Tumor Stromal Architecture Can Define the Intrinsic Tumor Response to VEGF-Targeted Therapy


Abstract

**Purpose:** The aim of the study was to investigate the vascular and stromal architecture of preclinical tumor models and patient tumor specimens from malignancies with known clinical outcomes to VEGFi treatment, to gain insight into potential determinants of intrinsic sensitivity and resistance.

**Experimental Design:** The tumor stroma architecture of preclinical and clinical tumor samples were analyzed by staining for CD31 and α-smooth muscle actin (α-SMA). Tumor models representative of each phenotype were then tested for sensitivity to the VEGFR2-blocking antibody DC101.

**Results:** Human tumor types with high response rates to VEGF inhibitors (e.g., renal cell carcinoma) have vessels distributed amongst the tumor cells (a “tumor vessel” phenotype, TV). In contrast, those malignancies where single-agent responses are lower, such as non–small cell lung cancer (NSCLC), display a complex morphology involving the encapsulation of tumor cells within stroma that also supports the majority of vessels (a “stromal vessel” phenotype). Only 1 of 31 tumor xenograft models displayed the stromal vessel phenotype. Tumor vessel models were sensitive to VEGFR2-blocking antibody DC101, whereas the stromal vessel models were exclusively refractory. The tumor vessel phenotype was also associated with a better Response Evaluation Criteria in Solid Tumors (RECIST) response to bevacizumab + chemotherapy in metastatic colorectal cancer (CRC).

**Conclusion:** The tumor stromal architecture can differentiate between human tumor types that respond to a VEGF signaling inhibitor as single-agent therapy. In addition to reconciling the clinical experience with these agents versus their broad activity in preclinical models, these findings may help to select solid tumor types with intrinsic sensitivity to a VEGFi or other vascular-directed therapies.

Introduction

The early promise of agents that target the VEGF signaling axis has failed to translate widely in the clinic. Although VEGF signaling plays a pivotal role in angiogenesis, and some patients receive benefit, not all patients are responsive to VEGF inhibitor (VEGFi) therapy, and those who do respond will eventually become refractory to treatment (1, 2). The expectation that the use of VEGFi therapy would eventually become refractory to treatment is based largely upon the broad efficacy of these agents in a variety of preclinical tumor models in vivo (3, 4). Given this discrepancy, further insight into potential determinants of VEGFi efficacy is warranted.

The discordance between preclinical and clinical responses and the initial lack of markers identifying intrinsic resistance to a VEGFi has been a significant focus of research. Additional studies in preclinical models have revealed a number of putative mechanisms that may contribute to both intrinsic and acquired VEGFi resistance. One of the first mechanisms proposed as mediating resistance to VEGF-A neutralization was the presence or recruitment of inflammatory infiltrate, specifically CD11b, GR-1–positive cells derived from the bone marrow, in response to upregulation of tumor-derived factors (5, 6). A complementary resistance mechanism is the expression of factors that drive alternative angiogenic pathways such as high expression of the potent angiogenic stimulus FGF-2 (7). Structural features of the vessel have also been shown to influence the response to VEGF signaling inhibitors, with mature vessels supported by pericytes or myofibroblast-like cells being less sensitive to VEGFi treatment. Furthermore, models of acquired resistance to VEGFi are characterized by the recruitment of pericytes, possibly through an elevated EGFR signaling response (8), or platelet-derived growth factor (PDGF)-

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**Corresponding Authors:** Neil R. Smith, Oncology iMed, AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, United Kingdom. Phone: 440-1625233731; Fax: 440-1625510097; E-mail: Neil.R.Smith@astrazeneca.com; and Simon T. Barry, Oncology iMed, AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, United Kingdom. Phone: 440-1625513350; Fax: 440-1625519749; E-mail: Simon.T.Barry@astrazeneca.com

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Translational Relevance

VEGF pathway inhibitors (VEGFi) are approved for the treatment of advanced cancer. Although these deliver some clinical benefit, questions remain as to which tumor types are most sensitive, which patients receive greatest benefit, and why the broad preclinical activity seen with these agents did not translate to the clinic. Here, we differentiate human tumor types based on their stromal architecture into tumor vessel or stromal vessel phenotypes. The tumor vessel phenotype is associated with tumor types (renal and thyroid cancer) with better single-agent clinical response to a VEGFi than the stromal vessel phenotype (colorectal and non–small cell lung cancers). The tumor vessel phenotype is also evident in many human tumor xenograft models commonly used to evaluate efficacy, whereas a model displaying the stromal vessel phenotype was found to be refractory to treatment with the VEGFR2-blocking antibody DC101. These data suggest an association between stromal architecture and intrinsic tumor sensitivity to VEGFi therapy.

mediated signals (9–11). Determining the potential clinical relevance of such resistance mechanisms requires a closer examination of the preclinical models to determine which aspects are representative of human cancer.

We have previously studied the diversity in the angiogenic response represented by a panel of preclinical tumor xenograft models and found that while the human tumor cells may show different expression of angiogenic genes, the host response is similar between models (12). To build on this study, we have profiled a number of different human tumors to examine the histologic relationship between tumor and stroma, to determine whether these features are present within a panel of histologically diverse human tumor xenografts. We found that based on the tumor stromal architecture human disease could be broadly subdivided into 2 phenotypes that have different sensitivity to VEGFi therapy as a single agent. The significance of the phenotype is explored in the context of both preclinical modeling and clinical samples.

Materials and Methods

Human and xenograft tumor tissues

Formalin-fixed, paraffin-embedded (FFPE) human primary cancer resection blocks, both whole and formatted into tumor microarrays (TMA), were sourced under approved legal contract from commercial tissue suppliers, Asterand, Cytomyx, and TriStar Technology Group and Wales Cancer Bank. Appropriate consents, licensing, and ethical approval were obtained for this research. The suitability of each specimen for immunohistochemical (IHC) analyses was determined by pathology assessment of tissue morphology and preservation [hematoxylin and eosin (H&E)] and the general extent of antigen preservation (pan p-tyr immunostains). Tumor xenograft tissue was derived from experiments carried out as described (13) with licenses issued under the UK Animals (Scientific Procedures) Act 1986.

DC101 tumor growth studies

Calu-6 and Calu-3 human lung tumor xenografts established in nude and severe combined immunodeficient (SCID) female mice, respectively. HT-29 and SW620 tumors were established in nude mice. Once tumors reached a mean volume of about 0.2 to 0.3 cm$^3$ (Calu-3), mice were then intraperitoneally injected twice weekly with either 15 mg/kg of DC101 (Cell Essentials Inc.) or an isotype control antibody for the times indicated. DC101 is a monoclonal neutralizing antibody raised to murine VEGF receptor-2 (flk-1) that was chosen on the basis of its specificity as a VEGFi. Following treatment, tumors from each group were excised and divided; one half being snap frozen in liquid nitrogen and stored at –80°C until required, and the other fixed in neutral-buffered formalin for 24 hours.

Histopathologic staining

The following antibodies were used in IHC and immunofluorescent (IF) analyses: rabbit anti-mouse CD31 (AstraZeneca, CHG-CD31-P1; ref. 13); mouse anti-human α-smooth muscle actin (α-SMA; Sigma, 1A4); rabbit anti-human PDGFRβ (Epitomics 1469-1); mouse anti-human CD68 (Dako, M0876); mouse anti-human Neutrophil Elastase (Dako, M0752); rabbit anti-human E-cadherin (Cell Signaling Technology, 3195); and mouse anti-human vimentin (Dako, M0725), rat anti-mouse Gr-1 (BD Pharmingen, 550291), rat anti-mouse F4/80 (Serotec, MACP497), and mouse anti-human Ki67 (Dako, M7240). Tissues were sectioned onto glass slides, dewaxed, and rehydrated. For both IHC and IF, all incubations were conducted at room temperature and TBS containing 0.05% Tween (TBST) used for washes. Antigen retrieval was conducted in pH 6 retrieval buffer (S1699, Dako) at 110°C for 5 minutes in an RHS-1 microwave vacuum processor (Milestone), then endogenous biotin (Vector, SP-2002, neutrophil elastase, E-cadherin, Gr-1 and F4/80 only), peroxidase activity (3% hydrogen peroxide for 10 minutes), and nonspecific binding sites in kit, was added for 30 minutes. Sections were washed and

For single marker analyses, antibodies raised to CD31, α-SMA, PDGFRβ, CD68, neutrophil elastase, E-cadherin, vimentin, Gr-1, F4/80, and Ki67 were diluted, 1:400, 1:1,000, 1:2,000, 1:200, 1:200, 1:100, 1:1,000, 1:1,000, and 1:100, respectively, in antibody diluent (Dako, S0809) and applied to sections for 1 hour. Mouse Envision secondary (Dako, K4007) for α-SMA, vimentin, CD68, and Ki67, rabbit Envision secondary (Dako, K4003) for CD31, biotinylated rabbit anti-mouse IgG (Dako, E0464) for neutrophil elastase, goat anti-rabbit IgG (Dako, E0432) for PDGFRβ, and E-cadherin or rabbit anti-rat IgG (Dako, E0488) for Gr-1 and F4/80 were added for 30 minutes. For neutrophil elastase, vimentin, Gr-1, and F4/80, Vectastain Elite ABC solution (Vector, PK-6100), diluted as instructed in kit, was added for 30 minutes. Sections were washed and
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developed in diaminobenzidine for 10 minutes (Dako, K3466) and then counterstained with Carazzi’s hematoxylin. Appropriate no primary antibody and isotype controls were conducted for each antibody. Chromogenic CD31-α-SMA co-stain was conducted using the Envision G/2 doublestain kit (Dako, K3561) following the manufacturer’s recommendations with antigen retrieval and primary antibody dilution as described above. CD31-α-SMA co-IF was conducted as previously described (13).

Pathology scoring and computer-assisted image analysis

Chromogenic or fluorescent images were captured using the ×20 objective of either an Aperio image scan (Leica Biosystems) or Pannoramic SCAN (3DHISTECH), respectively. Scanned images were scored by a trained pathologist and 2 scientists using simple subjective reporting procedures. Both human and xenograft tumors were scored for stromal vessel and tumor vessel (tumor phenotypes) using H&E or CD31-α-SMA stains. The tumor vessel phenotype was defined as a tumor structure where vessels are embedded throughout the tumor cell mass and the stromal vessel phenotype classed as tumor cell nests surrounded by well-developed stromal structures which contain the majority of the vessels. A tumor was classified as either tumor vessel or stromal vessel based on the predominant phenotype (cutoff of >60% tumor area), whereas a tumor composed of 40% to 60% of both phenotypes was scored as intermediate. These criteria were prospectively defined by an expert pathologist to clearly differentiate between tumors that were predominantly tumor vessel (>60%) or stromal vessel (>60%) and those that were intermediate (40%–60%) and difficult to classify as one or the other phenotype. On the basis of CD68 marker immunostaining, macrophage infiltrate into the tumor compartment was classified as negative, low (>0 but ≤ 1% CD68+ cells: tumor cells) or medium-high (>1% CD68+ cells: tumor cells). Tumor epithelial-to-mesenchymal transition (EMT) status was scored using epithelial (E-cadherin) and mesenchymal (vimentin) markers to define epithelial (E-cadherin/vimentin−), mesenchymal (E-cadherin/vimentin+), or intermediate phenotypes (E-cadherin+vimentin−).

Computer-assisted image analysis was conducted on digitally acquired chromogenic images. For images of human tumors immunostained for CD31-α-SMA, the tumor compartment, including associated stroma, was selected by hand using the Aperio Image Viewer Software (Leica Biosystems). Downstream image analyses of the annotated areas were conducted using Aperio image analysis software (Leica Biosystems). Microvessel density (MVD, number of vessels per mm² viable tumor), using CD31 as a marker, was determined using the Aperio microvessel analysis algorithm (Leica Biosystems). Myofibroblast levels based on α-SMA were determined using the Aperio color deconvolution algorithm to measure percentage of α-SMA-positive pixels per total number of pixels. Genie (Leica Biosystems), a pattern recognition software tool, was trained to segment stained images of the Calu-3 xenograft into tumor, stroma, and necrotic regions and used to determine the percentage of each compartment per tumor. This Genie classifier was combined with the appropriate Aperio image analysis algorithm to analyze biomarker parameters in stromal- or tumor-classified regions of Calu-3 tumor s. Calu-3 stromal or tumor MVD, based on CD31 immunostain, was determined using the microvessel analysis algorithm. Stromal myofibroblast content (α-SMA and PDGFRβ) or macrophage (F4/80) and neutrophil (Gr-1) infiltrate into the stroma or tumor was analyzed using the color deconvolution algorithm (Leica Biosystems) to determine the percentage of positive pixels per total number of pixels. Tumor proliferative index [number of Ki67-positive tumor cells/total number of tumor cells (hematoxylin-positive nuclei)] and tumor cell density [total number of tumor cells (hematoxylin-positive nuclei)/tumor area] were determined using a nuclear algorithm (Leica Biosystems). To measure tumor nest size, the diameter of 44 to 220 distinct nests were measured for each α-SMA–immunostained Calu-3 section (to highlight hematoxylin-positive tumor nests) using the Aperio image viewer measuring tool (Leica Biosystems). Biomarker data were analyzed using the Student 2-tailed t test to determine statistical significance between treatment groups.

TaqMan fluidigm gene expression profiling

RNA was isolated from 30 to 50 mg frozen tumor using an RNeasy Lipid Tissue Mini Kit (Qiagen, 74104), according to manufacturer’s protocol. On-column DNase digestion was conducted using the RNase-free DNase Kit (Qiagen, 79254). RNA concentration was measured using the NanoDrop ND1000 (Thermo Fisher Scientific). Human- and mouse-specific assays were designed and supplied by Applied Biosystems, whereas eukaryotic 18S rRNA was used as the endogenous control (Supplementary Table S4). Total RNA (50 ng) was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) in final volume of 20 μL, according to the manufacturer’s instruction. cDNA (1.25 μl) was pre-amplified using a pool of TaqMan primers at a final dilution of 1 in 100 and Pre-amplification Master Mix (Applied Biosystems, 4391128) in a final volume of 5 μL. Samples were diluted 1 in 5 with 1× TE and stored at −20°C. Sample and assay preparation for 48.48 dynamic arrays was conducted according to the manufacturer’s instruction (Fluidigm).

Data were collected and analyzed using the Fluidigm Real-Time PCR Analysis 2.1.1 software (v.2.1.3). Species-specific normalization of the expression data to 18S rRNA was conducted as reported (12). Gene expression values were calculated using the comparative Ct (ΔΔCt) method as previously described in User Bulletin #2 ABI PRISM 7700 Sequence Detection System 10/2001, using the corrected 18S rRNA Ct values for normalization of the tumor transcript and the original values for the stroma. To determine genes altered by DC101 treatment student and differentially expressed between Calu-3 versus the others models, t tests and fold change were calculated with significantly altered genes identified by having a P<0.05 and a fold change >1.5.
Results
Tumor stromal architecture defines human tumor types that respond to VEGF signaling inhibitors as monotherapy

To assess the tumor stromal architecture of different solid human malignances, we used a number of tumor TMAs comprising renal cell carcinoma (RCC), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSCC), non–small cell lung carcinoma (NSCLC), ovarian (OvC), thyroid (ThC) subtypes, colorectal (CRC), prostate (PC), and breast (BC) cancers (Fig. 1; Supplementary Table S2). For the purposes of this analysis, we have viewed responsive disease as high incidence of tumor response by Response Evaluation Criteria in Solid Tumors (RECIST) assessment (tumor shrinkage) and/or a large increase in progression free survival on long-term therapy reported.

Figure 1. Two dominant phenotypes based on tumor stromal architecture stratify tumor types. Tumor vessel phenotype (A; a tumor structure where vessels are embedded throughout the tumor cell mass) and stromal vessel phenotype (B; a tumor structure dominated by a pattern of tumor cell nests surrounded by well-developed stromal structures which contain the majority of the vessels). Also shown are IF-stained FFPE human tumors [CD31-AF488 (green), α-SMA-AF555 (red), and DAPI counterstain (blue)], indicative of tumor vessel (RCC, GBM, HCC, ThC, OvC, and HNSCC) and stromal vessel (CRC, NSCLC, PC, and BC). C, percentage ratios of tumor vessel: stromal vessel phenotypes for RCC, GBM, OvC, HCC, ThC, HNSCC, CRC, NSCLC, PC, and BC. Phenotypes were scored from CD31-α-SMA or H&E-stained TMAs (1 TMA per tumor type, number of patient samples per tumor type are shown).
with different therapies targeting VEGF signaling. RCC (14) and GBM (15, 16) are diseases we have taken as representative of cancers known to respond to VEGFi as a single-agent therapy (Supplementary Table S1). CRC (17) and NSCLC (18) are diseases where VEGFi inhibitors show little single-agent responses and hence are trialed in combination with cytotoxic therapy. Tumors were screened by IF to visualize the relative distribution of myofibroblast-like cells (α-SMA), blood vessels (CD31), and tumor cells [4',6-diamidino-2-phenylindole (DAPI)]. This revealed 2 dominant morphologies: (i) a "tumor vessel" phenotype which describes a tumor structure with vessels embedded throughout the tumor cell mass represented in RCCs, GBMs, and hepatocellular carcinoma (HCC; Fig. 1A, Supplementary Fig. S1 and Supplementary Table S2); (ii) a "stromal-vessel" phenotype in which the tumor structure is dominated by a pattern of tumor cell nests surrounded by well-developed stromal structures containing the majority of the vessels (Fig. 1B), as evident in CRCs, NSCLCs, and PC (Fig. 1B, Supplementary Fig. S1 and Supplementary Table S2). Using this classification, cores from multiple tumor types were scored for either a tumor vessel or stromal vessel phenotype (Fig. 1C). The tumor vessel phenotype was observed in RCCs, GBMs, OvC, HCC, ThC, and head and neck squamous cell carcinoma (HNSCC). The stromal vessel phenotype was dominant in CRC, NSCLC, PC3, and BCs.

As the phenotype ratios for each tumor type were derived initially from TMA cores, full tumor sections were also classified for each phenotype to ensure the results were representative of larger diagnostic tumor samples [RCCs, NSCLCs and PC (n = 10); GBM, ThC, CRC, and BC (n = 9); and NSCLC (n = 19); Fig. 2A, Supplementary Fig. S2 and Supplementary Table S3]. Where sections exhibit regions of both phenotypes, the predominant phenotype (>60%) was scored (Supplementary Fig. S2a). The frequency of the phenotypes representing diagnostic specimens agreed with the original TMA dataset (Supplementary Fig. S2b and S2c). These data establish that the tumor stromal architecture of the tumor can be used to cluster human tumor types into 2 groups. Significantly, those clinical tumor types associated with response to a VEGFi alone [CRC (14), ThC (19, 20), GBN (15, 16)] were dominated by a tumor vessel phenotype. In contrast, tumors where a VEGFi has shown modest activity in combination [CRC (17), PC (21), NSCLC (18), BC (22)] were dominated by the stromal vessel phenotype.

Additional biomarker analyses were conducted on the diagnostic tumor panel (n = 32 tumor vessel, n = 35 stromal vessel) to further examine the properties of the two phenotypes (Fig. 2B–D). Although immature and mature pericyte covered vessels (CD31+ endothelial cells with tightly associated α-SMA+ cells) were associated with both phenotypes, tumor-embedded vasculature of the tumor vessel phenotype tended to be pericyte-free, whereas stromal vessels in the stromal vessel phenotype were often pericyte covered or associated with α-SMA+ fibroblasts (Fig. 2B). Computer-assisted image analysis of CD31 immunostained lesions revealed that the tumor vascular density in the combined tumor and stromal compartments was higher in tumor vessel (mean ± SEM = 105.3 ± 21.3) than in stromal vessel (47.7 ± 7.5; Fig. 2C and Supplementary Fig. S3b). α-SMA analysis showed myofibroblasts were greater in stromal vessel (mean ± SEM = 10.0 ± 1.8) than in tumor vessel (1.4 ± 0.3) phenotype tumors (Fig. 2C and Supplementary Fig. S3b) tumors. EMT status was determined by immunostaining serial sections for E-cadherin and vimentin (Fig. 2D and Supplementary Fig. S3b). Tumor vessel tumors were generally more mesenchymal (E-cadherin+/vimentin−) than stromal vessel tumors (E-cadherin+/vimentin+). Qualitative histologic assessment of CD68-immunostained tumors from the panel showed that the tumor vessel phenotype has higher levels of macrophage tumor infiltrate (Supplementary Fig. S3b and S3c).

Neuroendocrine pancreatic cancer has recently been shown to respond to the VEGFi sunitinib (23), whereas pancreatic adenocarcinoma has proven comparatively resistant to such approaches (24–26). Comparing available samples of neuroendocrine pancreatic cancer with pancreatic adenocarcinoma revealed them to represent tumor vessel and stromal vessel phenotypes, respectively (Supplementary Fig. S3a), consistent with our hypothesis on VEGFi sensitivity.

The tumor vessel phenotype is common to tumor xenograft models

To evaluate the relevance of the 2 phenotypes in preclinical models, a panel of human tumor xenografts grown subcutaneously and representing a broad range of different tumor types of origin [i.e., lung (8), colon (5), breast (4), prostate (3), brain (2), stomach (1), ovary (1), pancreas (1), skin (1), pharynx (1), uterus (1), vulva (1), and blood (1) tumor and fibrosarcoma (1) cell lines] were classified for the 2 phenotypes based on the approach used for human tumor s (Fig. 3). The majority (30 of 31) exhibited a tumor vessel phenotype (Fig. 3A and B). Only Calu-3 (lung adenocarcinoma) had a stromal vessel phenotype (Fig. 3A and C). These data indicate that the tumor vessel phenotype is common to subcutaneously grown tumor xenograft models.

Intrinsic gene expression differences between stromal vessel and tumor vessel xenograft tumors

Gene expression analysis of human transcripts was used to investigate the relationship between Calu-3, Calu-6, SW620, and HT-29 tumor s. The difference in expression of a range of 180 genes associated with angiogenesis, inflammation, and invasive growth was determined (Fig. 3D and E). This gene set differentiated Calu-3 from the other models; genes showing the highest differential expression in Calu-3 were MMP7, LPAR3, CT55, IL1RN, and SPP1 (osteopontin). Compared to other models analyzed, Calu-3 xenograft tumor s were enriched in genes associated with recruitment of the stromal cells, in particular fibroblast growth factor (FGF) and PDGF ligands (Fig. 3D). To validate that Calu-3 tumors showed a different expression
profile, they were also compared to a broader panel of tumor xenograft models, again the same genes were over-expressed relative to other models. This expression analysis supports the conclusion that Calu-3 tumor cells exhibit high expression of a number of transcripts that may contribute to their phenotype.

The tumor vessel phenotype has greater sensitivity to VEGFR2 inhibitor, DC101, than the stromal vessel phenotype, in vivo

To determine whether the tumor stromal architecture could influence tumor response to VEGF, we used the murine VEGFR2-blocking antibody, DC101 to assess the...
**Figure 3.** Prevalence and intrinsic gene expression differences between the 2 phenotypes in tumor xenograft models. A, classification of 31 subcutaneously implanted human tumor xenograft models for tumor vessel and stromal vessel phenotypes. Data were derived from the scoring of CD31-α-SMA–immunostained TMAs (4 FFPE tumors per model, 3 cores per tumor). B and C, diagrams and fluorescent immunostained [CD31-AF488 (green), α-SMA-AF555 (red), and DAPI counterstain (blue)] images representing preclinical xenograft. B, tumor vessel (Calu-6—low myofibroblast content) and (C) stromal vessel (Calu-3). D and E, transcript profiling of tumor vessel (Calu-6, HT-29, and SW620) and stromal vessel (Calu-3) tumor xenograft models. Genes with high (D) and low (E) expression levels in Calu-3 compared to Calu-6, HT-29 and SW620 are presented. ddCT = \[\frac{\text{average ddCT Calu-3}}{\text{average ddCT (Calu-6 + SW620 + HT-29)}}\].
effects of pruning neovasculature on tumor growth in tumor vessel (Calu-6, HT-29 and SW620) compared to stromal vessel (Calu-3) tumor xenograft models. DC101 was dosed intraperitoneally at 15 mg/kg twice weekly. Calu-6, HT-29, and SW620 xenografts exhibited the classic tumor growth phenotype is more sensitive to VEGFR2 signaling inhibitor, DC101, than the stromal vessel phenotype in vivo. A and B, geometric mean tumor volume growth curves (cm³) for DC101 (15 mg/kg twice weekly; red) and IgG1 (control; blue) treated arms of (A) Calu-6, HT-29, SW620 and (B) Calu-3 (growth curve 1, growth curve 2) tumor xenograft studies. Geometric mean ± SEM are shown for growth curves. C, MVD (number of CD31 vessels per mm²) of IgG1 control and DC101-treated Calu-6 and Calu-3 tumor studies (growth curves 1 and 2). D, significant gene changes induced in the tumor (human) and stromal (stromal) vascular modulation and invasion transcript profile on DC101 treatment of Calu-6 and Calu-3 (growth curve 1) xenograft tumors. Tumor transcripts are normalized to human 18S, whereas stromal transcripts are normalized to total 18S. Only significant gene changes are included (P > 0.05 and fold change > 1.5). Each column is an individual tumor and each color gradient is scaled independently within each gene.

The gross vascular response to DC101 is similar between tumor vessel and stromal vessel tumor xenografts

To compare the total vascular response of tumor vessel and stromal vessel models to DC101, MVD analysis was conducted on CD31-immunostained control and DC101-treated samples from the Calu-6 and Calu-3 (replicates 1 and 2) growth studies. The effect of DC101 on MVD reduction was similar between Calu-6 and Calu-3 models (Fig. 4C). Furthermore, species-specific reverse transcription quantitative polymerase chain reaction (RT-qPCR)
using genes associated with angiogenesis and tumor cell invasion (Fig. 4D) revealed that human tumor gene changes were unique to each tumor xenograft model, but a range of murine genes associated with endothelial cells, were reduced in both. Therefore, while there is a clear vascular response to DC101 in both models this does not translate to growth inhibition in the Calu-3 xenograft.

DC101 targets the stromal vasculature in a stromal vessel phenotype model, which leads to a reduction in the stromal compartment but with negligible effect on tumor size

Tumor-embedded vessels were not detected in the Calu-3 model; however, vasculature was detected at a high density in the stroma (Fig. 5A and B). Stromal vasculature was reduced by >50% in response to DC101 with the remaining stromal vessels exhibited a greater mean vessel area than those in untreated Calu-3 tumors (Fig. 5A). Qualitative assessment of CD31-α-SMA IF-stained tumors revealed the proportion of pericyte-covered stromal vessels increased in treated tumors (Fig. 5B). These data indicate that while treatment with DC101 reduces small immature vessels, it fails to reduce the larger more mature vasculature within the stroma.

To examine the influence of the stromal vessel phenotype on the response to DC101, we conducted a detailed biomorphometric analysis of DC101-treated Calu-3 xenograft tumors. In Calu-3 tumors, DC101 treatment led to reduction in the area of the stromal compartment and an increase in the area occupied by tumor cells with negligible effect on tumor size (Fig. 5C). Analysis of the stromal compartment for myofibroblasts using α-SMA and PDGFRβ staining revealed a significant reduction in stromal myofibroblast levels (Fig. 5D). DC101 treatment also led to a significant increase in the mean diameter of tumor nests (from 200 to 450 μm, P = 0.04); however, effects on the proliferative index and density of the tumor were negligible at this time point (Supplementary Fig. S4a and S4c). The recruitment of inflammatory cells was also assessed using F4/80 (macrophages) and Gr-1 (neutrophils). DC101 treatment resulted in a small but significant increase in the tumor macrophage content and a significant accumulation of Gr-1-positive cells in the stroma (Supplementary Fig. S4b and S4c). In conclusion, the reduction of stromal angiogenic vasculature by DC101 in the Calu-3 model had the greatest impact on the architecture and cellular composition of the stroma, rather than the tumor compartment, but with a negligible effect on the overall tumor mass.

The two phenotypes correlate with different clinical efficacies to bevacizumab and FOLFIRI in metastatic CRC

The relationship between phenotype and RECIST response to a combination of VEGFi and oxaliplatin-based chemotherapy in patients with metastatic CRCs was investigated. Two TMAs consisting of surgical tumor samples from 56 patients with metastatic CRC (Tristar) were chromogenically stained for CD31-α-SMA and scored for the tumor vessel and stromal vessel phenotypes by 2 observers. Forty-two patient samples were classified as the stromal vessel phenotype and 14 as the tumor vessel phenotype (Fig. 6). RECIST response information for FOLFIRI and bevacizumab (Avastin) treatment as first- or second-line therapy post-surgery was available for each patient. Fisher exact test was used to determine the significant associations between the phenotype and best RECIST response categories [progressive disease (PD) and other RECIST outcome] during patient treatment periods (for this dataset, best RECIST responses also had longest duration). The probability of the stromal vessel phenotype group having a poorer response (PD category) to bevacizumab and FOLFIRI than that of the tumor vessel phenotype group was statistically significant [P = 0.0487; 95% confidence interval (CI), 1.24–∞]. Although it is not possible to conclude that the differential response is a direct result of the treatment with bevacizumab alone, these data suggest that the tumor vessel phenotype appears to be more sensitive to the combination therapy than the stromal vessel phenotype.

Discussion

This study suggests that human tumor types can be broadly categorized according to the spatial distribution of their blood vessels in relation to the tumor cells and other stromal components. Across a broad panel of human tumor types, independent of disease type, tumors largely adopted either a tumor vessel or stromal vessel phenotype. The tumor vessel phenotype appears to be indicative of tumors that are likely to show greater single-agent responses to anti-angiogenic drugs targeting the VEGF signaling axis. Although we were able to separate different tumor types using this approach, no tumor type studied was exclusively one type of architecture. For example, although RCC tumors are predominantly of the tumor vessel phenotype, some exhibit a stromal vessel architecture. Conversely, while CRC tumors are dominated by the stromal vessel phenotype, a subset exhibits the tumor vessel architecture. Heterogeneity is also evident within individual tumors. In particular, tumor types with a stromal vessel architecture have regions with tumor vessel architecture, which may represent more angiogenic regions of the tumor. Tumor vessel vasculature is embedded within the tumor cell mass. This forms a major structural feature of the tumor, facilitating intratumoral blood flow. In contrast, in stromal vessel tumors, the vascular supply develops almost exclusively in the stromal compartment, which separates the tumor cells from the vessels, potentially creating more mature vessels.

The tumor vessel phenotype was associated with differences in the distribution of other cells, specifically the presence of high levels of macrophages. In stromal vessel tumors, these macrophages did not commonly associate with the tumor cells perhaps indicating a different role for the macrophage in these tumors. A possible explanation for these observations is that the intratumoral vasculature observed in the tumor vessel phenotype, but absent from...
the stromal vessel phenotype, would facilitate extravasation of immune infiltrate into the tumor. In contrast, in the stromal vessel, phenotype macrophages would extravasate into the dense stroma surrounding the tumor nests which may impair their migration into the tumor. On the basis of E-cadherin loss and vimentin gain, the tumor vessel phenotype was more associated with tumor cells of a mesenchymal phenotype. It is conceivable that the tissue of origin or subsequent progression of tumor to a more mesenchymal phenotype could influence reciprocal signaling between the tumor and the tumor microenvironment, thereby influencing the extent of stromal recruitment and its subsequent architecture. However, we do not currently have a mechanism to explain this.

While it remains challenging to acquire human tumor samples with associated outcome data following treatment with VEGF signaling inhibitors, we were able to obtain TMAs of samples from patients with CRCs treated with bevacizumab + FOLFIRI. Although these samples were limited in number, and only RECIST response category information was available, they did allow initial exploration of the hypothesis that tumor vessel and stromal vessel tumor s may respond differently to therapy. The analysis from the TMA is valid as previous data on baseline samples sets showed good concordance between the whole tumor sample and the TMA. The distribution of tumor vessel versus stromal vessel was largely representative of the larger datasets for CRCs. Interestingly, in this dataset, the tumor vessel phenotype was associated with good response, with no examples of PD associated with this phenotype, suggesting that the tumor vessel phenotype may be more sensitive to the combination therapy. This supports the idea that the tumor vessel or stromal vessel should be considered when differentiating response of these tumors to angiogenic therapy. It would be interesting to understand in late-stage CRCs, where VEGF inhibitors can be used as a single agent, whether differential response or disease control is associated with the differences in phenotype. As progression-free survival (PFS) and overall survival (OS) information was not available for these samples, further analyses to correlate phenotypes with these survival outcomes are required to understand the use of this approach to predict long-term outcome in diseases dominated by stromal vessel morphology. Only primary tumor samples were analyzed in this study. Often archival diagnostic samples from the primary tumor are the only tissue available to evaluate the tumor biomarker status of a patient. It will be important to establish whether the phenotypes are maintained during disease progression (e.g., metastatic lesions) and on progression following therapeutic intervention to determine the use of stratifying tumor types based on phenotype using archival samples.

Preclinical tumor xenografts have been used to define many of the mechanisms with the potential to influence response to VEGFi, other angiogenic factors (7), bone marrow–derived cells (5, 6), or by recruitment of stromal fibroblasts/periocytes into the tumor which drive the maturation of tumor vessels (8–10). Our analysis suggests that those mechanisms of resistance defined in preclinical tumor xenografts may be most relevant to those diseases that display a tumor vessel phenotype. It is probable that in selecting tumor models that grow quickly to facilitate drug testing, stromal-rich tumors have been severely underrepresented by virtue of their growth characteristics.

Figure 5. The stromal neovascularization is targeted by DC101 in a tumor model of the stromal vessel phenotype, Calu-3 which leads to stromal changes with negligible effect on tumor size. A, MVD (number of CD31 vessels per mm²) and microvessel area (MVA, total CD31 area (μm²/total number of CD31 vessels) of Genie classified stromal compartment of IgG1 control and DC101-treated Calu-3 tumor s (growth curve study 1). B, chromogenic (CD31, DAB, brown) and fluorescent [CD31-AF488 (green), α-SMA -AF555 (red), and DAPI counterstain (blue)] immunostained control and DC101-treated Calu-3 tumor s (growth curve 1). For CD31 images, black or gray arrows point to small and large stromal vessels, respectively (S, stroma; T, tumor). For CD31-α-SMA images, white or gray arrows indicate naked and pericyte-covered vessels, respectively; C, Genie (Aperio Technologies Ltd) classification and quantification of tumor, stromal, and necrotic areas (% number of tumor, stromal, or necrotic pixels/total number of pixels) using digitally captured images of hematoxylin-stained sections for DC101 (15 mg/kg twice weekly, n = 6) and IgG1 (control, n = 6) treated arms of Calu-3 growth studies [1 and 2 (data not shown)]. Hematoxylin (blue) staining of control and DC101-treated Calu-3 tumors and overlay of Genie classified tumor, stromal, and necrotic compartments for Calu-3 growth curves 1 and 2 (data not shown). D, stromal myofibroblast content (percentage number of αSMA–PDGFRI+ pixels/total number of pixels) within Genie classified regions of control and DC101-treated tumor s (growth curve 1). Mean ± SEM and P values are shown for all biomarkers.

Figure 6. Correlation of phenotype with RECIST response to bevacizumab and FOLFIRI in metastatic CRC. Data were derived from a CD31-α-SMA immunostained TMA consisting of 56 metastatic CRC samples (n = 2 cores per tumor) from patients before treatment. Tumors were classified as either tumor vessel or stromal vessel phenotypes by 2 observers and compared to best RECIST outcome for the treatment period of each patient. CR, complete response; PR, partial response; SD, stable disease. On the basis of the dataset, Fisher exact test showed that the stromal vessel phenotype group has higher probability of having a poor response (PD category) to bevacizumab and FOLFIRI than that of the tumor vessel phenotype group with statistical significance (P = 0.0487; 95% CI, 1.24–∞).

Tumor Stromal Phenotypes Define VEGF Sensitivity
Understanding the implications of the stromal vessel architecture on tumor cell function and response to drugs will be important. The Calu-3 model (stromal vessel phenotype), which exhibited a slower growth rate and showed a poor response to the specific VEGFR2-blocking antibody DC101, expressed higher levels of human genes thought to play a role in recruitment of stroma than models representative of the tumor vessel phenotype. These genes, PDGF-A, C and D, FGF-2, interleukin (IL)-8 are known to influence fibroblast function as well as resistance to VEGF signaling inhibitors (9–12, 27–30). The high stromal content would generate a distinct microenvironment where the mature vessels in the stroma may be resistant to therapy, an effect observed with VEGF signaling inhibitors in preclinical tumor xenografts (3, 11), the stromal phenotype may be a more extreme representation of this resistance mechanism. Interestingly, Calu-3 tumor stroma had a high local vessel density that was reduced following DC101 treatment in this model, but a significant number of established mature residual vessels remained. In addition, although treatment with DC101 led to a decrease in the density of the stroma, the general architecture of the stroma was maintained. This suggests that in Calu-3 tumors, there may be a role for the vasculature in maintaining or changing the tumor compartment following a reduction in vessels leading to a reduction in reactive stroma. Treating human stromal vessel tumors with VEGFi may change the apparent vessel permeability, perfusion or blood flow as determined by imaging or reduces vessels as measured by other biomarker approaches, but without reaching a threshold that is sufficient to impact upon tumor cell growth or survival. In stromal vessel tumors, pruning of immature vasculature and retention of larger mature vessels in the stroma by VEGFi could alter interstitial pressure within the tumor consistent with the vascular normalization hypothesis (31) and influence drug delivery. Tumor types that are predominantly stromal vessel (e.g., CRC, NSCLC, BC) do not exhibit dramatic responses to VEGFi monotherapy in the clinic but alterations in stromal density may give benefit in combination or with single-agent therapy in late-stage disease. Although we have only conducted a limited analysis, it will be interesting to examine how reductions in tumor vasculature influence tumor cell survival or growth in other models with a high stromal content. Here, we focused on testing VEGF signaling inhibition by using an antibody that specifically antagonized VEGFR2 on the murine vasculature. Many small-molecule VEGFR inhibitors have also been developed, but these compounds have broader pharmacology profiles and inhibit additional kinase such as PDGFR and related family members which impart additional effects in the stroma or tumor. It will be of future interest to also model such small-molecule inhibitors preclinically taking into account their clinical exposure profiles, to determine whether any differential effects are delivered with these mixed pharmacology agents on tumor vessel and stromal vessel tumors.

Our findings offer an alternative way to interpret the effects we have seen with VEGFi therapy in the clinic. If tumors with either a tumor vessel or stromal vessel architecture are viewed as distinct from the point of view of tumor angiogenesis, then it also challenges whether we have interpreted current biomarkers appropriately. For example, while biomarker changes in vessel numbers or vascular function in response to VEGFi treatment could conceivably be observed in tumors with either a tumor vessel or stromal vessel phenotype, it is possible that these may only translate into therapeutic benefit in the former. Given that predominantly stromal vessel diseases (e.g., CRC) will still have a small proportion of tumors with a tumor vessel phenotype, it would also be interesting to determine whether differences in response are seen within these tumor subsets. This segmentation of disease may offer an approach to prioritize tumor types that are likely to show a clinical response to VEGFi therapy and potentially other vascular directed agents. We suggest that diseases that predominantly display a tumor vessel phenotype would show good single-agent response to such agents.

This study has a number of limitations. Although the preclinical evaluation of the concept uses several tumor vessel models treated with DC101, because of the scarcity of tumor xenografts that represent the stromal vessel phenotype, we were limited to one stromal vessel model (Calu-3). Studies using additional VEGFR antagonists at different exposures in models that represent a range of phenotypes, including additional stromal vessel phenotypes and models heterogeneous for both phenotypes, would be important in investigating this concept further. In addition, a more comprehensive evaluation of the phenotypes in specific tumor types is required to better understand (i) prevalence and heterogeneity, (ii) prognostic and predictive value, and (iii) phenotype changes associated with disease progression or following therapy.

The vascular architecture is not the only parameter that is likely to determine the response of an individual tumor to VEGFi therapy. Within the context of each phenotype, other factors may further influence the outcome following therapy. For example, with a vascular targeting therapy, it is reasonable to expect factors that determine the metabolic status of the cells, or the degree of environmental stress the tumor cell can withstand, would also influence the likelihood of obtaining an objective tumor response. In particular, the stromal architecture may influence features such as the intrinsic metabolic status of the tumor cells either directly, or by creating a dependency on metabolic coupling between the stromal fibroblast and tumor to promote anabolic growth (the "reverse Warburg effect"; ref. 32). It is unclear what features of the tumor cell determine the vessel phenotypes, but a better understanding of the underlying mechanisms could help further refine patient selection strategies for VEGFi therapy.

In conclusion, we suggest that consideration of the tumor stromal architecture may be an important determinant of whether tumors will be therapeutically susceptible to
treatment with VEGFi monotherapy and potentially other vascular modulating agents. The ability to stratify VEGFi responses in preclinical models and human disease on the basis of a tumor vessel or stromal vessel phenotype warrants wider evaluation.

Disclosure of Potential Conflicts of Interest

All authors are current or former AstraZeneca employees and shareholders. M. Farrar was employed as a Post Doc and S. Wedge as Senior Principal Scientist. J. Kendrew and S.T. Barry have Ownership Interest (including patents) as AstraZeneca Shareholder. No potential conflicts of interest were disclosed.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.R. Smith, M. Farrar, A.J.C. Pommier, R. Swann, K. McDaid, J. Kendrew, C. Womack

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.R. Smith, M. Farrar, A.J.C. Pommier, X. Wang, S. Mistry, J. Kendrew, C. Womack, S. Wedge, S.T. Barry

Writing, review, and/or revision of the manuscript: N.R. Smith, A.J.C. Pommier, J. Kendrew, C. Womack, S. Wedge, S.T. Barry

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.R. Smith, D. Baker, A.J.C. Pommier, K. McDaid, S.T. Barry

Study supervision: N.R. Smith, S.T. Barry

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