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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 demonstrated 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 utilized to characterize treatment-induced changes in a bevacizumab responsive and a nonresponsive 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, while the inhibition was

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 Materials and methods.

temporary in the luminal-like model. Integrating the bevacizumab-induced dynamic changes in protein levels with bioinformatic modeling predicted inhibition of 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.

1. Introduction

Angiogenesis represents a critical step in cancer growth, invasion and metastasis, with vascular endothelial growth factor (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 pro-stimulatory pathway is therefore likely to be compensated by the activation of other pathways to sustain tumor growth [5]. This was demonstrated in a pancreatic islet cancer, where inhibition of VEGFR signaling resulted in higher expression of pro-angiogenic factors, like FGF, when the tumors relapsed [6]. Subsequent targeting of FGF in combination with VEGFR signaling attenuated the revascularization and inhibited tumor growth, demonstrating 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 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 employed RPPA arrays 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.

2. Materials and methods

2.1 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, while 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, while the luminal-like did not respond to bevacizumab treatment [7]. Tumors were harvested at day 3, 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 Reverse Phase Protein Array (RPPA) analysis were performed.

A second animal experiment with mice carrying the basal-like xenograft were treated with either bevacizumab (5 mg/kg, twice weekly (Roche-Genentech)), NVP-BEZ235 (45 mg/kg, daily peroraly (Selleck Chemicals)) and Iressa (100 mg/kg, daily peroraly (G-4408; LC Labs)), and two groups of animals receiving bevacizumab in combination with either BEZ235 or Iressa. 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 Iressa containing treatment groups were allowed to grow beyond day 19. However, when Iressa monotherapy treated mice had to be sacrificed at day 26, all the remaining groups were also sacrificed. Treatment with BEZ235 was given 0.5-3 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 Iressa 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 were performed.

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 performed and *in vivo* growth curves were generated from mean relative tumor volumes from both experiments ± standard error of the mean (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 performed.

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].

2.2 Reverse Phase Protein Arrays (RPPA)

The first RPPA analysis was performed 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), Iressa treated (n=4) and Iressa + 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, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na3VO4, 10% glycerol, protease inhibitor (Roche Applied Science, 05056489001) and phosphatase inhibitor (Roche Applied Science 04906837001), adjusted protein concentration after BCA measurements and denatured the samples in a 4x SDS Sample Buffer (40% Glycerol, 8% SDS, 0.25M TrisHCL pH6.8, 10% beta-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 DakoCytomation-catalyzed system (DAKO) and quantitated using Microvigene software (Vigene Tech Inc, Carlise, MA) to measure spot intensity. The latter was processed by the R package SuperCurve [13], and protein concentrations were derived by curve-fitting of each lysates dilution curve to the supercurve on the slide. Linear data values were utilized 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 utilized to study treatment-induced responses. Proteins with less than 10 % fold changes at all time points were excluded from the analysis. Average inter-assay CV values between control tumors from two separate experiments, as well as intra CV values between control tumors from the same experiment were calculated, and both were found to be below 9 %.

2.3 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].

2.4 Western blot analysis

To confirm proteins of interest obtained by RPPA analysis, tumor lysate was analysed 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) was utilized.

Results

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

Previous studies demonstrated the basal-like xenograft as bevacizumab-responsive, while 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 analysed using RPPA.

RPPA results demonstrated 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 Epidermal Growth Factor Receptor (EGFR) and Insulin-like Growth Factor 1 Receptor beta (IGF-1Rb) (Supplementary table 1). 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 1). 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 (Figure 1A and B). This signaling was, however, reversed and upregulated towards 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, non-responsive 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 (Figure 1C), and to a degree in the luminal-like tumor (Figure 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.

3.2 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 administrated 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 was compared to doxorubicin monotherapy. Reflected by the upregulation of phospho-$S6^{S240}$and elF4E, bevacizumab induced an early activation of mTOR signaling in combination treated basal-like tumors (Figure 2A and Supplementary table 2). 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 (Figure 2A and Supplementary table 2).

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 (Figure 2B and Supplementary table 2). 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 (Figure 2B). The Raf/MEK/ERK cascade showed a peak in upregulation at day 10 in the basal-like tumor, but this regulation was transient and downregulated towards the end of the experiment (Figure 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 (Figure 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 towards later time points (Supplementary table 2). In contrast, luminal-like tumors did not respond to treatment with a similar alteration in the cell adhesion molecules.

3.3 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 illustrated in Figure 3 provides a measure of how fast the signal of bevacizumab treatment is transduced across the VEGFR2 network. From the analysis, three pathway components were identified as critical nodes in luminal-like tumor growth after bevacizumab monotherapy; MEK, MAPK/ERK and TSC2 (Figure 3A). In contrast, the basal-like tumor responded to bevacizumab treatment by activating several pathway components (Figure 3B), with MEK, MAPK/ERK, STAT3, AMPK, TSC2 and AKT as potential critical nodes. As indicated by the median reaction rates, bevacizumab-induced signaling was faster in the luminal-like, compared to the basal-like tumor.

3.4 Mechanistic modeling predicts drugable targets with additive effects when combined with bevacizumab

The mathematical model was further utilized to predict the effect of inhibiting every node in the network. By calculating how the signal transduction across the whole network would be affected after targeting individual molecules, the model provided an indication of what drug inhibitor should be used to better increase inhibition of the whole signaling across the VEGFR2 network. That is; which target could have an additive effect when combined with bevacizumab. For both xenografts the model predictions suggested that inhibition of the MAPK/ERK pathway or the PI3K pathway would increase inhibition of VEGF/VEGFR2 signaling (data not shown). Furthermore, the model predicted that Akt could reactivate the

signaling cascades due to feedback loops. Thus, Akt inhibition in combination with bevacizumab would be a potential intervention strategy.

3.5 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 Iressa, 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 rational for such treatment in the basal-like tumor.

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

3.6 RPPA analysis on tumor tissue from bevacizumab and BEZ235 treated xenografts

Tumor tissue harvested at the end of the *in vivo* experiments in Figure 4 were lysed and analysed by RPPA. Interestingly, a difference in protein regulation was seen between the tumors harvested 0.5-3 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 (Figure 5), compared to 24 hours after last dose (Supplementary Table 3). 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 3).

As demonstrated in Figure 4, the combination of bevacizumab and BEZ235 induced a significant and prolonged downregulation of basal-like tumor growth, compared to controls and monotherapies. To evaluate if 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 post treatment) upregulation in pro-apoptotic proteins, as well as the early downregulation of the mTOR downstream effectors, pAkt and PI3K, were BEZ235-induced (Supplementary Table 3).

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 demonstrated modest treatment effects. This may be explained by the bevacizumab-induced upregulation of alternative growth factor receptors, like EGFR and IGF-R1b, 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 to 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 versus day 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, compared to 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 et al. [23] a higher glycolytic activity was found in the luminal-like model, compared to 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 Figure 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 demonstrated their significant and superior growth inhibitory effects, both compared to 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 was downregulated (figure 5). The stronger effect of BEZ235 on S6 phosphorylation compared to 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 downregulation of S6 compared to 4EBP1 is expected from this treatment modality. Differential effect on 4EBP1 versus S6K from mTOR inhibition with rapamycin was also seen in a paper by Choo et al. [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 due to 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, beta 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) [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 Iressa alone, or in combination with bevacizumab. The lack of effect from Iressa monotherapy in the *in vivo* experiments may be explained by the low baseline level of PTEN expression in the basal-like, compared to 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 Iressa effect may 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 Iressa, and other upstream activators, is probably to combine the drug with a downstream inhibitor, like components of the PI3K or the MAPK pathway.

In conclusion, the present study suggests biological 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 evolves 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 modelling, such as the Raf/MEK/ERK cascade, should also be evaluated as drugable targets able to increase the therapeutic efficacy of bevacizumab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure of Potential Conflicts of Interest

O.E. is the principal investigator of a clinical study investigating molecular changes in breast cancer patients treated with neoadjuvant therapy with and without bevacizumab, supported by Roche Norway (Oslo, Norway) (NCT00773695).

Abbreviations

VEGF	Vascular Endothelial Growth Factor
EGFR	Epidermal Growth Factor Receptor
IGF1Rb	insulin-like growth factor 1 receptor beta
IGFBP2	insulin-like growth factor binding protein 2
Raf C	Raf proto-oncogene serine/threonine-protein kinase
MEK1	mitogen-activated protein kinase kinase 1
ERK	mitogen-activated protein kinase 1
PI3K	Phosphoinositide-3 kinase
PDK1	3-phosphoinositide-dependent protein kinase 1

GSK3	Glycogen synthase kinase-3
TSC2	Tuberin
mTOR	mammalian target of rapamycin
p70S6K	p70 S6 kinase
S6	S6 Ribosomal protein
eIF4E	Eukaryotic initiation factor 4E
PTEN	Phosphatase and tensin homologue deleted on chromosome ten

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Translational relevance

Antiangiogenic therapy with bevacizumab has demonstrated 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 utilized 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 demonstrates 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.

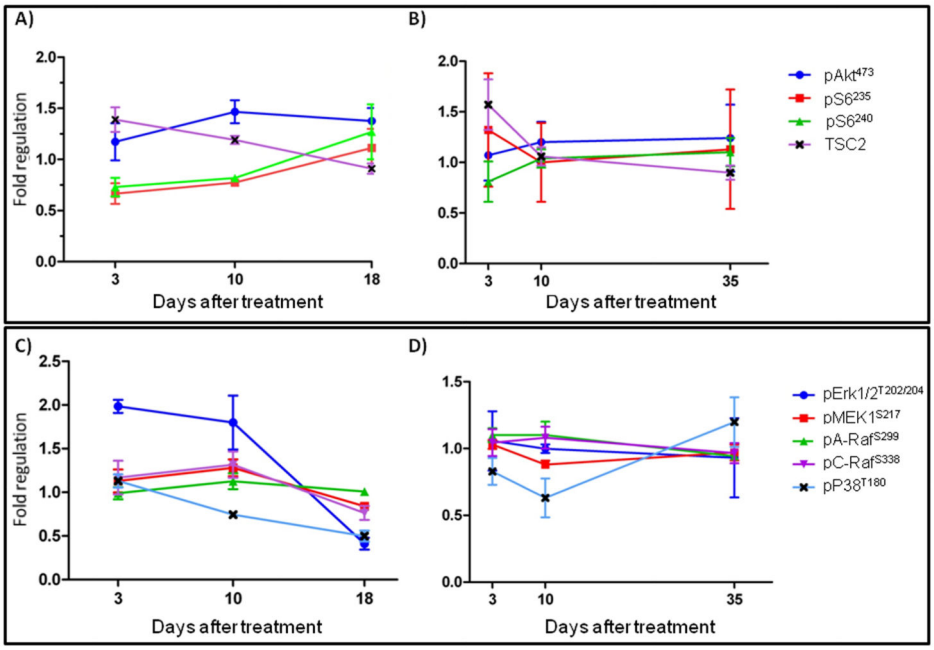

Figure 1. Proteomic effects from bevacizumab monotherapy, as measured by RPPA

Bevacizumab induced an early downregulation of mTOR signaling in both the basal-like (A) and the luminal-like (B) tumor models, which was reversed and activated at the end of the experiment. Furthermore, an early activation of Raf/MEK/ERK signaling was seen in the basal-like tumor, followed by a gradual downregulation (C). An early activation of this cascade was also seen in the luminal-like model (D), followed by a gradual decrease. However, a notable increase in p38 MAPK was observed at the end of the experiment. Error bars indicate ± SEM for bevacizumab treated normalized to control.

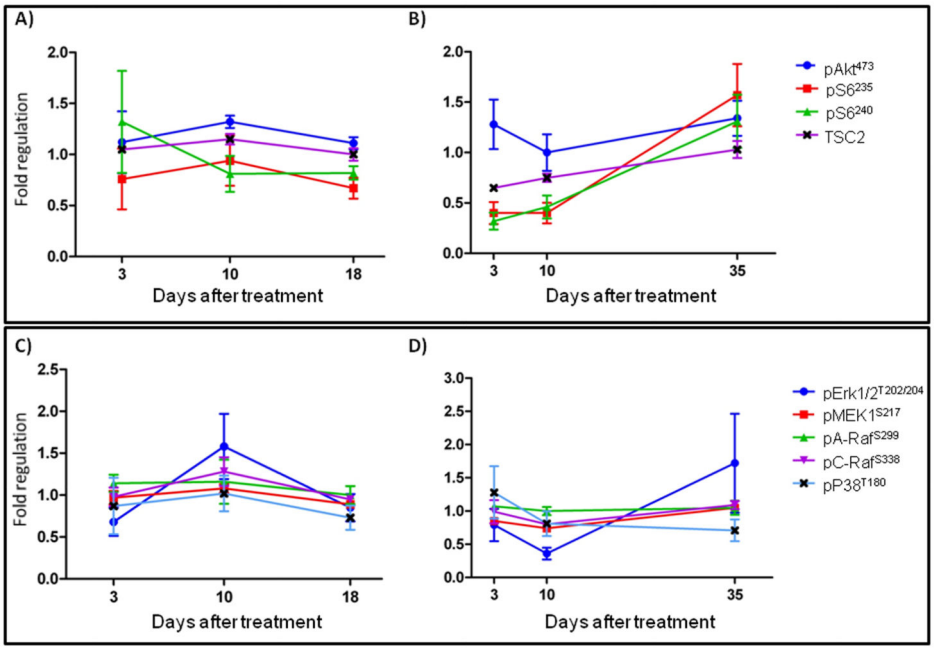

Figure 2. Proteomic effects from bevacizumab and doxorubicin combination therapy, compared to doxorubicin monotherapy

Adding doxorubicin to bevacizumab treatment attenuated mTOR signaling in both the basal-like (A) and luminal-like (B) model. However this effect was only temporary in the luminal-like model (B). A peak in Raf/MEK/ERK signaling was seen 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 day 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 combination treated normalized to doxorubicin treated.

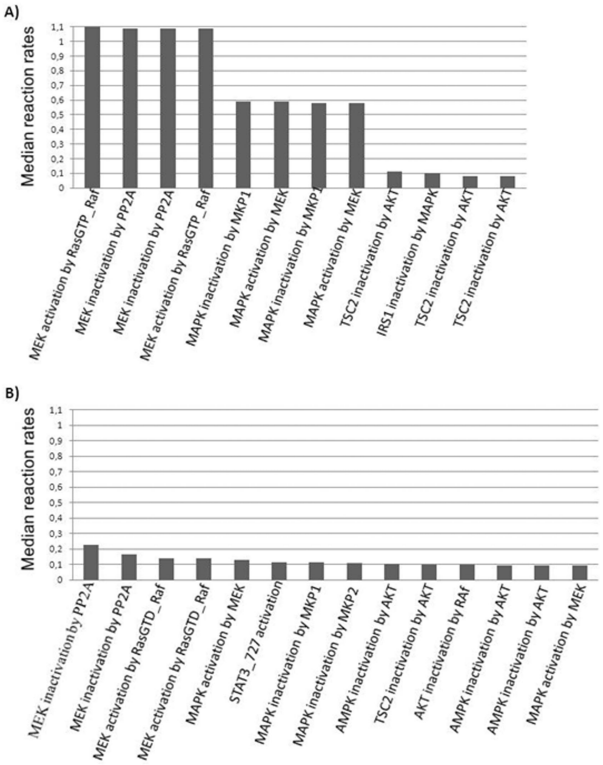

Figure 3. Visualization of the response rate across the VEGFR2 signaling network after bevacizumab treatment in the luminal-like (A) and basal-like (B) breast cancer xenografts

By combining the dynamic changes in phospho-protein expression after bevacizumab treatment (as measured through RPPA) with mathematical modeling, a measure of how fast the signaling was transduced could be outlined. The median reaction rates reflect the level of protein change after bevacizumab treatment at the three different timepoints analysed, and indicates that the effects of bevacizumab treatment is faster in the luminal- (A), compared to 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).

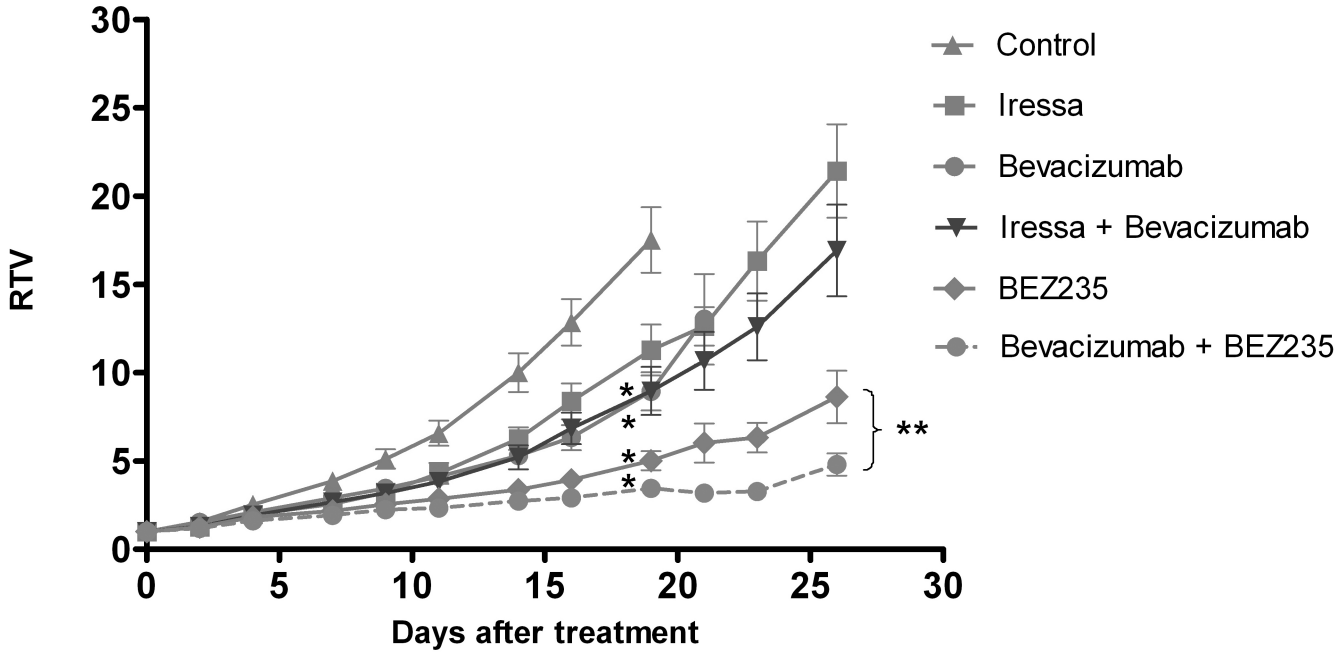

Figure 4. Bevacizumab and BEZ235 combination therapy shows additive growth inhibitory effects in the basal-like tumor model

Mice carrying the basal-like tumor were treated with the indicated drugs. Growth curves were generated from two independent animal experiments, and Mann-Whitney U test was performed between individual groups. All treatments, except for Iressa monotherapy ($p = 0.0630$), showed a significant growth inhibition as compared to controls (* $p < 0.05$). Furthermore, the combination of bevacizumab and BEZ235 was superior and significantly better than all other treatment regimens (**$p = 0.014$).

RTV = Relative tumor volume

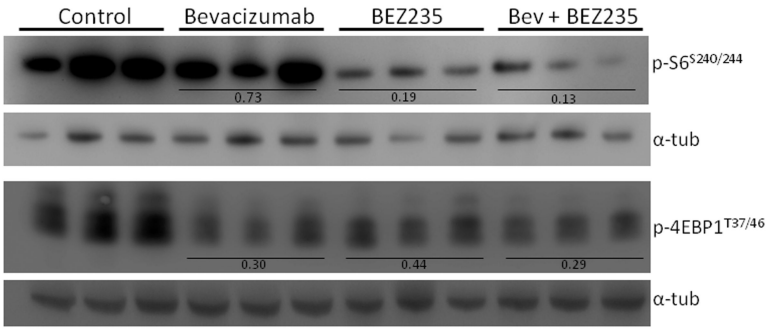

Figure 5. Bevacizumab and BEZ235 combination therapy attenuated the mTOR feedback signaling in basal-like xenografts

RPPA results demonstrated that phosphorylation of the mTOR downstream effectors S6 and 4EBP1 were downregulated after BEZ235 and bevacizumab combination treatment. These results were confirmed for tumors harvested 0.5-3 hours after last BEZ235 dose, through western immunoblotting with antibodies targeting the indicated proteins. Values indicate fold regulation compared to control, as measured by densitometric measurements, and normalized for loading control.

Bev = Bevacizumab