Identification and Analysis of \textit{in vivo} VEGF downstream Markers Link VEGF
Pathway Activity with Efficacy of Anti-VEGF Therapies

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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 genes or VDV) 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.

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 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 pre-treatment clinical samples with the subsequent clinical efficacy of bevacizumab (anti-VEGF) containing therapy.

Results. We show that VEGF-dependent vasculature (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 indicates that patients with higher VDV expression in pre-treatment 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 pre-treatment 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.
Vascular endothelial growth factor (VEGF) signaling through activation of the endothelial cell-specific tyrosine kinase receptor (RTK) VEGFR-2 is indispensable for developmental angiogenesis (1, 2) and required for tumor neo-vascularization and growth in many pre-clinical cancer models (3, 4). Although VEGF inhibition is lethal during embryonic development, its neutralization is well tolerated in adults (5, 6), coincident with post-natal vascular maturation and differentiation in various tissues.

Not all vessels in the adult react equally to VEGF deprivation, however. While 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 sub-compartment is currently unknown.

Following observations that VEGF blockade is sufficient to inhibit angiogenesis and tumor growth in pre-clinical tumor models (4, 12), a humanized VEGF blocking monoclonal antibody (mAb) bevacizumab (Avastin®), was tested in clinical trials and subsequently FDA-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 VEGF-driven vasculature for tumor progression may modulate responses to these anti-angiogenic 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.
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 10mg/kg twice weekly in immuno-compromised mice or weekly (at 5mg/kg) in RIP-TβAg mice. Sunitinib and axitinib were dosed at 60mg/kg daily by oral gavage. Wound healing assays were carried out as described (21).

Gene signature derivation and application. Log2 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) down-regulated 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’s t test (unless stated otherwise below) with p values < 0.05 considered significant. Of 1017 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, delta-delta Ct 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(two 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 two-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) (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 anti-vascular 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 (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 seven 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 up-regulation of gene expression, suggesting that gene expression
changes were primarily driven by physical elimination of VEGF dependent tumor-associated endothelial cells (Fig 1B, table S1).

The genes with decreased expression were enriched for known endothelial specific genes (table S1) and implicated in blood vessel development (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 (Figure 1).

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 while 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, 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; 30-33). 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 two 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). While 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-βAg 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 (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 two 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, while 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 (23-25). 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, while 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 VEGF dependent vasculature.
ProxVDV genes are VEGF/VEGFR-2 downstream targets expressed in tumor-associated endothelial cells (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®; (26)), a small-molecule tyrosine kinase inhibitor (TKI) that targets VEGFR-2 among other RTKs, or axitinib (27, 28), a more specific VEGFR-2 inhibitor. Tumors were collected at 8, 16 or 72 hours after treatment (Fig 4A, upper panel). Consistent with prior observations, all three inhibitors induced a significant reduction of MVD in tumors collected 72 hours after treatment (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 three VEGF pathway inhibitors (Fig. 4A, and S2B). Importantly, the expression of Vegf and of the non-vascular markers E-cad (epithelial) and Cd45 (hematopoietic) was not markedly affected by any of the VEGF pathway inhibitors tested (Fig S2B), suggesting that the changes in proxVDV 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 post-treatment were the effects of the two SMIs 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 post treatment.

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 (Fig S2C). This is consistent with Esm1 being a bona fide proxVDV gene. However, because ESM1 is regulated by stimuli besides VEGF (29), and since ESM1 may be occasionally expressed in tumor cells (30), 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 (FACS). 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 three 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 (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, while 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 tumor-associated endothelial cells 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 pre- and 21-days-post-treatment biopsies taken from 19 inflammatory breast cancer patients treated with one dose of bevacizumab as a single agent (31). Although conventional bioinformatics analyses failed to identify most of the specific vascular gene expression changes in response to bevacizumab a priori (32), 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 pre-
treatment (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 pre-treatment 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) (33). 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”) (34). To test if VDV gene expression correlates with PFS, we analyzed VDV gene expression levels in the available pre-treatment 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. Due to 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 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% 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 to 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 (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.
Within both treatment arms, the differences in PFS for high vs. low VDV patients were not significant \( (p=0.23, \text{ XELOX+placebo}; \ p=0.081, \text{ XELOX+bevacizumab}) \), and in the “VDV low” population (Fig 6D), the combination of bevacizumab and chemotherapy conferred only modest gains as compared to chemotherapy alone \( (HR, 0.88; 95\% \text{ CI, 0.47 to 1.62; } p=0.67) \). In stark contrast, in “VDV high” patients (Fig 6D, solid lines), the addition of bevacizumab to chemotherapy vs chemotherapy alone provided a marked and significant PFS benefit \( (HR, 0.36; 95\% \text{ CI, 0.17 to 0.77; } p=0.0079) \), and the interaction between treatment and marker status shows a significant predictive effect for PFS \( (p=0.036) \).

Overall survival (OS) was a secondary endpoint of this clinical study and was not significant in the intent-to-treat (ITT) population. The OS improvement from bevacizumab for our VDV-high subpopulation was significant, however the interaction between treatment and biomarker classification was not. (See discussion and 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.
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IDENTITY AND RELEVANCE OF ANTI-VEGF BIOMARKERS

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 is 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 two sets of genes in the signature suggests that at least two 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 VEGF dependent vasculature. This model implies that the tumor endothelial compartment is not homogeneously sensitive to VEGF deprivation, but that anti-VEGF pathway inhibitors target a sub-compartment (perhaps isomorphic to the neovasculature) within tumors. Furthermore, the genes expressed within this VEGF-dependent vasculature can be partitioned into two 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 (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 (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 (for example, 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, while 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 anti-angiogenic 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 OS upon combination treatment of bevacizumab with cytotoxic agents are likely to be more complex. In patients with advanced breast and ovarian cancer and glioblastoma (an approved indication) combinations of bevacizumab and cytotoxic agents increase PFS without increasing OS; while in some other approved indications such as advanced CRC, and NSCLC combinations of chemotherapy and anti-VEGF treatment have been shown to increase PFS and also OS. In the NO16966 trial, however, bevacizumab plus chemotherapy did not show a significant increase in OS (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 (Fig S6A vs B), 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 OS data analyzed here, such as small sample size, post-study patient crossover, limited time on bevacizumab treatment or the relative underpowering of OS assessment compared to the PFS. Alternatively, OS outcome may be influenced by additional variables including evasive resistance, myeloid cell infiltration, combinatorial effects with cytotoxic agents (e.g. vascular normalization; (43)) or a possible (but highly disputed) increased invasiveness induced by anti-VEGF agents (44, 45), but see also (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 VEGF dependent vascular compartment inform on VEGF downstream bioactivity. The positive correlation between pre-treatment 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.
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Author contributions: M. Singh and C.B. conceptualized, planned, and performed experiments, analyzed data, and wrote the manuscript. M.J.B., and NF also contributed to the writing of the manuscript. M.J.B. and J.S.K. provided key intellectual input, performed gene expression and statistical analyses. Z.M., C.D.J., H., and N.F. designed experiments, provided key reagents, and intellectual input. G.Z., M. Schmidt, J.Y., X.W., S.S.J., G.K., T.O., R.N.W.T., and J.H.C. performed experiments and analyzed data. R-F.Y. and P.Y. carried out gene expression and statistical analyses of clinical data. S.X.Y. and S.M.S. designed and conducted the inflammatory breast cancer clinical trial, and provided patient biopsy material. A.J. carried out histopathological analyses,
ISH and provided intellectual input. P.H., D.C., and S.J.S. provided the NO16966 patient samples and critical input on the analysis thereof.

**Competing interests:** All authors affiliated with Genentech, Inc. are current or former employees and stockholders of Roche, AG. C.B., M. Singh, M.J.B., and J.S.K. are inventors on US Patent application number 20110117083, published on 05/19/2011.

**References:**


Figure legends:
Fig 1. Identifying a VEGF-dependent tumor endothelial compartment in a mouse PNET model.  (A) Representative images from histological analysis of tumor vessel density via MECA-32 staining at various times following anti-VEGF treatment (20X magnification). Nuclei are counterstained with DAPI (blue). Quantitation from 4-6 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 (grey 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 three independent biological replicates. Error bars = log2 standard deviation. Gene expression changes are for treatment relative to anti-Ragweed MAb or vehicle controls.

Fig 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 post-treatment and U87 (B) intracranial orthotopic
tumor samples collected 13-42 days post-treatment with the anti-VEGF antibody B20-4.1.1, compared to control treatment. (A-B) Genes in the VDV signature (red lines) decrease significantly relative to all genes (shown as a grey histogram) in the stroma (upper graph, mouse chip, p<0.0001 for A and p = 0.0105 for B), but not in the tumor cells (lower graph, human chip, both experiments show no significant differences). VDV genes 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-B). N = 5-10 instances for each treatment cohort shown here. Gene expression changes are for treatment relative to anti-Ragweed Ab or vehicle controls.

Fig 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, (upper graph, p=0.0125) and the converse up-regulation, when comparing recombinant VEGF applied for 12 hours (p<0.0001). (B) In contrast to anti-VEGF downregulation of the VDV gene signature (right, upper graph, p<0.0001), anti-Dll4 treatment causes a upregulation of a majority of VDV genes (right, lower 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 plots (A-B). VDV genes 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.

Fig 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 (sunitinib and axitinib). Values represent the mean of the log2 fold change in relative gene expression induced by VEGF/VEGFR-2 inhibitor compared to control treatment. (51) Gene expression data represent the log2 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 log₂ fold change of 3 replicates. Error bars represent standard deviation. Gene expression changes are for treatment relative to anti-Ragweed MAb or vehicle controls.

Fig 5. VDV gene expression analyses in bevacizumab-treated human breast and colon cancer biopsies. (A) Change in gene expression (post- vs pre-treatment) in biopsy samples from 19 inflammatory breast cancer patients. Genes in the VDV signature (red lines) decrease significantly relative to all genes (grey histogram), p=0.0275. VDV genes 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.

Fig 6. Higher expression levels of VDV genes in pre-treatment 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 –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.
Figure 3

A

Wound Healing
(12 Hours of Treatment)

Density

In log2 Field Change (vs Control)

B

MDA-MB-231 Tumors
(48 Hours of Treatment)

Density

In log2 Field Change (vs Control)
Figure 4
Effect of bevacizumab on VDV expression in human tumor biopsies

Mouse Microarray

- 
- 7 days

- \alpha\text{-}VEGF or control treatment

Mouse VDV signature

RIP-TβAg tumor-bearing mice

Mouse VDV signature

Human Microarray

- 21 Days

- Bevacizumab 15 mg/kg

- Bevacizumab 15 mg/kg pre- and post-treatment samples

- Subsequent chemotherapy

Inflammatory and locally advanced breast cancer patients (n=21)

Effect of bevacizumab on VDV expression in human tumor biopsies

- log2FC (post− vs pre−treatment)

- Density

- ESM1

- U181

- RASGRF3

- LAMA4

- NID2

- PRND

- KCNE3

- COL4A2

- MEST

- APLN

- APLMR

- HLX

- ABCC9

- CCL2

- MCAM

- GBP4

- LILRB1

- Research.
Figure 6

A. Effect of treatment on PFS

B. Interaction between treatment and marker status (p=0.036)
Identification and Analysis of in vivo VEGF downstream Markers Link VEGF Pathway Activity with Efficacy of Anti-VEGF Therapies


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