Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Identification and Analysis of *In Vivo* VEGF Downstream Markers Link VEGF Pathway Activity with Efficacy of Anti-VEGF Therapies

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Abstract

Purpose: The aim of this study was to identify conserved pharmacodynamic and potential predictive biomarkers of response to anti-VEGF therapy using gene expression profiling in preclinical tumor models and in patients.

Experimental Design: Surrogate markers of VEGF inhibition [VEGF-dependent genes or VEGF-dependent vasculature (VDV)] were identified by profiling gene expression changes induced in response to VEGF blockade in preclinical tumor models and in human biopsies from patients treated with anti-VEGF monoclonal antibodies. The potential value of VDV genes as candidate predictive biomarkers was tested by correlating high or low VDV gene expression levels in pretreatment clinical samples with the subsequent clinical efficacy of bevacizumab (anti-VEGF)-containing therapy.

Results: We show that VDV genes, including direct and more distal VEGF downstream endothelial targets, enable detection of VEGF signaling inhibition in mouse tumor models and human tumor biopsies. Retrospective analyses of clinical trial data indicate that patients with higher VDV expression in pretreatment tumor samples exhibited improved clinical outcome when treated with bevacizumab-containing therapies.

Conclusions: In this work, we identified surrogate markers (VDV genes) for *in vivo* VEGF signaling in tumors and showed clinical data supporting a correlation between pretreatment VEGF bioactivity and the subsequent efficacy of anti-VEGF therapy. We propose that VDV genes are candidate biomarkers with the potential to aid the selection of novel indications as well as patients likely to respond to anti-VEGF therapy. The data presented here define a diagnostic biomarker hypothesis based on translational research that warrants further evaluation in additional retrospective and prospective trials. *Clin Cancer Res; 19(13); 3681–92. ©2013 AACR.*

Introduction

VEGF signaling through activation of the endothelial cell-specific receptor tyrosine kinase (RTK) VEGFR-2 is indispensable for developmental angiogenesis (1, 2) and required for tumor neo-vascularization and growth in many preclinical cancer models (3, 4). Although VEGF inhibition is lethal during embryonic development, its neutralization is well tolerated in adults (5, 6), coincident with postnatal vascular maturation and differentiation in various tissues.

Not all vessels in the adult react equally to VEGF deprivation, however. Although some blood vessels are severely pruned, others are refractory or only marginally affected by VEGF neutralization (7). Similarly, inhibition of VEGF signaling has been shown to disrupt neoplastic angiogenesis and inhibit the growth of early-stage tumors while having a less pronounced effect on late-stage carcinomas (8–10), in which only a fraction of the total tumor vasculature is pruned (8, 11). However, the molecular identity of this distinct tumor vascular subcompartment is currently unknown.

Following observations that VEGF blockade is sufficient to inhibit angiogenesis and tumor growth in preclinical tumor models (4, 12), a humanized VEGF blocking

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Translational Relevance

Several VEGF pathway inhibitors have been approved for treatment of advanced cancer. Despite the overall clinical success of bevacizumab and other VEGF pathway inhibitors, clinical efficacy is variable and some individual patients and tumor types seem to be refractory or to acquire resistance to these therapies. Thus, pharmacodynamic and predictive biomarkers are urgently required to improve patient selection for VEGF-targeted agents. The data provided here identify a conserved collective group of *in vivo* VEGF signaling downstream markers [VEGF-dependent vasculature (VDV) genes] and indicate a link between preexistent VEGF pathway activity in human tumor vessels and the subsequent clinical efficacy of bevacizumab (anti-VEGF)-containing therapy. These findings identify VDV genes as candidate biomarkers and support prospective validation of the VDV gene profile as a possible strategy for the selection of indications and/or patients more likely to benefit from anti-VEGF therapy.

monoclonal antibody (mAb) bevacizumab (Avastin) was tested in clinical trials and subsequently U.S. Food and Drug Administration approved for the treatment of multiple malignancies including cancer (13–15). Despite the overall clinical success of bevacizumab and other VEGF pathway inhibitors, clinical efficacy is variable, and some individual patients and tumor types seem to be refractory or to acquire resistance to these therapies (16).

The factors restricting the clinical efficacy of bevacizumab are largely unknown. Intrinsic factors such as variable levels of dependency on VDV for tumor progression may modulate responses to these antiangiogenic agents (17). Other proposed additional mechanisms, not investigated here, include evasive resistance (8) and myeloid cell infiltration (18) may also limit clinical response to agents targeting the VEGF pathway.

Further optimization of anti-VEGF therapies depends upon the identification of pharmacodynamic and predictive markers of VEGF pathway response (13, 14). Discovery of such biomarkers has been difficult due to several factors, among them: (i) the small percentage of cells in a tumor that form part of the vasculature and that are directly targeted by anti-VEGF therapy, (ii) the limited understanding of the molecular identity of the cellular compartment targeted by anti-VEGF agents, and (iii) a lack of proximal surrogate markers for VEGF bioactivity. Here, through the characterization of the vascular compartment directly targeted by anti-VEGF treatment in neoplasia (i.e., the VEGF-dependent tumor vascular compartment), we report the identification of *in vivo* surrogate markers of anti-VEGF activity and assess the extent to which the intrinsic levels of VEGF bioactivity in tumors may influence the clinical efficacy of anti-VEGF therapies.

Materials and Methods

Treatment regimens

All dosing regimens and animal protocols followed according to IACUC guidelines. B20-4.1.1 and anti-DLL4 Mabs are full-length (phage derived) reverse-chimeric antibodies with murine constant (igG2a) domains. B20-4.1.1 is cross-reactive with human and mouse VEGF and it was developed for long-term administration in immunocompetent mice (19).

B20-4.1.1 (anti-VEGFA), anti-Ragweed, and anti-Dll4 were prepared and purified in-house as described (20), and dosed by i.p. injection at 5 or 10 mg/kg twice weekly in immuno-compromised mice or weekly (at 5 mg/kg) in RIP-TβAg mice. Sunitinib and axitinib were dosed at 60 mg/kg daily by oral gavage. Wound healing assays were carried out as described (21).

Gene signature derivation and application

Log_2 ratio intensity values from Agilent WMG microarrays and RMA normalized intensities from Affymetrix Mouse430.2 microarrays were imported into R expression sets using the package "Biobase." Linear models were fit to each feature using "lmFit" and "eBayes" from the "limma" package (22). This corresponds to a gene-by-gene comparison of treatment to control samples, followed by moderation of the *t*-statistic using an empirical Bayes method to adjust *P*-values for multiple comparisons. EntrezGene identifiers were retained for features significantly (adjusted $P < 0.05$) downregulated in anti-VEGF-A compared to control treatments. The union of these genes was taken as representative of the VEGF-A-responsive vasculature. Variation in this VDV gene expression signature was determined for other experiments by fitting a linear model to the microarray data as above and calculating the mean of the *t*-statistics from genes in the signature.

Statistical analyses

Data sets were compared using Student *t* test (unless stated otherwise later) with *P* values < 0.05 considered significant. Of 1,017 patients from the XELOX containing arms in the NO16966 trial, 109 had sufficient RNA from archival tumor material to be analyzed for the gene expression of VDV genes. After qPCR analysis via Fluidigm chip, ΔΔCt values were calculated after normalization to housekeeping genes and relative to a universal reference. The value for each of the 22 VDV genes [*ESM1*, *NID2*, *COL4A1*, *COL4A2*, *LAMA4*, *VEGFR3*, *DLL4*, *VEGFR2*, *CD144*, *CD34*, *EFNB2*, *EGFL7*, *NG2*, *NRP1 (2 isoforms)*, *NRP2*, *NOTCH1*, *RGS5*, *SEMA3f*, *TSP1*, *VEGFR1*, and *VIM*] was mean centered and variance scaled across samples, resulting in a *z*-score for each gene. The average of VDV gene scores was calculated for each of the 103 samples that had data for all VDV genes. To compare the clinical outcomes between marker and treatment subgroups, log-rank and Cox regression tests were used, median time calculated by the Kaplan–Meier analysis. All statistical tests were 2-sided.

Results

Identification of acute markers of VEGF pathway inhibition

We first characterized the biological consequences of VEGF neutralization in a transgenic murine model of highly vascularized pancreatic neuroendocrine tumors (PNETs; ref. 3). Histological analysis of *RIP-TβAg* late-stage tumors showed anti-VEGF monoclonal antibody treatment causing a rapid reduction in microvascular density (MVD), reaching a plateau of approximately 50% at day 7. The anti-VEGF induced pruning of this fraction of the tumor vasculature—hereinafter referred to as "VEGF-dependent vasculature" (VDV)—is not significantly reversed nor increased at later treatment time points (Fig. 1A). In contrast to these rapid antivascular effects, a decrease in the tumor proliferative index in the anti-VEGF–treated group was not observed at day 7, becoming obvious only by day 14, and a consequent reduction in tumor burden that was only evident at day 21 (Supplementary Fig. S1). We reasoned that the separation in time of the direct and indirect tumor effects of anti-VEGF treatment would allow us to characterize the specific gene expression response of the vascular compartment that was sensitive to VEGF blockade.

Microarray analysis of whole tumors from animals treated for 7 days with anti-VEGF or control anti-ragweed antibody showed that a small population of genes responded to anti-VEGF treatment with a significant (adjusted-$P < 0.01$) decrease in transcript abundance. We observed no corresponding upregulation of gene expression, suggesting that gene expression changes were primarily driven by physical elimination of VEGF-dependent tumor-associated endothelial cells (TAEC; Fig. 1B, Supplementary Table S1).

The genes with decreased expression were enriched for known endothelial-specific genes (Supplementary Table S1) and implicated in blood vessel development (Supplementary Table S2). In addition, the median fold-change in expression for this gene set was similar to that seen in the pan-vascular markers Cd31 and Plvap, and consistent with the extent of MVD decrease as measured by immunofluorescence of the pan-vascular marker Plvap (Fig. 1).

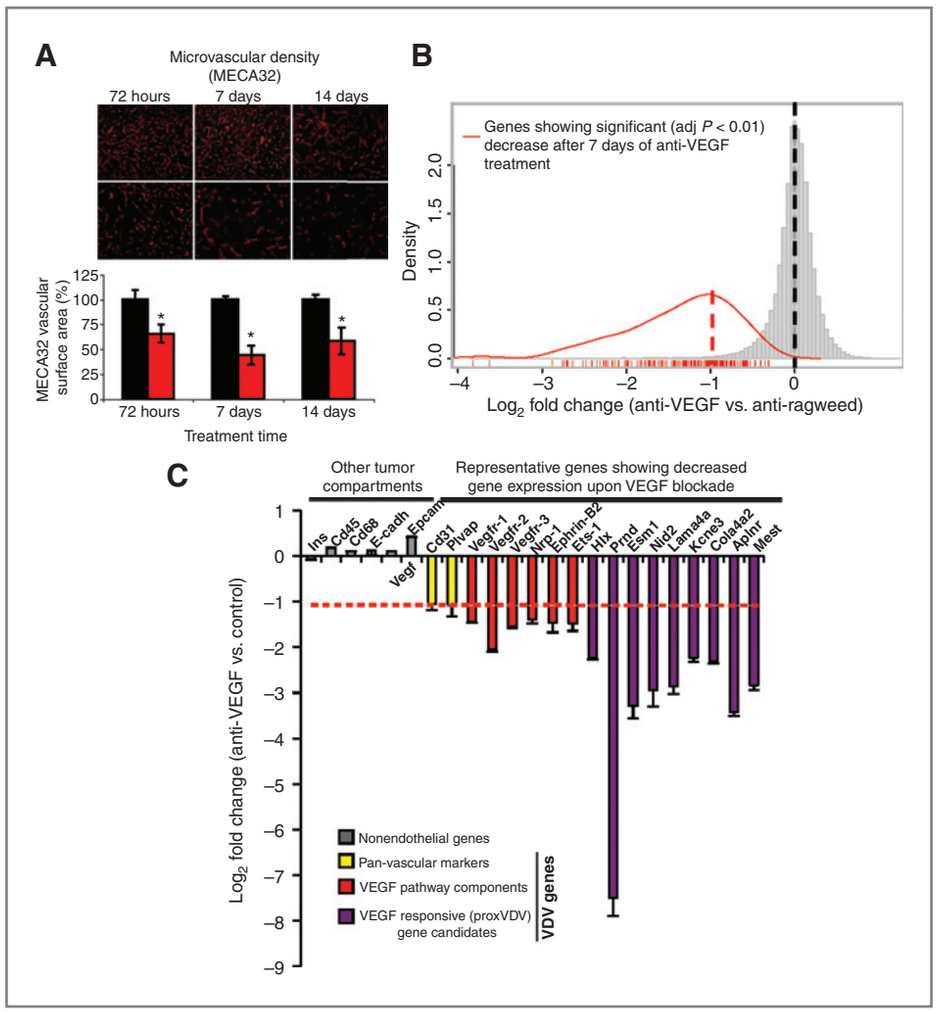

Figure 1. Identifying a VEGF-dependent tumor endothelial compartment in a mouse PNET model. A, representative images from histological analysis of tumor vessel density via MECA32 (Plvap) staining at various times following anti-VEGF treatment (20× magnification). Nuclei are counterstained with DAPI (blue). Quantitation from 4 to 6 tumors in each case is shown in the bar graphs below as mean ± SEM. *, $P < 0.05$; NS = not significant. B, density plots from microarray analysis of B20-4.1.1 anti-VEGF antibody treated tumors ($n = 5$/cohort). Expression levels of genes (shown as red lines) decrease significantly relative to all genes (gray histogram). The dashed red line indicates the mean change for these selected genes. The black dashed line indicates the mean fold change for the rest of the genes. C, qPCR validation of select individual VDV gene expression change for the above comparison. Dashed red line indicates the mean expression change for pan-vascular marker transcripts. Bars represent mean expression from 3 independent biological replicates. Error bars, $\log_2$ SD. Gene expression changes are for treatment relative to anti-Ragweed MAb or vehicle controls.

Quantitative PCR (qRT-PCR) of whole tumors from anti-VEGF or control-treated animals confirmed the microarray results, verifying that markers specific to: tumor (insulin), epithelial (E-Cad and Epcam), pan-hematopoietic (Cd45), or macrophage (Cd68) cells are not significantly changed by anti-VEGF treatment. qRT-PCR also confirmed that although VEGF transcript levels are not significantly changed, all tested endothelial markers are downregulated by this treatment (Fig. 1C).

We identified a subset of genes whose downregulation in response to anti-VEGF was more pronounced than other genes in the signature (Fig. 1C, Supplementary Table S1), suggesting that some of the signature genes might be selectively expressed in the vessels that are sensitive to anti-VEGF treatment. In contrast to low- and intermediate-responding genes, this set of highly responsive genes includes tip-cell markers and, in the context of developmental retinal angiogenesis, known VEGF targets (Fig. 1C; 23–26). Interestingly, endothelial tip cells are known to be highly responsive to VEGF signaling. Thus, we postulated that these genes are candidate proximal biomarkers of VEGF pathway inhibitor activity (proxVDV genes), and likely to be VEGF targets more selectively expressed in the VEGF-dependent tumor vasculature. These results suggest that VDV gene expression signature reflects at least 2 related biological processes: (i) direct VEGF downstream signaling inhibition, and (ii) the subsequent loss of vessels that are dependent on VEGF signaling for survival. According to this hypothesis, VDV endothelial genes include proximal (proxVDV) as well as more distal (distVDV) downstream surrogate markers of VEGF signaling inhibition.

VDV responses to VEGF signaling blockade are stromal specific and conserved across multiple tumor models

We looked for this VDV transcriptional signature response in other tumor models, first by analyzing whole-tumor responses to anti-VEGF treatment in samples from an established subcutaneous human breast carcinoma tumor model (MDA-MB-231). Although unsupervised expression analysis failed to distinguish between anti-VEGF and control treatment samples (not shown), short-term anti-VEGF treatment was sufficient to induce a significant downward shift in expression of the vast majority of the VDV genes relative to all other genes ($P < 0.0001$; Fig. 2A). Consistent with the hypothesis that VDV genes are endothelial specific, expression changes in these genes were detected only by probes on the murine, but not the human microarrays, corresponding to the distinction between stromal and tumor cells (Fig. 2A). Also, in agreement with the data from the *RIP-TβAg* GEMM, anti-VEGF treatment of the subcutaneous xenograft breast cancer tumors induced a steeper downregulation of candidate proxVDV gene candidates relative to pan-vascular markers and other VDV genes. These acute changes in gene expression suggest that besides reflecting changes in MVD, some of these markers may report VEGF transcriptional activity.

We investigated the effects of long-term anti-VEGF administration in an orthotopic, intracranial U87 glioblastoma model. The anti-VEGF treatment resulted in decreased VDV gene expression, with exclusive detection by the mouse-specific probes; and with more marked downregulation of candidate proxVDV genes (Fig. 2B). Comparable VDV patterns of response to anti-VEGF antibody were also observed across multiple anti-VEGF–treated tumor models tested (Supplementary Table S3).

Regardless then of the tumor model tested and implantation site, and independent of the length of antibody treatment, the VDV signature enables consistent detection of gene expression changes that reflect vascular downstream biological consequences of VEGF pathway inhibition in whole tumor mRNA samples.

VEGF signaling induces VDV gene expression

We assessed the extent to which VEGF stimulation might affect endothelial VDV gene expression in 2 different pathophysiological contexts: wound healing and increased tumor angiogenesis in response to blockade of the Dll4/Notch1 signaling pathway.

In an *in vivo* mouse skin-wounding assay, topical addition of recombinant VEGF (rVEGF) for 12 hours increased expression of a majority of VDV genes at the skin wound site, whereas anti-VEGF treatment had the opposite effect (Fig. 3A). Consistent with the response seen in tumors, the effects of VEGF blockade and topical VEGF were most marked for most proxVDV gene candidates.

The Dll4/Notch1 pathway increases nonproductive angiogenesis partly via enhancement of VEGF signaling (27–29). We characterized these effects by treating MDA-MB-231 tumor bearing mice for 48 hours with either anti-Dll4, anti-VEGF, or anti-ragweed antibody. As expected, anti-VEGF treatment induced pruning of tumor vasculature, whereas treatment with anti-Dll4 resulted in increased MVD (Fig. 3B). The expression of VDV genes changed concordantly, decreasing upon anti-VEGF treatment and increasing in response to blockade of Dll4. Again, as in other models, the change in expression of the proxVDV gene candidates is more pronounced than of the other VDV genes.

Overall, these data suggest that most VDV genes are expressed in VEGF-driven neo-vasculature, and that their collective expression likely reflects VEGF biological activity as well as the relative abundance of VDV.

ProxVDV genes are VEGF/VEGFR-2 downstream targets expressed in TAECs

Next we investigated the effects on proxVDV gene candidates of other drugs that directly target VEGFR-2 signaling. Mice bearing established MDA-MB-231 tumors were treated with a control mAb, anti-VEGF mAb (a surrogate for bevacizumab), sunitinib (Sutent; ref. 30), a small-molecule tyrosine kinase inhibitor that targets VEGFR-2 among other RTKs, or axitinib (31, 32), a more specific VEGFR-2 inhibitor. Tumors were collected at 8, 16, or 72 hours after treatment (Fig. 4A, top). Consistent with prior observations, all 3 inhibitors induced a significant reduction of MVD in tumors collected 72 hours after

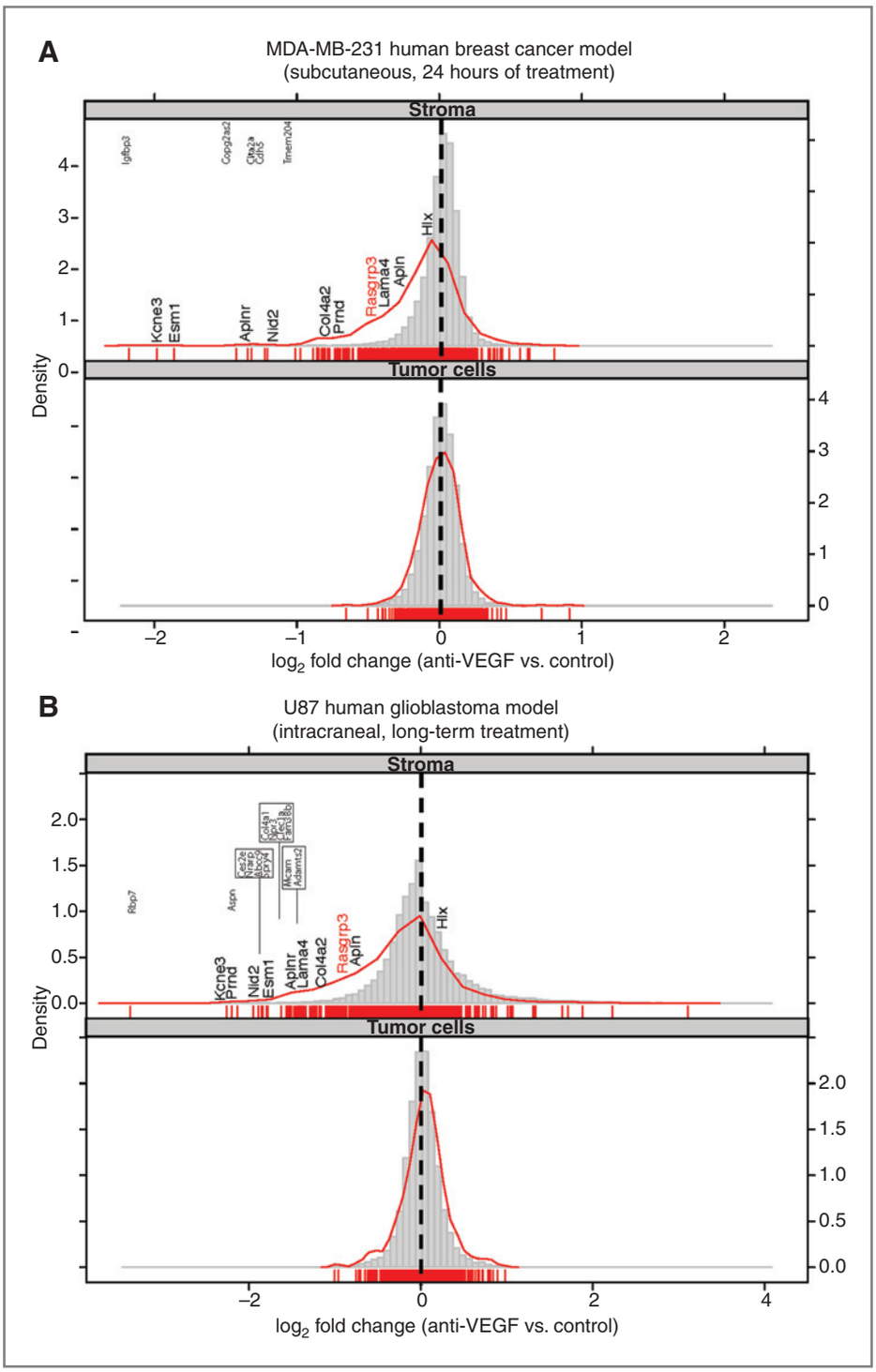

Figure 2. Conservation of VDV gene response to VEGF blockade in xenograft tumor models. Microarray data from MDA-MB-231 (A) subcutaneous xenograft tumor samples collected 24 hours posttreatment and U87 (B) intracranial orthotopic tumor samples collected 13 to 42 days posttreatment with the anti-VEGF antibody B20-4.1.1, compared to control treatment. A and B, genes in the VDV signature (red lines) decrease significantly relative to all genes (shown as a gray histogram) in the stroma (top graph, mouse chip, $P < 0.0001$ for A and $P = 0.0105$ for B), but not in the tumor cells (bottom graph, human chip, both experiments show no significant differences). VDV gene names annotated above the intensity plots correspond to gene transcripts with a log fold change (respect to control) of less than −1.5-fold (black letters, small font size). ProxVDV gene candidates are also indicated (black letters, big font size). RasGRP3, a proxVDV gene candidate previously reported to be a VEGF target expressed in tumor vasculature is included in red letters as a reference control. A and B, $N = 5$ to 10 instances for each treatment cohort shown here. Gene expression changes are for treatment relative to anti-Ragweed Ab or vehicle controls.

treatment (Supplementary Fig. S2A). All tested proxVDV gene candidates with the exception of Lama4a and Col4a2 showed evident downregulation by anti-VEGF after only 8 hours of treatment and consistently greater decrease in expression than that seen for the pan-vascular markers Cd31 and Plvap for the later time-points (16 and 72 hours) for all 3 VEGF pathway inhibitors (Fig. 4A, Supplementary Fig. S2B). Importantly, the expression of VEGF and of the nonvascular markers E-cad (epithelial) and Cd45 (hematopoietic) was not markedly affected by any of the VEGF pathway inhibitors tested (Supplementary Fig. S2B), suggesting that the changes in proxVDV

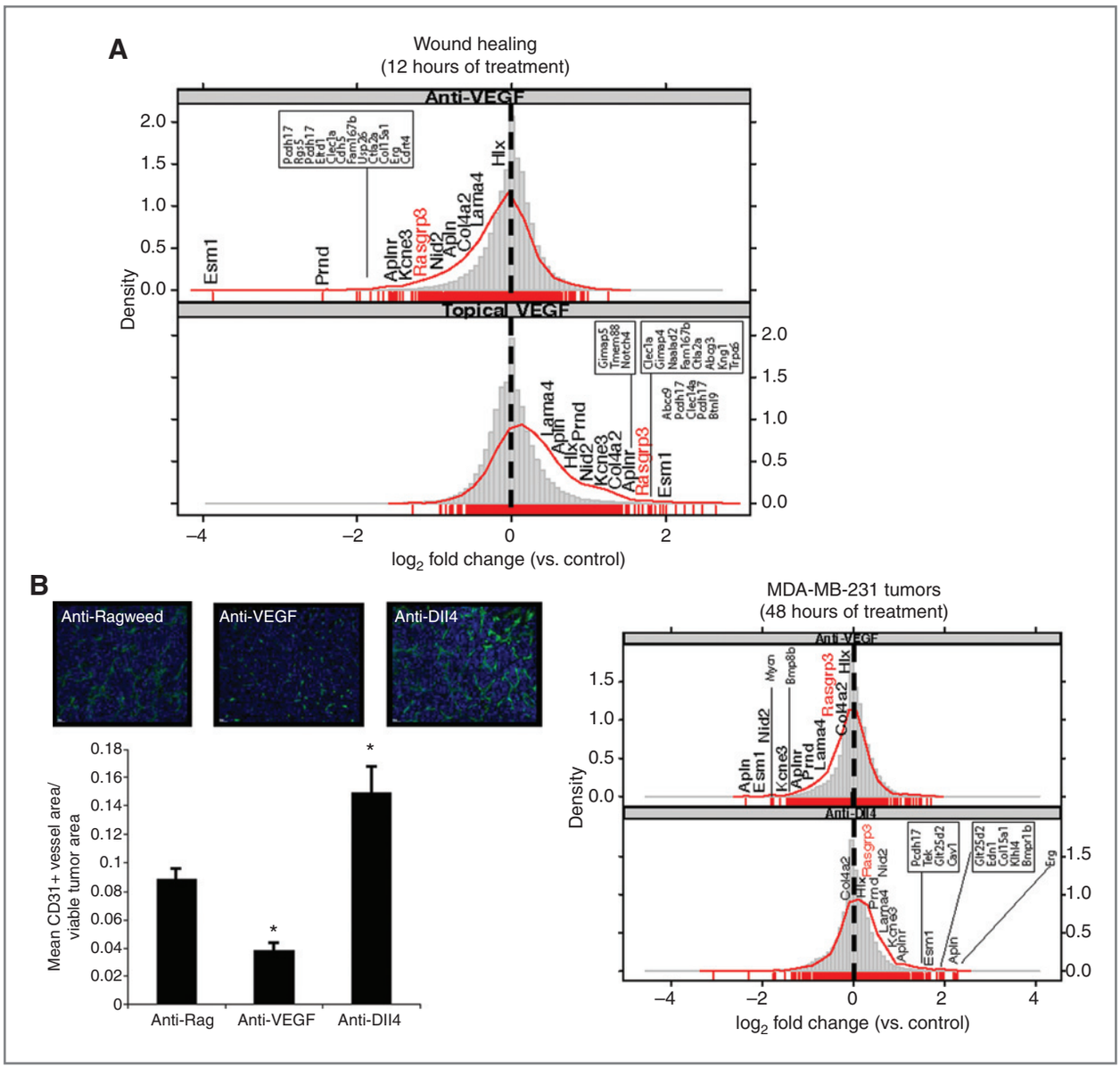

Figure 3. VEGF signaling induces VDV gene expression. A, VDV downmodulation observed upon topical application of anti-VEGF MAb B20-4.1.1 to a skin wound (top graph, $P = 0.0125$) and the converse upregulation, when comparing recombinant VEGF applied for 12 hours ($P < 0.0001$). B, In contrast to anti-VEGF downregulation of the VDV gene signature (right, top graph, $P < 0.0001$), anti-Dll4 treatment causes an upregulation of a majority of VDV genes (right, bottom graph, $P < 0.0001$) after 48 hours in an MDA-MB-231 model. This is consistent with hypervascularization evident by immunofluorescent staining for CD31/PECAM as compared to control treatment (left). Individual proxVDV transcript fold changes are annotated in black letters in each microarray density plot (A–B). VDV gene names annotated above the intensity plots correspond to gene transcripts with a log fold change (respect to control) of less than −1.5-fold (black letters, small font size). ProxVDV gene candidates are also indicated (black letters, big font size). RasGRP3, a proxVDV gene candidate previously reported to be a VEGF target expressed in tumor vasculature, is included in red letters as a reference control. $N = 5–10$ instances for each treatment cohort shown here. Gene expression changes are for treatment relative to anti-Ragweed MAb or vehicle controls.

gene expression are likely "on-target" and endothelial specific. At 72 hours, sunitinib seems to be the strongest VEGF pathway inhibitor (Fig. 4A).

The dynamics of the transcriptional response observed with small molecule inhibitors were different from those in anti-VEGF antibody-treated groups. No significant changes in gene expression were induced by sunitinib or axitinib after 8 hours of treatment (not shown). Only at 16 hours posttreatment were the effects of the 2 small molecule inhibitors on downregulating proxVDV gene expression apparent, and this effect increased at 72 hours after treatment (Fig. 4A). In contrast, downregulation of proxVDV (but not distVDV) genes by anti-VEGF mab is obvious at 8 hours after dosing, reaching its peak by 16 hours posttreatment.

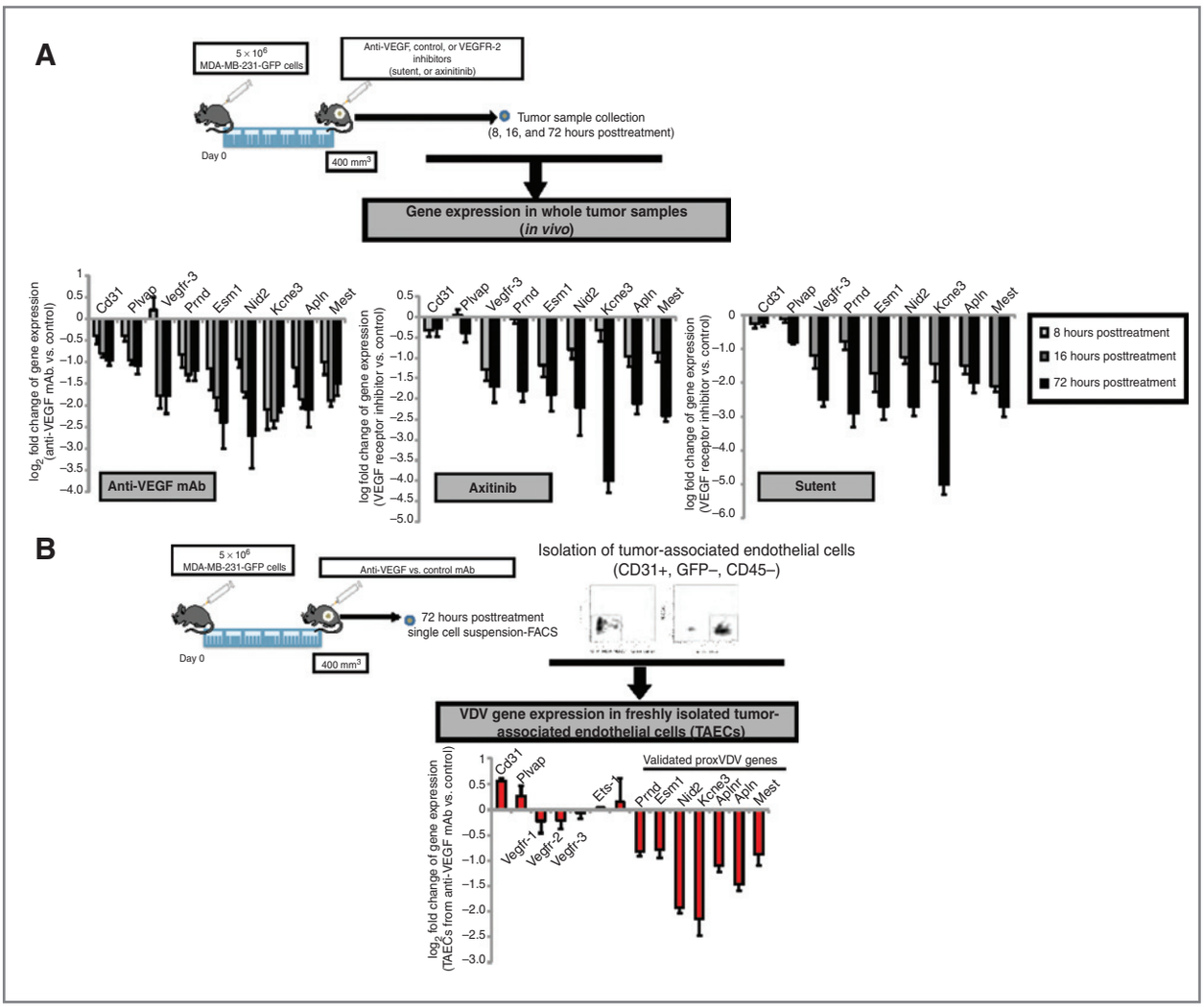

Figure 4. proxVDV are markers of VEGF downstream bioactivity in endothelial cells. A, consistent proxVDV downregulation by multiple VEGF pathway inhibitors. Analysis of gene expression in MDA-MB-231 xenograft tumors collected 8, 16, or 72 hours after treatment with VEGF and VEGFR-2 inhibitors (sutent and axitinib). Values represent the mean of the $\log_2$ fold change in relative gene expression induced by VEGF/VEGFR-2 inhibitor compared to control treatment. A, gene expression data represent the $\log_2$ mean of 8 biological replicates for each treatment. Error bars represent standard deviation. B, quantification of proxVDV gene expression by qRT-PCR in endothelial cells sorted from MDA-MB-231 xenograft tumors treated with ragweed or anti-VEGF mAb. Values represent the mean of the $\log_2$ fold change of 3 replicates. Error bars represent SD. Gene expression changes are for treatment relative to anti-Ragweed MAb or vehicle controls.

In situ hybridization (ISH) indicated that Esm1 is highly expressed in a significant fraction of vessels from HM7 colon xenograft tumors, while being nearly undetectable in vessels from the same model treated with anti-VEGF (Supplementary Fig. S2C). This is consistent with Esm1 being a *bona fide* proxVDV gene. However, because ESM1 is regulated by stimuli besides VEGF (33), and because ESM1 may be occasionally expressed in tumor cells (23), we validated additional proxVDV gene candidates as a more specific means to gauge *in vivo* consequences of VEGF signaling inhibition. To investigate which proxVDV gene candidates are regulated by VEGF signaling in TAECs, we treated MDA-MB-231-GFP tumor-bearing animals with anti-VEGF or control antibody, and then isolated TAECs (Cd31+, Cd45−, GFP−) by fluorescence-activated cell sorting. *Ex vivo* gene expression of those proxVDV gene candidates that were rapidly downregulated by anti-VEGF at 8 hours and consistently downregulated at later time-points by all 3 VEGF-targeted agents was compared between the treatment groups. We found that all pan-vascular markers and proxVDV genes tested were highly enriched on endothelial cells, compared to other sorted cell populations (Supplementary Fig. S3). In agreement with the *in vivo* VEGF pathway inhibitor data, the ProxVDV genes Prnd, Esm1, Nid2, Kcne3, Aplnr, Apln, and Mest were consistently downregulated in TAEC cells isolated from anti-VEGF–treated animals relative to controls, whereas Cd31, Plvap, Ets-1, and Hlx (a *RIP-TβAg* candidate proxVDV that was not

markedly in most tested models) were not (Fig. 4B). This confirms that Prnd, Esm1, Nid2, Kcne3, Apln, Aplnr, and Mest are proximal and sensitive biomarkers of VEGF bioactivity and candidate reporters for the direct inhibition of the pathway in TAECs *in vivo*.

VDV expression enables detection of the anti-VEGF activity of bevacizumab treatment in human biopsies

We examined the effect of VEGF blockade on VDV signature expression in tumor biopsies from bevacizumab (Avastin)-treated patients using published microarray data from matched pretreatment and 21 days posttreatment biopsies taken from 19 inflammatory breast cancer patients treated with one dose of bevacizumab as a single agent (24). Although conventional bioinformatics analyses failed to identify most of the specific vascular gene expression changes in response to bevacizumab *a priori* (25), our focused expression analyses of the human orthologs of the VDV gene set showed a clear downregulation of VDV transcripts in post-bevacizumab treatment clinical samples compared with pretreatment (Fig. 5). As in the murine tumor models, the expression of several proxVDV genes such as *ESM1*, *NID2*, *PRND*, *KCNE3*, and *MEST* decreases more markedly upon VEGF treatment. The VDV gene set we identified in preclinical models thus enables the detection of an evolutionary conserved vascular response to VEGF signaling inhibition in clinical tumor samples.

Higher expression levels of VDV genes in pretreatment colorectal cancer samples correlate with bevacizumab clinical efficacy

Our data indicate that human tumor vessels enriched in VDV genes are uniquely responsive to VEGF signaling inhibition. We next tested the hypothesis that a higher expression of these markers could actually predict responsiveness to anti-VEGF therapy.

Bevacizumab in combination with chemotherapy is an approved treatment for patients with metastatic colorectal carcinoma (CRC; ref. 26). In the first-line metastatic CRC trial NO16966, patients received oxaliplatin-based chemotherapy (XELOX or FOLFOX-4) in combination with either placebo or bevacizumab. The addition of bevacizumab to chemotherapy significantly improved the primary endpoint of PFS (defined in the original study as time to first documentation of disease progression "per investigator assessment"; ref. 34). To test if VDV gene expression correlates

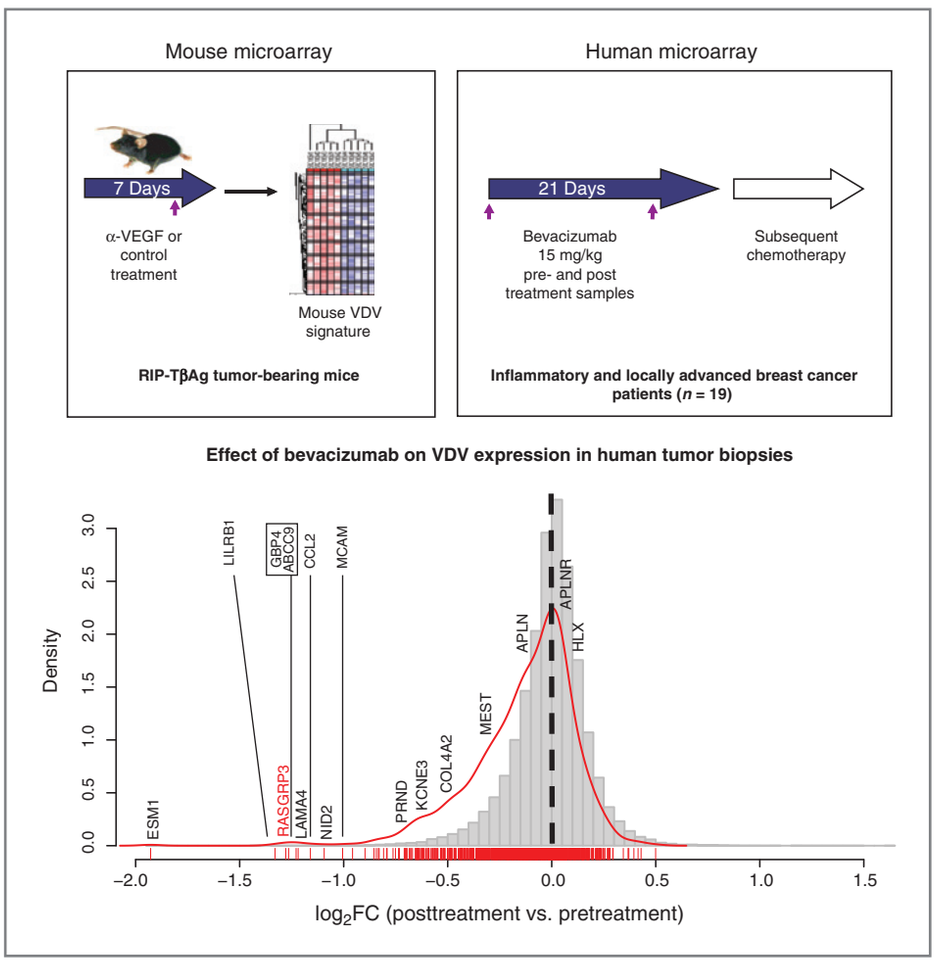

Figure 5. VDV gene expression analyses in bevacizumab-treated human breast and colon cancer biopsies. A, change in gene expression (posttreatment vs. pretreatment) in biopsy samples from 19 inflammatory breast cancer patients. Genes in the VDV signature (red lines) decrease significantly relative to all genes (gray histogram), $P = 0.0275$. VDV gene names annotated above the intensity plots correspond to gene transcripts with a log fold change (respect to control) of less than −1.5-fold (black letters, small font size). ProxVDV gene candidates are also indicated (black letters, big font size). RasGRP3, a proxVDV gene candidate previously reported to be a VEGF target expressed in tumor vasculature is included in red letters as a reference control.

with PFS, we analyzed VDV gene expression levels in the available pretreatment archival tumor tissue from 103 patients (biomarker evaluable subpopulation) from the XELOX-containing arms. These patients had no known clinical or demographic covariates that distinguished them from the remaining patients in the treatment arm. Because of limited quality of RNA from these FFPE tumor samples, gene expression was analyzed in a previously validated "angiogenesis" Fluidigm qRT-PCR chip (Fig. 6B) that included 4 different housekeeping genes, VEGF, and 22 representative proximal and distal VDV genes (see Supplementary Fig. S4A for validation of this compacted 22 VDV gene signature).

As expected, in the biomarker evaluable population, the addition of bevacizumab to chemotherapy provided a statistically significant PFS [HR = 0.59; 95% confidence interval (CI), 0.37 to 0.93; $P = 0.024$; Fig. 6A]. (This compares to the value for patients in the XELOX-containing arms of the study: HR = 0.77; 97.5% CI, 0.63–0.94; $P = 0.0026$; ref. 34.) As the expression of these 22 VDV genes is correlated (mean Pearson correlation = 0.419), we classified samples as based on a median VDV expression score and then tested the correlation of "high" or "low" VDV gene expression with PFS (Fig. 6B). The stratification of the treatment cohorts by pretreatment VEGF-A mRNA levels alone did not show differential effects on PFS in the bevacizumab-treated patients (Supplementary Fig. S4B). Similarly, bevacizumab-treated patients classified into "high" and "low" subsets by the expression levels of a single distVDV gene (CD31) did not show marked differences in PFS, nor did the interaction between VEGF or CD31 and treatment show any predictive effect.

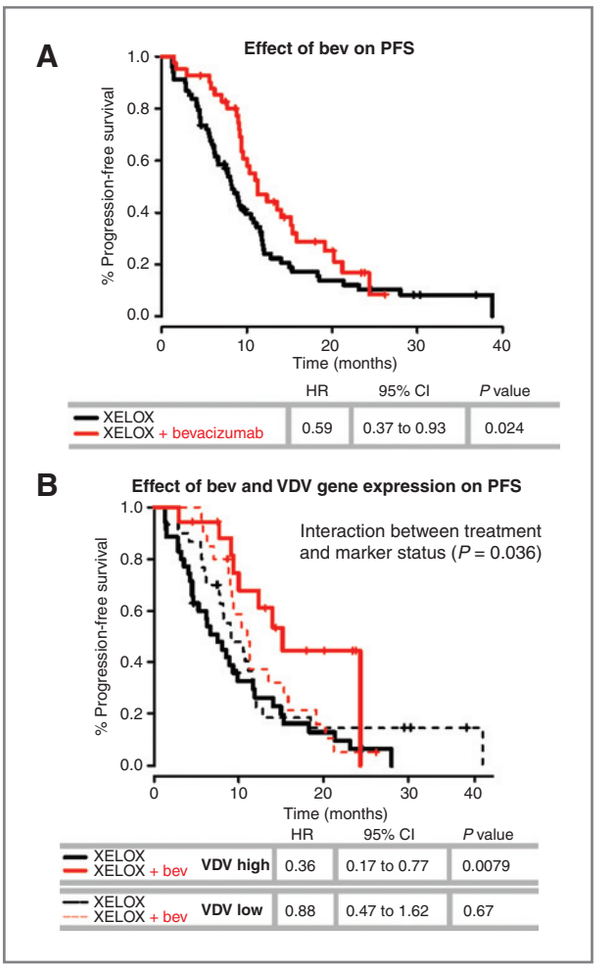

Figure 6. Higher expression levels of VDV genes in pretreatment colorectal cancer samples correlate with PFS in response to bevacizumab. A, progression-free survival of the 103 colorectal cancer patients (no stratification) in the biomarker available population of the NO16966 trial. B, the 22-gene VDV signature was used to stratify the biomarker-available population into VDV-high and VDV-low samples. Shown are progression-free survival curves of patients with "VDV-high" (solid lines) versus "VDV-low" samples (dashed lines), treated with XELOX (black) or XELOX + bevacizumab.

Within both treatment arms, the differences in PFS for high vs. low VDV patients were not significant ($P = 0.23$, XELOX + placebo; $P = 0.081$, XELOX + bevacizumab), and in the "VDV low" population (Fig. 6D), the combination of bevacizumab and chemotherapy conferred only modest gains as compared to chemotherapy alone (HR = 0.88; 95% CI, 0.47–1.62; $P = 0.67$). In stark contrast, in "VDV high" patients (Fig. 6D, solid lines), the addition of bevacizumab to chemotherapy versus chemotherapy alone provided a marked and significant PFS benefit (HR = 0.36; 95% CI, 0.17 to 0.77; $P = 0.0079$), and the interaction between treatment and marker status shows a significant predictive effect for PFS ($P = 0.036$).

Overall survival was a secondary endpoint of this clinical study and was not significant in the intent-to-treat population. The overall survival improvement from bevacizumab for our VDV-high subpopulation was significant however the interaction between treatment and biomarker classification was not (see discussion and Supplementary Fig. S6).

Discussion

We have characterized the transcriptome of a distinct tumor vascular compartment directly responsive to VEGF signaling inhibition. Identification of the genes comprising this response enables the detection of vascular-specific anti-VEGF downstream effects in tumor models where not otherwise apparent. We show conservation of the core VDV gene expression response to VEGF signaling inhibition across multiple murine tumor models and in the orthologous signature in biopsies of patients treated with bevacizumab. The consistency of the results observed across the tested experimental models and clinical data suggest that, the core of the response to VEGF blockade and the molecular identity of the VDV compartment are conserved across multiple mouse tumor vascular beds and in human cancers.

When VEGF was neutralized or VEGFR-2 downstream signaling was inhibited with small molecule inhibitors, we found that the expression levels of known pan-vascular genes were moderately changed in comparison to a specific subset of genes that includes *Esm1*, *Prnd*, *Nid2*, *Kcne3*, *Apln*, *Aplnr*, and *Mest*. The differential magnitude of

transcriptional response between these 2 sets of genes in the signature suggests that at least 2 processes comprise the response of tumor vasculature to VEGF pathway blockade. We interpret the greater magnitude of decrease as representing an actual transcriptional response to Vegfr blockade, combined with signal representing ablation of VDV. This model implies that the tumor endothelial compartment is not homogeneously sensitive to VEGF deprivation, but that anti-VEGF pathway inhibitors target a subcompartment (perhaps isomorphic to the neovasculature) within tumors. Furthermore, the genes expressed within this VDV can be partitioned into 2 groups: constitutively expressed and VEGF signaling modulated genes.

Adding rVEGF to endothelial cells *in vitro* did not provide sufficient information to determine if a gene is indeed a bona-fide VEGF *in vivo* target. Indeed, we found that rVEGF stimulation of HUVECs does *not* induce transcription of most of the proxVDV genes that we identified in this study (Supplementary Fig. S6). This discrepancy should not be surprising, considering the systemic role of the vasculature as an integrator of many stimuli, including blood flow and mechanical forces, and the dose and context dependence of VEGF response. However, other VDV genes were consistently upregulated by VEGF *in vitro* (Supplementary Fig. S5), indicating that the proxVDV genes set presented here is unlikely to be exhaustive.

Several proxVDV genes such as *Esm1*, *Nid2*, and *Apln*, and proxVDV gene candidates (e.g., as *Vegfr3*) and *Lama4* have been already implicated either as markers of endothelial-tip cells and/or as *in vivo* VEGF targets (e.g., during retinal vascular development in mice (35–40). Notably, tip–endothelial cells are highly responsive to VEGF stimulation and required for initiating sprouting angiogenesis. Collectively, these data suggest that proxVDV genes are highly expressed in an immature neo-vascular compartment that is highly sensitive to VEGF inhibition. Thus, we propose that the VDV signature score represents a molecular (gene expression) correlate of the VEGF-driven immature tumor vessel content. The association between high VDV gene expression and increased benefit from bevacizumab is in agreement with an emerging large body of evidence that suggests that anti-VEGF therapy selectively targets the immature tumor vasculature subcompartment (17, 41). It is also important to note that the proxVDV genes Prnd and Apln have been previously identified as specific markers of angiogenic tumor-associated endothelium (42), confirming that at least some of the distinct characteristics of tumor vasculature may be driven by supraphysiological levels of VEGF signaling.

The endothelial-specific downregulation of the VDV genes seen in response to VEGF pathway inhibition *in vivo* was corroborated *ex vivo* by expression analyses of freshly isolated TAECs from anti-VEGF–treated animals. This experiment confirmed that, although most VDV genes are indirect surrogate markers that simply reflect VDV ablation (i.e., they are more distal in the response process), a subset of these genes (proxVDV genes) comprises *in vivo* proximal biomarkers of VEGF/VEGFR-2 signaling inhibition in TAECs. As such, these genes should be useful to help guide the establishment of optimized dosing clinical schedules for evaluation VEGF inhibitors in combination with other targeted agents and/or for novel VEGF pathway inhibitors.

In addition to the pharmacodynamics biomarker function of proxVDV genes, the connection between the expression of VDV genes and proximal and distal VEGF downstream biological activity suggests that these genes have potential clinical predictive value in distinguishing patients that may benefit from VEGF-blocking therapies. In our assessment of predictive value, the stratification of PFS benefit by VDV gene expression was clearly significant. Given that efficacious antiangiogenic treatment is expected to reduce tumor growth rate, increased PFS is likely a direct consequence of the mechanism of action of anti-VEGF. The mechanisms underlying increased overall survival upon combination treatment of bevacizumab with cytotoxic agents are likely to be more complex. In patients with advanced breast and ovarian cancer and glioblastoma, combinations of bevacizumab and cytotoxic agents increase PFS without increasing overall survival; whereas in some other approved indications such as advanced CRC, and non–small cell lung carcinoma combinations of chemotherapy and anti-VEGF treatment have been shown to increase PFS and also overall survival. In the NO16966 trial, however, bevacizumab plus chemotherapy did not show a significant increase in overall survival (a secondary end point of this particular study). Nonetheless, when we stratified NO16966 available patients via VDV gene expression we found a marked improvement in the OS hazard ratio of "VDV high" patients treated with bevacizumab (Supplementary Fig. S6A vs. Supplementary Fig. S6B), although the increased benefit on the "VDV high" patients did not reach statistical significance. It is unclear if this is a consequence of inherent factors limiting the interpretation of the overall survival data analyzed here, such as small sample size, poststudy patient crossover, limited time on bevacizumab treatment, or the relative underpowering of overall survival assessment compared to the PFS. Alternatively, overall survival outcome may be influenced by additional variables including evasive resistance, myeloid cell infiltration, combinatorial effects with cytotoxic agents (e.g., vascular normalization; ref. 43) or a possible (but highly disputed) increased invasiveness induced by anti-VEGF agents (44, 45), but see also refs. 46–49. Finally, VDV expression analysis in the CRC samples was restricted to probes (corresponding to 22 VDV genes) available in a previously clinically validated qRT-PCR array assay, and analyses of additionally selected VDV gene subsets may further improve the predictive value of these markers. Overall, these data strongly support further retrospective and prospective evaluation of VDV genes as predictive biomarkers in appropriate larger clinical data sets (with known clinical outcomes) as these samples become available in the near future.

It has been recently published that high plasma levels of the short $VEGF^{121}$ and $VEGF^{110}$ isoforms correlate with

responses to bevacizumab in some clinical trials. Based on this evidence, it has been postulated that circulating levels of the more diffusible short isoforms (as opposed to $VEGF^{165}$ protein levels) may more accurately reflect levels of bioactive VEGF in tumors (50). This predictive biomarker hypothesis is consistent with the data presented here that suggest that higher levels of VEGF downstream bioactivity (in tumor tissue) may correlate with increased responses to bevacizumab-containing therapies.

In conclusion, our data indicate that genes expressed in the VDV compartment inform on VEGF downstream bioactivity. The positive correlation between pretreatment VDV gene set enrichment in tumor biopsies and clinical outcome following bevacizumab treatment observed in our analyses supports further evaluation of these candidate markers as potential guides for the selection of patient subpopulations and/or novel indications likely to derive benefit from anti-VEGF therapy.

Disclosure of Potential Conflicts of Interest

All authors affiliated with Genentech, Inc. are current or former employees and stockholders of Roche, AG. C. Bais, M. Singh, M.J. Brauer, and J.S. Kaminker are inventors on US Patent application number 20110117083, published on 05/19/2011.

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