Identification of colorectal cancer metastasis markers by an angiogenesis-related cytokine-antibody array

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Abstract

AIM: To investigate the angiogenesis-related protein expression profile characterizing metastatic colorectal cancer (mCRC) with the aim of identifying prognostic markers.

METHODS: The expression of 44 angiogenesis-secreted factors was measured by a novel cytokine-antibody array methodology. The study evaluated vascular endothelial growth factor (VEGF) and its soluble vascular endothelial growth factor receptor (sVEGFR)-1 protein levels by enzyme immunoassay (EIA) in a panel of 16 CRC cell lines. mRNA VEGF and VEGF-A isoforms were quantified by quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) and vascular endothelial growth factor receptor (VEGFR)-2 expression was analyzed by flow cytometry.

RESULTS: Metastasis-derived CRC cell lines expressed a distinctive molecular profile as compared with those isolated from a primary tumor site. Metastatic CRC cell lines were characterized by higher expression of angiopoietin-2 (Ang-2), macrophage chemoattractant proteins-3/4 (MCP-3/4), matrix metalloproteinase-1 (MMP-1), and the chemokines interferon γ inducible T cell α chemoattractant protein (I-TAC), monocyte chemoattractant protein I-309, and interleukins interleukin (IL)-2 and IL-1α, as compared to primary tumor cell lines. In contrast, primary CRC cell lines expressed higher levels of interferon γ (IFN-γ), insulin-like growth factor-1 (IGF-1), IL-6, epidermal growth factor (EGF), placental growth factor (PIGF), transforming growth factor β1 (TGF-β1) and VEGF-D, as compared with the metastatic cell lines. VEGF expression does not significantly differ according to the CRC cellular origin in normoxia. Severe hypoxia induced VEGF expression up-regulation but contrary to expectations, metastatic CRC cell lines did not respond as much as primary cell lines to the hypoxic stimulus. In CRC primary-derived cell lines, we observed a two-fold increase in VEGF expression between normoxia and hypoxia as compared to metastatic cell lines. CRC cell lines express a similar pattern of VEGF isoforms (VEGF121, VEGF165 and VEGF189) despite variability in VEGF expression, where the major transcript was VEGF121. No relevant expression of VEGFR-2 was found in CRC cell lines, as compared to that of human umbilical vein endothelial cells and sVEGFR-1 expression did not depend on the CRC cellular origin.

CONCLUSION: A distinct angiogenesis-related expression pattern characterizes metastatic CRC cell lines. Factors other than VEGF appear as prognostic markers and intervention targets in the metastatic CRC setting.

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths. The prognosis of CRC is dependent upon the extent of disease and approximately 60% of patients develop metastases after surgical resection. With a 5-year survival rate of less than 10% in patients with distant metastatic disease, targeting the metastatic process and sites should provide an effective treatment[8]. The progressive growth of colon cancer and subsequent metastatic process is dependent on an angiogenic network[9]. Thus, anti-angiogenic strategies have emerged as effective therapies in patients with colon cancer, especially in the metastatic setting of the disease[10-13]. Yet, differences in the magnitude of survival benefit point to alternative pathways in the tumor microenvironment as responsible for inconsistent outcomes[7].

Angiogenesis is a complex process dependent on the angiogenic factors secreted by the tumor and stroma cells[8]. Vascular endothelial growth factor is considered the major pro-angiogenic factor[8]. The vascular endothelial growth factor (VEGF) gene encodes for six alternatively spliced isoforms[14] with differential diffusion potential and binding to receptors[15]. The question currently consists of understanding the significance of VEGF/vascular endothelial growth factor receptor (VEGFR) signaling in cancer cells[12,18]. The VEGF isoforms and VEGF receptor expression pattern would drive the activity and functionality of the VEGF/VEGFR pathway in both tumor and endothelial cells. The multistep process of angiogenesis accompanies the multistage development of a tumor[16]. The switch into the metastatic phenotype brings a number of changes within the tumor microenvironment, including acquisition of hypoxia-tolerance mechanisms[17]. While up-regulation of VEGF expression is activated mainly under hypoxia[8], recent reports reflect on the question of whether metastatic tumors rely as much on angiogenesis and VEGF as primary tumors.[18]

Other studies report that tumors in more advanced stages do not rely on a unique angiogenesis driver[12]. A network of multiple cytokines and growth factors create a crosstalk within the tumor microenvironment which ultimately drives tumor angiogenesis[12,16]. The mediators of vessel wall remodeling matrix metalloproteinases, macrophage chemoattractant proteins and angiopeptin, involved in invasion and metastasis processes, exert pro-angiogenic signals[8,17]. Chemokines such as interleukin (IL)-1α and IL-8 play an important role in colon cancer progression and angiogenesis[18], and IL-8 up-regulates MMPs[19]. VEGF expression actually determines the activity of Ang-1/Ang-2 and the expression of MCPs[20,21].

Great efforts have been made to characterize biomarkers in CRC[22]. However, the question of biomarkers of CRC metastasis remains currently unresolved. On this basis, the aim of this study was to characterize the protein factors behind the angiogenic potential of CRC cell lines of metastatic origin.

MATERIALS AND METHODS

Cell cultures and conditioned media

We used 16 CRC cell lines: HT29, WiDr, HCT116, RKO, SW480, Colo320, Caco2, SW1116, LS174T, SW1417, DLD-1, LS513, HCT15, SW620, LoVo and T84 (all from American Type Culture Collection, Manassas, VA) (Table 1). The cell lines were maintained in the recommended growth media supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). For harvesting conditioned media, CRC lines cells were grown approximately to 70% confluence in serum free media. The conditioned media, CRC lines cells were grown approximately to 70% confluence in serum free media. The conditioned media were collected after 24 h of incubation, centrifuged and kept frozen.

VEGF and sVEGFR1 protein detection by quantitative immunoassay

VEGF-A in supernatant was determined using the Human VEGF Quantikine® EIA kit (R and D Systems) and soluble vascular endothelial growth factor receptor (sVEGFR)-1 was quantified by EIA (Human sVEGFR

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Key words: Colorectal cancer metastasis; Cytokine-antibody array; Angiogenesis; Vascular endothelial growth factor; Biomarkers

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Table 1 Colorectal cancer cell lines origin

| Cell line | Type/origin |
|-----------|-------------|
| SW620     | Colon adenocarcinoma. Derived from: metastasis to lymph node |
| T84       | Colon carcinoma. Derived from metastasis to lung |
| LoVo      | Derived from metastatic site: left supraclavicular region |
| SW480     | Colon adenocarcinoma |
| WiDr      | Colon adenocarcinoma |
| RKO       | Colon carcinoma |
| HT29      | Colon adenocarcinoma |
| HCT15     | Colon adenocarcinoma |
| HCT116    | Colon carcinoma |
| SW1116    | Colon adenocarcinoma |
| SW1417    | Colon adenocarcinoma |
| LS174T    | Colon adenocarcinoma |
| LS513     | Colon carcinoma |
| Caco2     | Colon adenocarcinoma |
| DLD-1     | Colon adenocarcinoma |
| LS411N    | Colon adenocarcinoma |
| Colo320   | Colon adenocarcinoma |
**Table 2** Primer and probe sequences for vascular endothelial growth factor isoforms quantitative reverse-transcription polymerase chain reaction

| VEGF end-point and cloning | Antisense primer | Taqman probe | Amplicon size (bp) |
|----------------------------|------------------|--------------|--------------------|
| GAPDH end-point and cloning | ACTGCCATCCAATCGAGACGC | GATGCCCTTGAAAGTGACTCTGATACTTC | 189 |
| VEGF-1 mRNA | TGGTATCGTGGAAGGACTCATGAC | ATGCCGCTTGAACTCATGAC | 121 |
| VEGF-2 mRNA | GATGGCTTGAAGATGTACTCGATCT | CTTCCCTTGAACTCATGAC | 189 |
| VEGF-3 mRNA | ACTGCCATCCAATCGAGACC | AGGCCAAGGAACTCATGAC | 65 |

VEGF: Vascular endothelial growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

R1/Ft-1 Quantikine®, R and D Systems), according to the manufacturer’s instructions. We normalized VEGF and sVEGFR-1 protein levels per number of cells. Results are the average of replicates.

**Total VEGF and isoforms mRNA determination by quantitative reverse-transcription polymerase chain reaction**

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). Single strand DNA was synthesized from 1 μg total RNA using the cDNA Archive kit (Applied Biosystems). Quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) for total VEGF was performed using primers and probes purchased from Applied Biosystems (HS00900054_m1). RNA18s (HS99999901_s1) was used as an endogenous control and data obtained was represented as 2^-ΔΔCt.

VEGF isoforms were determined by Q-RT-PCR using primers designed specifically for VEGF-121, VEGF-164, and VEGF-189, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (Table 2). The relative quantification of samples was performed using a standard curve by dilution of a specific plasmid for each isoform (ranging from 10 pg to 1 fg). Human VEGF cDNA for each isoform and GAPDH were cloned from total RNA isolated from lung cancer tissue and point and cloning (GAPDH end-point and cloning). Each array was incubated with 1.2 mL of medium at 4°C. Following the incubation, unbound anti-VEGFR-2 antibody was removed by washing the cells twice in 4 mL PBS buffer. The human umbilical vein endothelial cells (HUVEC) cell line was used as a positive control.

**Secreted angiogenic profile by cytokine antibody-array**

The secretion of angiogenic factors by CRC cell lines was evaluated in duplicate using a protein array method (RayBio® Human Angiogenesis Antibody Array, RayBioTech C Series 1000, RayBiotech, Inc Norgross, GA). This assay is capable of simultaneously detecting 44 different angiogenic factors (spotted in sub-arrays I and II) with high specificity. The sensitivity of the antibodies present in the arrays ranged from 1-2000 pg/mL. Conditioned media was obtained after the incubation of 2 × 10^6 cells in serum-free medium for 20 h at 37°C and 5% CO2. Each array was incubated with 1.2 mL of medium at 4°C overnight, and bound antigens were detected according to the manufacturer’s instructions. To determine the relative concentrations of angiogenic factors in the media, the densities of individual spots were measured using Imagegene 4.1 software (Biodiscovery Inc., Marina Del Rey, United States) for image capture and analysis.

**Statistical analysis**

Statistical analysis was carried out with SPSS 13.0 soft-
RESULTS

Distinct angiogenesis-related expression pattern in primary and metastatic colorectal cancer cell lines

To identify the angiogenesis-related “secretome” of CRC cell lines in normoxia, we analyzed 44 angiogenesis-related cytokines and growth factors by an antibody-array in primary (Caco2, SW1417, DLD1, HT29 and SW480) and metastatic (SW620 and T84) CRC cell lines. K-means analysis classified CRC cell line angiogenesis-related secreted factors according to their level of secretion (Figure 1A). Cluster I showed a homogeneous high expression of the pro-angiogenic IL-8, MMP-1, MCP-1, growth related oncogene (GRO)-α, regulated upon activation, normal T expressed and secreted (RANTES), urokinase-type plasminogen activator-receptor (uPAR), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-10 (IL-10), interleukin-12 (IL-12), platelet-derived growth factor (PDGF), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-6 (IL-6), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-10 (IL-10), interleukin-12 (IL-12), platelet-derived growth factor (PDGF), interleukin-4 (IL-4), and transforming growth factor-β1 (TGF-β1). Negative control; Pos: Positive control.

Figure 1  Angiogenesis-related factors expression profile in colorectal cancer cell lines as determined by cytokine antibody-array. A: K-means (n = 4) clustering grouped the angiogenesis-related proteins according to level of expression; B: Unsupervised-hierarchical clustering of the factors with a significantly different expression in primary and metastatic colorectal cancer (CRC) cell lines after detection and processing; IL: Interleukin; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinases; GRO: Growth related oncogene; MCP: Macrophage chemoattractant proteins; RANTES: Regulated upon activation normally T-expressed and secreted; uPAR: Urokinase-type plasminogen activator-receptor; G-CSF: Granulocyte colony-stimulating factor; PIGF: Phosphatidylinositol glycan, class F; TNF-α: Tumor necrosis factor-α; GM-CSF: Granulocyte macrophage colony-stimulating factor; IFN-γ: Interferon-γ; IGF: Insulin-like growth factor; PECAM: Platelet-endothelial cell adhesion molecule; I-TAC: Inducible T cell α chemoattractant protein; ENA: Epithelial neutrophil activating protein; EGF: Epidermal growth factor; PDGF-BB: Platelet-derived growth factor; β polypeptide; TGF-β1: Transforming growth factor β1; Neg: Negative control; Pos: Positive control.

Relative expression

CRC cell lines

Cluster 1-9 genes

Cluster 3-11 genes

Cluster 2-9 genes

Cluster 4-10 genes

Figure 1A: K-means (n = 4) clustering grouped the angiogenesis-related proteins according to level of expression.

Figure 1B: Unsupervised-hierarchical clustering of the factors with a significantly different expression in primary and metastatic colorectal cancer (CRC) cell lines after detection and processing.

Figure 1C: Images of subarrays Ⅰ and Ⅱ of the primary Caco2 and the metastatic T84 CRC cell lines after detection and processing.
normal T-cell expressed, and secreted protein (RANTES), urokinase-type plasminogen activator-receptor (uPAR) and VEGF; and the anti-angiogenic tissue inhibitor of metalloproteinases tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 (Figure 1A, cluster Ⅰ). Cluster Ⅱ integrated angiogenic factors not secreted by CRC cell lines in normoxia, including VEGF family proteins placental growth factor (PlGF) and sVEGFR-2 and inflammatory cytokines with pro-angiogenic properties granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor-α (TNF-α) (Figure 1A, cluster Ⅱ). Primary tumor- and metastasis-derived CRC cell lines were characterized by a distinct angiogenesis-related molecular pattern in normoxia, including VEGF family proteins placental growth factor (PIGF) and sVEGFR-2 and inflammatory cytokines with pro-angiogenic properties granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor-α (TNF-α) (Figure 1A, cluster Ⅱ). Primary tumor- and metastasis-derived CRC cell lines were characterized by a distinct angiogenesis-related molecular pattern in normoxia, including VEGF family proteins placental growth factor (PIGF) and sVEGFR-2 and inflammatory cytokines with pro-angiogenic properties granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor-α (TNF-α), IGF-1, IL-6, leptin, EGF, PIGF, thrombopoietin, TGF-β1 and VEGF-D (P < 0.05), as compared with the metastatic ones (Figure 1B). Interestingly, VEGF-A (VEGF) was not found among the proteins differentially expressed according to the cellular source of isolation. Figure 1C illustrates processed antibody-arrays and the images captured of Caco2 (primary CRC cell line) and T84 (metastatic CRC cell line).

**VEGF expression in primary and metastatic colorectal cancer cell lines**

The antibody array data showed no significant changes in VEGF secretion between primary and metastasis-derived CRC cell lines (Figure 1B). To validate the antibody array results, we analyzed VEGF levels by EIA. The results were confirmed by a statistically significant positive correlation between VEGF protein as determined by the antibody-array and by EIA (r_Spearman = 0.7, P < 0.05) (Figure 2A).

In a second step, VEGF secretion by EIA and VEGF mRNA expression was analyzed in a larger panel of 16 CRC cell lines. As shown in Figure 2B and C, we did not detect any significant difference in VEGF expression according to the primary or metastatic CRC cell lines (mean of 28.9 pg/mL and 22.7 pg/mL VEGF protein; 0.011 and 0.009 (relative quantification) VEGF mRNA, respectively). Further, a strong correlation (r = 0.65, P <
0.01) was detected between VEGF protein (by EIA) and VEGF mRNA expression (Figure 2D) in CRC cell lines, indicative of the major role of transcriptional mechanisms in the regulation of VEGF expression.[23]. A similar correlation was observed in hypoxia between VEGF protein (by EIA) and VEGF mRNA expression (Figure 3A). Severe hypoxia induced different levels of VEGF expression up-regulation depending on the CRC cellular origin. Surprisingly, the fold change normoxia-hypoxia in VEGF expression of metastatic CRC cell lines was ≤ 1.5 in the majority of time points tested, as compared with the > 1.5-4.0 fold change in primary cell lines for both protein and mRNA VEGF (Figure 3A).

VEGF isoforms have differential angiogenic and tumorigenic activities and their expression pattern may also define the CRC cell angiogenic capacity.[24]. Primary and metastatic CRC cell lines had a similar expression pattern of the three major isoforms VEGF121, VEGF165 and VEGF189, despite variability in VEGF expression (Figure 3B), implying a similar mechanism of regulation. VEGF121 was the predominant isoform expressed by CRC cell lines (58.23% ± 5.05% of total VEGF mRNA), as compared to VEGF165 and VEGF189 (15.13% ± 2.71% and 26.6% ± 6.5% of VEGF transcripts, respectively). In line with a previous study on tumor tissue,[25], the expression of the three isoforms was significantly associated with total VEGF protein; $r = 0.55$, $P < 0.05$ for VEGF121 and furthermore, VEGF165 and VEGF189 showed higher correlation ($r = 0.67$ and $r = 0.69$, $P < 0.01$, respectively) (Table 3).

Table 3 Association between vascular endothelial growth factor mRNA isoforms and vascular endothelial growth factor protein secretion

| VEGF protein | VEGF121 mRNA | VEGF165 mRNA | VEGF189 mRNA |
|--------------|--------------|--------------|--------------|
| VEGF121 mRNA | $r = 0.55$   | $P = 0.034$  |              |
| VEGF165 mRNA | $r = 0.67$   | $r = 0.93$   | $P = 0.007$  |
| VEGF189 mRNA | $r = 0.69$   | $r = 0.95$   | $r = 0.92$   |

VEGF: Vascular endothelial growth factor.

Vascular endothelial growth factor expression regulation. A: Modulation of vascular endothelial growth factor (VEGF) expression (mRNA and protein) in response to severe hypoxia in primary and metastatic colorectal cancer (CRC) cell lines; B: Expression of VEGF isoforms 121, 189 and 165 by CRC cells in normoxia.

VEGFR expression in colorectal cancer cell lines

While the role of the VEGF/VEGFR pathway in endothelial cells is well characterized, its functionality and expression by tumor cells is still controversial.[3]. Soluble VEGFR-1 was quantified in CRC cell line supernatants at a lower range than VEGF (mean 8.3 and 27.8 pg/mL respectively) and no differences were found according to the cellular origin (7.57 ± 2.12 and 10.67 ± 3.1, in primary and metastatic CRC cell lines, respectively) (Figure 4A). In agreement with other studies,[26], a trend was observed for
an inverse correlation between sVEGFR-1 and VEGF expression (data not shown), indicative of the angiogenesis inhibiting role of sVEGFR-1[13].

In our CRC cell lines panel, the antibody array data showed a lack of expression of sVEGFR-2 (Figure 1A). Given the hypothesis that earlier tumor stages are more dependent on the VEGF/VEGFR signaling pathway[15], we analyzed surface VEGFR-2 expression in CRC cells of primary origin. Flow cytometry revealed a general lack of surface VEGFR-2 expression in CRC cells of medium to high VEGF expression, as compared to HUVEC expression (Figure 4B). These findings add to the stock of controversial results to date[27,28].

DISCUSSION

Identifying the proteins responsible for the different behavior of more advanced CRC tumors seems warranted in order to more effectively use current treatment options. Furthermore, there is a need to characterize definite biomarkers of CRC metastasis to serve as prognostic indicators and novel interventional targets. As derived from our findings in vitro, the tumor microenvironment of CRC metastases would be different to that of primary tumors, because of the effect of the CRC cells secreted factors. Metastatic CRC cell lines are characterized by a greater expression of cytokines majorly involved in metastasis, migration and invasion, while being proven pro-angiogenic effectors. MMP-1 plays an important role in CRC tumor invasion and metastasis[29] and MMP-9 has proved to be of prognostic value in stage II colon cancer patients, where tumors with higher protein expression had a higher recurrence rate[30]. The monocyte attractant chemokine I-309 has been shown to stimulate chemotaxis and invasion of endothelial cells and the roles of IL-1α in colon cancer angiogenesis and of IL-2 in inflammation and apoptosis, seem also consistent with the metastatic phenotype[18,31,32].

Hypoxia is widely recognized as the major transcription effector for VEGF expression[9]. However, the greater (two-fold increase) induction of VEGF expression in hypoxia observed in primary CRC as compared to metastatic cell lines is an interesting finding which agrees with recent hypotheses. Tolerance to hypoxia is frequently acquired by tumor cells progressing towards more advanced phenotypes[13]. Our finding suggests the metastatic CRC molecular phenotype provides some intrinsic resistance to the hypoxic induction of VEGF expression. Some authors have shown that hypoxia would select more malignant metastatic cells, less sensitive to anti-angiogenic treatment[13], to yield poorer patients outcomes[34,35]. The community still agrees that angiogenesis is a hallmark of cancer in metastatic stages[36]. However, given the broad angiogenic network in the tumor microenvironment, research should move in the direction of investigating the mechanisms by which metastatic tumors depend on VEGF, since they seem to be different to those exploited by primary tumors[15]. Furthermore, with the objective of individualized care in mCRC, the distinct metastatic “sec-

Figure 4 Vascular endothelial growth factor receptors expression in colorectal cancer cell lines. A: Soluble vascular endothelial growth factor receptor (sVEGFR)-1 expression measured by EIA is not significantly different between primary and metastatic colorectal cancer (CRC) cell lines; B: Flow cytometry of the surface expression of vascular endothelial growth factor receptor (VEGFR)-2 in human umbilical vein endothelial cells (HUVEC) and the primary CRC cell lines HCT116, Caco2 and RKO under normoxic conditions reveals a general lack of VEGFR-2 expression on the surface of CRC cells as compared to HUVEC. NS: Not significant.
retome” proteins emerge as alternative targets to consider in the management of advanced disease. Further to the VEGF expression profile, the pattern of VEGF isoforms represents the next step to identifying intrinsic differences to guide treatment choice. However, the similar expression of VEGF isoforms across cell lines does not offer clarification. Further to this finding, it would be of interest to explore how VEGF transcription factors modulate the ratio of VEGF isoforms as disease progresses, given the changes on VEGF dependence. Interestingly, a novel class of VEGF isoforms, VEGFxxxb, generated through alternative splicing of exon 8, has been recently described[17]. Studies suggest anti-angiogenic or weak angiogenic properties for these isoforms[8,9,10]. Not exempt from controversy, this discovery will help in further defining the role of VEGF/VEGFR signaling in CRC, yet still the testing techniques need refinement in specificity between the two classes.

Emerging data suggest VEGF to be a growth factor also for tumor cells and VEGF/VEGFR signaling to regulate their expression. However, this hypothesis remains unproven until consolidated results on VEGF receptor expression on tumor cells become available[12,20]. Extensive work has been done on the activity of VEGF/VEGFR-1 signaling in CRC cells showing that it mediates cell motility and invasiveness but not cell proliferation[18]. While this would involve VEGF/VEGFR-1 in CRC progression and metastatic processes, sVEGFR-1 secretion was not found of significant relevance in metastasis-derived CRC cells. In contrast, not so much is known about the activity of VEGF/VEGFR-2 in cancer cells. Reports suggest an involvement in the sensitivity of CRC cells to inhibition of VEGF-related survival pathways[8,9]. However, controversial results on the VEGFR-2 expression on tumor cells to date[27,28], to which our results add, do not help to resolve this question. Definite confirmation of the expression and functionality of this pathway is necessary in order to shed more light on the mechanism of action of anti-VEGF therapies[40].

Consistent with the key role of VEGF in the “angiogenic switch” and the hypoxia-resistance mechanisms in metastatic stages, CRC cell dependence on VEGF in more advanced settings seems attenuated in favor of other cytokines in the progression of metastasis. Further investigation of these findings and testing the significance of the distinct “secretome” of CRC metastases at the clinic side seems warranted given the implications for patient outcomes.

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REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96
2. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249-257
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70
4. Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, Griffing S, Bergsland E. Phase II, randomized trial comparing bevazucizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 2003;21:60-65
5. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fylde G, Rogers B, Ross R, Kabbinavar F, Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335-2342
6. Emmanouilides C, Pegram M, Robinson R, Hecht R, Kabbivanavar F, Isacoff W. Anti-VEGF antibody bevacizumab (Avastin) with SFU/LV as third line treatment for colorectal cancer. Tech Coloproctol 2004;8 Suppl 1: 80-82
7. Kerbel RS. Tumor angiogenesis. N Engl J Med 2008;358:2039-2049
8. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 2007;6:273-286
9. Ferrara N. VEGF as a therapeutic target in cancer. Oncology 2005;69 Suppl 3: 11-16
10. Akiri G, Nahari D, Finkelstein Y, Le SY, Elroy-Stein O, Levi BZ. Regulation of vascular endothelial growth factor (VEGF) expression is mediated by internal initiation of translation and alternative initiation of transcription. Oncogene 1998;17:227-236
11. Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into
the subepithelial extracellular matrix and bioactivity of extra-
cellular matrix-bound VEGF. Mol Biol Cell 1993; 4: 1317-1326

Cheng J, Slavin RE, Gallagher JA, Zhu G, Biehl TR, 
Swanstrom LL, Hansen PD. Expression of vascular endo-
theelial growth factor and receptor flk-1 in colon cancer liver 
metastases. J Hepatoobili Pancreat Surg 2004; 11: 164-170

Fan F, Wey JS, McCarty MF, Belchaeva A, Liu W, Bauer TW, 
Somcio RJ, Wu Y, Hooper A, Hicklin DJ, Ellis LM. Expression 
and function of vascular endothelial growth factor recep-
tor-1 on human colorectal cancer cells. Oncogene 2005; 24: 
2647-2653

Poon RT, Fan ST, Wong J. Clinical implications of circu-
ing angiogenic factors in cancer patients. J Clin Oncol 2001; 19: 
1207-1225

Brahimi-Horn MC, Chiche J, Pouyysségur J. Hypoxia and 
cancer. J Mol Med (Berl) 2007; 85: 1301-1307

Acevedo VD, lttmann M, Spencer DM. Paths of FGFR-driven 
tumorigenesis. Cell Cycle 2009; 8: 580-588

Shin JE, Jung SA, Kim SE, Joo YH, Shin KN, Kim TH, Yoo 
K, Moon IH. [Expression of MMP-2, HIF-1alpha and VEGF 
in colon adenoma and colon cancer]. Korean J Gastroenterol 
2005; 50: 9-18

Matsuo T, Sawai H, Ma J, Xu D, Ochi N, Yasuda A, Taka-
hashi H, Funahashi H, Takeyama H. IL-1alpha secreted by 
colon cancer cells enhances angiogenesis: the relationship 
between IL-1alpha release and tumor cells' potential for liver 
metastasis. J Surg Onc 2009; 99: 361-367

Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly 
enhanced endothelial cell survival, proliferation, and matrix 
metalloproteinases production and regulated angiogenesis. J 
Immunol 2003; 170: 3369-3376

Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial 
growth factor activates nuclear factor-kappaB and induces 
monocyte chemoattractant protein-1 in bovine retinal endo-
theelial cells. Diabetes 1999; 48: 1131-1137

Lobov IB, Brooks PC, Lang RA. Angiopoietin-2 displays 
VEGF-dependent modulation of capillary structure and en-
dothelial cell survival in vivo. Proc Natl Acad Sci USA 2002; 
99: 11205-11212

Jain RK, Duda DG, Willett CG, Sahani DV, Zhu AX, Loef-
fler JS, Batchelor TT, Sorensen AG. Biomarkers of response 
and resistance to antiangiogenic therapy. Nat Rev Clin Oncol 
2009; 6: 327-338

Loureiro RM, D’Amore PA. Transcriptional regulation of 
vascular endothelial growth factor in cancer. Cytokine Growth 
Factor Rev 2005; 16: 77-89

Zhang HT, Scott PA, Morbidelli L, Peak S, Moore J, Turley H, 
Harris AL, Ziche M, Bicknell R. The 121 amino acid isoform 
of vascular endothelial growth factor is more strongly tu-
morigenic than other splice variants in vivo. Br J Cancer 2000; 
83: 63-68

Cressrey R, Wattananupong O, Lertrprasertsuke N, Vinikut-
kenmuang U. Alteration of protein expression pattern of 
vascular endothelial growth factor (VEGF) from soluble to cell-
associated isoform during tumourogenesis. BMC Cancer 2005; 
5: 128

Shibuya M. Differential roles of vascular endothelial growth 
factor receptor-1 and receptor-2 in angiogenesis. J Biochem 
Mol Biol 2006; 39: 469-478

Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ash-
ton SE, Sproat G, Swann R, Gray N, Ryan A, Jürgensmeier 
JM, Womack C. Vascular endothelial growth factor receptors 
VEGFR-2 and VEGFR-3 are localized primarily to the vascu-
larature in human primary solid cancers. Clin Cancer Res 2010; 
16: 3548-3561

Kim SJ, Lee JH, Lee YJ, Yoon JH, Choi CW, Kim BS, Shin 
SW, Kim YH, Kim JS. Autocrine vascular endothelial growth 
factor/vascular endothelial growth factor receptor-2 growth 
pathway represents a cyclooxygenase-2-independent target 
for the cyclooxygenase-2 inhibitor NS-398 in colon cancer 
cells. Oncology 2005; 68: 204-211

Bendardaf R, Buhrmeida A, Ristamäki S, Syrjänen K, 
Pyrhönen S. MMP-1 (collagenase-1) expression in primary 
colorectal cancer and its metastases. Scand J Gastroenterol 
2007; 42: 1473-1478

Buhrmeida A, Bendardaf R, Hilfska M, Collan Y, Laato M, 
Syrjänen S, Syrjänen K, Pyrhönen S. Prognostic significance 
of matrix metalloproteinase-9 (MMP-9) in stage II colorectal 
carcinoma. J Gastrointest Cancer 2009; 40: 91-97

Bernardini G, Spinetti G, Ribatti D, Camarda G, Morbidelli 
L, Ziche M, Santoni A, Capogrossi MC, Napolitano M. I-309 
binds to and activates endothelial cell functions and acts as 
an angiogenic molecule in vivo. Blood 2003; 96: 4039-4045

Bae J, Park D, Lee YS, Jeoung D. Interleukin-2 promotes an-
giogenesis by activation of Akt and increase of ROS. J Micro-
biol Biotechnol 2008; 18: 377-382

Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS. Effect of 
p53 status on tumor response to antiangiogenic therapy. 
Science 2002; 295: 1526-1528

Giantonio BJ, Catalano PJ, Meropol NJ, O’Dwyer PJ, Mitch-
ell EP, Alberts SR, Schwartz MA, Benson AB. Bevacizumab 
in combination with oxaliplatin, fluorouracil, and leucovorin 
(FOLFOX4) for previously treated metastatic colorectal can-
cer: results from the Eastern Cooperative Oncology Group 
Study E3200. J Clin Oncol 2007; 25: 1539-1544

Lièvre A, Samalin E, Mitry E, Assenat E, Boyer-Gestin C, Le-
pere C, Bachet JB, Portales V, Vaillant JN, Ychou M, Rougier 
P. Bevacizumab plus FOLFIRI or FOLFOX in chemotherapy-
refractory patients with metastatic colorectal cancer: a retro-
spective study. BMC Cancer 2009; 9: 347

Verheul HM, Hammers HI, van Erp K, Wei Y, Sanni T, 
Salumbides B, Qian DZ, Yancopoulos GD, Pili R. Vascular 
endothelial growth factor trap blocks tumor growth, me-
tastasis formation, and vascular leakage in an orthotopic 
murine renal cell cancer model. Clin Cancer Res 2007; 13: 
4201-4208

Ladomery MR, Harper SJ, Bates DO. Alternative splicing in 
angiogenesis: the vascular endothelial growth factor para-
digm. Cancer Lett 2007; 249: 133-142

Catena R, Montes R, Pio R, Montuenga LM, Calvo A. VEGF 
binds to and activates endothelial cell functions and acts as 
a positive autocrine growth factor in the survival of hypoxic 
colon cancer cells. Cancer Res 2000; 60: 1309-1315

Fan F, Wey JS, McCarty MF, Belchaeva A, Liu W, Bauer TW, 
Somcio RJ, Wu Y, Hooper A, Hicklin DJ, Ellis LM. Expression 
and function of vascular endothelial growth factor recep-
tor-1 on human colorectal cancer cells. Oncogene 2005; 24: 
2647-2653

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