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Heterogeneity of Vascular Endothelial Growth Factor Receptors 1, 2, 3 in Primary Human Colorectal Carcinoma

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Abstract. *Background: The vascular endothelial growth factor (VEGF) pathway plays an important role in growth and progression of human cancer, including colorectal carcinomas (CRC). The key mediators of VEGF signaling are VEGFR1, VEGFR2, and VEGFR3, part of a family of related receptor tyrosine kinases. The relative expression, activity, or interplay among these receptors may determine the response of CRC patients to anti-angiogenic therapies. Materials and Methods: We developed technically sound immunohistochemical (IHC) assays to quantify VEGFR1, 2 and 3, and using a well-annotated CRC tissue microarray (TMA), we carried out comprehensive comparative evaluation of the three VEGFRs in archival primary CRC tissues (n=84). For each TMA core, tumor cell VEGFR1 expression was reported as H-score (range=0-300); vascular VEGFR2/VEGFR3 expression was manually scored as the number of receptor-positive tumor stromal vessels. Each case was defined as VEGFR1/ VEGFR2/VEGFR3-negative, low, medium or high. Results: Based on the differential expression of the three VEGFRs, eight VEGFR staining profiles were observed: Triple VEGFR positive (n=12, 14%), VEGFR1 predominant (n=17, 20%), VEGFR2 predominant (n=7, 8%), VEGFR3 predominant (n=1, 1%), VEGFR1/2 predominant (n=39, 46%), VEGFR1/3 predominant (n=2, 2%), VEGFR2/3 predominant (n=3, 4%), and triple-VEGFR-negative (n=3, 4%). Conclusion: Herein we demonstrated heterogeneity of expression of VEGFRs in human CRC stromal vessels and tumor cells. The observed VEGFR expression-based subsets of human CRCs may reflect differences in biology of pathologic angiogenesis in primary CRC tissues. Furthermore, the heterogeneity of expression of VEGFRs unraveled in this analysis merits independent validation in larger cohorts of primary and metastatic human CRC tissues and in pertinent experimental models treated with various anti-angiogenic therapies.*

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Angiogenesis is the process of new blood vessel formation from a pre-existing vascular bed, and is essential for the growth of primary tumors and their metastases. Successful treatment of established human tumors may not only require inhibition of further angiogenesis but also regression of the existing tumor vessels to reduce the existing tumor mass (1). Biologically, tumor angiogenesis is a highly regulated and complex process involving a variety of pro-angiogenic (VEGFs, fibroblast growth factors, platelet-derived growth factors, insulin-like growth factors and transforming growth factors) and anti-angiogenic (thrmobospondin-1, angiostatin and endostatin) factors (2).

Vascular endothelial growth factor-A (VEGF-A) plays important roles in vascular development and in diseases involving irregular growth of blood vessels. VEGF-A is also involved in the development of lymphatic vessels and disease-related angiogenesis. While VEGF-A is required to sustain newly-formed (immature) blood vessels, this survival factor is dispensable for the mature vasculature (3). Pathological angiogenic and lymphangiogenic signaling is largely mediated by three vascular endothelial growth factor receptors (VEGFRs) 1, 2 and 3. Among these, VEGFR2 is the predominant mediator of VEGF-A signaling to regulate angiogenesis. Interaction between VEGF-A and VEGFR2 results in activation of a number of intracellular signals that result in endothelial cell survival, proliferation, migration, differentiation and increased vascular permeability (4-6). VEGFR1 is a receptor for VEGF-A in addition to VEGF-B and placental growth factor and is a potent positive regulator of physiologic and developmental angiogenesis and, like VEGFR2, is also thought to be important for endothelial cell migration and differentiation (7, 8). VEGFR1 has been shown to be present and functional on CRC cells and its activation by VEGF ligands can activate processes involved in tumor progression and metastases (9). VEGFR3 binds with highest

Table I. *Clinicopathological characteristics of colonic carcinoma cases subjected to VEGFR1, 2 and 3 profiling (n=85).*

Category	Subcategory	Value
Age	Years	31-89 (mean=68)
Gender	Male	48 (56.5%)
	Female	37 (43.5%)
Histopathological subtype	Adenocarcinoma (ADC)	80 (94.1%)
	Mucinous ADC, grade 2	1 (1.25%)
	ADC with signet ring cell areas	1 (1.25%)
	Squamous cell carcinoma	1 (1.25%)
	Squamous cell carcinoma with basaloid features	1 (1.25%)
	Mixed carcinoma (ADC and squamous cell carcinoma with basaloid features)	1 (1.25%)
Histologic Grade	1	14 (16.5%)
	2	59 (69.4%)
	3	12 (14.1%)
Stage (AJCC 2006)	II	21 (24.7%)
	III	18 (21.2%)
	IV	46 (54.1%)

affinity to VEGF-C and VEGF-D and is a mediator of lymphangiogenesis. Its expression is largely restricted to lymphatic vessel and endothelial tip cells and has been associated with the dissemination of tumor cells to regional lymph nodes (10-12). Of the three VEGFRs, VEGFR2 is globally expressed by vascular endothelial cells, while VEGFR1 and VEGFR3 expression is restricted to vascular endothelial cells in distinct locations (13).

The VEGF signaling pathway plays an important role in the growth and progression of human cancer, including colorectal carcinoma (CRC). Inhibition of tumor angiogenesis can prevent the initial growth of primary tumors, reduce the occurrence and growth of metastases and can make conventional chemotherapy agents more effective (14-16). Since VEGFR1, VEGFR2, and VEGFR3 are the key mediators of VEGF signaling, the relative expression, activity or interplay among these receptors may determine the response of CRC patients to various anti-angiogenic therapies. Therefore, using technically sound immunohistochemical assays developed in our laboratory, we designed retrospective analyses of the three VEGF receptors in a well-characterized cohort of primary human CRC tissues with the following aims: i) To characterize the immunohistochemical expression of the three VEGFRs (1, 2 and 3) in archival primary human CRC tissues; and ii) To identify the various subsets of primary human colorectal cancers, based on the observed VEGF receptor profiles.

Materials and Methods

Patients, tissue specimens, and ethics statement. Commercially-available tissue microarrays (TMAs) were purchased from TriStar Technology Group (Rockville, MD, USA) for this study. The acquisition of human tissue samples was guided by TriStar's standard operating procedures and included informed donor consent, IRB/EC approval, patient anonymity, and compliance with international and European Union regulations. Briefly, primary human colorectal carcinoma tissues from 85 adult patients, who had undergone resection of their primary tumors followed by 5-fluorouracil-based chemotherapy regimens with or without bevacizumab, were included in this study. All primary CRC tissues were fixed in neutral buffered formalin and sampled to form the TMA paraffin block (TA1324, TriStar). Pertinent clinico-pathological data is summarized in Table I. Mean patient follow-up time was 40 months (range=8-83 months). Based on independent and blinded review of the evaluable CRC tissue in each TMA core, the submitted histopathologic diagnoses and histologic grades (well, moderately and poorly-differentiated) were independently confirmed or revised by an American Board-certified anatomic pathologist with subspecialty expertise in gastrointestinal pathology (AN). For each case staging information was provided by the contributor, using the American Joint Committee on Cancer (AJCC)-the Union Internationale Contre Le Cancer (UICC)-TNM staging system (17), as summarized in Table I.

Cell culture and processing of cell lines. H441 (lung adenocarcinoma, papillary), HUVEC (endothelial) and HeLa (cervix adenocarcinoma) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All media were supplemented to include 1% (v/v) penicillin/streptomycin and 10% fetal bovine serum (FBS). H441 cells were expanded using RPMI-1640 with 10 mM HEPES, and 1 mM sodium pyruvate. HUVEC cells were propagated with F-12K medium adjusted to contain 0.04 mg/ml endothelial cell growth supplement (ECGS) and 0.1 mg/ml heparin. HeLa cells were grown using Eagle's Minimum Essential Medium with Earle's Balanced Salt solution supplemented with both 0.5 mM sodium pyruvate and 1% non-essential amino acids. All cells were propagated at 5% CO_2 and 37˚C, harvested by incubation in 0.25% trypsin with 0.53 mM EDTA at 37˚C, and then collected by pipetting. Cells were pooled and aliquoted for western blotting by pelleting and lysing as described below, or for paraffin embedding by fixation in 10% neutral buffered formalin (NBF) for 24 h. Histotechnological preparation of cells was performed as described previously (18).

Western blots. Whole-cell extracts were prepared by re-suspending in protease and phosphatase inhibitor-supplemented RIPA buffer (Thermo Scientific, Waltham, MA, USA). Samples were combined with loading buffer containing dithiothreitol (DTT) and sodium dodecyl sulfate (SDS), held at 95˚C for 3 min, and then separated on NuPage 4-12% bis-tris polyacrylamide gels (Thermo Scientific). Recombinant VEGFR1 protein (NP_002010.2, a.a. 27–656, rECD) was expressed in a baculovirus system then purified on Ni-NTA (nitrilotriacetic acid) affinity and size exclusion chromatography columns. Recombinant protein was added as a control. Gels were transferred to nitrocellulose membranes and probed with an anti-VEGFR1 primary antibody (Abcam [Epitomics], Burlingame, CA, USA; 1303-1) overnight at 4˚C with agitation. Blots were incubated with species-specific, HRP-conjugated secondary antibodies for 1 h then visualized using enhanced chemiluminescence detection (Pierce, Thermo). Anti-GAPDH primary antibody (CST, clone 14C10) was used to verify protein loading on gels. Primary antibodies were applied at a 1:1,000 working dilution.

Immunohistochemical assays for VEGFR1, VEGFR2, VEGFR3. We developed and optimized immunohistochemical assays for evaluation of VEGFR1, VEGFR2, and VEGFR3 in archival human tissues. Staining methodologies for VEGFR2 IHC (using a rabbit monoclonal IgG, Cell Signaling Technology [CST], Danvers, MA, USA; 55B11) and VEGFR3 IHC (using a mouse monoclonal IgG , Millipore, Billerica, MA, USA; clone 9D9F9) were performed as previously described by our group (18, 19). To detect VEGFR1, optimal FFPE sections of the CRC TMA were cut, dried, and baked at 60˚C prior to staining. Slides were de-paraffinized and rehydrated on the Bond III automated stainer (Leica Biosystems, Buffalo Grove, IL, USA), and antigens were retrieved at 100˚C for 40 min in EDTA-based buffer at pH 9.0 (ER2). Endogenous peroxidases were treated with Peroxide Block (Leica) for 5 min and PowerVision IHC Super Block (Leica) for 10 min. The anti-VEGFR1 rabbit monoclonal IgG (1303-1) was applied at a concentration of 0.08 μg/mL in antibody diluent (Leica), and allowed to incubate for 45 min. Bond Refine HRP (horseradish peroxidase) Polymer (Leica) was applied for 8 min, 3, 3-diaminobenzidine (DAB) substrate solution (Refine Chromogen, Leica) for 10 min, and finally hematoxylin (Leica) was applied for 5 min. Slides were removed from the stainer and dehydrated by sequential submersion in 95% ethanol, 100% ethanol, and xylene on an automated linear stainer following routine process. The slides were then coverslipped using mounting media (Sakura; Torrance, CA, USA).

Controls. For all IHC assays, optimal positive and negative tissue and reagent controls were included in each staining run. Reagent-negative controls with isotype specific IgG were used to assess non-specific staining in the stained tissues.

Pre-absorption experiments (VEGFR1, VEGFR2, VEGFR3). Synthetic peptides were designed for 12- to 24-mer spans of residues near the N-terminus of VEGFR1 (NP_002010.2; Midwest Bio-Tech, Fishers, IN, USA). The diluted anti-VEGFR1 antibody was combined with a 200-fold molar excess of peptides in antibody diluent, and then incubated overnight at 4˚C with rocking before immunohistochemical staining.

Brightfield in situ hybridization (BRISH) for VEGFR1. Dry slides were loaded on the Bond RX, and deparaffinization, rehydration, and pre-treatment using EDTA buffer solution (ER2, pH9, Leica) was performed for 15 min at 100˚C. RNAscope LS reagent kit (Advanced Cell Diagnostics [ACD], Hayward, CA, USA) was used for detection of mRNA. Briefly, protease was applied at 40˚C for 20 min, and then peroxide was applied for 10 min. FLT1 (VEGFR1) RNAscope LS probe 2.0 (ACD) was hybridized for 2 h at 40˚C. The signal amplification steps were performed per manufacturer's instructions for Amp 1-6, with the addition of a 2 × SSC stringency wash performed before Amp 2. DAB and hematoxylin were applied as part of the modified Bond Polymer Refine Detection kit (Leica). Slides were dehydrated and coverslipped as described above.

Immunohistochemical staining for vascular and lymphatic endothelial markers. Serial sections of tissue microarray (TMA) were stained at a CLIA-certified reference laboratory (Clarient, Aliso Viejo, CA, USA) using their well-established IHC staining protocols for the vascular (CD34, QBEnd/10) and lymphatic (podoplanin, D2-40) endothelial markers.

Immunohistochemical scoring of VEGF receptor expression. All stained slides were reviewed and scored by an experienced solid tumor pathologist (AN) with subspecialty expertise in Gastrointestinal Pathology without prior knowledge of the clinico-pathologic characteristics.

VEGFR1 immunoreactivity was reported as an H-score. Briefly, an objective assessment of the intensity of VEGFR1 staining (0, no staining; 1+, weak staining; 2+, moderate staining; 3+, intense staining) was made in viable tumor cells. The percentage of tumor cells staining for each intensity was determined. The value of each staining level (0, 1, 2 or 3) was multiplied by the respective percentage of tumor cells at that intensity level. A total H-score represents the sum of the three scores, reported on a continuous scale of 0-300.

Stromal vessels in viable areas of the tumor tissue that showed unequivocal brown VEGFR2 staining were counted manually. For each case the absolute vessel count was normalized by the number of CD34-positive vessels and reported as VEGFR2 vascular positivity index (VEGFR2-VPI, range=0-100).

VEGFR3-positive vessels were counted in each TMA core and reported as an absolute value. These counts were not normalized since the number of VEGFR3+ vessels in a given TMA core was generally not as high as for VEGFR2+ vessels.

Pathologic classification of primary human colorectal cancers based on VEGF receptor profiles. Based on the level of immunohistochemical/expression of the three VEGF receptors, each primary CRC tissue was categorized as follows: VEGFR1 H-scores were categorized as low/negative (0-50), medium (51-100) or high (>100). VEGFR2-vascular positivity index was categorized as low/negative (≤25), medium (26-49) or high (≥50). VEGFR3-positive vessel counts were categorized as low/negative (≤5), medium (6-10) or high (>10).

Based on relative expression levels of the three VEGFRs, each primary CRC tissue was placed into 1 of 8 subclasses as follows: I) Triple VEGFR positive, with medium or high expression of all 3 VEGFRs; II) VEGFR1 predominant, with medium or high expression VEGFR1 and low or negative expression of VEGFR2 and VEGFR3; III) VEGFR2 predominant, with medium or high expression VEGFR2 and low or negative expression of VEGFR1 and VEGFR3; IV) VEGFR3 predominant, with medium or high expression of VEGFR3 and low or negative expression of VEGFR1 and VEGFR2; V) Mixed VEGFR1/2 predominant, with medium or high expression of VEGFR1 and VEGFR2, and low or negative expression of VEGFR3; VI) Mixed VEGFR1/3 predominant, with medium or high expression

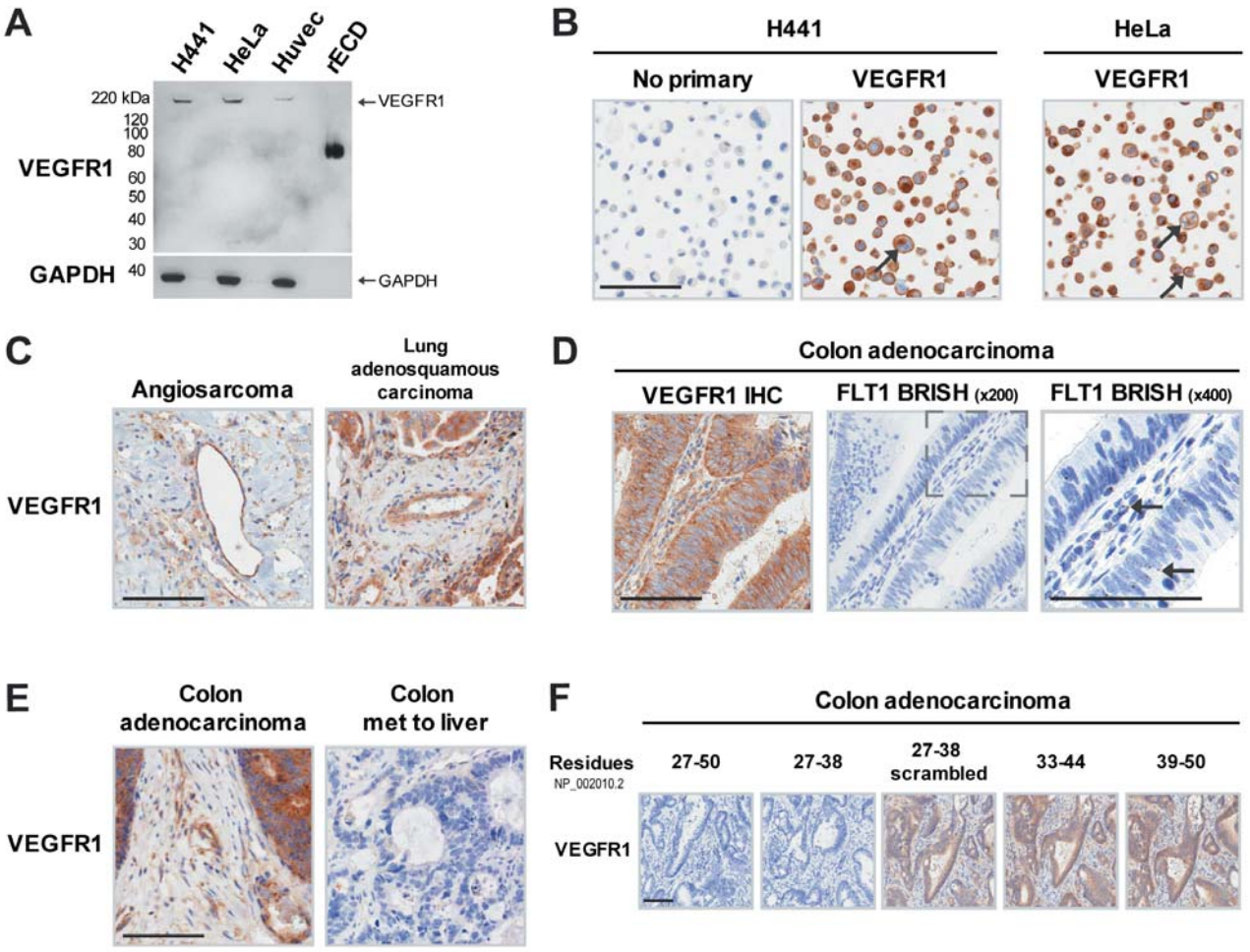

Figure 1. *An optimized IHC assay developed in our laboratory reliably detects VEGFR1 protein levels in FFPE tissues. Immunoblot probed with the anti-VEGFR1 antibody (A) shows a single, discrete band at the expected molecular weight for three different human cell lines. A recombinant protein harboring a region of the extracellular domain of VEGFR1 (rECD) was included as a control. VEGFR1 IHC on FFPE cell pellets (B) shows representative immunoreactivity (brown). No primary, anti-VEGFR1 antibody was substituted with diluent. Black arrows represent membranous accentuation of VEGFR1 staining. VEGFR1 IHC on human tumor specimens shows distinct VEGFR1 immunoreactivity in endothelial cell cytoplasm and confluent immunoreactivity in epithelial tumor cells (C). In human tumor tissue, VEGFR1 IHC staining is consistent with the mRNA localization by in situ hybridization (D). Dashed line in middle panel is magnified in rightmost panel. Black arrows show representative positive cells. VEGFR1 expression showed a range of expression within any given tumor ranging from strong, confluent positive to negative staining, suggesting that cytoplasmic staining is not merely an artifact of FFPE tissue (E). Preabsorption assays suggest that the anti-VEGFR1 antibody used is specific for the immunogen from which it was generated and binds within a 12 residue region within the extracellular domain of VEGFR1 (F). Slides were counterstained with hematoxylin (blue). Original magnification of all IHC panels, ×200. Black scale bars: 100 μm.*

of VEGFR1 and VEGFR3, and low or negative expression of VEGFR2; VII) Mixed VEGFR2/3 predominant, with medium or high expression of VEGFR2 and VEGFR3, and low or negative expression of VEGFR1; and VIII) Triple VEGFR negative, with low to negative expression of all three VEGFRs.

Photomicrography. Representative images of CRC tissues showing immunohistochemical expression of various VEGF receptor expression profiles, podoplanin (D2-40) and CD34 were obtained as high-resolution ×200 digital scans (Scanscope XT; Aperio Technologies, Vista, CA) and were exported as image files to illustrate pertinent pathologic findings at required magnifications (×100 or ×200).

Statistics. Immunoreactivity scores for the three VEGFRs were compared by box plots generated using the Tukey method in Prism (v6.03; GraphPad Software; La Jolla, CA, USA). Unpaired, two-tailed *t*-tests were performed for associations between tumor grades (Grade 1, Grade 2, Grade 3, and Grades 2+3 combined). For VEGFR1 and VEGFR3 scores parametric tests were used. For VEGFR3, a non-parametric test was used. *p*-Values and 95% confidence intervals (CI) were determined.

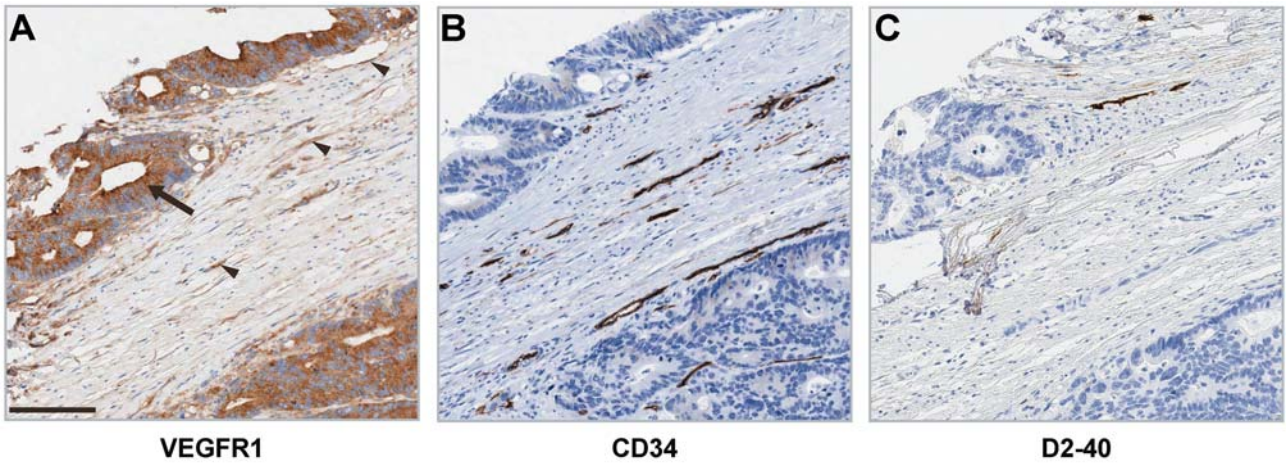

Figure 2. *Representative VEGFR1 IHC in CRC. (A) Invasive colonic adenocarcinoma (Grade 2) showing distinct localization of VEGFR1 immunoreactivity in the neoplastic glandular epithelium (black arrow), and in the tumor stromal vessels (black arrowheads). Most of the vessels showing VEGFR1 immunoreactivity in their endothelial lining are CD34-positive (B), and D2-40-negative (C), suggesting that they are tumor stromal blood vessels. Original images were captured at ×100. Scale bar=100 μm, applicable to all panels.*

Results

Performance verification of VEGFR1 immunohistochemistry (IHC). Following a comprehensive assay development paradigm, we demonstrated the specificity, and appropriate immunoreactivity pattern of VEGFR1 IHC, as was previously reported for VEGFR2 and VEGFR3 IHC assays by our laboratory (18, 19). The rabbit monoclonal IgG was first profiled by immunoblot (Figure 1A). In H441, HeLa, and HUVEC cell lysates, a predominant band was observed at the expected molecular weight of 180 kDa. Representative IHC in FFPE cell lines showed an unequivocal and full range of cytoplasmic localization of VEGFR1 with frequent membranous accentuation (Figure 1B). In archival human angiosarcoma and adenosquamous carcinoma of the lung tissues, VEGFR1 was observed in both the endothelial and epithelial tumor cell cytoplasm (Figure 1C), likely due to detection of soluble VEGFR1 isoforms. VEGFR1 immunoreactivity in the cytoplasm of epithelial tumor cells is consistent with the localization of FLT1 (*VEGFR1*) mRNA in colorectal carcinoma (CRC, Figure 1D). VEGFR1 showed a full range of expression in epithelial tumor cells, including some VEGFR1-negative cases, suggesting that that staining is not merely an artifact of antibody performance in FFPE tissue (Figure 1E). Synthetic peptides harboring extracellular domain residues of VEGFR1 were preabsorbed with the anti-VEGFR1 antibody before application to IHC. Two peptides harboring a 12- or 24-amino acid sequence were able to abolish immunoreactivity in CRC tissues (Figure 1F). A BLAST search of the 12 amino acid sequence that was able to completely block the immunoreactivity, n-SKLKDPELSLKGTQ-c, returned no hits with 100% identity spanning the 12 residue region other than VEGFR1. After confirming the optimal performance of the VEGFR1 IHC assay and demonstrating the specificity of the anti-VEGFR1 antibody used on a known VEGFR1-positive cell line (Figure 1B) and human tissue controls (Figure 1C), including the reagent-negative controls (Figure 1B), we applied our optimized VEGFR1 IHC staining protocol to the larger cohort of CRC tissues in these analyses, along with the VEGFR2 and VEGFR3 IHC staining protocols. In our hands, the performance of the three antibodies used for the three VEGF receptor IHC assays in our laboratory, was found to be reproducible.

Colorectal cancer tissue distribution and subcellular localization of VEGF receptors. This study was carried out on archival primary colorectal cancer tissues from 85 patients whose clinico-pathologic characteristics are summarized in Table I. In the primary CRC tissues, we observed different patterns of distribution of the three VEGF receptors, as follows:

VEGFR1. In the vast majority of CRC tissues evaluated, VEGFR1 immunoreactivity was mainly localized to tumor cell cytoplasm and also in variable numbers of tumor stromal vessels (Figure 2). Overall, 70 of 84 (83.3%) of CRC tissues showed distinct cytoplasmic expression of VEGFR1 in tumor cells, 17 (20.2%) cases also showed distinct VEGFR1 expression in tumor stromal vessels while in 42 (50.0%) cases vascular VEGFR1 immunoreactivity was weak. No vascular VEGFR1 immunoreactivity was found in the remaining 11 (13.1%) cases (Table II). Ten out of 84 (11.9%) cases exhibited low cytoplasmic expression of VEGFR1 in invasive tumor cells, and half of these had negative/low expression in tumor stromal vessels.

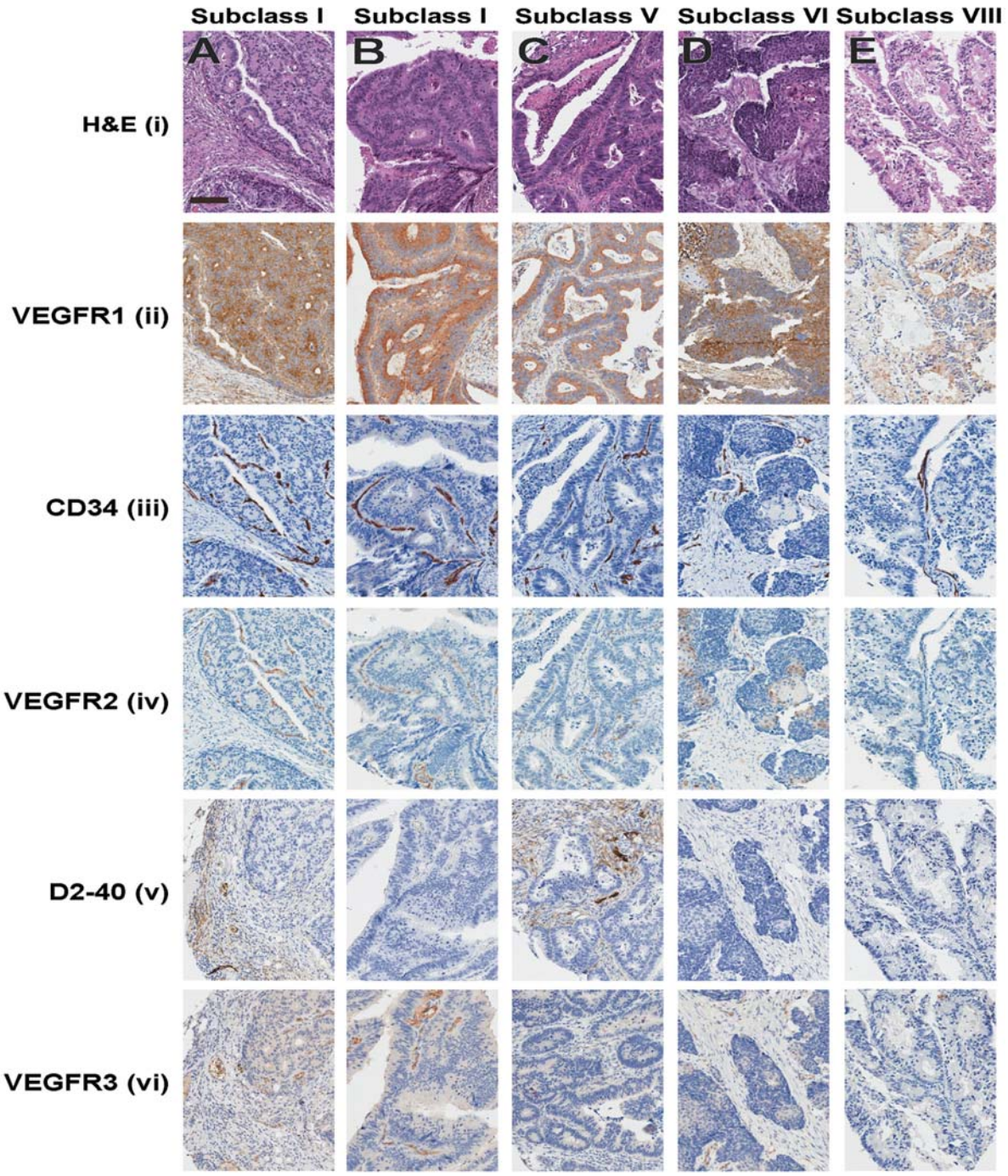

Figure 3. *Histomorphologic and pathologic findings representative of some of the new VEGFR-based subclasses of human colorectal cancer tissues, based on tumor cell expression of VEGFR1 and vascular expression of VEGFR2 and VEGFR3. Invasive colonic adenocarcinoma (A, Case 1H) representing triple VEGFR-positive profile (subclass I) featuring distinct tumor cell staining for VEGFR1 (panel ii) and vascular expression of VEGFR2 (iv) and VEGFR3 (vi). The majority of the tumor stromal vessels outlined by CD34 (iii) and D2-40 (v) is also immunoreactive for VEGFR2 (iv) and VEGFR3 (vi). Another invasive colonic adenocarcinoma (B, Case 3K), representing triple VEGFR-positive profile (subclass I) with findings similar to case A. Invasive colonic adenocarcinoma (C, Case 1F), representing VEGFR1/2-predominant (subclass V) profile. Although several tumor stromal vessels are positive for lymphatic marker, D2-40 (panel v), there is no vascular VEGFR3 expression (vi). Invasive squamous cell carcinoma with basaloid features (D, Case 5H), representing mixed VEGFR1/3 predominant profile (subclass VI). There is distinct tumor cell positivity for VEGFR1 (panel ii) and vascular positivity for VEGFR3 (vi). Invasive colonic adenocarcinoma (E, Case 3A), representing triple-VEGFR-low-negative profile (subclass VIII), based on negative or low expression of all three VEGF receptors (panels ii, iv, and vi). Slides were counterstained with hematoxylin. Images captured at original magnification of ×200. Scale bar=100 μm, applicable to all panels.*

Case	VEGFR1 tumor cell H-score	VEGFR2 VPI	VEGFR3+ vessels	Subclass
1A	60	54.9	2	V
1B	35	26.9	3	III
1C	165	42.1	4	V
1D	100	25.0	5	II
1E	40	56.0	1	III
1F	85	36.2	5	V
1G	60	5.8	0	II
1H	100	64.9	24	I
1I	165	33.3	11	I
1J	95	no data	2	II
1K	70	no data	0	II
1L	75	37.8	2	V
1M	30	no data	13	IV
1N	no data	0.0	0	VIII
1O	195	0.0	0	II
1P	110	30.3	0	V
1Q	75	38.5	1	V
3A	15	0.0	0	VIII
3B	35	34.1	0	III
3C	90	75.0	0	V
3D	100	37.9	1	V
3E	90	61.8	0	V
3F	100	no data	0	II
3G	75	5.2	0	II
3H	130	27.3	3	V
3I	100	20.7	0	II
3J	no data	no data	no data	na
3K	140	50.0	11	I
3L	50	38.8	8	VII
3M	125	32.4	0	V
3N	85	38.8	2	V
3O	100	2.9	0	II
3P	75	35.6	2	V
3Q	80	33.8	1	V
5A	75	45.7	5	V
5B	80	26.8	0	V
5C	90	37.5	0	V
5D	60	0.0	0	II
5E	120	69.6	1	V
5F	140	90.9	0	V
5G	80	25.0	0	II
5H	120	25.0	17	VI
5I	100	49.2	2	V
5J	80	38.9	0	V
5K	70	23.8	2	II
5L	130	85.7	3	V
5M	70	37.9	0	V
5N	60	66.7	7	I
5O	130	76.9	0	V
5P	no data	25.5	no data	III
5Q	80	88.5	6	I
7B	90	33.3	0	V
7C	70	35.3	5	V
7D	95	40.6	6	I
7E	40	38.1	0	III
7F	135	47.6	0	V
7G	100	30.8	2	V
7H	70	62.9	14	I
7I	75	27.3	7	I
7J	115	61.9	1	V
7K	165	21.6	2	II
7L	140	31.6	0	V
7M	125	33.3	9	I
7N	125	6.3	1	II
7O	105	67.3	16	I
7P	80	46.4	0	V
7Q	15	73.3	0	III
8A	95	no data	5	II
9B	75	55.6	6	I
9C	15	50.0	0	III
9D	75	58.1	4	V
9E	115	0.0	4	II
9F	0	0.0	0	VIII
9G	120	83.3	1	V
9H	110	50.0	3	V
9I	120	38.9	2	V
9J	70	32.6	5	V
9K	50	50.0	11	VII
9L	95	44.8	3	V
9M	no data	55.6	12	VII
9N	80	14.6	16	VI
9O	75	50.0	4	V
9P	85	60.7	0	V
9Q	135	16.0	0	II
10A	80	35.7	8	I

Color Key: Neg/Low | Medium | High

Figure 4. *Quantification of expression of VEGF receptors (1, 2, 3) in colon adenocarcinoma (n=84 [85-1]; in one case, no evaluable tumor was present on any slide). VEGFR1 H-score, VEGFR2 VPI, and VEGFR3 vessel counts were categorized as negative, low, medium and high. Blue cells, high expression; green cells, intermediate expression; yellow cells, low/negative expression; white cells, represent TMA cores in which no evaluable tissue was present. Red cases are those represented in Figure 3.*

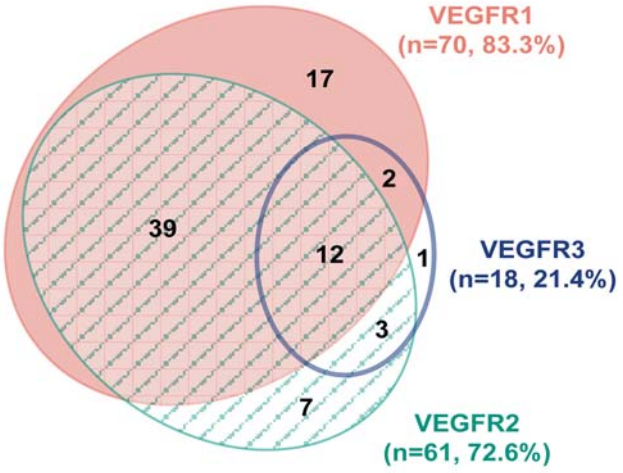

Figure 5. *Area-proportional 3-Venn diagram of VEGFR profiling in CRC. Black numbers represent number of cases that intersect between or among VEGFR1, VEGFR2, and VEGFR3 positive sets based on data described in Figure 4 and Table III.*

VEGFR2. As illustrated in Figure 3, in the CRC adenocarcinoma tissues examined, VEGFR2 immunoreactivity was only localized to tumor stromal vessels, and no unequivocal VEGFR2 immunoreactivity was found in adenocarcinoma cells (Figure 3A, panel iv). However, distinct VEGFR2 staining was found in some of the infiltrating tumor cells in a case of anal squamous cell carcinoma with basaloid features (Figure 3D, panel iv). While tumor stromal vessels in this case were VEGFR2-negative, variable VEGFR2 immunoreactivity was found in some of the tumor cell nuclei and cytoplasm.

VEGFR3. Similar to VEGFR2, VEGFR3 immunoreactivity was also localized to the tumor stromal vessels in which distinct staining of endothelial cells was noted (Figure 3A, panel vi). No tumor cell expression of VEGFR3 was found in any of the 84 CRC tissue specimens examined.

Distribution of VEGFR2 and VEGFR3 staining in tumor blood vessels and lymphatics. Based on staining of serial TMA sections for a lymphatic-specific marker D2-40, along with CD34, VEGFR1, VEGFR2, and VEGFR3, we were able to distinguish intra-tumoral lymphatics (D2-40-positive) from tumor stromal blood vessels (D2-40-negative) and could determine lymphatic or blood vascular distribution of the three VEGF receptors in the primary CRC tissues. Vascular staining of VEGFR2 was mainly localized to CD34-positive/D2-40-negative vessels – consistent with a blood vascular phenotype (compare panels iii and iv in Figure 3A and 3B). In some instances, VEGFR2 immunoreactivity was also noted in D2-40-positive lymphatics. Conversely, vascular staining of VEGFR3 was mainly localized to CD34-negative/D2-40-positive vessels,

Table II. *Subsets of CRC cases evaluated for IHC expression of VEGFR1 in invasive carcinoma cells and tumor stromal vessels (blood vessels, lymphatics).*

VEGFR1 immunoreactivity in invasive carcinoma cells	VEGFR1 immunoreactivity in tumor stromal vessels	N (%)
Distinct and unequivocal immunoreactivity		70 (83.3)
	Unequivocal immunoreactivity	17 (20.2)
	Low immunoreactivity	42 (50.0)
	No immunoreactivity	11 (13.1)
Low immunoreactivity		10 (11.9)
	Unequivocal immunoreactivity	5 (5.9)
	Low immunoreactivity	4 (4.8)
	No immunoreactivity	1 (1.2)
No immunoreactivity		1 (1.2)
	No immunoreactivity	1 (1.2)
Excluded due to absence/inadequacy of invasive carcinoma cells		3 (3.6)
Total Cases		84 (100)

consistent with a lymphatic vascular phenotype (Figure 3A, panels v and vi). In some instances, VEGFR3 immunoreactivity was also noted in CD34-positive/D2-40-negative vessels, consistent with a blood vascular phenotype.

Quantification of immunohistochemical expression of VEGFR1, 2 and 3 in CRC tissues. Based on prior evaluation of large numbers of multiple human cancer tissues during development of immunohistochemical assays for VEGFR1, 2, and 3, we were able to characterize the range of expression of the three VEGF receptors in those tumors. Taking into consideration our findings on multi-tumor tissues and on the CRC tissues in current analyses, we defined cut-points (see Materials and Methods section) to categorize each CRC case as low/negative, medium or high in expression of each of the three VEGF receptors (1, 2, 3) (Figure 4). About three-fourths of the cases had medium or high expression of both vascular VEGFR2 and tumor cell expression of VEGFR1, while only about one-fourth of the cases had medium to high levels of vascular VEGFR3 expression. The heterogeneity of VEGF receptor expression is depicted in Figure 5.

Classification of colorectal cancers based on the observed VEGF receptor profiles. Based on the differential (high, medium, low/negative) expression of the three VEGF receptors, and variation in their distribution in the CRC tissues, heterogeneity of the observed VEGF receptor profiles was clearly evident. Taking into consideration the various VEGF receptor (1, 2, 3) expression/co-expression patterns, we could classify each primary CRC case in this analysis (n=84) into one of the eight possible subclasses (or VEGFR profiles) (Table III). The main CRC subclasses with various VEGF receptor profiles were mixed VEGFR1/2 predominant (46.4%), VEGFR1-predominant (20.2%), triple VEGFR positive (14.3%) and VEGFR2-predominant (8.3%). The remaining cases were represented by a heterogeneous set of uncommon VEGFR profiles (Table III). Representative histomorphologic and pathologic findings of some of the observed VEGFR profiles are illustrated in Figure 3. As an example of the most frequent (46.4%; mixed VEGFR1/2 predominant) profile, a CRC case demonstrates unequivocal cytoplasmic expression of VEGFR1 in almost all invasive CRC glands, variable expression of VEGFR1 in tumor stromal vessels (Figure 3C, panel ii), distinct VEGFR2 expression in CD34-positive blood vessels (Figure 3C, panel iv), while most of the D2-40-positive lymphatics are VEGFR3-negative (Figure 3C, panel vi). Some of the other lesser frequent VEGF receptor profiles are also illustrated in Figure 3. The reliability of VEGF-receptor (VEGFR1, 2, 3) profiling data presented in this study is backed up by the technically sound immunohistochemical assays developed and optimized in our laboratory.

Table III. *Proposed subclasses of colorectal carcinomas based on relative immunohistochemical expression of the three VEGF receptors (VEGFR1, VEGFR2, VEGFR3).*

VEGFR IHC subclass of CRC	VEGF receptor profiles	N (%)
I	Triple-VEGFR-Positive	12 (14.3)
II	VEGFR1-predominant	17 (20.2)
III	VEGFR2-predominant	7 (8.3)
IV	VEGFR3-predominant	1 (1.2)
V	Mixed, VEGFR1/2 predominant	39 (46.4)
VI	Mixed, VEGFR1/3 predominant	2 (2.4)
VII	Mixed, VEGFR2/3 predominant	3 (3.6)
VIII	Triple-VEGFR-Negative	3 (3.6)
-	Total Cases	84 (100)

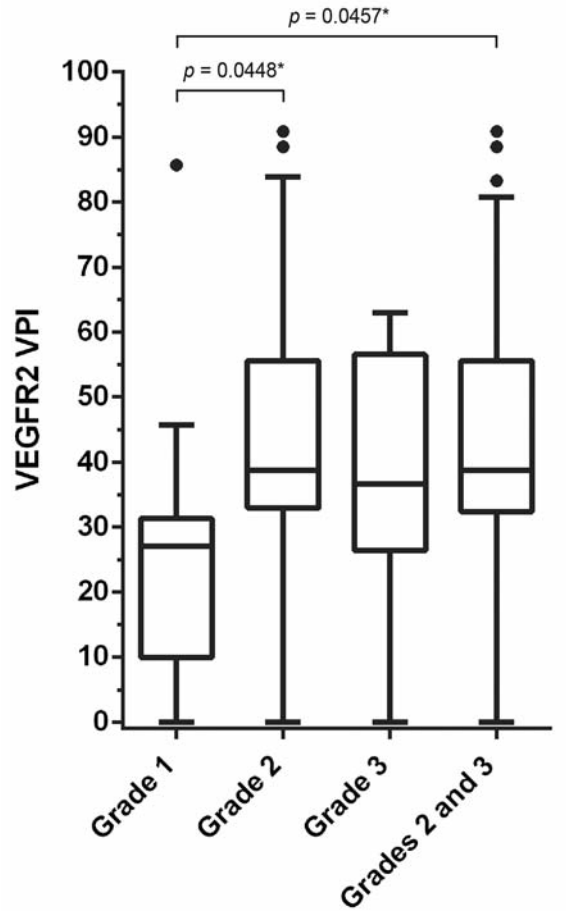

Figure 6. *VEGFR2 Vascular Positivity Index (VEGFR2-VPI) is positively associated with histologic grade of colorectal carcinomas. Boxes show medians, 25th, and 75th percentiles. Whiskers were calculated by Tukey's method. Values outside of whiskers, shown as points, are outliers. p-Values from unpaired t-tests are shown for associations between Grade 1 and Grade 2 (95% CI of difference: 0.3414 to 28.49) and between Grade1 and Grades 2+3 combined (95% CI of difference: 0.2743 to 27.43). Asterisks indicate statistically significant p-values.*

VEGF receptors and histologic grades in colorectal cancer. For VEGFR1 H-scores, VEGFR2 VPI, and VEGFR3 vessel counts independently, comparative analyses were carried out between median immunoreactivity scores and the histologic grade of the CRC tissues. Median VEGFR2 VPIs and tumor grade are positively associated and showed significant differences between Grade 1 and Grade 2 and between Grade 1 and Grades 2 + 3 combined (Figure 6). No significant differences were observed between histologic grades of the primary CRC tissues and VEGFR1 or VEGFR3 expression levels.

Discussion

Angiogenesis inhibitors targeting the VEGF signaling pathway have shown demonstrable therapeutic efficacy in mouse models of cancer and in an increasing number of human malignancies (20). However, not all treated patients respond to anti-VEGF therapy, and patients who do respond may eventually experience progressive disease (21). In colorectal cancer, a number of studies have shown improvement in progression-free (PFS) and/or overall survival (OS) (15, 22-28) with various combined anti-angiogenic and chemotherapy regimens. In a recently published large phase III trial, compared to placebo plus FOLFIRI, combination of ramucirumab (anti-VEGFR2 therapeutic antibody) and FOLFIRI showed significant improvement in overall survival as second-line treatment for metastatic CRC patients who had progressed during or after first-line therapy with bevacizumab, oxaliplatin and flouropyrimidine (28), resulting in an approval by the FDA. In contrast, in several other trials (29-32), anti-angiogenic/anti-VEGF therapies have failed to improve PFS and/or OS in CRC patients.

In order to explain such inconsistent or transient clinical benefits with anti-angiogenic cancer therapies, a number of cellular and molecular mechanisms of intrinsic or acquired resistance to anti-angiogenic therapies have been proposed. These include heterogeneity of tumor blood vessels, alternative pro-angiogenic pathways, role of stromal cells as part of tumor microenvironment, alternative mechanisms of tumor vascularization, increased tumor aggressiveness, VEGF signaling in different cell types (endothelial cells of normal vasculature, dendritic cells, myeloid cells, pericytes and tumor cells) and interactions between VEGF receptors and other cell surface receptors/co-receptors, including neuropilin-1 (33) integrins (34) TGFR-beta (35) and MET (36). While it is important to investigate the biologic basis of the clinical challenges of anti-angiogenic therapies in CRC patients, in order to maximize their clinical efficacy, there is also an urgent need to identify and analytically validate biologically relevant biomarkers.

A number of clinical trials in CRC patients have evaluated one or more of the VEGF receptors or ligands as biomarkers (27, 37-39), but the expression of all three VEGF receptors has not be systematically assessed (40). In order to determine the relative expression of the three principal VEGF receptors in human cancer tissues, we have developed and optimized technically sound IHC assays in our laboratory (18, 19, 41-43). In this study, we applied these IHC assays to characterize the levels of expression/co-expression and heterogeneity of the three principal VEGF receptors (VEGFR1, 2, and 3) on primary CRC tissues from the same patients.

Overall, we observed significant variability in the pattern of distribution (vascular vs. tumor cell) and the level of expression of the three VEGF receptors in the primary CRC tissues analyzed. In pathologic vasculature of the primary CRC tissues, VEGFR2 was the most frequently expressed VEGF receptor, while VEGFR1 and VEGFR3 showed relatively lower levels of vascular expression. These findings

are in line with global expression of VEGFR2 in endothelial cells, whereas VEGFR1 and VEGFR3 are more restricted to endothelial cells in distinct locations (13). The most frequently observed VEGF receptor profiles were mixed VEGFR1/2 predominant (46.4%), VEGFR1-predominant (20.2%), and triple VEGFR positive (14.3%). The remaining CRC tissues exhibited a number of other heterogeneous and less common VEGFR profiles.

The majority of CRC tissues showed intermediate to high VEGFR1 expression in tumor cells, localized mainly to the tumor cell cytoplasm with variable expression of VEGFR1 in the tumor vasculature. Furthermore, a significant proportion of CRCs (39/84, 46.4%) were classified as mixed VEGFR1/2 predominant. These findings may be biologically relevant, since even though VEGFR2 mediates most of the known angiogenic responses to VEGF-A, VEGFR1 may modulate the function of VEGFR2 (44). Furthermore, inhibition of VEGFR1 (FLT1) has been shown to suppress migration and invasion of cultured malignant cells (9), to inhibit tumor angiogenesis, growth and metastasis *in vivo* (45) and to block endothelial cell proliferation, migration and tube formation in response to VEGF-A, PLGF and VEGF-B (46). In a clinical setting, however, the inhibition of VEGFR1 alone may not be sufficient to block tumor growth and may require simultaneous inhibition of VEGFR2 (47). In a recent analysis, high expression levels of VEGFR1, KDR (VEGFR2) and their ligand, VEGFA were associated with poor prognosis is CRC patients (48). The recognition of a sizeable subset of mixed VEGFR1/2 predominant CRCs (46.4%) in the present study underscores the need for further evaluation of prognostic and predictive value of co-expression of VEGFR2 and VEGFR1 in large independent series of human CRCs.

In all primary CRC tissues analyzed, VEGFR2 expression was localized only to the tumor stromal vessels. No distinct VEGFR2 immunoreactivity was identified in the tumor cells. Similar pattern of vascular localization of VEGFR2 in CRC tissues has been shown in other studies (49, 50), one of which (49) used the same clone of anti-VEGFR2 monoclonal antibody (55B11) as the present study. Our findings are, however, in contrast to other studies (51, 52) that showed localization of VEGFR2 to human colorectal cancer cells. The observed differences in the pattern of distribution of VEGFR2 in CRC tissues in various studies may in part be explained by the differences in the affinity or specificity of the primary anti-VEGFR2 antibodies used. Other factors that may contribute to such variable findings include differences among individual laboratories regarding IHC staining protocols, reagents used, personnel training and expertise and the level of rigor in optimization of various IHC assay parameters, including selection of optimal tissue and reagent controls to standardize interpretation and scoring of the IHC assay results. In order to build reliable VEGFR2 IHC datasets, it will be important for the laboratories to ensure the use of best-in-class, high-affinity, specific, monoclonal anti-VEGFR2 antibodies and to rigorously standardize the VEGFR2 IHC staining protocols.

In our analysis of the relative expression of VEGFR1, 2, and 3 on tumor tissue from the same patients, one of the major findings was the identification of CRC subsets with higher levels of vascular VEGFR2, alone or in combination with one or more of the other VEGFRs (61 out of 84 cases [72.6%]). Since VEGFR2 is known to be the principal receptor driving pathologic angiogenesis in human cancer, frequent expression of higher levels of vascular VEGFR2 (72.6%) in CRC tissues, as demonstrated in the present study, is of great interest to us because of its relevance to some of the newer anti-angiogenic therapies like ramucirumab and, therefore, merits further investigation. A relatively smaller, yet biologically relevant subset of CRCs in our analyses comprising 23 of 84 cases (27.4%), in which vascular VEGFR2 was absent or only weakly expressed (VEGFR2-negative CRC) with or without the presence of VEGFR1 and VEGFR3). In contrast, a similar analysis of differential expression of VEGFRs in primary NSCLC tissues showed a much higher proportion of cases (57.3%) to be VEGFR2 low/negative (data shown in a parallel manuscript). Based on substantial differences in the frequency of VEGFR2 expression in these two cancer types, in future studies it will be prudent to evaluate expression/co-expression of other biologically relevant markers of resistance to anti-VEGF therapies, like c-MET, PDGFs/PDGFRs, bFGF, and FGFRs (20, 36, 53-55). VEGFR2-negative CRCs should also be evaluated for neuropilin-1 and neuropilin-2, the co-receptors that can transmit VEGF signaling even in the absence of VEGFRs through their interactions with other types of tyrosine kinases (56).

Similar to VEGFR2, in our experience, VEGFR3 expression was also localized to the tumor stromal vessels rather than tumor cells. These observations are in line with some earlier reports (9, 49), but in contrast to other studies, in which VEGFR3 was found in tumor cells (57-61). In CRC cells, absence of VEGFR2 and VEGFR3 protein and mRNA has also been shown by RT-PCR, ELISA and western blot (9). Therefore, exclusive localization of VEGFR2 and VEGFR3 to tumor vasculature suggests that the primary target of anti-VEGFR2 and anti-VEGFR3 therapies in human CRC will be the tumor vasculature and not the tumor cells.

VEGFR3 is highly expressed in angiogenic sprouts, and VEGFR3 blockade with monoclonal antibodies results in decreased sprouting, vascular density, vessel branching and endothelial cell proliferation in mouse angiogenesis models (62). Furthermore, stimulation of VEGFR3 augmented VEGF-induced angiogenesis and sustained angiogenesis even in the presence of VEGFR2 inhibitors, whereas antibodies against VEGFR3 and VEGFR2 in combination resulted in additive inhibition of angiogenesis and tumor growth, implying that effective inhibition of tumor progression may require simultaneous inhibition of multiple angiogenic targets (63)

rather than a single ligand or receptor. Therefore, we believe further analyses of larger cohorts of human CRC tissues to determine their VEGF receptor profiles and patterns of co-expression of markers of resistance to angiogenic therapies will be required. Furthermore, it will be valuable to compare the various VEGF receptor profiles between matched primary and metastatic CRC tissues.

Based on immunohistochemical expression of VEGFR2 and VEGFR3 in CD34-positive/D2-40-negative blood vessels and/or CD34-negative/D2-40-positive lymphatic vessels in CRC stroma, interesting patterns of similarities and differences in distribution of these two receptors emerged from case to case. In CRC, VEGFR2 is known to be present on both intra-tumoral blood and lymphatic vessels, while VEGFR3 is predominantly localized to lymphatic vessels (49). In this study, vascular staining of VEGFR2 was mainly localized to CD34-positive/D2-40-negative tumor blood vessels (compare panels iii and iv in Figure 2A and 2B). In some instances, VEGFR2 staining was also noted in D2-40-positive lymphatics. Conversely, vascular staining of VEGFR3 was mainly localized to CD34-negative/D2-40-positive tumor lymphatics (Figure 3A, panels v and vi), although a predominant blood vascular localization of VEGFR3 has also been reported (64). In some instances, VEGFR3 immunoreactivity was also noted in CD34-positive/D2-40-negative tumor blood vessels. The finding that VEGFR3 can sustain low levels of tumor angiogenesis even in the presence of VEGFR2 inhibitors (62), suggests that CRCs expressing high levels of VEGFR3 in tumor vessels may be less sensitive to therapies that target VEGFR2 alone. Furthermore, emerging anti-angiogenic approaches to combine anti-VEGFR2 and anti-VEGFR3 therapies (65) will likely achieve higher levels of clinical efficacy, if tailored to CRC patients with relevant VEGF receptor profiles. Therefore, recognition of a subset of CRCs with higher co-expression of VEGFR2 and VEGFR3 on tumor blood vessels in the present study supports further investigation of these findings in independent cohorts of human CRC tissues and experimental models of CRC treated with individual or combined anti-VEGFR2 and/or anti-VEGFR3 anti-angiogenic therapies.

Although triple VEGFR-negative was not a major subset of CRC tissues (4%) in this study, this subset will need further assessment of its prevalence in larger independent cohorts to investigate the extent of VEGFR-independent angiogenesis and the expression of various novel biomarkers that have been associated with resistance to anti-angiogenic therapies. Since histologic grade is known to have prognostic value in human CRC, our observation of higher VEGFR2 expression in higher histologic grade primary colorectal carcinomas suggests that higher vascular VEGFR2 expression may also have prognostic significance in human colorectal cancer – an area that merits further investigation in independent series of human CRC tissues.

Summary

The VEGF receptor profiling approach presented in this study revealed multiple heterogeneous patterns of differential expression the three VEGF receptors (1, 2, 3) that have not been fully appreciated until this systematic analysis of human CRC tissues. Compared to VEGFR1 and VEGFR3, VEGFR2 was the more frequently and widely expressed VEGF receptor in the human CRC vasculature. The majority of human CRC tissues exhibited intermediate to high levels of VEGFR1 in tumor cells, localized mainly to the tumor cell cytoplasm. CRC tissues with higher levels of vascular VEGFR2, alone or in combination with one or more of the other VEGFRs comprised a major subset (>70%). Based on the differential expression of VEGFRs 1, 2, 3 in primary CRC tissues, we have identified newer subclasses (VEGF receptor profiles) of human CRCs, especially mixed VEGFR1/2 predominant (46.4%), VEGFR1-predominant (20.2%), triple VEGFR positive (14.3%) and triple VEGFR low/negative (3.6%) CRCs. Since VEGFR2 is the main receptor for VEGF-A/VEGFR2-mediated tumor angiogenesis, frequent expression of higher levels of vascular VEGFR2 in CRC tissues suggests that pathologic angiogenesis in human CRCs may be amenable to therapeutic inhibition by anti-VEGFR2 monoclonal antibodies. Although VEGFR2-negative CRCs were fewer in this analysis of primary CRC tissues, this CRC subset will need further evaluation of its prevalence in larger cohorts of human CRC tissues.

Conclusion

Based on this systematic disease characterization analysis, we observed substantial heterogeneity in the immuno-histochemical expression of VEGF receptors 1, 2 and 3 in primary human CRC tissues. We propose a new sub-classification approach of human colorectal cancers, based on our current understanding of VEGFR biology, which merits independent validation on larger series of primary and metastatic colorectal cancers. We conclude that the observed heterogeneity of VEGF receptor profiles in primary human CRC tissues points to the underlying complexity of VEGF receptor biology in this cancer type. These original observations in our analyses may form the basis of data-driven hypotheses to test the status of pathologic angiogenesis in human CRCs as well as relevant pre-clinical models.

Future Directions

Using the bi-directional model of translational research, potential future implications of the novel VEGFR-based CRC classification proposed in this study may be in the form of forward or reverse translation. Clinical relevance of the VEGF receptor-based classes of human CRCs in this study can be

evaluated by the application of robust tissue-based VEGF receptor methodologies on high-quality human CRC tissues from patients treated with various FDA-approved anti-angiogenic therapies, especially ramucirumab (Cyramza) (forward translation). Similarly, VEGF receptor profiling of various pre-clinical models of CRC may allow an assessment of the response or resistance of those models to various anti-angiogenic therapies in experimental setting (reverse translation).

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