Human Cancer Biology

Vascular Endothelial Growth Factor Receptors VEGFR-2 and VEGFR-3 Are Localized Primarily to the Vasculature in Human Primary Solid Cancers

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Abstract

Purpose: Vascular endothelial growth factor (VEGF) signaling is key to tumor angiogenesis and is an important target in the development of anticancer drugs. However, VEGF receptor (VEGFR) expression in human cancers, particularly the relative expression of VEGFR-2 and VEGFR-3 in tumor vasculature versus tumor cells, is poorly defined.

Experimental Design: VEGFR-2– and VEGFR-3–specific antibodies were identified and used in the immunohistochemical analysis of human primary cancers and normal tissue. The relative vascular localization of both receptors in colorectal and breast cancers was determined by coimmunofluorescence with vascular markers.

Results: VEGFR-2 and VEGFR-3 were expressed on vascular endothelium but not on malignant cells in 13 common human solid tumor types (n > 400, bladder, breast, colorectal, head and neck, liver, lung, skin, ovarian, pancreatic, prostate, renal, stomach, and thyroid). The signal intensity of both receptors was significantly greater in vessels associated with malignant colorectal, lung, and breast than adjacent nontumor tissue. In colorectal cancers, VEGFR-2 was expressed on both intratumoral blood and lymphatic vessels, whereas VEGFR-3 was found predominantly on lymphatic vessels. In breast cancers, both receptors were localized to and upregulated on blood vessels.

Conclusions: VEGFR-2 and VEGFR-3 are primarily localized to, and significantly upregulated on, tumor vasculature (blood and/or lymphatic) supporting the majority of solid cancers. The primary clinical mechanism of action of VEGF signaling inhibitors is likely to be through the targeting of tumor vessels rather than tumor cells. The upregulation of VEGFR-3 on tumor blood vessels indicates a potential additional antiangiogenic effect for dual VEGFR-2/VEGFR-3–targeted therapy.

Vascular endothelial growth factor (VEGF) signaling is key to physiologic and pathologic angiogenesis and lymphangiogenesis, and is an established target in the development of anticancer therapeutics. VEGF-A is an important driver of the neovascular growth required to support solid tumor progression (1). The primary signaling receptor for VEGF-A is VEGF receptor (VEGFR)-2 (Flk-1, KDR; refs. 2, 3), and activation of VEGFR-2 by VEGF-A on endothelial tip and stalk cells directs the migration and extension of sprouting vessels, respectively (4). VEGFR-3 (Flt-4) is the receptor for VEGF-C and VEGF-D (5). Expression of VEGFR-3 is largely confined to lymphatic endothelium in adult tissues (6), and its activation induces lymphangiogenesis (7). VEGFR-3 was recently also found expressed on the endothelial tip cells of angiogenic tumor vasculature, and blockade of the receptor in tumor models induces blood vessel endothelial cell sprouting and vessel branching (8).

Agents that inhibit VEGF signaling and are already registered for the treatment of cancer include the VEGF-A neutralizing antibody bevacizumab (9) and small-molecule multikinase inhibitors sorafenib, sunitinib, and pazopanib (10, 11). Other small-molecule VEGFR inhibitors under evaluation in clinical trials include vandetanib and cediranib. Cediranib is a potent inhibitor of VEGF signaling, targeting VEGFR-1, VEGFR-2, and VEGFR-3 and angiogenesis (12). Recent publications have shown that VEGFR-3 inhibition by cediranib reduces lymphangiogenesis as well as the incidence of lymphatic metastases in vivo (13, 14). The combined blockade of VEGFR-2 and VEGFR-3 has an additive antiangiogenic and antitumor effect.
Translational Relevance

Vascular endothelial growth factor (VEGF) signaling inhibitors have shown clinical efficacy in a range of solid tumor types, including colorectal, lung, and breast cancer. A better understanding of the expression and localization of VEGF receptors is needed to understand the potential for direct antitumor effects from VEGF signaling inhibitors in the clinic and to help identify patients who might gain greatest benefit from treatment. Our findings indicate that VEGFR-2 and VEGFR-3 are primarily localized to tumor vessels in a broad range of solid tumors, suggesting that tumor endothelial cells and not tumor cells are the likely primary target for anti-VEGFR-2 and anti-VEGFR-3 therapy in cancer. Furthermore, the expression of VEGFR-3, as well as VEGFR-2, on tumor blood vessels suggests that dual VEGFR-2/VEGFR-3 inhibitors may have additional antiangiogenic benefits in some patients.

in vivo over inhibition of VEGFR-2 alone (8). In addition to reducing lymphangiogenic metastases, dual VEGFR-2 and VEGFR-3 inhibitors may improve the antiangiogenic response in patients.

An understanding of the VEGFR-2 and VEGFR-3 status of human tumors may help to identify patients who would benefit most from treatment with dual VEGFR-2 and VEGFR-3 inhibitors and clarify the clinical mechanism of action of these agents. Presently, there is no clear agreement from the literature on the location (vasculature and/or cancer cells) or extent of expression of either VEGFR-2 or VEGFR-3 in human solid tumors. For example, using immunohistochemistry, VEGFR-2 has been detected on tumor vessels supporting human colorectal (CRC; ref. 15), breast (BC; refs. 16, 17), and non–small cell lung carcinomas (NSCLC; refs. 18, 19). However, tumor cell expression has also been reported in 40% to 100% of CRC (20, 21), BC (22–24), and NSCLC (18, 19, 25). Similarly, some reports confine VEGFR-3 to tumor vessels, primarily lymphatic but also blood endothelium in CRC (26), BC (27–30), and NSCLC (19, 31–33), whereas others localize the receptor to tumor cells [CRC (34), BC (23, 35), and NSCLC (19, 25, 32, 33, 36, 37)]. Recently, Petrova et al. (38) have provided compelling evidence that poor antibody specificity is likely to be responsible for some of the claims that VEGFR-3 is found on human tumor cells (38). Using a validated antibody, they confirm that VEGFR-3 is confined primarily to blood and lymphatic vessels in solid tumors (38).

This report aimed to resolve the issues around the relative extent and location of VEGFR-2 and VEGFR-3 expression in human primary solid tumors and, in doing so, better define the clinical mechanism of action of VEGF-targeted therapy. To do this, we determined the cross-reactivity of human VEGF antibodies using multiple screening platforms and identified two antibodies, 55B11 (VEGFR-2; Cell Signaling Technology) and AF349 (VEGFR-3; R&D Systems), with credible specificity. These were used in immunohistochemical and communofluorescent analyses of archival human tumor samples to understand the expression of the two receptors between (a) tumor endothelium and malignant cells and (b) tumor and normal endothelium and investigate the relative localization of VEGFR-2 and VEGFR-3 on tumor vasculature.

Materials and Methods

Antibodies

The following antibodies were used: custom rabbit polyclonal antibody raised to COOH-terminal peptide of mouse CD31 (CHG-CD31-P1; AstraZeneca); mouse monoclonal antibodies to human CD31 (IC70A; Dako), α-smooth muscle actin (α-SMA; I44; Sigma), phosphotyrosine (P-Tyr-100; Cell Signaling Technology), and rabbit glyceraldehyde-3-phosphate dehydrogenase (AM4300; Ambion); rabbit monoclonal against human VEGFR-2 (55B11); goat polyclonal against human VEGFR-3 (AF349); and rabbit polyclonal against human LYEVE-1 (102-PA50AG; Reliatech). Other VEGF antibodies are listed in Supplementary Table S1.

Cell lines

Cell lines were maintained at 37°C with 5% CO2: 1T-29 (obtained from the European Collection of Animal Cell Cultures) in DMEM with 5% fetal bovine serum (FBS) and 1% nonessential amino acids; Colo-205 (European Collection of Animal Cell Cultures) in RPMI 1640/10% FBS; A549 (American Type Culture Collection) in DMEM/10% FBS/10% M1; PC-9 (a kind gift from Dr. K Nishio, National Cancer Centre of Japan) in RPMI 1640/10% FBS/10% M1; MCF-7 (Imperial Cancer Research Fund) in DMEM/10% FCS; aortic VSMC (C-12533; Promocell) in Promocell VSMC growth media (C-22062); M-07e (Deutsche Sammlung von Mikroorganismen und Zellkulturen) in RPMI 1640/10% FBS/10% M1/interleukin-3 (10 ng/mL)/granulocyte macrophage colony-stimulating factor (10 ng/mL); PAE (Ludwig Institute for Cancer Research) in Ham’s F12/10% FBS/l-glutamine; and NIH-3T3 (Jefferson Cancer Institute) in DMEM/10% FBS/l-glutamine. Full-length human cDNA for VEGFR-1, VEGFR-2, and CSFIR was PCR amplified from nm_002019.3, nm_002253.2, and nm_005211.3, respectively, using the following primer pairs: VEGFR-1, 5′-GGTATACCTTTAAGAAGGAGATATAACCATCGTCCAGCTACTGACCCGGG-3′ (forward) and 5′-CTATAGGTCCTCCTCCTGATTAGCTTCTGCTGATGGTTGGGGAGATTTAGCTTTTGCTC-3′ (reverse); VEGFR-2, 5′-ATTAACCTTTAAGAAGGAGATATAACCCAGAAGGGTCTGCTGGCGCGTCCGGC-3′ (forward) and 5′-TCTATAGGTCCTCCTGATTAGCTTCTGCTC-3′ (reverse); and CSF1R, 5′-TCTATAGGTCCTCCTGATTAGCTTCTGCTC-3′ (reverse) and 5′-TCTATAGGTCCTCCTGATTAGCTTCTGCTC-3′ (reverse).
(forward) and 5′-ATTTAAGTAAAGAGATATAAC-CATGGCCCCAGGATTTGCTGTCCTGCC-3′ (reverse).

VEGFR-3 (cDNA cloned into pDONR221), accession number nm_182925, was obtained from GeneArt. DNA fragments were subcloned into the retroviral vector pBIN CIP SIN via Gateway technology (Invitrogen) and cotransfected with constructs expressing the viral envelope protein VSVG (Clontech) into a packaging cell line, Phoenix-Hek 293 (Garry P. Nolan, Stanford University School of Medicine). Retrovirals were used to infect NIH-3T3 and PAE cells, and stable lines were selected for resistance to puromycin.

**Tumor xenografts**

Tumor xenograft tissue was derived from experiments conducted with licenses issued under the UK Animals (Scientific Procedures) Act 1986 and after local ethical review and approval. Cell lines were maintained in the recommended growth medium and implanted s.c. into the left flank of immunocompromised mice: nude, scid, or scid-bg (see Supplementary Table S2 for details). Tumors were grown to ~1 cm³ volume and then collected and fixed in formalin for 24 hours before being embedded in paraffin.

**Patient tissue samples**

Formalin-fixed, paraffin-embedded (FFPE) human primary cancer resection samples and tissue microarrays (TMA) from patients with untreated human primary bladder, breast, colorectal, head and neck, liver, lung, skin, ovarian, pancreatic, prostate, renal, stomach, and thyroid cancers and matched adjacent normal tissue (for colorectal, lung, and breast cancers) were sourced under approved legal contract from three commercial tissue suppliers (Asterand, Cytomyx, and TriStar Technology Group) and a hospital tissue bank (Wales Cancer Bank). Appropriate consents, licensing, and ethical approval were 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 (CD31 and p-Tyr immunostains).

**Western blot analysis**

Subconfluent (70-80%) cell lines were scraped into ice-cold lysis buffer [20 mmol/L Tris (pH 7.5), 137 mmol/L NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 50 mmol/L NaF, 1 mmol/L Na₂VO₄, 1 protease inhibitor tablet/25-mL buffer (Boehringer Ingelheim)], incubated on ice for 30 minutes, aspirated, and cleared by centrifugation. Frozen tumor xenograft tissues were homogenized in ice-cold lysis buffer using an Ultraturrax T25 homogenizer (Janke and Kunkel) before incubation on ice. Lysate samples (250 μg) were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane (Invitrogen) by Western blotting. Membranes were blocked in TBS containing 0.05% Tween (TBST) and 5% nonfat dry milk (Marvel) for 1 hour at room temperature and then incubated for 16 hours at 4°C in VEGFR-2 antibodies (55B11, A3, sc-504, and sc-315; diluted 1:1,000 in block), VEGFR-3 antibody (AF349; 1:1,000), CD31 antibody (CHG-CD31-P1; 1:500), or glyceraldehyde-3-phosphate dehydrogenase antibody (AM4300; 1:10,000). Immunoblots were incubated in a 1:2,000 dilution of horseradish peroxidase–conjugated anti-rabbit (New England Biolabs), anti-mouse (New England Biolabs), or anti-goat (Dako) antibody and visualized using the SuperSignal West Pico Substrate method of detection (Perbio Science).

**Immunohistochemistry and immunocytochemistry**

All incubations were at room temperature and washes were done with TBST. FFPE tissues sectioned at 4 μm onto slides were dewaxed and rehydrated. Antigen retrieval was done in a pH 9 retrieval buffer (S1699; Dako) for p-Tyr, CD31, and VEGFR-3; or in pH 9 retrieval buffer (S2367; Dako) for VEGFR-2. Endogenous biotin was blocked using an avidin-biotin kit (SP-2002; Vector Laboratories, Inc.), endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, and nonspecific binding sites were blocked with serum-free protein blocking agent (X0908; Dako) for 20 minutes. Primary antibodies to p-Tyr (P-Tyr-100), CD31 (IC70A and CHG-CD31-P1), VEGFR-2 (55B11), and VEGFR-3 (AF349) were diluted 1:500, 1:500, 1:600, and 1:200, respectively, in antibody diluent (S0809; Dako) and incubated with sections for 1 hour. Either mouse Envision secondary (K4007; Dako) for p-Tyr and CD31, rabbit Envision secondary (K4003; Dako) for VEGFR-2 and CD31 (CHG-CD31-P1), or biotinylated rabbit anti-goat immunoglobulin antibodies (E0466, diluted 1:400 in TBST; Dako), followed by Vectastain Elite ABC solution (PK-6100; Vector Laboratories), for VEGFR-3 were added for 30 minutes each. Sections were developed in diaminobenzidine for 10 minutes (K3466; Dako) and counterstained with Carazzi’s hematoxylin. Appropriate no primary antibody and isotype controls were done for each antibody.

For immunocytochemical analyses, subconfluent cells were transferred to 10% neutral buffered formalin for 24 hours at 4°C. Cells were centrifuged to a pellet, washed in 80% ethanol, processed into 1% agarose, and embedded into paraffin blocks. Cell pellets were sectioned at 4 μm, and 55B11 and AF349 antibodies were evaluated using the immunohistochemical conditions without the antigen retrieval.

**Immunofluorescence**

Immunofluorescence was done on dewaxed and rehydrated FFPE sections. Antigen retrieval was carried out as above in pH 9 retrieval buffer. After blocking in 20% horse serum, sections were incubated for 1 hour with antibody pairs diluted in serum: 55B11 (1:20, VEGFR-2) and AF349 (1:20, VEGFR-3); 55B11 and IC70A (1:20, CD31); AF349 (1:20) and IC70A (1:20); 55B11 (1:20) and 1A4 (1:1,000, α-SMA); AF349 (1:20) and 1A4 (1:1,000); and IC70A (1:20) and 102-PA50AG (1:60). Appropriate secondary
antibody pairs combined 1:800 in serum were added for 30 minutes: 55B11 was detected with donkey anti-rabbit IgG conjugated to Alexa Fluor 555 or Alexa Fluor 488 (A31572 or A21206; Molecular Probes); AF349 with donkey anti-goat IgG Alexa Fluor 555 (A21432; Molecular Probes); NP056 with donkey anti-mouse IgG Alexa Fluor 488 or Alexa Fluor 555 (A21202 or A31570; Molecular Probes); 1A4 with donkey anti-mouse IgG Alexa Fluor 488; and 102-PA50AG with donkey anti-rabbit IgG Alexa Fluor 488. Sections were counterstained with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (P36931; Molecular Probes), and fluorescent images were scanned and captured using a MIRAX scan (Carl Zeiss).

**Image analysis and pathology review**

The mean number of VEGFR-2- and CD31-positive vessels per mm² viable tumor for each xenograft tumor model was quantified as described previously (39). The percentage of VEGFR-2-positive vessels per tumor model was calculated as follows: number VEGFR-2 vessels per mm²/number of CD31 vessels per mm² × 100. All human tissue sections were scored by a pathologist using a light microscope. A subjective reporting procedure was implemented, recording location (tumor cell, normal epithelium and vessel) and intensity [(– (none), + (weak), ++ (medium), and +++ (high)] of VEGFR-2, VEGFR-3, and CD31 staining.

**Statistical analysis**

For each tissue sample, the maximum VEGFR-2 or VEGFR-3 staining intensity was recorded on an ordinal scale (0, +, ++, +++) separately for the epithelium and endothelium in each sample where possible. A Wilcoxon signed-rank test was used to compare the relative intensity of staining between (a) tumor epithelium and endothelium in a sample and (b) tumor and normal endothelium for matched pairs of tumor and normal tissue. The Bonferroni-Holm procedure was used to account for the multiple tests being carried out in several tissue types and to control the family-wise error rate at 0.05 for each of VEGFR-2 and VEGFR-3. Those tests where the null hypothesis (of no difference between groups) would be rejected are highlighted in Table 1. The proportion of samples with positive VEGFR staining (+, ++, or ++++) was also estimated in each tissue type, and an exact 95% binomial confidence interval was given.

**Results**

**Evaluation of VEGFR antibodies**

A total of 12 antibodies, raised to either human VEGFR-2 (n = 8) or VEGFR-3 (n = 4), were selected for evaluation based on the supplier’s recommendation and/or a literature precedence for their use in immunohistochemistry (Supplementary Table S1). Of the eight VEGFR-2 antibodies tested, only 55B11 was VEGFR-2 specific and qualified for use in immunohistochemistry (Supplementary Table S1). By Western blot analysis, 55B11 detected a 230-kDa doublet band indicative of fully and partially glycosylated VEGFR-2 (40) in NIH-3T3 and PAE cells engineered to overexpress human VEGFR-2 but detected nothing in the untransfected parental cell lines (Fig. 1A). A band ∼150 kDa was also detected in VEGFR-2–overexpressing PAEs, which is likely to be unglycosylated VEGFR-2 protein (40). These data were confirmed by immunocytochemical analysis of FFPE parental and VEGFR-overexpressing PAE cells (Fig. 1B). 55B11 did not cross-react with VEGFR–related human kinases [VEGFR-1, VEGFR-3, platelet-derived growth factor receptor-β (PDGFR-β), c-Kit, or CSF1R] as determined by Western blot analysis of PAE and NIH-3T3 cells engineered to overexpress human VEGFR-1 (150 kDa), VEGFR-3 (75/120/195 kDa), or CSF1R (130/150 kDa) and human aortic VSMCs and M-07e, which express high levels of endogenous PDGFR-β (180 kDa; ref. 41) and c-Kit (145 kDa; ref. 42), respectively (Fig. 1A). Furthermore, 55B11 cleanly detected a 230-kDa VEGFR-2 doublet band by Western blotting in more complex heterogeneous protein lysates (e.g., human cancer xenograft lysates; Fig. 2C).

In our hands, the VEGFR-2 antibodies 55B11, A3, sc-315, and sc-304 commonly used to investigate the expression of VEGFR-2 in human tumors (18–24) did not qualify as specific. All three detected multiple bands, running at different sizes that corresponded to the expected expression of VEGFR-2, in lysates prepared from cell lines expressing VEGFR family members (Fig. 1A) and tumor xenografts and matched parental tumor cell lines (Fig. 2C). Because cross-reactivity of these antibodies with other proteins could potentially confound any analysis of VEGFR-2 expression by immunohistochemistry, they were not used in the analysis of human tumor samples.

Of the four antibodies evaluated, both AF349 and 9D9F9 were specific for VEGFR-3 and suitable for immunohistochemistry (Supplementary Table S1). AF349 was selected for further analyses over 9D9F9, as this was raised in goat and was therefore more suitable for use in coimmunofluorescence experiments with mouse monoclonal antibodies to CD31 and α-SMA. AF349 detected a 195-kDa protein, representing immature (nonproteolytically cleaved) VEGFR-3, in VEGFR-3–overexpressing PAE and NIH-3T3 cells, which was absent from the parental cell lines (Fig. 1A). Bands running at approximately 120 and 75 kDa were also detected in VEGFR-3–overexpressing PAEs. These represent the denatured mature protein, as fully processed mature VEGFR-3 consists of 120- and 75-kDa proteins linked by a disulfide bridge (43). In addition, AF349 did not cross-react with VEGFR-3–related kinases VEGFR-1, VEGFR-2, PDGFR-β, c-Kit, or CSF1R (Fig. 1A). The specificity of AF349 for VEGFR-3 was confirmed by immunocytochemical analysis of engineered PAE cells (Fig. 1B).

**VEGFR-2 expression in human tumor xenografts grown in immunocompromised mice**

TMAs were generated from 29 histologically diverse human tumor xenografts grown to ~1 cm³. Serial sections
Table 1. Maximum VEGFR-2 and VEGFR-3 histopathology intensity scores for human tumors

<p>| Tissue                     | VEGFR-2 | | VEGFR-3 | | | |
|----------------------------|---------|------------|---------|------------|---------|------------|---------|------------|---------|------------|---------|------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>T/N Endo</th>
<th>T cells/ N Epi</th>
<th>Endo positives</th>
<th>T Endo vs. T cell (P)</th>
<th>T Endo vs. N Endo (P)</th>
<th>Prop +ve</th>
<th>Exact 95% CI</th>
<th>Prop +ve</th>
<th>Exact 95% CI</th>
<th>Prop +ve</th>
<th>Exact 95% CI</th>
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<tr>
<td>Bladder cancer*</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>0.0020</td>
<td>(0.692-1.000)</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BC*</td>
<td>36</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>18</td>
<td>14</td>
<td>0.0001</td>
<td>(0.813-0.993)</td>
<td>31</td>
<td>1</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>CRC*</td>
<td>41</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>15</td>
<td>24</td>
<td>0.0001</td>
<td>(0.914-1.000)</td>
<td>41</td>
<td>1</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
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<td>11</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>11</td>
<td>0.0010</td>
<td>(0.715-1.000)</td>
<td>11</td>
<td>2</td>
<td>5</td>
<td>4</td>
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<td>Liver cancer*</td>
<td>22</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>22</td>
<td>0.00001</td>
<td>(0.546-0.922)</td>
<td>21</td>
<td>1</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
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<td>90</td>
<td>1</td>
<td>4</td>
<td>46</td>
<td>39</td>
<td>90</td>
<td>0.0001</td>
<td>(0.940-1.000)</td>
<td>90</td>
<td>4</td>
<td>9</td>
<td>34</td>
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<td>9</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>0.0039</td>
<td>(0.664-1.000)</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>5</td>
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<tr>
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<td>3</td>
<td>1</td>
<td>8</td>
<td>6</td>
<td>18</td>
<td>0.0001</td>
<td>(0.586-0.964)</td>
<td>15</td>
<td>1</td>
<td>6</td>
<td>6</td>
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<td>5</td>
<td>5</td>
<td>8</td>
<td>20</td>
<td>0.0001</td>
<td>(0.683-0.988)</td>
<td>16</td>
<td>0</td>
<td>4</td>
<td>6</td>
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<td>7</td>
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<td>12</td>
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<td>4</td>
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<td>3</td>
<td>3</td>
<td>13</td>
<td>19</td>
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<td>(0.824-1.000)</td>
<td>17</td>
<td>1</td>
<td>1</td>
<td>6</td>
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<tr>
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<td>1</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>0.0078</td>
<td>(0.631-1.000)</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>0.0039</td>
<td>(0.664-1.000)</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BC‡</td>
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<td>1</td>
<td>6</td>
<td>22</td>
<td>55</td>
<td>84</td>
<td>0.0039</td>
<td>(0.935-1.000)</td>
<td>56</td>
<td>5</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
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<td>10</td>
<td>17</td>
<td>20</td>
<td>3</td>
<td>50</td>
<td>0.80001</td>
<td>(0.663-0.900)</td>
<td>50</td>
<td>17</td>
<td>17</td>
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<tr>
<td>CRC‡</td>
<td>65</td>
<td>0</td>
<td>2</td>
<td>34</td>
<td>29</td>
<td>65</td>
<td>0.0001</td>
<td>(0.945-1.000)</td>
<td>40</td>
<td>0</td>
<td>1</td>
<td>13</td>
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<tr>
<td>Matched normal colorectal‡</td>
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<td>0</td>
<td>4</td>
<td>28</td>
<td>13</td>
<td>33</td>
<td>0.0059</td>
<td>(0.894-1.000)</td>
<td>33</td>
<td>7</td>
<td>13</td>
<td>9</td>
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<tr>
<td>NSCLC‡</td>
<td>31</td>
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<td>0</td>
<td>9</td>
<td>22</td>
<td>31</td>
<td>0.0001</td>
<td>(0.888-1.000)</td>
<td>31</td>
<td>0</td>
<td>3</td>
<td>15</td>
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<tr>
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<td>31</td>
<td>10</td>
<td>16</td>
<td>5</td>
<td>0</td>
<td>31</td>
<td>0.0001</td>
<td>(0.846-0.833)</td>
<td>31</td>
<td>7</td>
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</tbody>
</table>

NOTE: VEGFR-2 and VEGFR-3 expression levels were scored in tumor endothelium (T Endo), normal endothelium (N Endo), tumor cells (T cells), and normal epithelium (N Epi), where + = weak, ++ = medium, +++ = high. The highest intensity score was used when a sample exhibited a range of expression levels. n = number of unique samples in each group.

*Data were generated from TMA.
†Statistically significant differences according to the Bonferroni-Holm procedure.
‡Data were generated from whole tumor and matched normal samples.
were evaluated by immunohistochemistry for murine CD31 and murine and human VEGFR-2 using 55B11. As determined by CD31, murine vessels were associated with all xenograft tumors. VEGFR-2 localized to a subpopulation of murine intratumoral vessels but could not be detected in any of the human xenograft tumor cells (Fig. 2B). CD31 and VEGFR-2 microvessel density analysis was done for each tumor, and the percentage of VEGFR-2-positive tumor vessels was calculated (Fig. 2A). Less than 50% of the supporting CD31-positive vasculature was VEGFR-2 positive for the majority of tumor xenograft models. The respective presence and absence of VEGFR-2 in tumor vasculature and tumor cells were confirmed by comparing the receptor expression in a selection of five xenograft tumor

Fig. 1. Specificity of VEGFR-2 and VEGFR-3 antibodies. A, the specificity of antibodies raised to VEGFR-2 (55B11, sc-315, sc-504, and sc-6251) and VEGFR-3 (AF349) was determined by Western blot analysis of protein extracts from PAE and NIH-3T3 cells engineered to overexpress VEGFR-1, VEGFR-2, VEGFR-3, and CSF1R and cells expressing endogenous PDGFR-β (VSMC) and c-Kit (M-07e). B, 55B11 and AF349 were evaluated by immunocytochemical analysis of FFPE PAE cells engineered to overexpress VEGFRs.
Fig. 2. VEGFR-2 expression in human tumor xenograft models. TMAs representing 29 different xenograft models of human tumors (n = maximum 12 cores per model) were analyzed by immunohistochemistry for VEGFR-2 and CD31. A, using an ACIS II at ×10 magnification, the mean CD31+ and VEGFR-2+ vessel number per mm² of viable tumor (microvessel density [MVD]) and the ratio of VEGFR-2+ to CD31+ vessels for each model was calculated and plotted. B, typical VEGFR-2 immunostaining of tumor vasculature in xenograft models. C, evaluation of VEGFR-2 antibodies 55B11, A3, sc-315, and sc-504 by Western blot analysis of xenograft tumor lysates compared with their matched parental cell lines.
lysates [HT-29 and Colo-205 (colon), A549 and PC-9 (lung), and MCF-7 (breast)] compared with their matched parental cell lines (Fig. 2C). The 230-kDa VEGFR-2 doublet was present in each of the xenograft models tested but could not be detected in the parental cell lines, suggesting that the receptor was present in murine stroma but not in the human tumor. Moreover, VEGFR-2 levels in xenograft tumors paralleled that of CD31, indicating a vascular association.

The antibody AF349 reacts with only human VEGFR-3 and was therefore used to assess whether VEGFR-3 was expressed in human tumor xenografts. Using AF349, VEGFR-3 could not be detected in any of the human xenograft tumor cells (data not shown).

Quality assessment of human archival tumor tissue
To ensure that only suitable archival FFPE human tissues was used for immunohistochemical analyses, tissue samples were subjected to a series of morphologic (H&E) and immunohistochemical (CD31 and p-Tyr) checks to determine tissue quality and the extent of antigen preservation, respectively. Morphologic assessment of H&E sections for each specimen was done by a pathologist. CD31 and total protein tyrosine phosphorylation immunostaining were examined to assess antigen preservation in the vasculature and general preservation of phosphorylated proteins, respectively. Specimens with insufficient material, poor morphology, or poor antigen preservation were not used in this study (14% of cases).

Expression of VEGFR-2 and VEGFR-3 in human primary solid tumors
TMAs were constructed from archival whole tissue samples representing 13 different human primary solid tumor types: bladder, breast, colorectal, head and neck, liver, lung, skin, ovarian, pancreatic, prostate, renal, stomach, and thyroid cancers (3 × 0.6-mm-diameter cores per tumor sample). Serial sections from the TMAs were analyzed by immunohistochemistry for CD31, VEGFR-2, and VEGFR-3. Both VEGFR-2 and VEGFR-3 were found on intratumoral vasculature; depending on tumor morphology, vessels were either embedded within tumor and/or present in stroma adjacent to tumor (Table 1; Fig. 3A and B). Neither receptor was detected on malignant cells in all 13 tumor types. Differential localization of the receptors between tumor vessels and...
malignant cells was significant for all tumor types ($P < 0.05$; Table 1).

**VEGFR-2 and VEGFR-3 distribution in human primary colorectal, breast, and lung cancers compared with matched normal tissue**

Archival whole samples representing CRC (adenocarcinoma and Dukes’ A-D), BC (ductal, lobular, and mucinous carcinomas, stage I-III), and NSCLC (squamous cell carcinoma and adenocarcinoma, stage I-IV) and morphologically normal matched adjacent tissues were analyzed by immunohistochemistry for CD31, VEGFR-2, and VEGFR-3 expression (Table 1).

Although not precise, vessels having thick walls and containing erythrocytes are more likely to be blood vessels (BEC), whereas those typified by thin walls and a lack of RBCs are more likely to be of lymphatic origin (LEC). Weak to medium VEGFR-2 expression was observed primarily on putative BECs, supporting mucosa (+) and submucosa (+ to ++) in 33 matched normal colorectal samples (Fig. 4A). VEGFR-2 expression on putative lymphatic vessels in the submucosa was either absent or weak.

VEGFR-2 was localized to intratumoral vasculature, primarily with a blood vessel morphology, but was not detected on malignant cells in all 65 CRC samples analyzed (Fig. 4A). The vascular signal intensity of VEGFR-2 was higher in tumor compared with normal tissue ($P = 0.0059$; Table 1). In normal colon, VEGFR-3 was most prominently expressed (+/++) on the endothelium of vessels likely to be of lymphatic origin, whereas expression on putative BECs was generally weak (+) or absent (Fig. 4A). VEGFR-3 was confined to tumor vasculature in all CRCs analyzed ($n = 40$, ++ to +++), with the signal intensity significantly greater than that of normal vasculature ($P < 0.0001$; Table 1). VEGFR-3 tumor cell expression was not evident in any CRC.

A VEGFR-2 or VEGFR-3 signal was absent from many CD31+ vessels supporting normal breast tissue (Table 1; Fig. 4B). When vascular expression for either receptor was observed, this was weak-medium (+/++) in intensity (Table 1). VEGFR-2 and VEGFR-3 were expressed on intratumoral vasculature, morphologically resembling blood vessels, in all BCs analyzed ($n = 84$ and 56, respectively). VEGFR-2 signal intensity was generally greater than VEGFR-3 being strong (+++) compared with medium (+; Table 1). The signal intensity for both receptors was significantly greater on vasculature associated with malignant breast tissue than that from matched normal tissue ($P < 0.0001$; Fig. 4B). Similar to colon cancers, no tumor cell expression was observed in any of the cases.

Despite the high blood vessel content of normal lung, as determined by CD31 analysis, VEGFR-2- and VEGFR-3-positive vessels were mainly absent or rare in 31 matched normal samples (occasional weakly positive alveoli capillary; Table 1; Fig. 4C). Stronger VEGFR-2 signal was observed in neovasculature around lymphoid aggregates in five normal samples. Both receptors were expressed on intratumoral vasculature (++/+++)) but not on tumor cells in 31 NSCLCs, with signal levels significantly greater on tumor vessels compared with normal vasculature ($P < 0.0001$; Table 1; Fig. 4C). Specific VEGFR-2 and VEGFR-3 expression was not detected in malignant lung cells.

**Relative vascular localization of VEGFR-2 and VEGFR-3 in primary CRC and BC**

To confirm the endothelial cell localization of VEGFR-2 and VEGFR-3, serial sections from six CRC (adenocarcinoma and Dukes’ A-C) and six BC (ductal carcinoma, grade 1-3) samples were immunofluorescently costained for VEGFR-2 (red) or VEGFR-3, (red) in combination with CD31 (green) and α-SMA (green). Both VEGFR-2 and VEGFR-3 were localized to CD31+ endothelial cells but were absent from α-SMA+ pericytes associated with tumor blood vessels (Fig. 5A). The distinction between BEC and LEC based on vessel morphology alone is subjective, so tumor vessels were characterized further by combining pan–endothelial cell marker, CD31, and LEC marker, LYVE-1, in coimmunofluorescence analyses of CRC and BC samples ($n = 6$ each). CD31+ LYVE-1− and CD31+ LYVE-1+ intratumoral vessels were classified as lymphatic and blood, respectively. A section, serial to each CD31/ LYVE-1 section, was analyzed for VEGFR-2/VEGFR-3 and compared with the former to determine the relative blood and lymphatic vascular localization of VEGFR-2 and VEGFR-3.

In CRC, intratumoral vasculature could be subclassified into CD31+ LYVE-1− (blood vessels, gray arrows in Fig. 5B) and less abundant CD31+ LYVE-1+ (lymphatic vessels, white arrows). VEGFR-2 was present on both types of vasculature, although the VEGFR-2 signal was generally weaker for CD31+ LYVE-1− vessels than for CD31+ LYVE-1− (Fig. 5B, blue arrows). VEGFR-3 was localized predominantly to intratumoral lymphatic vessels (Fig. 5B, white and blue arrows) but was also observed on the occasional small CD31+ LYVE-1− vessel. In addition, VEGFR-2 was expressed on vessels transporting erythrocytes, whereas VEGFR-3 was not. In BC, all intratumoral vessels were CD31+ LYVE-1− and were likely to be blood vessels (Fig. 5C). Although intratumoral lymphatic vessels (CD31+ LYVE-1+) could not be detected, peritumoral lymphatics (distal to tumor in normal stroma) were detected in one larger sample. A subpopulation of CD31+ LYVE-1+ vessels was also VEGFR-2+ and, to a lesser extent, VEGFR-3+, indicating that both receptors are expressed on a subset of blood vessels in BC (Fig. 5C, white arrows). Indeed, both VEGFR-2 and VEGFR-3 were found on breast tumor vessels carrying RBCs.

**Discussion**

Published literature presents conflicting reports to the extent and localization of VEGFR-2 and VEGFR-3 expression in human tumors. Although some studies confine
Fig. 4. VEGFR-2 and VEGFR-3 expression in human primary colorectal, lung, and breast cancers compared with matched normal tissue. Archival whole colorectal (adenocarcinoma), lung (squamous cell carcinoma and adenocarcinoma), and breast (ductal, lobular, and mucinous carcinoma) cancer samples and matched morphologically normal tissues were immunostained for CD31, VEGFR-2, and VEGFR-3, and vascular and tumor cell staining was scored by a histopathologist (Table 1). Typical VEGFR-2, VEGFR-3, and CD31 immunostains of colorectal adenocarcinoma (CRC), well-moderately differentiated Duke’s B (A), breast ductal carcinoma, stage I (B), and lung adenocarcinoma (C) and their respective matched normal tissues. A, CD31 is strongly expressed on the majority of vasculature supporting normal and malignant tissue. VEGFR-2+ superficial capillaries in normal colorectal mucosa and VEGFR-2+ vessels associated with CRC. Black arrow, putative VEGFR-3+ lymphatic vessel; white arrow, VEGFR-3− blood vessel containing erythrocytes. Typical VEGFR-3 expression in CRC vessels. B, VEGFR-2+ vessels in normal lung. C, despite the presence of a dense capillary network in normal lung, VEGFR-2− and VEGFR-3− positive capillaries are rare (black arrows). In contrast, strong VEGFR-2 and VEGFR-3 vascular expression is associated with lung adenocarcinomas.
one or the other receptor to tumor vessels (15, 16, 24–31), the majority report expression in malignant cells (18–25, 32–37). Antibodies from a single source have been used to produce the majority of data, suggesting that tumor cells express VEGFR-2. In our hands, these behave nonspecifically in multiple platforms, which leads us to conclude that the high frequency of tumor cell staining observed with these antibodies may be due to cross-reactivity with nonspecific protein antigens. Similarly, the majority of studies reporting VEGFR-3 expression in tumor cells use the same antibodies, and Petrova et al. (38) have shown that the VEGFR-3 antibody sc-321, used in some of these reports, is also nonspecific.

We have provided evidence to show that antibodies 55B11 and AF349 are specific for VEGFR-2 and VEGFR-3, respectively. Using these validated antibodies in combination with quality-controlled archival tissues, we have shown for a wide range of common human primary solid cancers ($n > 400$, bladder, breast, colorectal, head and neck, liver, lung, skin, ovarian, pancreatic, prostate, renal, stomach, and thyroid cancers) that VEGFR-2 and VEGFR-3 are primarily localized to tumor vasculature and cannot be detected on malignant cells. We cannot discount the possibility that there would be incidences of tumor cell expression for either receptor when larger sample cohorts or other tumor types are analyzed, but our data indicate that this occurrence is generally much lower than previously reported.

Given the data presented herein, we suggest that 55B11 should be used more widely to determine the VEGFR-2 status of human tumors. There is literature precedence for the use of AF349, and our findings are in accordance with the majority of publications using this antibody (29, 44–46). However, there are reports suggesting that AF349 also detects malignant cells in addition to tumor vessels (32, 47, 48). In addition to antibody specificity, other
We used vascular markers CD31 and LYVE-1 to better classify blood and lymphatic vessels, respectively, and confirm our original morphologic observations that CRC and BC differ with respect to localization of VEGFR-2 and VEGFR-3 on these two vascular subpopulations. Our data indicate that in CRC, VEGFR-2 is present on both intratumoral blood and lymphatic vessels, whereas VEGFR-3 is predominantly localized to lymphatic vessels. In BC, intratumoral lymphatic vessels are scarce and we have shown that both VEGFR-2 and VEGFR-3 are localized to blood vessels. This vascular localization pattern combined with in vivo functional data implicating VEGFR-3 in sprouting angiogenesis (8) suggests that VEGFR-3, in combination with VEGFR-2, may have a stronger angiogenic role in BC than CRC. In addition, the relative balance of the two receptors between blood and lymphatic vessels could influence the tumor response to VEGF therapy and warrants further investigation.

Our data may also provide some insights into the mechanism of action of VEGF signaling inhibitors in the clinic. Tumor endothelial cells, both lymphatic and blood, and not tumor cells are the likely direct cellular targets for anti–VEGFR-2 and anti–VEGFR-3 therapy in many primary solid tumors. The upregulation of VEGFR-3 on blood vessels suggests that dual VEGFR-2/VEGFR-3 inhibitors may have additional antiangiogenic benefits. This and in vivo findings indicating that VEGFR-3 can sustain low-level angiogenesis even in the presence of VEGFR-2 inhibitors (8) suggest that tumors expressing high levels of VEGFR-3 on blood vessels may be less sensitive to VEGFR therapies that target VEGFR-2 alone.

An accurate understanding of the expression levels and localization of drug targets in disease is important for elucidating the mechanism of action of drugs in the clinic and, potentially, for identifying patients who would gain most benefit from treatment with VEGFR signaling inhibitors. Because immunohistochemical methodology has many potential pitfalls and confounding factors, our work suggests that greater antibody validation and tissue quality control measures than has hitherto generally been the case would give greater confidence in immunolocalization results for VEGFR-2 and VEGFR-3 in clinical samples.

Disclosure of Potential Conflicts of Interest

All authors are full-time employees of AstraZeneca and hold shares in the company.

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