Discovery and Validation of Biomarkers that Respond to Treatment with Brivanib Alaninate, a Small-Molecule VEGFR-2/FGFR-1 Antagonist

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Abstract
The process of neovascularization from preexisting blood vessels, referred to as angiogenesis, plays a critical role in both tumor growth and dissemination in multiple cancer types. Currently, there exists a need to identify biomarkers that can both indicate biological activity and predict efficacy at the molecular level for antiangiogenesis drugs which are anticipated to result in tumor stasis rather than regression. To identify such biomarkers, athymic mice bearing L2987 human tumor xenografts were treated with the antiangiogenic agent brivanib alaninate, which is currently under clinical evaluation. This was an orally available and selective tyrosine kinase inhibitor that targets the key angiogenesis receptors vascular endothelial growth factor receptor 2 (VEGFR-2) and fibroblast growth factor receptor 1. In the described studies, tumor samples were collected from these xenografts and RNA was extracted for gene expression profiling on Affymetrix 430A mouse GeneChips. Statistical analysis was done using a defined set of genes identified to be coexpressed with VEGFR-2 from a clinical tumor gene expression profiling database and between tumor samples isolated from brivanib alaninate–treated and untreated mice. Tyrosine kinase receptor 1 (Tie-1), collagen type IV α1 (Col4A1), complement component 1 q subcomponent receptor 1 (C1qr1), angiotensin receptor–like 1 (Agtrl1), and vascular endothelial-cadherin (Cdh5) were all identified to be significantly modulated by treatment with brivanib alaninate. These genes, which may be potentially useful as markers of brivanib alaninate activity, were further studied at the protein level in two separate in vivo human colon tumor xenograft models, HCT116 and GE0, using immunohistochemistry-based approaches. [Cancer Res 2007;67(14):6899–906]

Introduction
The process of angiogenesis in which new blood vessels are formed from the preexisting vascular system has clearly been shown to be a fundamental and highly dynamic component in multiple disease states including, most notably, tumor growth and metastasis (1, 2). This process is critically important for maintaining tumor oxygenation and for delivery of nutrients, including growth factors and hormones, and serves as a system for tumor metabolic waste removal. In the absence of angiogenesis, tumors are considered to be in a dormant state and are generally devoid of actively developing blood vessels (3). This highly complex process is known to be regulated by positive and negative factors that both influence and are influenced by the tumor host microenvironment (4). However, when an imbalance of these factors occurs in such a way that proangiogenic factors are expressed in favor of antiangiogenic factors, the “dormant” tumor can undergo the so-called “angiogenic switch,” resulting in rapid tumor growth and increased potential for metastasis (5). Multiple preclinical studies have shown the important role vascular endothelial growth factor (VEGF) plays in driving the angiogenic process through its cognate receptors, the VEGF receptor (VEGFR) family of transmembrane protein tyrosine kinases. The VEGF receptor 2 (VEGFR-2) signaling pathway, in particular, has been experimentally supported to be a major driver of tumor angiogenesis (6). Importantly, it is now a clinically validated pathway in cancer therapy based on the recent approvals of Sutent and sorafenib that directly inhibit VEGFR-2 enzymatic activity and Avastin that indirectly inhibits this signaling pathway by preventing VEGF ligand binding to its receptor (7, 8). Brivanib alaninate has recently entered clinical trials and is a prodrug of BMS-540215. This small-molecule has shown potent inhibition of VEGFR-2 as well as inhibition of fibroblast growth factor receptor 1 (FGFR-1), another receptor protein tyrosine kinase underlying the angiogenic pathway (9). Consistent with its antiangiogenic activity, BMS-540215 inhibits both VEGF- and basic fibroblast growth factor–stimulated human umbilical vascular endothelial cell growth in vitro but does not inhibit tumor cell growth in vitro. In multiple preclinical models of human tumor xenografts, both brivanib alaninate and its solubilized parent have shown potent antitumor activity when dosed orally on a once-a-day schedule with complete induction of tumor stasis. With the termination of dosing, tumor xenografts resume growth (ref. 9; Fig. 1C). Consequently, the anticipated effects of this therapeutic approach predict tumor stasis rather than regression and, therefore, antiangiogenic agents are expected to be used either in conjunction with standard therapy or in an adjuvant setting. As a result, the identification of a suitable and easily measured marker of antitumor activity in the clinical setting would facilitate the rapid clinical development of these agents.

One approach to this problem is the use of cDNA microarrays to identify novel biomarkers that respond to treatment. The development of microarray technologies for large-scale characterization of mRNA expression pattern has made it possible to systematically search for molecular markers and to categorize cancers into distinct subgroups (10–15). Such technologies and molecular tools have made it possible to monitor the expression levels of a large number of transcripts within a cell population at
any given time (16–19). Recent studies show that gene expression information generated by microarray analysis of human tumors can predict clinical outcome (20–22). These findings raised the expectation that cancer treatment will be vastly improved by better predicting and monitoring the response of individual patients to therapy.

In this article, two types of transcriptional profiling were done, one in human samples and another in xenografts. The first transcriptional profile found human genes that are coregulated with VEGFR-2. The second approach identified targets that are modulated by the specific VEGFR-2 antagonist brivanib alaninate. By merging these two approaches, genes were discovered that are coregulated with VEGFR-2 in humans and are modulated by brivanib alaninate in tumor models. These potential biomarkers were then validated by immunohistochemistry and ELISA. One of the markers, collagen type IV, fulfills all of the aforementioned criteria, is coexpressed with VEGFR-2, is down-regulated by a VEGFR-2 antagonist, and changes in the same way in two xenograft models. Furthermore, ELISA also shows the same variation in the blood of the mice, thus validating the discovery made via transcriptional profiling. This approach to discovery and validation provides a new way to identify biomarkers for specific compounds and can be used for a wide variety of compounds entering clinical trials (23).

Materials and Methods

Human cancer tumor samples used for clinical gene expression profile database. The use of these samples was approved by the Local Ethics Committee and informed consent was given by all patients before surgery. The study population consisted of 118 patients (age range, 27–91 years; mean, 69 ± 13.67 years) with primary colorectal tumors resected at the Royal London Hospital between December of 1997 and March of 2003.
Ninety-two patients underwent a potentially curative surgery, and 24 patients underwent noncurative or palliative surgery. The following variables were recorded for all patients with primary colorectal cancer: age, cancer site (right sided or left sided), type of surgical resection (curative or noncurative), tumor-node-metastasis classification and Dukes' stage, degree of histologic differentiation (well, moderate, or poor), therapies received before and after surgery, recurrences, and survival time after resections.

RNA isolation and gene expression profiling. Colon tumor samples obtained from cancer patients were snap frozen in liquid nitrogen within 20 min of resection and stored at −80°C until use. For mouse xenograft samples, tumors were split into two halves. One half was mixed in a vial of 10% formalin to be used for immunohistochemistry and the other half was mixed in 1.0 mL of RNA Later RNA Stabilization Reagent (Ambion, Inc.), held at room temperature for 30 min, and then stored at −80°C. Total RNA was extracted from the tumor samples using the RNeasy Kit according to the manufacturer’s instructions (Qiagen). One microgram of total RNA from the samples was used to prepare biotinylated probe according to the Affymetrix GeneChip Expression Analysis Technical Manual, 2001. Targets were hybridized to Affymetrix high-density oligonucleotide human U133A and mouse 430A arrays. The arrays were then washed and stained using the GeneChip Fluidics station according to the manufacturer’s instructions.

Mice xenograft tumor models. Tumors were propagated in nude mice as s.c. transplants using L2987, HCT116, and GEO cancer cell lines. Each group of animals was weighed before the initiation of treatment (Wt1) and then again following the last treatment dose (Wt2). The difference in body weight (Wt2 − Wt1) provided a measure of treatment-related toxicity. No weight loss was observed in treated groups when compared with untreated controls. Each group of animals was weighed before the initiation of treatment (Wt1) provided a measure of treatment-related toxicity. No weight loss was observed in treated groups when compared with untreated controls.

Transcriptional profiling data analysis. A total of 118 human colon cancer gene expression profiles were used to discover and to validate the expression of genes coexpressed with VEGFR-2. Affymetrix MAS 5.0 software was used for normalization of the arrays. The human colon cancer samples were randomized and then split into two separate groups, each consisting of 59 samples. A filter was first applied to the 59 human colon cancer gene expression data used for the analysis to remove any probe sets with no or low expression. This was accomplished by removing probe sets labeled with absent calls across all samples as determined by the Affymetrix MAS 5.0 software. This resulted in the reduction of the number of available probes for analysis from 22,284 to 17,343. A linear regression model with Pearson correlation metric was then used to compute the gene expression values for each probe set on the human U133A gene chip with the selected VEGFR-2 probe set (probe ID 203934_at). All probe sets included in the HU133A gene chip data were ranked in descending order of their coefficient values for correlation with VEGFR-2 (probe ID 203934_at). Probe sets having correlation with VEGFR-2 ≥ 0.55 (correlation coefficient) were considered to be strongly coexpressed with VEGFR-2. We identified 94 probes that were strongly coexpressed with VEGFR-2 (probe ID 203934_at) from 59 colon tumor gene expression profiles. The second set of 59 human colon cancer gene expression profiles was used as an independent validation set to see if the 94 VEGFR-2 coexpressed gene signature was reproducible. This was accomplished by use of hierarchical clustering. For the clustering results, the data were first log transformed to the median center of each gene. Average linkage clustering was applied using an uncentered correlation distance metric for both the genes and arrays. The gene expression pattern obtained from the hierarchical clustering results using the second set of 59 colon cancer samples was visualized with TreeView software. The results showed a very similar gene expression pattern for the 94 VEGFR-2 coexpressed genes to the results observed from the first set of 59 colon cancers, which was used for discovery (data not shown). These results suggested that the genes coexpressed with VEGFR-2 and its expression pattern may be conserved across independent samples. The 94 coexpressed VEGFR-2 genes identified from 59 human colon cancers profiled on HU133A arrays were then used to identify their corresponding mouse orthologue from mouse xenograft tumors profiled on mouse Affymetrix 430A arrays. This was achieved through the use of Affymetrix NetAffy analysis center software. Once the corresponding mouse probes were identified, a Student's t test (two-tailed distribution and equal variance) was done on the gene expression profile data generated from the untreated and brivanib alaninate–treated xenograft tumors (four replicates in each group) that were profiled on the mouse Affymetrix 430A arrays. A total of 18 probes were identified from the list of 94 coexpressed VEGFR-2 genes studied, which significantly changed in mRNA abundance after treatment with brivanib alaninate (P < 0.05, t test).

The transcriptional profile data have been submitted to GEO repository, with accession no. GSE7208 for 59 colon tumors profiled on human U133A GeneChips used for the discovery of genes coexpressed with VEGFR-2 and accession no. GSE7209 for four control (nontreated) and four brivanib (drug)-treated L2987 xenograft tumor samples profiled on mouse 430A GeneChips.

Immunohistochemistry. Tissue specimens collected for immunohistochemistry were fixed in 10% formalin. After fixation, the specimens were submerged in 70% ethanol before being embedded in paraffin blocks. Five-micron tissue sections were cut from each block and placed on positively charged microscope slides. The tissue slides were deparaffinized by immersing in xylene. The slides were rehydrated in a descending series of 100% ethanol, 95% ethanol, and 70% ethanol. After rehydration, the slides were washed several times with deionized water at room temperature. Staining optimization was done in an automated BioGenex i6000 immunohistochemistry system for each of the candidate marker antibodies independently. Both treated and untreated xenografts were stained in the same run to minimize slide-to-slide variations. The following antigen retrieval conditions were used for the markers: for vascular endothelial-cadherin (VE-cadherin) and Tie-1, AR10 at 95°C for 15 min (BioGenex); for collagen type IV, AGTR1, and C1QR1 CitraPlus at 95°C for 15 min (BioGenex). The antibodies used were as follows: Tie-1, at 0.5 μg/mL dilution (Santa Cruz Biotechnology); collagen type IV, at 1:1,000 dilution (Biodesign); AGTR1, at 2 mg/mL (Novus Biologicals); and C1QR1, at 2 mg/mL (R&D Systems). For detection, the Envision kit from DAKO was used, except for C1QR1, where the ABC system was used. Rabbit or mouse control immunoglobulin G was used as a control. All the negative controls gave no staining in the xenografts (data not shown).

ELISA. For collagen type IV, ELISA kits were obtained from Kamiya Biomedical Company and Exocell, Inc. The methods for processing the ELISA assays were as described in the manufacturers’ protocols. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from standard dilutions and protein of interest. Sample concentration was determined relative to the standard.

Results

Identification of a signature that is coexpressed with VEGFR-2. One hundred eighteen primary colon tumors were profiled (see Materials and Methods for a description). Fifty-nine tumor samples were used for discovery whereas the remainder were used for verification of the results. The aim of these studies was to identify genes that were coexpressed with VEGFR-2. To that end, the expression profile of these tumors was assessed and 94 biomarkers were identified that are coexpressed with VEGFR-2 having a Pearson correlation ≥ 0.55 (see Fig. L4 for a flowchart of the process). Once this signature was identified in humans, it was then tested to see if it could be modulated on inhibition of VEGFR-2. To address it, brivanib alaninate was used. Brivanib alaninate is

* Cluster & TreeView software programs are available at http://rana.lbl.gov/EisenSoftware.htm.

* http://www.affymetrix.com/analysis/index.affx
a small-molecule antagonist of VEGFR-2 that was previously shown to have in vivo efficacy (9). To see if the compound had any effect on the markers, the level of these markers was tested using xenografts in athymic mice treated with compound. L2987 was chosen as a model because it is a lung carcinoma tumor cell line that is highly vascularized and grows well in athymic mice. Tumors were allowed to grow to a predetermined size (usually between 100 and 200 mm³, with tumors outside the range excluded) and animals were treated with brivanib alaninate (100 mg/kg 1 qd × 14 days). Tumors were then excised from the animals. Half of the...
The tumor was fixed in formalin and embedded in paraffin for later use in immunohistochemistry. The other half of the tumor was used for RNA isolation and subsequent transcriptional profiling with the mouse 430 Affymetrix chip (Fig. 1C).

Of the original 94 probes, only 18 probes showed a significant ($P < 0.05$) difference in expression between pretreatment and brivanib alaninate–treated L2987 tumors (Fig. 1B). Of these 18, the top 5 were chosen for validation using immunohistochemistry because their fold change and $P$ value differences were among the highest between pretreatment and posttreatment. The markers identified were Tie-1, collagen type IV, C1QR1, angiotensin receptor–like 1, and VE-cadherin (Fig. 1B). The fold change for Tie-1 was 11.4 ($P = 0.001033$); for collagen type IV, 6.4 ($P = 0.000018$); C1QR1, 4.7 ($P = 0.000005$); angiotensin receptor–like 1, 3.8 ($P = 0.000555$); and VE-cadherin, 2.4 ($P = 0.001154$; Fig. 2). All of the markers found were down-regulated on treatment with the compound. Up-regulated markers were also identified but their fold change and $P$ values were not significant.

Validation of the markers in L2987 xenografts. A key question to address was if the difference in mRNA levels, observed with transcriptional profiling on compound treatment, translated to a difference in protein expression. To test this hypothesis, immunohistochemistry assays were developed with all the markers shown in Fig. 2. After optimizing each of the antibodies (see Materials and Methods for optimized protocols), they were tested in xenografts before and after compound treatment (Figs. 3–5). Interestingly, collagen type IV and C1QR1 showed the same changes with immunohistochemistry as seen with the transcription profile. Both were reduced on treatment with the compound (Fig. 3, compare A–B and C–D). The other markers tested, VE-cadherin, Tie-1, and angiotensin receptor–like 1, showed no consistent differences between the pretreatment and posttreatment samples (Fig. 4A–F). All of those markers had high levels with immunohistochemistry before treatment and there was not a significant difference observed after treatment (Fig. 4, compare A, C, and E to B, D, and F, respectively).

The apparent difference between the result from the mRNA analysis and the immunohistochemistry expression could be due to several reasons, among them mRNA half-life, protein degradation, and posttranslational modifications are just a few. All of those could potentially explain these differences.

Validation of the markers in HCT116 and GEO xenografts and decreased vascularization. To further validate the immunohistochemistry assays developed and the ability of the markers to be modified by brivanib alaninate treatment, additional xenograft models were analyzed by immunohistochemistry. Two human xenograft models were analyzed:

Figure 4. Immunohistochemistry of L2987 tumors with anti–VE-cadherin pretreatment (A) and posttreatment (B), anti–Tie-1 pretreatment (C) and posttreatment (D), and anti-AGTRL1 pretreatment (E) and posttreatment (F). Brown, presence of the protein; blue, hematoxylin counterstain. Images (A) to (D) were taken with a 20× objective and images (E) to (F) with a 10× objective.
colon xenografts, HCT116 and GEO, were treated with brivanib alaninate. Collagen type IV staining was high before treatment in HCT116 xenografts (Fig. 5A). On treatment with the compound, a similar decrease of staining was seen as previously observed with L2987 (Fig. 5B). Tumor xenograft down-regulation of the protein was also observed in GEO xenografts (data not shown). C1QR1 staining in GEO xenografts exhibited the same behavior (Fig. 5C and D). The same was observed when that marker was used to stain HCT116 xenografts (data not shown), indicating that the compound treatment affects the biomarker regardless of the origin of the xenograft.

We noticed that the staining from collagen type IV was restricted to the endothelial cells and surrounding cells in the pretreatment sample (Fig. 5E). On treatment with the compound, such staining disappears (Fig. 5F). Similar observations were noted with C1QR1. Interestingly, this correlated with the visible absence of endothelial cells in the vascular areas of the tumor on treatment. To test if this was due to the treatment with brivanib alaninate, tumors were stained with anti-CD34, a marker for endothelial cells. The mean vascular density was calculated for samples taken before and after treatment (Fig. 6A and B). A marked reduction was observed in two xenografts models, L2987 and HCT116, which was statistically significant ($P < 0.005$), indicating that the compound brivanib alaninate inhibits the new growth of endothelial cells. This perhaps explains that the markers detected are secreted by endothelial cells on stimulation from the tumor cells, and because the compound reduces this cell type, it may indirectly reduce the level of both markers. Furthermore, because the observation is also encountered in the L2987 tumor, we can conclude that the endothelial cells are responsible for the expression of these markers.

**Collagen type IV expression in blood from treated xenografts.** On the observation that collagen type IV was diminished in the xenograft tumors on treatment with brivanib alaninate, the next step was to determine if this marker could be present in the blood of mice bearing the xenografts. To that end, an ELISA assay was developed that is able to measure collagen type IV in the blood of mice. On brivanib alaninate treatment of the mice bearing L2987 xenografts, a marked decrease of the biomarker in peripheral blood was observed (Fig. 6C). This decrease was only seen in tumor-bearing mice, suggesting that collagen type IV is increased on tumor formation but is brought back to within normal levels with compound exposure. This overexpression of collagen type IV in tumor environments has previously been reported (24, 25).
ELISA to VE-cadherin was also developed and no changes in the levels of the protein in blood were found (data not shown), thus confirming the result obtained with immunohistochemistry for this marker.

Discussion

A new method for the identification and validation of biomarkers that are modulated by a small-molecule inhibitor of angiogenesis is described. Transcriptional profiling of human samples identified genes that change at the same time that VEGFR-2 gene changes occur. That signature was further validated by human cell lines treated with an antagonist to VEGFR-2, brivanib alaninate. The protein encoded by the mRNA of those genes was then assessed via immunohistochemistry. The ability of detecting two biomarkers (i.e., collagen type IV and C1QR1) with this assay showed that the protein change was behaving in similar ways to mRNA change. Furthermore, collagen type IV was present in the blood of mice and it also decreased on treatment with brivanib alaninate. Interestingly, several of the biomarkers showed clear positive staining by immunohistochemistry around tumor blood vessels before treatment. After treatment with brivanib alaninate, the amount of immunohistochemical staining significantly decreased as did the overall vascular density within the tumors, indicating that the endothelial component was potentially responsible for producing these effects.

The two biomarkers that maintained the same behavior in transcriptional profiling and immunohistochemistry were collagen type IV and C1QR1. Collagen type IV has previously been implicated to play an important role in angiogenesis and tumor progression (26). Small-molecule inhibitors of collagen IV biosynthesis are capable of preventing endothelial tube formation and tumor growth (27). Its encoding mRNA was also reduced on treatment with DC101, an antibody that targets human VEGF (28). In addition, collagen IV abnormalities have been detected in the tumor microenvironment, and it may be linked to a possible mechanism of resistance for antiangiogenesis therapies by providing a scaffold for endothelial cell regrowth (29, 30). Our data further support the hypothesis that collagen type IV may be an important biomarker of angiogenesis in cancer. Brivanib alaninate may provide an approach that can be used in the clinic to suppress collagen type IV expression and delay tumor progression. Here we show that brivanib alaninate, a small-molecule inhibitor of VEGFR-2, has a similar effect on collagen type IV, thus validating this biomarker and reproducing results that have already been observed with other antiangiogenic agents. Collagen type IV is present in the vessels and in the blood, and it decreases on treatment, thus supporting it as a surrogate marker for dose treatment.

That the rest of the biomarkers discovered using transcriptional profiling did not translate to the protein level is not necessarily unexpected. It is our experience that only ~30% of the biomarkers discovered using transcriptional profiling translates to protein changes. There are several reasons for this observation. First, transcriptional profiling is much more sensitive than immunohistochemistry and ELISA, which are the methods used to validate the findings. If the mRNA is present in small quantities, we may not be able to detect it with the conventional methods used to detect proteins. Second, the mRNA change may not correlate to a protein change due to secondary protein modifications. Third, we cannot discount the possibility that the antibodies used may have different binding capacities for the targeted proteins.

The most interesting point of the method that we have described for the discovery and validation of biomarkers is the potential ability of these biomarkers to be surrogate end points in clinical trials for the antiangiogenic activity of brivanib alaninate or other VEGFR-targeted compounds. This will be an interesting question that will be addressed in the near future.

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References

20. United States patent 5990388 to Ashby et al.
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