Tumour-stromal architecture can define the intrinsic tumour response to VEGF-targeted therapy

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Abbreviations: VEGFi, Vascular Endothelial Growth Factor inhibitor; PDGF, Platelet Derived Growth Factor; TV, tumour vessel; SV, stromal vessel; TMA, tissue microarray; MVD, microvessel density

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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 tumour types are most sensitive, which patients receive greatest benefit and why the broad pre-clinical activity seen with these agents did not translate to the clinic. Here we differentiate human tumour types based on their stromal architecture into tumour vessel (TV) or stromal vessel (SV) phenotypes. The TV phenotype is associated with tumour types (renal and thyroid cancer) with better single agent clinical response to a VEGFi than the SV phenotype (CRC and NSCLC). The TV phenotype is also evident in many human tumour xenograft models commonly used to evaluate efficacy, whereas a model displaying the SV phenotype was found to be refractory to treatment with the VEGFR2 blocking antibody DC101. These data suggest an association between stromal architecture and intrinsic tumour sensitivity to VEGFi therapy.
Abstract

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

Experimental design: The tumour-stroma architecture of pre-clinical and clinical tumour samples were analysed by staining for CD31 and α-SMA. Tumour models representative of each phenotype were then tested for sensitivity to the VEGFR2 blocking antibody DC101.

Results: Human tumour types with high response rates to VEGF inhibitors (e.g. renal cell carcinoma), have vessels distributed amongst the tumour cells (a “tumour 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 tumour cells within stroma that also supports the majority of vessels (a "stromal vessel" phenotype, SV). Only 1 out of 31 tumour xenograft models displayed the SV phenotype. TV models were sensitive to VEGFR2 blocking antibody DC101, whereas the SV model was exclusively refractory. The TV phenotype was also associated with a better RECIST response to bevacizumab + chemotherapy in metastatic CRC.

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

The early promise of agents that target the Vascular Endothelial Growth Factor (VEGF) signalling axis has failed to translate widely in the clinic. Although VEGF-signalling plays a pivotal role in angiogenesis, and some patients receive benefit, not all patients are responsive to VEGF-inhibitor (VEGFi) therapy, and those that do respond will eventually become refractory to treatment (1, 2). The expectation that the use of VEGFi therapy would transform the way in which many solid human tumours are managed clinically was based largely upon the broad efficacy of these agents in a variety of pre-clinical tumour models \textit{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 neutralisation was the presence or recruitment of inflammatory infiltrate, specifically CD11b, GR-1 positive cells derived from the bone marrow, in response to upregulation of tumour 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-signalling 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 characterised by the recruitment of pericytes, possibly through an elevated EGFR signalling response (8), or PDGF-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 pre-clinical tumour xenograft models, and found that while the human tumour 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 tumours to examine the histological relationship between tumour and stroma, to determine whether these features are present within a panel of histologically diverse human tumour xenografts. We found that based on the tumour-stromal architecture human disease could be broadly sub-divided into two phenotypes that have different sensitivity to VEGFi therapy as a single agent. The significance of the phenotype is explored in the context of both pre-clinical modelling and clinical samples.
Material and Methods

Human and xenograft tumour 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 was obtained for this research. The suitability of each specimen for immunohistochemical analyses was determined by pathology assessment of tissue morphology and preservation (H&E) and the general extent of antigen preservation (pan p-Tyr immunostains). Tumor xenograft tissue was derived from experiments conducted as described (13) with licenses issued under the UK Animals (Scientific Procedures) Act 1986.

DC101 tumour growth studies

Calu-6 and Calu-3 human lung tumour xenografts, established in nude and SCID female mice respectively. HT29 and SW620 tumours were established in nude mice. Once tumours reached a mean volume of ~0.2cm$^3$ to ~0.3cm$^3$ (Calu-3), mice were then intraperitoneally injected twice-weekly with either 15mg/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, tumours 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.

Histopathological staining

The following antibodies were employed in immunohistochemical (IHC) and immunofluorescence (IF) analyses: rabbit anti-mouse CD31 (AstraZeneca, CHG-CD31-P1, 13); mouse anti-human $\alpha$-smooth muscle actin ($\alpha$SMA; Sigma, 1A4); rabbit anti-human PDGFR$\beta$ (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); 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 performed at room temperature and TBS containing 0.05% Tween (TBST) used for washes. Antigen retrieval was performed in pH 6 retrieval buffer (S1699, Dako) at 110°C for 5min in a 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 10min) and non-specific binding sites (Dako, X0909) blocked.

For single marker analyses, antibodies raised to CD31, αSMA, PDGFRβ, CD68, Neutrophil elastase, E-cadherin, vimentin, Gr-1, F4/80 and Ki67 were diluted, 1:400, 1:1000, 1:2000, 1:200, 1:200, 1:200, 1:100, 1:100, 1:100 and 1:100, respectively in antibody diluent (Dako, S0809) and applied to sections for 1hr. Mouse Envision secondary (Dako, K4007) for αSMA, 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 30min. For Neutrophil elastase, vimentin, Gr-1 and F4/80, Vectastain Elite ABC solution (Vector, PK-6100), diluted as instructed in kit, was added for 30min. Sections were washed and developed in diaminobenzidine for 10min (Dako, K3466) then counterstained with Carazzi’s hematoxylin. Appropriate no primary antibody and isotype controls were performed for each antibody. Chromogenic CD31-αSMA costain was performed using the Envision G/2 doublestain System (Dako, K5361) following the manufacturers recommendations with antigen retrieval and primary antibody dilution as described above. CD31-αSMA co-immunofluorescence was performed as previously described (13).

**Pathology scoring and computer-assisted image analysis**

Chromogenic or fluorescent images were captured using the x20 objective of either an Aperio image scan (Leica Biosystems) or Pannoramic SCAN (3DHISTECH), respectively. Scanned
images were scored by a trained pathologist and two scientists using simple subjective reporting procedures. Both human and xenograft tumours were scored for stromal-vessel (SV) and tumour-vessel (TV) phenotypes using H+E or CD31-αSMA stains. The TV phenotype was defined as a tumour structure where vessels are embedded throughout the tumour cell mass and the SV phenotype classed as tumour cell nests surrounded by well developed stromal structures which contain the majority of the vessels. A tumour was classified as either TV or SV based on the predominant phenotype (cut off of >60% tumour area) while a tumour composed of 40-60% of both phenotypes was scored as intermediate. These criteria were prospectively defined by an expert pathologist to clearly differentiate between tumours that were predominantly TV (>60%) or SV (>60%) and those that were intermediate (40-60%) and difficult to classify as one or the other phenotype. Based on CD68 marker immunostaining, macrophage infiltrate into the tumour compartment was classified as negative, low (>0 but ≤1% CD68+ cells: tumour cells) or medium-high (>1% CD68+ cells: tumour cells). Tumour 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 performed on digitally acquired chromogenic images. For images of human tumours immunostained for CD31-αSMA, the tumour compartment, including associated stroma, was selected by hand using the Aperio image viewer software (Leica Biosystems). Downstream image analysis of the annotated areas were performed using Aperio image analysis software (Leica Biosystems). Microvessel density (MVD, number of vessels per mm² viable tumour), using CD31 as a marker, was determined using the Aperio microvessel analysis algorithm (Leica Biosystems). Myofibroblast levels based on αSMA were determined using the Aperio color deconvolution algorithm to measure % αSMA positive pixels/total number of pixels. Genie™ (Leica Biosystems), a pattern recognition software tool, was trained to segment stained images of the Calu-3 xenograft into tumour, stroma and necrotic regions and used to determine the % of each compartment per tumour. This Genie classifier was combined with the appropriate Aperio image analysis algorithm to analyze biomarker parameters in stromal or tumour classified regions of Calu-3 tumours. Calu-3 stromal or tumour MVD, based on CD31
immunostain, was determined using the microvessel analysis algorithm. Stromal myofibroblast content (αSMA and PDGFRβ) or macrophage (F4/80) and neutrophil (Gr-1) infiltrate into the stroma or tumour was analyzed using the color deconvolution algorithm (Leica Biosystems) to determine % positive pixels / total number of pixels. Tumour proliferative index (number of Ki67 positive tumour cells/ total number of tumour cells (haematoxylin positive nuclei)) and tumour cell density (total number of tumour cells (haematoxylin positive nuclei)/tumour area) was determined using a nuclear algorithm (Leica Biosystems). To measure tumour nest size, the diameter of 44-220 distinct nests were measured for each αSMA immunostained Calu-3 section (to highlight haematoxylin positive tumour nests) using the Aperio image viewer measuring tool (Leica Biosystems). Biomarker data was analysed using the Student's two tailed t-test, to determine statistical significance between treatment groups.

**TaqMan® Fluidigm Gene Expression Profiling**

RNA was isolated from 30-50mg frozen tumour using an RNeasy Lipid Tissue Mini Kit (QIAGEN, 74104), according to manufacturer's protocol. On-column DNase digestion was performed 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 while eukaryotic 18S rRNA was used as the endogenous control (Supplementary Table 4). Total RNA (50ng) was converted into cDNA using the High Capacity cDNA Reverse transcription kit (Applied Biosystems, 4368814) in final volume of 20μl, 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 5μl. Samples were diluted 1 in 5 with 1x TE and stored at -20°C. Sample and assay preparation for 48.48 dynamic arrays was performed according to the manufacturer's instruction (Fluidigm).

Data was collected and analysed 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 performed as
Gene expression values were calculated using the comparative CT (ΔCT) method as previously described in User Bulletin #2 ABI PRISM 7700 Sequence Detection System 10/2001, using the corrected 18S rRNA Ct values for normalisation of the tumour 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

Tumour-stromal architecture defines human tumour types that respond to VEGF-signaling inhibitors as monotherapy

To assess the tumour-stromal architecture of different solid human malignances we used a number of tumour tissue micro-arrays (TMA) 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 2). For the purposes of this analysis we have viewed responsive disease as high incidence of tumour response by RECIST assessment (tumour shrinkage) and/or a large increase in progression free survival on long term therapy reported with different therapies targeting VEGF-signalling. 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 1). CRC (17) and NSCLC (18) are diseases where VEGF inhibitors show little single agent responses, and hence are trialed in combination with cytotoxic therapy. Tumours were screened by immunofluorescence to visualize the relative distribution of myofibroblast like cells (αSMA), blood vessels (CD31) and tumour cells (DAPI). This revealed two dominant morphologies: (1) a “tumour-vessel” phenotype (TV) which describes a tumour structure with vessels embedded throughout the tumour cell mass represented in RCC, GBM and HCC (Fig. 1a, Supplementary Fig. 1, and Table 2); (2) a “stromal-vessel” phenotype (SV) in which the tumour structure is dominated by a pattern of tumour cell nests surrounded by well developed stromal structures containing the majority of the vessels (Fig. 1b), as evident in CRC, NSCLC and PC (Fig. 1b and Supplementary Fig. 1 and Table 2). Using this classification, cores from multiple tumour types were scored for either a TV or SV phenotype (Fig. 1c). The TV phenotype was observed in RCC, GBN, OvC, HCC, ThC and HNSCC. The SV phenotype was dominant in CRC, NSCLC, PC and BC.

Since the phenotype ratios for each tumour type were derived initially from TMA cores, full tumour sections were also classified for each phenotype to ensure the results were...
representative of larger diagnostic tumour samples (RCC, HCC and PC (n=10), GBM, ThC, CRC and BC (n=9) and NSCLC (n=19)); Fig. 2a, Supplementary Fig 2, Supplementary Table 3). Where sections exhibit regions of both phenotypes, the predominant phenotype (>60%) was scored (Supplementary Fig. 2a). The frequency of the phenotypes representing diagnostic specimens agreed with the original TMA dataset (Supplementary Fig. 2b and c). These data establish that the tumour-stromal architecture of the tumour can be used to cluster human tumour types into two groups. Significantly, those clinical tumour types associated with response to a VEGFi alone (RRC (14), ThC (19, 20), GBN (15, 16) were dominated by a TV phenotype. In contrast, tumours where a VEGFi has shown modest activity in combination (CRC (17), PC (21) NSCLC, (18), BC (22)) were dominated by the SV phenotype.

Additional biomarker analyses were performed on the diagnostic tumour panel (n=32 TV, n=35 SV) 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 αSMA+ cells) were associated with both phenotypes, tumour embedded vasculature of the TV phenotype tended to be pericyte-free while stromal vessels in the SV phenotype were often pericyte covered or associated with αSMA+ fibroblasts (Fig. 2b). Computer-assisted image analysis of CD31 immunostained lesions revealed that the tumour vascular density in the combined tumour and stromal compartments was higher in TV (mean ± s.e.m = 105.3 ± 21.3) than SV (47.7 ± 7.5; Fig. 2c and Supplementary Fig. 3b). αSMA analysis showed myofibroblasts were greater in SV (s.e.m = 10.0 ± 1.8) compared to TV (1.4 ± 0.3) phenotype tumours (Fig. 2c and Supplementary Fig 3b) tumours. EMT status was determined by immunostaining serial sections for E-cadherin and vimentin (Fig. 2d and Supplementary Fig 3b). TV tumours were generally more mesenchymal (E-cadherin+/vimentin+) than SV tumours (E-cadherin+/vimentin-). Qualitative histological assessment of CD68 immunostained tumours from the panel showed that the TV phenotype has higher levels of macrophage tumour infiltrate (Supplementary Fig 3b, c).

Neuroendocrine pancreatic cancer has recently been shown to respond to the VEGFi sunitinib (23) while pancreatic adenocarcinoma has proven comparatively resistant to such approaches (24, 25, 26). Comparing available samples of neuroendocrine pancreatic cancer with
pancreatic adenocarcinoma revealed them to represent TV and SV phenotypes, respectively (Supplementary Fig. 3a), consistent with our hypothesis on VEGFi sensitivity.

The TV phenotype is common to tumour xenograft models

To evaluate the relevance of the two phenotypes in pre-clinical models, a panel of human tumour xenografts grown subcutaneously and representing a broad range of different tumour 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), uterous (1), vulva (1) and blood (1) tumour and fibrosarcoma (1) cell lines) were classified for the two phenotypes based on the approach employed for human tumours (Fig. 3). The majority (30/31) exhibited a TV phenotype (Fig. 3a,b). Only Calu-3 (lung adenocarcinoma) had a SV phenotype (Fig. 3a,c). This data indicates that the TV phenotype is common to subcutaneously grown tumor xenograft models.

Intrinsic gene expression differences between SV and TV xenograft tumours

Gene expression analysis of human transcripts was used to investigate the relationship between Calu-3, Calu-6, SW620, and HT29 tumours. The difference in expression of a range of 180 genes associated with angiogenesis, inflammation and invasive growth was determined (Fig. 3d, 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, SPP1 (osteopontin). Compared to other models analysed, Calu-3 xenograft tumours were enriched in genes associated with recruitment of the stromal cells, in particular FGF and PDGF ligands (Fig 3d). To validate that Calu-3 tumours showed a different expression profile they were also compared to a broader panel of tumour xenograft models, again the same genes were over-expressed relative to other models. This expression analysis supports the conclusion that Calu-3 tumour cells exhibit high expression of a number of transcripts that may contribute to their phenotype.
The TV phenotype has greater sensitivity to VEGFR-2 inhibitor, DC101 than the SV phenotype, in vivo

To determine whether the tumour-stromal architecture could influence tumour response to VEGF inhibition we used the murine VEGFR-2 blocking antibody, DC101 to assess the effects of pruning neovascularity on tumour growth in TV (Calu-6, HT-29 and SW620) compared to SV (Calu-3) tumour xenograft models (25). DC101 was dosed intraperitoneally at 15mg/kg twice-weekly. Calu-6, HT-29 and SW620 xenografts exhibited the classic tumour growth response to VEGF-signaling inhibitors (Fig. 4a) seen in multiple models (27, 28, 29). In contrast Calu-3 tumours exhibited a poor response, with DC101 having no effect on tumour growth (experiment 1), or inducing a small initial reduction in growth (0-3 days, experiment 2) followed by a rapid return to a growth rate similar to the controls (Fig 4b).

The gross vascular response to DC101 is similar between TV and SV tumour xenografts

To compare the total vascular response of TV and SV models to DC101, MVD analysis was performed on CD31 immunostained control and DC101 treated samples from the Calu-6 and Calu-3 (replicate 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 RT-qPCR using genes associated with angiogenesis and tumour cell invasion (Fig. 4d) revealed that human tumour gene changes were unique to each tumour 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 SV phenotype model which leads to a reduction in the stromal compartment but with negligible effect on tumour size**

Tumour embedded vessels were not detected in the Calu-3 model, however vasculature was detected at a high density in the stroma (Fig 5a, 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 tumours (Fig 5a). Qualitative assessment of CD31-αSMA immunofluorescent stained tumours revealed the proportion of pericyte covered stromal vessels increased in treated tumours (Fig. 5b). The data indicates 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 SV phenotype on the response to DC101 we performed a detailed biomorphometric analysis of DC101 treated Calu-3 xenograft tumours. In Calu-3 tumours, DC101 treatment led to reduction in the area of the stromal compartment and an increase in the area occupied by tumour cells with negligible effect on tumour size (Fig. 5c). Analysis of the stromal compartment for myofibroblasts using αSMA 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 tumours nests (from 200 to 450μm, P=0.04), however effects on the proliferative index and density of the tumour were negligible at this time point (Supplementary Fig. 4a, c). 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 tumour macrophage content and a significant accumulation of Gr-1 positive cells in the stroma (Supplementary Fig. 4b, c). 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 tumour compartment, but with a negligible effect on the overall tumour 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 metastatic CRC patients was investigated. Two TMAs consisting of surgical tumour samples from 56 metastatic CRC patients (Tristar) were chromogenically stained for CD31-αSMA and scored for the TV and SV phenotypes by two observers. 42 patient samples were classified as the SV phenotype and 14 as the TV phenotype (Fig. 6). RECIST response information for FOLFIRI and bevacizumab (Avastin) treatment as 1st or 2nd line therapy post surgery was available for each patient. Fisher's Exact Test was used to determine the significant associations between the phenotype and best RECIST response categories (PD and other RECIST outcome) during patient treatment periods (for this dataset best RECIST responses also had longest duration). The probability of the SV phenotype group having a poorer response (PD category) to bevacizumab and FOLFIRI than that of the TV phenotype group was statistically significant (P=0.0487; 95% CI, 1.24-∞). Although it is not possible to conclude that the differential response is a direct result of the treatment with bevacizumab alone, the data suggest that the TV phenotype appears to be more sensitive to the combination therapy than the SV phenotype.
Discussion

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

The TV phenotype was associated with differences in the distribution of other cells, specifically the presence of high levels of macrophages. In SV tumours these macrophages did not commonly associate with the tumour cells perhaps indicating a different role for the macrophage in these tumours. A possible explanation for these observations is that the intratumoural vasculature observed in the TV phenotype, but absent from the SV phenotype, would facilitate extravasation of immune infiltrate into the tumour. In contrast, in the SV phenotype macrophages would extravasate into the dense stroma surrounding the tumour nests which may impair their migration into the tumour. Based on E-cadherin loss and vimentin gain, the TV phenotype was more associated with tumour cells of a mesenchymal phenotype. It is conceivable, that the tissue of origin or subsequent progression of tumour to a more mesenchymal phenotype could influence reciprocal signaling between the tumor and the tumour...
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 tumour samples with associated outcome data following treatment with VEGF signalling inhibitors, we were able to obtain TMAs of samples from CRC patients 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 TV and SV tumours 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 tumour sample and the TMA. The distribution of TV vs SV was largely representative of the larger datasets for CRC. Interestingly in this dataset the TV phenotype was associated with good response, with no examples of progressive disease associated with this phenotype, suggesting that the TV phenotype may be more sensitive to the combination therapy. This supports the idea that the TV or SV should be considered when differentiating response of these tumours to angiogenic therapy. It would be interesting to understand in late stage CRC, where VEGF inhibitors can be used as a single agent, whether differential response or disease control is associated with the differences in phenotype. As PFS and OS information was not available for these samples, further analyses to correlate phenotypes with these survival outcomes are required to understand the utility of this approach to predict long term outcome in diseases dominated by SV morphology. Only primary tumour samples were analysed in this study. Often archival diagnostic samples from the primary tumour are the only tissue available to evaluate the tumour 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 utility of stratifying tumour types based on phenotype using archival samples.

Pre-clinical tumour 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/pericytes into the tumour which drive the maturation of tumour vessels (8, 9, 10). Our analysis suggests that those mechanisms of resistance defined
in pre-clinical tumour xenografts may be most relevant to those diseases that display a TV phenotype. It is probable that in selecting tumour models that grow quickly to facilitate drug testing, stromal rich tumours have been severely under-represented by virtue of their growth characteristics.

Understanding the implications of the SV architecture on tumour cell function and response to drugs will be important. The Calu-3 model (SV 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 TV phenotype. These genes, PDGF-A, C and D, FGF-2, IL-8 are known to influence fibroblast function as well as resistance to VEGF signalling inhibitors (9-12, 27-31). 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-signalling inhibitors in pre-clinical tumour xenografts (3, 11), the stromal phenotype may be a more extreme representation of this resistance mechanism. Interestingly Calu-3 tumour 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 tumours there may be a role for the vasculature in maintaining the stroma, or that changes in the tumour compartment following a reduction in vessels lead to a reduction in reactive stroma. Treating human SV tumours 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 tumour cell growth or survival. In SV tumours, pruning of immature vasculature and retention of larger mature vessels in the stroma by VEGFi could alter interstitial pressure within the tumour consistent with the vascular normalisation hypothesis (32) and influence drug delivery. Tumour types that are predominantly SV (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 performed a limited
analysis it will be interesting to examine how reductions in tumour vasculature influence tumour cell survival or growth in other models with a high stromal content. Here we focussed on testing VEGF signalling inhibition by using an antibody that specifically antagonised VEGFR-2 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 tumour. 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 TV and SV tumours.

Our findings offer an alternative way to interpret the effects we have seen with VEGFi therapy in the clinic. If tumours with either a TV or SV architecture are viewed as distinct from the point of view of tumour 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 tumours with either a TV or SV phenotype, it is possible that these may only translate into therapeutic benefit in the former. Given that predominantly SV diseases (e.g. CRC) will still have a small proportion of tumours with a TV phenotype, it would also be interesting to determine whether differences in response are seen within these tumour subsets. This segmentation of disease may offer an approach to prioritise tumour 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 TV 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 TV models treated with DC101, because of the scarcity of tumour xenografts that represent the SV phenotype we were limited to one SV model (Calu-3). Studies using additional VEGF antagonists at different exposures in models that represent a range of phenotypes, including additional SV 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 tumour 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 tumour 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 tumour cell can withstand, would also influence the likelihood of
obtaining an objective tumour response. In particular, the stromal architecture may influence
features such as the intrinsic metabolic status of the tumour cells either directly, or by creating a
dependency on metabolic coupling between the stromal fibroblast and tumour to promote
anabolic growth (the "reverse Warburg effect") (33). It is unclear what features of the tumour 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 tumour-stromal architecture may be an
important determinant of whether tumours 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 TV or SV phenotype
warrants wider evaluation.
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AUTHOR CONTRIBUTIONS

NRS, STB designed and directed experiments and their analyses and wrote the manuscript.

SM, DB, RS conducted experiments and reviewed manuscript

MF, JK designed and conducted experiments and reviewed manuscript

CW provided pathology review

XW provided statistical review

SRW interpreted study data and helped draft and critique the manuscript

COMPETING FINANCIAL INTERESTS

All authors are current or former employees of AstraZeneca.
FIGURE LEGENDS

Figure 1 Two dominant phenotypes based on tumour-stromal architecture stratify tumour types. (a,b) diagrams highlighting: (a) tumour-vessel phenotype (TV, a tumour structure where vessels are embedded throughout the tumour cell mass) and (b) stromal-vessel phenotype (SV, a tumour structure dominated by a pattern of tumour cell nests surrounded by well developed stromal structures which contain the majority of the vessels). Also shown are immunofluorescence stained FFPE human tumours (CD31-AF488 (green); αSMA-AF555 (red) and DAPI counterstain (blue)), indicative of TV (RCC, GBM, HCC, ThC, OvC and HNSCC) and SV (CRC, NSCLC, PC and BC). (c) Percentage ratios of TV:SV phenotypes for RCC, GBM, OvC, HCC, ThC, HNSCC, CRC, NSCLC, PC and BC. Phenotypes were scored from CD31-αSMA or H+E stained TMAs (1 TMA per tumour type, number of patient samples per tumour type are shown).

Figure 2 Biomarker analyses of the two phenotypes in whole tumour samples. (a) Percentage ratios of TV:SV:intermediate phenotypes for RCC, GBM, HCC, ThC, HNSCC, CRC, PC and BC. Phenotypes were scored from CD31-αSMA costained whole FFPE tumour sections (number of patient samples per tumour type are shown). (b) Pericyte coverage of vessels associated with TV (RCC and ThC) and SV (CRC and NSCLC) phenotypes, white or grey arrows indicate naked and pericyte covered vessels, respectively. Samples were analysed for: (c) microvessel density (MVD, number of CD31+ vessels per mm²) and myofibroblast content (percentage number of αSMA+ pixels/ total number pixels) and (d) EMT status (percentage ratio epithelial (E-cadherin+vimentin-): intermediate (E-cadherin+vimentin+): mesenchymal (E-cadherin- vimentin+)).

Figure 3. Prevalence and intrinsic gene expression differences between the two phenotypes in tumour xenograft models (a) Classification of 31 subcutaneously implanted human tumour xenograft models for TV and SV phenotypes. Data was derived from the scoring of CD31-αSMA immunostained TMAs (4 FFPE tumours per model, 3 cores per tumour). (b,c)
Diagrams and fluorescent immunostained (CD31-AF488 (green); αSMA-AF555 (red) and DAPI counterstain (blue)) images representing preclinical xenograft. **(b)** Tumour vessel (Calu-6 - low myofibroblast content) and **(c)** stromal vessel (Calu-3). **(d, e)** Transcript profiling of TV (Calu-6, HT29 and SW620) and SV (Calu-3) tumour xenograft models. Genes with high **(d)** and low **(e)** expression levels in Calu-3 compared to Calu-6, HT29 and SW620 are presented. ddCT = (Average dCT Calu-3) – (Average dCT (Calu-6 + SW620 + HT29)).

**Figure 4** The tumour vessel phenotype is more sensitive to VEGFR-2 signalling inhibitor, DC101 than the stromal-vessel phenotype in vivo. **(a, b)** Geometric mean tumour volume growth curves (cm³) for DC101 (15mg/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) tumour xenograft studies. Geometric mean ± s.e.m. are shown for growth curves. **(c)** Microvessel density (MVD, number of CD31⁺ vessels per mm²) of IgG₁-control and DC101 treated Calu-6 and Calu-3 tumour studies (growth curves 1 and 2). **(d)** Significant gene changes induced in the tumour (human) and stromal (stromal) vascular modulation and invasion transcript profile on DC101 treatment of Calu-6 and Calu-3 (growth curve 1) xenograft tumors. Tumour transcripts are normalized to human 18s while stromal transcript are normalized to total 18s. Only significant gene changes are included (P>0.05 and fold change greater than 1.5), Each column is an individual tumour and each colour gradient is scaled independently within each gene.

**Figure 5** The stromal neovasculature is targeted by DC101 in a tumour model of the SV phenotype, Calu-3 which leads to stromal changes with negligible effect on tumour size. **(a)** Microvessel density (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 tumours (growth curve study 1). **(b)** Chromogenic (CD31, DAB, brown) and fluorescent (CD31-AF488 (green); αSMA-AF555 (red) and DAPI counterstain (blue) immunostained control and DC101 treated Calu-3 tumours (growth curve 1). For CD31 images, black or grey arrows point to small and large stromal vessels, respectively (S=stroma, T=tumour). For CD31-αSMA images, white or grey arrows indicate naked and...
pericyte covered vessels, respectively. (c) Genie™ (Aperio Technologies Ltd) classification and quantification of tumour, stromal and necrotic areas (% number of tumour, stromal or necrotic pixels/ total number of pixels) using digitally captured images of haematoxylin stained sections for DC101 (15mg/kg/twice weekly, n=6) and IgG1 (control, n=6) treated arms of Calu-3 growth studies (1 and 2 (data not shown)). Haematoxylin (blue) staining of control and DC101 treated Calu-3 tumours and overlay of Genie classified tumour, stromal and necrotic compartments for Calu-3 growth curves 1 and 2 (data not shown). (d) Stromal myofibroblast content (percentage number of αSMA⁺ or PDGFRβ⁺ pixels/ total number pixels) within Genie classified regions of control and DC101 treated tumours (growth curve 1). Mean ± s.e.m. and P-values are shown for all biomarkers.

Figure 6 Correlation of phenotype with RECIST response to bevacizumab and FOLFIRI in metastatic CRC.

Data was derived from a CD31-aSMA immunostained TMA consisting of 56 metastatic CRC samples (n=2 cores per tumour) from patients prior to treatment. Tumours were classified as either tumour vessel (TV) or stromal vessel (SV) phenotypes by two observers and compared to best RECIST outcome for the treatment period of each patient (CR, complete response; PR, partial response; SD, stable disease and PD, progressive disease). Based on the dataset, Fisher’s Exact Test demonstrated that the SV phenotype group has higher probability of having a poor response (PD category) to bevacizumab and FOLFIRI than that of the TV phenotype group with statistical significance (P=0.0487; CI, 1.24-∞).
Fig. 1

(a) TV

(b) SV

(c) Phenotypes (% ratio)

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>TV</th>
<th>SV</th>
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<tr>
<td>RCC</td>
<td>0%</td>
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</tr>
<tr>
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</tr>
<tr>
<td>BC</td>
<td>10%</td>
<td>90%</td>
</tr>
</tbody>
</table>
Fig. 2

(a) Phenotype (% ratio) versus cancer type.

(b) Micrographs showing the effects of different treatments on cancer cell phenotypes.

(c) Graphs illustrating microvessel density and αSMA positivity.

(d) Bar chart showing EMT status (% ratio) for different cancer phenotypes.
Fig. 3

Tumour xenograft models (n=31)

- TV
- SV

a

b

TV

Calu-6

Calu-3

c

sv

d
e

Research.
Fig. 4

a) TV (Geometric mean tumour volume (cm³ ± s.e.m.) vs. Days of treatment)

b) SV (Geometric mean tumour volume (cm³ ± s.e.m.) vs. Days of treatment)

c) TV (Micronvessel density (number vessels per mm²) vs. Control vs. DC101 Treated)

Gene Human Fold Change P-Value Gene Human Fold Change P-Value
CDH8 5.17 0.005 PLAT 1.77 0.032
SGK2 2.57 0.029 VEGFA 1.61 0.009
GAS6 2.38 0.016 BMP2 1.53 0.037
LPA4R1 1.70 0.028 MST1 1.51 0.036
LPA4R1 1.98 0.034 FGF2 1.69 0.033
SPP1 -1.08 0.014
CSTA -4.06 0.010

Gene Human Fold Change P-Value Gene Human Fold Change P-Value
CDH8 5.17 0.005 PLAT 1.77 0.032
SGK2 2.57 0.029 VEGFA 1.61 0.009
GAS6 2.38 0.016 BMP2 1.53 0.037
LPA4R1 1.70 0.028 MST1 1.51 0.036
LPA4R1 1.98 0.034 FGF2 1.69 0.033
SPP1 -1.08 0.014
CSTA -4.06 0.010
Fig. 5

a) Calu-3 (1) Stroma MV density (number vessels per mm²)

b) Calu-3 (1) Stromal microvascular area (μm² per vessel)

c) Calu-3 (1) Relative proportions of xenograft compartments (%)

d) Calu-3 (1) % positivity αSMA/PDGFRβ
Tumour-stromal architecture can define the intrinsic tumour response to VEGF-targeted therapy


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