGF as one of the most potent proangiogenic factors. Various strategies have therefore been investigated to inhibit VEGF or its receptors, including the neutralizing anti-VEGF monoclonal antibody bevacizumab. The use of bevacizumab in breast cancer treatment has been debated, due to the significant, but modest increase in progression-free survival, and lack of survival benefit in the metastatic setting (1–3). Therefore, identification of factors identifying evolving bevacizumab resistance is pivotal for the future use of such therapy.

Angiogenesis is a complex process with many redundant pathways involved (4), possibly explaining why initial treatment responses often are transient and followed by development of resistance. Targeting one prostimulatory pathway is therefore likely to be compensated by the activation of other pathways to sustain tumor growth (5). This was shown in a pancreatic islet cancer, where inhibition of VEGFR signaling resulted in higher expression of proangiogenic factors, like fibroblast growth factor (FGF), when the tumors relapsed (6). Subsequent targeting of FGF in combination with VEGFR signaling attenuated the revascularization and inhibited tumor growth, showing the key role of several angiogenic factors in tumor progression. In the present study, we have identified signaling pathways associated with tumor progression on bevacizumab therapy in...
Translational relevance

Antiangiogenic therapy with bevacizumab has shown activity in patients in the neoadjuvant and metastatic treatment setting when used in combination with chemotherapy. However, markers selecting patients for such therapy do not exist, and resistance to such therapy regularly occurs in the metastatic setting. Two validated in vivo models representative for luminal- and basal-like breast cancer have been used to identify molecular differences driving response and/or resistance to antiangiogenic therapy. By combining high-throughput proteomic arrays and bioinformatic modeling, bevacizumab-induced treatment effects and pathways with potential key roles in acquired resistance was identified. The study shows that in vivo targeting of such compensatory signaling pathways may overcome the resistance and improve the efficacy of antiangiogenic therapy. These results are highly relevant for clinical optimization of antiangiogenic therapy in patients and for future clinical studies identifying response markers for antiangiogenic therapy.

two patient-derived breast cancer xenograft models. We have further investigated whether such pathways may be targeted to avoid acquired resistance and subsequently achieve continuous tumor growth inhibition. The tumor models, of basal- and luminal-like origin, have previously been characterized as bevacizumab-responsive and nonresponsive, respectively (7). Analyzing their differences in bevacizumab-induced molecular effects may therefore aid in identifying markers able to stratify patients likely to benefit from antiangiogenic treatment.

One of the advantages of protein-based platforms, in contrast to the more established RNA arrays, is that the enzymatic activity of key proteins can be detected by staining with phospho-specific antibodies. Hence, the actual protein signaling networks can be elucidated by measuring the level of phosphorylation/dephosphorylation, allowing the identification of activated pathways coinciding with acquisition of resistance. In the present study, we used reverse-phase protein arrays (RPPA) to study the proteomic response to antiangiogenic treatment, as this has proven to be a highly reliable and reproducible system for large-scale analysis of target identification (8–10). We also integrated high-throughput proteomic analyses with computational network modeling to reveal differences in the extent of activated pathways between the two breast cancer subtypes in response to bevacizumab. RPPA results and modeling predicted the PI3K/Akt/mTOR pathway as a target with potential additive effect when combined with bevacizumab. In subsequent in vivo experiments, the dual PI3K/mTOR inhibitor BEZ235 confirmed its additive effect in combination with bevacizumab in the basal-like model.

Materials and Methods

Animal models and treatments

Two breast cancer xenograft models, MAS98.06 and MAS98.12, derived from primary mammary adenocarcinoma specimens (MAS) have previously been described (11). Molecular characterization of the two xenografts has classified MAS98.06 as luminal-like and hormone receptor–positive, whereas MAS98.12 has been classified as basal-like and hormone receptor–negative. The two xenograft models have previously been treated with bevacizumab, doxorubicin, and a combination of these drugs, identifying the basal-like as antiangiogenic-responsive whereas the luminal-like did not respond to bevacizumab treatment (7). Tumors were harvested at days 3 and 10 and at endpoints (day 18 for basal-like and day 35 for luminal-like tumors), snap-frozen in liquid nitrogen, and stored at −80°C until RPPA analysis was conducted.

A second animal experiment with mice carrying the basal-like xenograft were treated with bevacizumab [5 mg/kg, twice weekly (Roche-Genentech)], NVP-BEZ235 [45 mg/kg, daily perorally (Selleck Chemicals)], and gefitinib [100 mg/kg, daily perorally (G-4408; LC Labs)] and 2 groups of animals receiving bevacizumab in combination with either BEZ235 or gefitinib. The in vivo experiments were repeated twice; in the first experiment, all animals were sacrificed and tumor tissue harvested at day 19, due to symptom onset in control mice. Last dose of BEZ235 was given 24 hours before sacrificing the animals. In the second experiment, BEZ235 and gefitinib containing treatment groups were allowed to grow beyond day 19. However, when gefitinib monotherapy-treated mice had to be sacrificed at day 26, all the remaining groups were also sacrificed. Treatment with BEZ235 was given 0.5 to 3 hours before sacrificing the animals in the latter experiment, based on experiments showing a superior mTOR-inhibiting effect after 3 hours (data not shown). Bevacizumab and gefitinib were given 24 hours before sacrificing the animals. Tumors were harvested, immediately snap frozen in liquid nitrogen, and stored at −80°C until RPPA analysis was conducted.

In all animal experiments, tumor growth was measured twice weekly, and tumor volumes were calculated using the formula length × width × width × 0.5. Two independent in vivo experiments were carried out and in vivo growth curves were generated from mean relative tumor volumes from both experiments ± SEM. Statistical significance of treatment effect was assessed by Mann–Whitney U test comparing controls and treatment groups at day 19, the last measurement where all mice from all treatment groups were alive. In addition, the statistical difference between BEZ235 monotherapy and BEZ235 and bevacizumab combination therapy was assessed at day 26. A statistical difference of P < 0.05 was considered significant for all analyses conducted.

All procedures and experiments involving animals were approved by The National Animal Research Authority and were conducted according to the European Convention for the Protection of Vertebrates used for Scientific Purposes. All
procedures and endpoints were in compliance with what has previously been described (12).

RPPA

The first RPPA analysis was conducted on tumor tissue from basal-like (passage 47) and luminal-like (passage 28) tumors, with n = 3 for each treatment group (controls, bevacizumab, doxorubicin, and bevacizumab plus doxorubicin combination treatment) and at each time point. In the second RPPA analysis, tumor tissue from controls (n = 8), bevacizumab-treated (n = 8), BEZ235-treated (n = 8), BEZ235 + bevacizumab-treated (n = 9), gefitinib-treated (n = 4), and gefitinib + bevacizumab-treated (n = 4) mice carrying the basal-like tumor was used for RPPA analysis. Tumor tissue was lysed in lysis buffer [1% Triton X-100, 50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L Na pyrophosphate, 1 mmol/L Na3VO4, 10% glycerol, protease inhibitor (Roche Applied Science, 05056489001), and phosphatase inhibitor (Roche Applied Science 04906837001), adjusted protein concentration after bicinchoninic acid (BCA) measurements and denatured the samples in a 4 × SDS sample buffer (40% glycerol, 8% SDS, 0.25 mol/L Tris-HCl, pH 6.8, 10% β-mercaptoethanol) and printed on nitrocellulose-coated slides in serial dilutions. Each slide was then probed with a primary antibody, followed by a biotin-conjugated secondary antibody. The signal was amplified using the DakoCyto-\text{mation-catalyzed system (DAKO) and quantitated using Microvigene software (Vigene Tech Inc.) to measure spot intensity.} The latter was processed by the R package SuperCurve (13), and protein concentrations were derived by curve fitting of each lysate dilution curve to the supercurve on the slide. Linear data values were used to calculate fold changes in phospho and total protein levels between different treatment regimens. After removal of mice antibodies, due to unspecific binding, a total of 122 antibodies (41 phospho-targeted) in the first RPPA experiment and 143 antibodies (46 phosphorylated) in the second experiment were used to study treatment-induced responses. Proteins with less than 10% fold changes at all time points were excluded from the analysis. Average inter-assay coefficient of variance (CV) values between control tumors from 2 separate experiments, as well as intra-assay CV values between control tumors from the same experiment were calculated, and both were found to be below 9%.

Integration of signaling pathways with computational network analysis for targeted therapy predictions

Computational modeling was integrated with the RPPA results on phospho-proteins from bevacizumab-treated mice to identify the most influenced molecules driving sustained tumor growth and to predict the optimal bevacizumab combinations to inhibit development of resistance. This computational procedure is described elsewhere (14).

Western blot analysis

To confirm proteins of interest obtained by RPPA analysis, tumor lysate was analyzed on Western immunoblots as described previously (7). Primary antibodies targeting phospho-S6 ribosomal protein Ser^240/244 and phospho-4E-BP1 Thr^37/46 (rabbit, Cell Signaling) were used.

Results

RPPA analysis of bevacizumab-treated tumors identifies signaling pathways involved in sustained tumor growth

Previous studies showed the basal-like xenograft as bevacizumab-responsive, whereas the luminal-like model did not respond to the antiangiogenic treatment (7). To characterize the proteomic response signature linked to bevacizumab monotherapy, primary tumors from treated mice were analyzed using RPPA.

RPPA results showed that both tumor models responded to bevacizumab monotherapy with an early (day 3) upregulation of several total and phosphorylated proteins, including growth factor receptors like EGF receptor (EGFR) and insulin-like growth factor 1 receptor β (IGF-1Rβ; Supplementary Table S1). Furthermore, both tumors showed an early bevacizumab-induced increase in downstream activators from these receptors, like insulin-like growth factor–binding protein 2 (IGFBP2), 3-phosphoinositide-dependent protein kinase 1 (PDK1), Akt, and glycogen synthase kinase-3 (GSK3; Supplementary Table S1). In contrast, the early upregulation of the mTOR inhibitor tuberin (TSC2), together with a downregulation of the mTOR effector phospho-S6, reflected an early bevacizumab-induced downregulation of mTOR signaling in both tumors (Fig. 1A and B). This signaling was, however, reversed and upregulated toward the end of the experiment, as reflected by the observed increase in phospho-S6 and decrease in TSC2. This activation was observed earlier in the luminal-like, nonresponsive tumor. Interestingly, Akt was continuously activated throughout the experiment in both models.

An early upregulation of the Raf/MEK/ERK cascade was seen in the basal-like (Fig. 1C) and to a lesser degree in the luminal-like tumor (Fig. 1D) in response to bevacizumab monotherapy. However, this signaling was reversed and downregulated at the end of the experiment in the basal-like tumor, whereas p38 MAPK was upregulated in the luminal-like tumor.

Bevacizumab in combination with doxorubicin shows additive effects by inhibiting feedback loops in the bevacizumab-responding, basal-like tumor model

Bevacizumab as single-agent therapy has limited effect on tumor growth and is therefore almost always administered with chemotherapy or other combination therapies. The superior effect of combination therapy was also evident in our previous in vivo experiments but was only manifested in the basal-like tumor model (7).

To isolate the bevacizumab-induced effects in the combination therapy, RPPA results from the latter were
compared with doxorubicin monotherapy. Reflected by the upregulation of phospho-S6S240 and eIF4E, bevacizumab induced an early activation of mTOR signaling in combination-treated basal-like tumors (Fig. 2A and Supplementary Table S2). However, this activation was transient and followed by a continuous downregulation from day 10 and until the end of the experiment. Phosphorylated or total EGFR, in addition to phospho-Akt, were on the other hand upregulated throughout the experiment (Fig. 2A and Supplementary Table S2).

As in basal-like tumors, luminal-like tumors responded with an early activation of EGFR and the downstream effector Akt, in combination versus doxorubicin-treated tumors (Fig. 2B and Supplementary Table S2). However, in contrast to basal-like tumors, combination versus doxorubicin induced an early and sustained downregulation of mTOR signaling that was reversed and activated at the end of the experiment (Fig. 2B). The Raf/MEK/ERK cascade showed a peak in upregulation at day 10 in the basal-like tumor after this treatment (C). In the luminal-like tumor, the latter cascade was downregulated from day 3 to 10, and then followed by an increase (D). In contrast to bevacizumab monotherapy, p38 MAPK decreased gradually throughout the experiment in the luminal-like model after combination versus doxorubicin therapy (D). Error bars indicate ± SEM for bevacizumab-treated normalized to control.
toward the end of the experiment (Fig. 2C). In contrast, the early downregulation of the Raf/MEK/ERK cascade in combination-treated luminal-like tumors was activated at the end of the experiment. p38 MAPK was, on the other hand, gradually downregulated (Fig. 2D).

A difference in cytoskeletal and adhesion molecules was also found between the two models; an early upregulation of E-cadherin, β-catenin, and claudin 7 in the basal-like model, followed by a decrease in E-cadherin, loss of β-catenin and claudin 7 regulation, and upregulation of P- and N-cadherin toward later time points (Supplementary Table S2). In contrast, luminal-like tumors did not respond to treatment with a similar alteration in the cell adhesion molecules.

**Mechanistic modeling elucidates the response rate to bevacizumab treatment**

By focusing solely on phosphorylated proteins, the specific activation status in response to bevacizumab monotherapy was further elucidated. This was done by combining the bevacizumab-induced dynamic changes in phospho-protein expression from day 3 to endpoints, with mathematical network modeling. The median reaction rates reflect the level of protein change after bevacizumab treatment at the 3 different time points analyzed and indicates that the effects of bevacizumab treatment is faster in the luminal-like (A), compared with the basal-like model (B). Furthermore, whereas the luminal-like tumor seems to be mainly driven through MEK and MAPK/ERK signaling (A), the basal-like tumor is driven through several additional pathways (B).

**Mechanistic modeling predicts druggable targets with additive effects when combined with bevacizumab**

The mathematical model was further used to predict the effect of inhibiting every node in the network. By calculating...
In the context of tumor growth inhibition, the model predicted that Akt could reactivate signaling cascades due to feedback loops. Thus, Akt inhibition in combination with bevacizumab would be a potential intervention strategy.

**Inhibition of PI3K/mTOR increases the activity of bevacizumab**

To evaluate the computational predictions, mice carrying the basal-like xenograft were treated with either bevacizumab, the dual PI3K/mTOR inhibitor BEZ235, or a combination of both. Two additional groups of mice were treated with the EGFR inhibitor gefitinib, alone or in combination with bevacizumab. The latter treatment was based on the observed bevacizumab-induced activation of EGFR in the present RPPA analysis, as well as in previous kinase activity analysis (7), suggesting a potential role for this receptor in the development of acquired resistance. EGFR has also been found to have a higher expression in basal/triple-negative breast cancers (TNBC) than in other types of breast cancers (15–18), supporting the rationale for such treatment in the basal-like tumor.

As illustrated in Fig. 4, all treatments, except gefitinib monotherapy, inhibited in vivo tumor growth significantly, as compared with controls (Mann–Whitney U test, P < 0.05). Furthermore, the combination of bevacizumab and BEZ235 was superior and significantly better than BEZ235 monotherapy (P = 0.0048) and bevacizumab (P < 0.0001) monotherapy. In contrast, the addition of gefitinib to bevacizumab treatment did not show any significantly improved effect compared with either of the 2 single agents.

**RPPA analysis on tumor tissue from bevacizumab and BEZ235-treated xenografts**

Tumor tissue harvested at the end of the in vivo experiments in Fig. 4 were lysed and analyzed by RPPA. Interestingly, a difference in protein regulation was seen between the tumors harvested 0.5 to 3 hours after last BEZ235 dose and the tumors harvested 24 hours after last BEZ235 treatment. That is the targeting effect of BEZ235 on mTOR signaling was stronger shortly after treatment (Fig. 5), compared with 24 hours after last dose (Supplementary Table S3). The same trend was also seen for other proteins, like the pro-apoptotic proteins Bak, Bax, and Bim; upregulated shortly after treatment, and downregulated 24 hours after last BEZ235 dose (Supplementary Table S3).

As shown in Fig 4, the combination of bevacizumab and BEZ235 induced a significant and prolonged downregulation of basal-like tumor growth, compared with controls and monotherapies. To evaluate whether the BEZ235 treatment could work as a strategy to prolong the growth-inhibitory effects of bevacizumab and doxorubicin combination treatment, the BEZ235-induced changes were isolated by comparing bevacizumab plus BEZ235 to bevacizumab monotherapy. These results showed that the early (0.5–3 hours posttreatment) upregulation in pro-apoptotic proteins, as well as the early downregulation of the mTOR upstream effectors, pAkt and PI3K, were BEZ235-induced (Supplementary Table S3).

**Discussion**

In this study, we have used two breast cancer xenografts of basal- and luminal-like origin to study mechanisms driving response and/or resistance to antiangiogenic treatment with bevacizumab. By the use of RPPA and computational...
modeling, we have identified the PI3K/Akt/mTOR pathway as a putative key player in driving resistance to bevacizumab, and activation of this pathway may therefore be suggested as a negative predictor for treatment response. Further studies of patient samples will be needed to determine whether mutation, copy number, or Akt pathway activation status predicts benefit from bevacizumab. As there are no effective predictors of benefit, this could be an important contribution in addition to the potential for rational combination therapy with PI3K pathway inhibitors.

The basal- and luminal-like tumor models have previously been shown responsive and resistant to bevacizumab treatment, respectively (7). However, even in the responsive model, single-agent bevacizumab showed modest treatment effects. This may be explained by the bevacizumab-induced upregulation of alternative growth factor receptors, like EGFR and IGF-R1, and downstream signaling pathways, like the PI3K/Akt/mTOR pathway. The obtained results are in line with several published papers, indicating that acquired resistance to targeted treatment may develop due to upregulation of alternative rescue pathways to compensate for attenuated signaling in the targeted pathway (5, 19).

The combination of bevacizumab and doxorubicin was significantly more active in the basal-like, compared with the luminal-like model. One explanation for this additive effect may be the observed attenuation of the bevacizumab-induced feedback activation of mtOR signaling at later time points (as measured by RPPA in the presence of doxorubicin). In contrast, a strong activation of this pathway was seen at endpoints in the less responsive luminal-like tumor after receiving combination therapy. This delayed activation of mtOR signaling in the luminal-like tumor resembles the earlier activation of this pathway after bevacizumab monotherapy and may therefore reflect a general bevacizumab-induced resistance mechanism. However, as luminal-like tumors were harvested later than basal-like tumors at endpoints (day 35 vs. 18), doxorubicin may have lost its effect in the former model as it was only given once at the start of the experiment.

To further investigate the molecular effects elicited by bevacizumab, the time-dependent flux in bevacizumab-induced protein activation, as identified through RPPA, was combined with mathematical network modeling. First, the luminal-like tumor was characterized to be mainly driven through the MEK/ERK pathway, whereas basal-like tumors responded to bevacizumab treatment by activating several pathways. These results correspond to gene expression analysis, showing that the basal-like subtype has a complex genomic phenotype, characterized by a high number of gains/losses (20, 21). This could also be linked to the aggressiveness of basal-like cancers and the challenges associated with targeted therapy, where simultaneous inhibition of several pathways seems necessary. As indicated by the median reaction rates, the signal transduction was faster in the luminal-like model, than in the basal-like. The faster reaction to bevacizumab therapy in the luminal-like model corresponds to the limited vascularization in this model (22). In a study by Grinde and colleagues (23), a higher glycolytic activity was found in the luminal-like model, compared with the basal-like. The high glycolytic activity was suggested to be an adaption to low glucose levels, due to fewer blood vessels. One can therefore speculate that an inhibition of the already scarce vasculature in this model requires a fast reaction to sustain glucose and oxygen supply.

Mechanistic modeling predictions identified the PI3K and MAPK/ERK pathways as molecular targets with potential additive effect when combined with bevacizumab. Furthermore, Akt was suggested to reactivate VEGFR2 signaling through feedback loops, indicating that an Akt inhibitor could have additive effect in combination with bevacizumab. This prediction correlated to our own observations in Figs. 1 and 2 of a sustained Akt activation. We chose to investigate the combined effect of bevacizumab and BEZ235, based on the computational modeling and the ability of this drug to inhibit the PI3K/Akt/mTOR signaling pathway. In vivo results in the basal-like tumor showed their significant and superior growth-inhibitory effects, both compared with controls and single-agent therapy. Consistent with the effects of BEZ235, the PI3K/Akt/mTOR pathway was found downregulated at the proteomic level through RPPA analysis. mtOR inhibition was confirmed through Western immunoblotting, where both S6- and 4EBP1 phosphorylation were downregulated (Fig. 5). The stronger effect of BEZ235 on S6 phosphorylation compared with the other treatment modalities, together with a somewhat similar effect on 4EBP1 phosphorylation, may be explained through the ability of PI3K to activate S6K directly via its effector PDK1, without the need for mtOR activation (24). As BEZ235 targets PI3K directly, a stronger down-regulation of S6 compared with 4EBP1 is expected from this treatment modality. Differential effect on 4EBP1 versus S6K from mtOR inhibition with rapamycin was also seen in an article by Choo and colleagues (25). Here, rapamycin inhibited S6K activity throughout the duration of treatment, whereas 4EBP1 recovered phosphorylation within 6 hours. This illustrates the complexity in mtOR signaling and inhibition.

The downregulation of phospho-Akt is of particular interest, as Akt was found continuously activated after bevacizumab and doxorubicin combination therapy. Akt signaling could therefore play a key role in driving sustained tumor growth after bevacizumab and doxorubicin combination treatment, and its targeting could suggest a potentially intervention strategy to prolong tumor growth inhibition. Evolving resistance to the bevacizumab and doxorubicin combination treatment was suggested because of the observations of a slight increase in basal-like tumor growth around day 15 (7). From the RPPA results, a bevacizumab-induced switch in cadherin expression was observed in combination-treated basal-like tumors, from early expression of E-cadherin, β-catenin and claudin 7 to induction of P- and N-cadherin at later time points. This classical cadherin switching may suggest escape from
treatment through induction of epithelial-to-mesenchymal transition (EMT; refs. 26, 27), supporting a potential development of resistance. EMT can be induced by several distinct pathways and molecules, including the PI3K/Akt pathway (28), which has been shown to repress E-cadherin transcription (29). The BEZ235-induced downregulation of this pathway could therefore possibly circumvent acquired resistance and prolong the tumor growth-inhibitory effect from bevacizumab and doxorubicin combination therapy.

Mice carrying the basal-like tumor were also treated with the EGFR inhibitor gefitinib alone or in combination with bevacizumab. The lack of effect from gefitinib monotherapy in the in vivo experiments may be explained by the low baseline level of PTEN expression in the basal-like compared with the luminal-like, model (30), as PTEN loss has been shown to confer resistance to EGFR kinase inhibitors (31). Another explanation for the lack of gefitinib effect may be the targeting of an upstream growth factor receptor, which may not be sufficient to inhibit tumor growth due to the redundancy in downstream pathway activators. As illustrated by the promising results from bevacizumab and BEZ235, a better strategy to increase the effectiveness of gefitinib, and other upstream activators, is probably to combine the drug with a downstream inhibitor, like components of the PI3K or the mitogen-activated protein kinase (MAPK) pathway.

In conclusion, the present study suggests biologic mechanisms underlying growth-inhibiting effects or acquired resistance to bevacizumab treatment, where activation of the Raf/MEK/ERK and the PI3K/Akt/mTOR pathway seems to be of particular importance. The additive effect of BEZ235 and bevacizumab in vivo, as well as the downregulation of these pathways after treatment, suggest that the significant growth-inhibitory effect from bevacizumab and doxorubicin combination therapy may be prolonged by adding BEZ235 to the treatment when the tumors evolve resistance. Future studies verifying these results are highly warranted, as the current study indicates a promising therapeutic option for patients responding to bevacizumab treatment. Other pathways identified by computational modeling, such as the Raf/MEK/ERK cascade, should also be evaluated as druggable targets able to increase the therapeutic efficacy of bevacizumab.

Ethical Standards
The authors declare that all experiments followed the ethical guidelines in Norway and the EU. All procedures and experiments involving animals were approved by The National Animal Research Authority and were conducted according to the European Convention for the Protection of Vertebrates used for Scientific Purposes; as stated in Methods.

Disclosure of Potential Conflicts of Interest
G. B. Mills has commercial research grant from AstraZeneca, Celgene, CelMines, Exelixis/Sanoﬁ Aventis, GSK, HanAll BioPharma Co. Korea, Roche Pharmaceuticals, SDL, and Wyeth/Pfizer/Puma; has ownership interest (including patents) in Catena Pharmaceuticals, PTV Ventures, and Spindle Top Ventures; and is a consultant/advisory board member of Arcxis Biotechnologies, AstraZeneca, Nuvolution, Targeted Molecular Diagnostics, LLC, Taur Therapeutics, Asuagen, Aushon, Catena, Dutchi Pharmaceutical, Foundation Medicine, HanAll BioPharma Co. Korea, Komen Foundation, and Novartis. O. Engebraaten is the principal investigator of a clinical study investigating molecular changes in patients with breast cancer treated with neoadjuvant therapy with and without bevacizumab, supported by Roche Norway (NCT00773695). No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: O. Engebraaten
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Kristian, G.B. Mills, O. Engebraaten
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.M. Lindholm, M. Krohn, S. Iadevaia, G.B. Mills, O. Engebraaten
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