Vascular endothelial growth factor 165b expression in stromal cells and colorectal cancer

Makoto Tayama, Tomohisa Furuhata, Yoshiko Inafuku, Kenji Okita, Toshihiko Nishidate, Toru Mizuguchi, Yasutoshi Kimura, Koichi Hirata

Makoto Tayama, Tomohisa Furuhata, Yoshiko Inafuku, Kenji Okita, Toshihiko Nishidate, Toru Mizuguchi, Yasutoshi Kimura, Koichi Hirata, the First Department of Surgery, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan

Author contributions: Tayama M and Furuhata T contributed equally to this work; Tayama M, Furuhata T, Inafuku Y, Okita K, Nishidate T, Mizuguchi T, Kimura Y and Hirata K designed the research; Tayama M, Furuhata T and Inafuku Y performed the research; Tayama M and Furuhata T provided new reagents/analytic tools; Tayama M, Furuhata T and Okita K analyzed data; and Tayama M, Furuhata T and Hirata K wrote the paper.

Correspondence to: Makoto Tayama, MD, the First Department of Surgery, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan. tayama@sapmed.ac.jp

Telephone: +81-11-6112111-3281 Fax: +81-11-6131678

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Abstract

AIM: To characterize the implications of vascular endothelial growth factor (VEGF)-A in stromal cells and colorectal cancer and the expression of VEGF-A splice variants.

METHODS: VEGF-A expression in tumor and stromal cells from 165 consecutive patients with colorectal cancer was examined by immunohistochemistry. The association between VEGF-A expression status and clinicopathological factors was investigated. Twenty fresh-frozen samples were obtained for laser capture microdissection to analyze the splice variants of VEGF-A.

RESULTS: VEGF-A was expressed in 53.9% and 42.4% of tumor and stromal cells, respectively. VEGF-A expression in tumor cells (t-VEGF-A) was associated with advanced clinical stage (stage 0, 1/9; stage 1, 6/16; stage 2, 33/55; stage 3, 22/66; stage 4, 5/19, P = 0.004). Multivariate analyses for risk factors of recurrence showed that only s-VEGF-A expression was an independent risk factor for recurrence (relative risk 0.309, 95% confidence interval 0.141-0.676, P = 0.0033). The five-year disease-free survival (DFS) rates of t-VEGF-A-positive and -negative cases were 51.4% and 62.9%, respectively. There was no significant difference in t-VEGF-A expression status. The five-year DFS rates of s-VEGF-A-positive and -negative cases were 73.8% and 39.9%, respectively. s-VEGF-A-positive cases had significantly better survival than s-VEGF-A-negative cases (P = 0.0005). Splice variant analysis revealed that t-VEGF-A was mainly composed of VEGF165 and that s-VEGF-A included both VEGF165 and VEGF165b. In cases with no venous invasion (v0), the level of VEGF165b mRNA was significantly higher (v0 204.5 ± 122.7, v1 32.5 ± 36.7, v2 2.1 ± 1.7, P = 0.03). The microvessel density tended to be lower in cases with higher VEGF165b mRNA levels.

CONCLUSION: s-VEGF-A appears be a good prognostic factor for colorectal cancer and includes VEGF165 and VEGF165b.

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Key words: Colorectal cancer; Vascular endothelial growth factor-A; Vascular endothelial growth factor165; Microvascular density; Stromal cell

Peer reviewer: Josep M Pique, MD, Department of Gastroenterology, Hospital Clinic of Barcelona, Barcelona 08036, Spain

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INTRODUCTION

The growth and metastasis of cancer depend on angiogenesis, and vascular endothelial growth factor (VEGF)-A. VEGF-A is known to be one of the most important angiogenic factors. VEGF-A protein was discovered by Ferrara in 1989 as a specific growth factor and a blood vascular permeability factor for endothelial cells. As a result of alternative splicing, 6 VEGF isoforms of 121, 145, 165, 183, 189 and 206 amino acids are produced from a single gene. Most studies suggest that VEGF165 is the most abundant and biologically active isoform. The biological effects of VEGF165 are mediated by tyrosine kinase receptors, i.e., VEGF receptor (VEGFR) 1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4). In colorectal cancer, VEGF-A is highly expressed in the case of hematogenous metastasis; therefore, VEGF-A is assumed to have value as a prognostic factor. VEGF-A and its receptor system are deeply involved in tumor angiogenesis. Thus, it is important to analyze the expression of VEGF165 and VEGF165b in colorectal cancer.

VEGF165b was recently isolated from kidney epithelial cells as an angiogenesis inhibitor. This variant is identical to VEGF165 except for the last six amino acids encoded by alternative splicing. VEGF165b also binds to both the VEGF receptor 1 (VEGFR-1) and the VEGF receptor 2 (VEGFR-2) with a similar affinity to that of VEGF165. VEGF165b was shown to bind to VEGFR-2, but not to stimulate phosphorylation, and to inhibit VEGF165-mediated phosphorylation in human umbilical vein endothelial cells.

We examined the association between VEGF-A expression status and clinicopathological characteristics in order to determine how VEGF-A in stromal cells affects tumor progression. We also analyzed the expression of VEGF-165 and VEGF165b using fresh-frozen specimens.

MATERIALS AND METHODS

Patients

Tumor specimens were obtained from 165 consecutive patients with colorectal cancer who underwent resection at the First Department of Surgery, Sapporo Medical University from 1997 through 2001. Of these 165 patients, 146 at stages 0-III received curative resection. None of the patients received radiation or chemotherapy before surgery. The pathological stages, depth, histology, venous invasion, and lymphatic invasion of the primary tumor are shown in Table 1. Venous invasion and lymphatic invasion were both classified into four grades according to the Japanese Classification of Colorectal Carcinoma. v0 and ly0 represent no invasion, v1 and ly1, slight invasion, v2 and ly2, moderate invasion, and v3 and ly3, high invasion. Immunohistochemical (IHC) analysis was performed in these 165 cases. We also obtained 20 fresh-frozen samples from patients with colorectal cancer in 2006-2007 to analyze the expression of VEGF165 and VEGF165b mRNAs.

Immunohistochemistry

For IHC staining, paraffin-embedded tissues were cut at 4 μm. Slides were deparaffinized in xylene for 3 min three times, 3 min in absolute alcohol, 3 min in 90% ethanol,
tion with xylene was conducted twice for 1 min each time, staining was performed three times with eosin. Dehydration was performed using 95% and 100% ethanol for 10 s in each case. Counterstaining was performed using hematoxylin for 30 s and rinsed in distilled water, followed by dehydration with 70% ethanol for 3 min and rinsed in 5% and 10% PBS for three times. After being deparaffinized, sections were incubated in 3% H2O2-methanol for 20 min to inactivate endogenous peroxidase. Deparaffinized and rehydrated sections were heated in DAKO Target Retrieval Solution (DAKO Japan, Tokyo, Japan) for 15 min in an autoclave at 105 °C. Nonspecific binding was blocked with 10% goat serum for 15 min at room temperature followed by incubation with the primary antibody in a moist chamber at 4 °C overnight. After rinsing in PBS for 3 min three times, the sections were incubated with a biotinylated secondary antibody, ENVISION + Mouse/HRP (Dako Japan, Tokyo, Japan), for 30 min. Sections were stained using aminoethylcarbazole (Dako Japan, Tokyo, Japan). Slides were mounted prior to observation under conventional light microscopy.

Monoclonal antibodies
The primary antibodies were mouse monoclonal antibodies against VEGF-A, anti-human VEGF (N5) (IBL, Takasaki, Japan), CD34, anti-human CD34 (QBEnd10) and mouse monoclonal antibody Dako N1632 (Dako, Japan, Tokyo, Japan).

Evaluation of immunohistochemistry
VEGF-A expression was examined under light microscope, and both the tumor and the stromal cells were separately classified into stained cells and unstained cells. Three sections of tumor cells and stromal cells were counted respectively at × 400 magnification for marginal cancer tissue to determine whether the cells were positive for VEGF-A, and the percentage of stained cells was averaged. Specimens were regarded as VEGF negative if less than 5% of the cells were stained and as VEGF positive if more than 5% were stained. These criteria were used in many previous reports\(^{25-27}\). Microvessel density (MVD) was assessed using light microscopy in invasive tumors containing the highest number of capillaries and small venules per unit area. Any single endothelial cell or cell cluster stained with CD34 was counted as a single vessel at × 400 magnification for marginal cancer tissue\(^{28}\). Three sections were counted in one case, and the number of vessels was averaged.

Laser capture microdissection
Laser capture microdissection (LCM) is a method for obtaining pure populations of cells from heterogeneous samples. Using this technique, colorectal tumor tissues were separated into tumor and stromal tissues. The frozen tissues were sectioned at a thickness of 8 μm using a cryostat and mounted on nonadhesive glass slides. Tissue sections were rehydrated using 70% ethanol for 3 min and rinsed twice in distilled water (Invitrogen Corp., Carlsbad, CA). They were then stained using hematoxylin for 30 s and rinsed in distilled water, followed by dehydration with 95% and 100% ethanol for 10 s in each case. Counters staining was performed three times with eosin. Dehydration with xylene was conducted twice for 1 min each time, followed by air drying for 20 min. The PixCell LM200 system (Arcturus Engineering, Mountain View, CA) was used to microdissect the tumor cells and the stromal cells from the colorectal tissue sections. Ten sections were used to obtain sufficient RNA for reverse transcription polymerase chain reaction (RT-PCR), and each section needed at least 10 000 pulses. Processing of the total RNA began immediately following LCM. Extraction and isolation were performed using a QIAGEN RNeasy Mini Kit (QIAGEN, Valencia, CA).

Real-time polymerase chain reaction
We constructed the following primers to amplify fragments of human VEGF165 and VEGF165b specifically. The forward primer was located in exon 7a (TGGTTTG TACAAGATCCCGAGACGTG). One reverse primer complementary to exon 8 (CTACCGCCTCGG CTTGTCACATGCAAGTACGGTT) detected VEGF165 but not VEGF165b, and the other reverse primer complementary to exon 9 (GTTTCGTACGTTCTCTCGTGAGAGATCTGCA) detected VEGF165b but not VEGF165. Denaturing was conducted at 96 °C for 30 s, with annealing at 55 °C for 30 s and extension at 72 °C for 60 s in reactions cycled 30 times. PCR products were run on 3% agarose gels containing 0.5 μg/mL ethidium bromide and visualized under a UV transilluminator. This reaction consistently resulted in amplicons of 121 bp consistent with VEGF165b and 119 bp consistent with VEGF165p. To confirm the amplification of VEGF165 and VEGF165b, we performed sequence analysis of these PCR products.

Real time PCR was performed on a LightCycler (Roche, Basel, Switzerland) for the semi-quantitation of VEGF165 and VEGF165b mRNA levels. The primer sequences were the same as those of the primers used for RT-PCR. The calculated amounts of VEGF165 and VEGF165b mRNAs were normalized to the endogenous reference control gene, human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH). All data were presented as the ratio of the target gene/GAPDH expression.

Statistical analysis
The χ\(^2\) test and Mann-Whitney U test were used to examine the association between the expression status of VEGF and clinicopathological characteristics. To analyze the risk factors for recurrence, logistic regression analysis was conducted. Survival curves were computed according to the Kaplan-Meier method. The log-rank test was used to compare the survival curves. P < 0.05 was considered statistically significant.

RESULTS
Expression of VEGF-A in tumor and stromal cells
VEGF-A expression in tumor cells was positive in 53.9% (89/165) of the cases (Figure 1A). VEGF-A immunoreactivity was observed mainly in the cytoplasm of tumor cells. VEGF-A expression in stromal cells was observed in 42.4% (73/165) of the cases (Figure 1B).
Association between VEGF-A expression status and clinicopathological characteristics

A summary of the correlation between VEGF-A expression and clinicopathological characteristics is shown in Table 2. Tumor VEGF-A (t-VEGF-A) expression rates in tumors were 11.1% (1/9) in stage 0, 12.5% (2/16) in stage I, 58.2% (32/55) in stage II, 57.6% (38/66) in stage III, and 84.2% (16/19) in stage IV. t-VEGF-A expression was associated with the clinical stage \( (P < 0.0001) \).

VEGF-A (s-VEGF-A) expression rates in stromal cells were 77.8% (7/9) in stage 0, 37.5% (6/16) in stage I, 60.0% (33/55) in stage II, 33.3% (22/66) in stage III, and 26.3% (5/19) in stage IV. The s-VEGF-A expression rate increased in the earlier clinical stage \( (P = 0.004) \).

The t-VEGF-A expression rate increased with the depth of invasion \( (P = 0.0002) \). Conversely, the s-VEGF-A expression rate decreased with the depth of invasion \( (P = 0.01) \).

There was no significant association between VEGF-A expression and the histological type. t-VEGF-A expression became significantly higher with the grade of venous and lymphatic invasion, while s-VEGF-A expression became significantly lower with the grade of venous and lymphatic invasion.

Microvessel density

MVD was calculated by counting CD34-positive vascular endothelial cells. The association between VEGF-A expression status and MVD is shown in Figure 2. The MVDs of t-VEGF-A and s-VEGF-A expression (+, +), (-, +), (-, -), and (+, -) were 58.5, 52.4, 51.2 and 119.0, respectively. In s-VEGF-A-positive cases, the low MVD score

![Figure 1](image1.png)

**Figure 1** Immunohistochemical of colorectal cancer tissues used the anti-vascular endothelial growth factor-A antibody. A: Vascular endothelial growth factor (VEGF)-A was expressed in tumor cells but not in stromal cells; B: VEGF-A was expressed in stromal cells but not in tumor cells.

![Figure 2](image2.png)

**Figure 2** Microvessel density of vascular endothelial growth factor-A expression status. In s-vascular endothelial growth factor (VEGF)-A positive cases, microvessel density (MVD) was maintained at a low score regardless of tumor VEGF-A (t-VEGF-A) expression. In s-VEGF-A negative cases, MVD was influenced by t-VEGF-A expression.

### Table 2  Association between vascular endothelial growth factor-A expression and clinicopathological characteristics \( n \) (%)

|                      | n | Tumor VEGF positive cases | Stromal VEGF positive cases |
|----------------------|---|---------------------------|----------------------------|
| **TNM stage**        |   |                           |                            |
| 0                    | 9 | 1 (11.1)                  | 7 (77.8)                   |
| I                    | 16| 2 (12.5)                  | 6 (37.5)                   |
| II                   | 55| 32 (58.2)                 | 33 (60.0)                  |
| III                  | 66| 38 (57.6)                 | 22 (33.3)                  |
| IV                   | 19| 16 (84.2)                 | 5 (26.3)                   |
| Total                | 165| 89 (53.9)                | 73 (44.2)                  |
| **T factor**         |   |                           |                            |
| Tis                  | 9 | 1 (11.1)                  | 7 (77.8)                   |
| T1                   | 7 | 0 (0.0)                   | 6 (85.7)                   |
| T2                   | 25| 9 (36.0)                  | 11 (44.0)                  |
| T3                   | 111| 70 (63.1)                | 45 (40.5)                  |
| T4                   | 13| 9 (69.2)                  | 4 (30.8)                   |
| Total                | 165| 89 (53.9)                | 73 (44.2)                  |
| **Histological differentiation** | | | |
| Well                 | 47| 15 (31.9)                 | 23 (48.9)                  |
| Moderate             | 95| 63 (66.3)                 | 41 (43.2)                  |
| Poor                 | 8 | 3 (37.5)                  | 4 (50.0)                   |
| Mucinous             | 10| 5 (50.0)                  | 2 (20.0)                   |
| Other                | 5 | 1 (20.0)                  | 3 (60.0)                   |
| Total                | 165| 89 (53.9)                | 73 (44.2)                  |
| **Venous invasion**  |   |                           |                            |
| v0                   | 46| 14 (30.4)                 | 27 (58.7)                  |
| v1                   | 73| 43 (58.9)                 | 29 (39.7)                  |
| v2                   | 32| 23 (71.9)                 | 15 (46.9)                  |
| v3                   | 14| 9 (64.3)                  | 2 (14.3)                   |
| Total                | 165| 89 (53.9)                | 73 (44.2)                  |
| **Lymphatic invasion** | | | |
| ly0                  | 53| 16 (30.1)                 | 26 (49.1)                  |
| ly1                  | 79| 48 (60.8)                 | 39 (49.4)                  |
| ly2                  | 28| 20 (71.4)                 | 7 (25.0)                   |
| ly3                  | 5 | 5 (100.0)                 | 1 (20.0)                   |
| Total                | 165| 89 (53.9)                | 73 (44.2)                  |

NS: Not significant; VEGF: Vascular endothelial growth factor.

**NS**: Not significant; **VEGF**: Vascular endothelial growth factor.

**Association between VEGF-A expression status and clinicopathological characteristics**

A summary of the correlation between VEGF-A expression and clinicopathological characteristics is shown in Table 2. Tumor VEGF-A (t-VEGF-A) expression rates in tumors were 11.1% (1/9) in stage 0, 12.5% (2/16) in stage I, 58.2% (32/55) in stage II, 57.6% (38/66) in stage III, and 84.2% (16/19) in stage IV. t-VEGF-A expression was associated with the clinical stage \( (P < 0.0001) \). VEGF-A (s-VEGF-A) expression rates in stromal cells were 77.8% (7/9) in stage 0, 37.5% (6/16) in stage I, 60.0% (33/55) in stage II, 33.3% (22/66) in stage III, and 26.3% (5/19) in stage IV. The s-VEGF-A expression rate increased in the earlier clinical stage \( (P = 0.004) \). The t-VEGF-A expression rate increased with the depth of invasion \( (P = 0.0002) \). Conversely, the s-VEGF-A expression rate decreased with the depth of invasion \( (P = 0.01) \). There was no significant association between VEGF-A expression and the histological type. t-VEGF-A expression became significantly higher with the grade of venous and lymphatic invasion, while s-VEGF-A expression became significantly lower with the grade of venous and lymphatic invasion.

**Microvessel density**

MVD was calculated by counting CD34-positive vascular endothelial cells. The association between VEGF-A expression status and MVD is shown in Figure 2. The MVDs of t-VEGF-A and s-VEGF-A expression (+, +), (-, +), (-, -), and (+, -) were 58.5, 52.4, 51.2 and 119.0, respectively. In s-VEGF-A-positive cases, the low MVD score...
was almost the same regardless of t-VEGF-A expression. t-VEGF-A-positive and s-VEGF-A-negative cases had significantly higher MVD scores.

**Recurrence**

Risk factors for recurrence in the 146 cases excluding stage IV cases were examined using logistic regression analysis. In univariate analysis, clinical stage, venous invasion, lymphatic invasion, t-VEGF-A positivity and s-VEGF-A negativity were risk factors for recurrence (Table 3). Multivariate analyses of these risk factors were performed, which showed that only s-VEGF-A expression was an independent risk factor for recurrence \( (P = 0.0033) \) (Table 3).

**Survival analysis**

Survival analysis was performed for stage II and III patients \( (n = 121) \). The five-year disease-free survival (DFS) rates of t-VEGF-A-positive \( (n = 70) \) and -negative \( (n = 51) \) were 51.4% and 62.9%, respectively. There was no significant difference in t-VEGF-A expression status (Figure 3A). The five-year DFS rates of s-VEGF-A-positive \( (n = 55) \) and -negative \( (n = 66) \) cases were 73.8% and 39.9%, respectively. s-VEGF-A-positive cases had significantly better survival than negative cases \( (P = 0.0005) \) (Figure 3B).

**Expression analysis of VEGF165 and VEGF165b**

Expression analysis of VEGF165 and VEGF165b was performed using specimens of 20 cases obtained by LCM. RT-PCR was performed using specific primer sets (exon7/exon8 and exon7/exon9) to investigate the expression of VEGF165 and VEGF165b. Sequence analysis revealed that the PCR products were VEGA165 and VEGF165b \( [26] \). IHC analysis was performed in the same 20 cases. Expression of s-VEGF-A and t-VEGF-A was positive in 40% (8/20) and 70% (14/20), respectively. mRNA levels of VEGF165 and VEGF165b were semiquantified by real time PCR for each VEGF-A expres-

### Table 3  Logistic regression analysis for recurrence in colorectal carcinoma except for stage IV cases

| Factor                  | \( n \) (Recurrence) | Relative risk | 95% CI          | \( P \) value | Relative risk | 95% CI          | \( P \) value |
|-------------------------|-----------------------|---------------|-----------------|--------------|---------------|----------------|--------------|
| Clinical stage          |                       |               |                 |              |               |                 |              |
| 0                       | 9 (1)                 | 2.120         | 1.302-3.451     | 0.0250       | 1.718         | 0.980-3.010     | 0.0586       |
| 1                       | 16 (3)                |               |                 |              |               |                 |              |
| Ⅱ                       | 55 (15)               |               |                 |              |               |                 |              |
| Ⅲ                       | 66 (32)               |               |                 |              |               |                 |              |
| Venous invasion         |                       |               |                 |              |               |                 |              |
| v0                      | 46 (12)               | 1.500         | 1.050-2.143     | 0.0260       | 0.812         | 0.504-1.307     | 0.3907       |
| v1                      | 63 (27)               |               |                 |              |               |                 |              |
| v2                      | 27 (6)                |               |                 |              |               |                 |              |
| v3                      | 10 (6)                |               |                 |              |               |                 |              |
| Lympathic invasion      |                       |               |                 |              |               |                 |              |
| ly0                     | 52 (13)               | 2.094         | 1.354-3.238     | 0.0010       | 1.27          | 0.714-2.261     | 0.4155       |
| ly1                     | 68 (24)               |               |                 |              |               |                 |              |
| ly2                     | 23 (12)               |               |                 |              |               |                 |              |
| ly3                     | 3 (2)                 |               |                 |              |               |                 |              |
| s-VEGF-A positive       | 68 (14)               | 0.269         | 0.135-0.535     | 0.0002       | 0.309         | 0.141-0.676     | 0.0033       |
| t-VEGF-A positive       | 73 (31)               | 2.340         | 1.218-4.495     | 0.0110       | 1.918         | 0.768-3.718     | 0.1918       |
| Total                   | 146 (51)              |               |                 |              |               |                 |              |

CI: Confidence interval.
sion status determined by IHC. In tumor tissues, only VEGF165 was expressed in t-VEGF-A-positive cases \( (P = 0.02) \) (Figure 4A). In stromal tissues, both VEGF165 and VEGF165b were expressed in s-VEGF-A-positive cases (Figure 4B).

**Correlation between VEGF165b expression in stromal tissues and venous invasion, VEGF165b expression in stromal tissues and lymphatic invasion**
The VEGF165b mRNA level in v0 cases was significantly higher than in v1 cases (Figure 5A). There were no significant differences of VEGF165b mRNA levels among various degrees of lymphatic invasion (Figure 5B).

**VEGF165 and VEGF165 mRNA levels and MVD in each case**
In cases with lower VEGF165b mRNA levels (numbers 1-8), MVD depended on the VEGF165 mRNA level, while in cases with higher VEGF165b mRNA levels (numbers 14-20), MVD did not reach a high score regardless of the VEGF165 mRNA level (Figure 6).

**DISCUSSION**
Neoangiogenesis plays an important role in the progression and metastasis of colorectal cancer, and VEGF-A, among many molecules, is known to be of paramount importance because VEGF-A secreted from tumor cells chiefly binds to VEGFR-2 and induces angiogenesis. In colorectal cancer, it is well known that VEGF-A is highly expressed in cases with hematogenous metastasis \[29,30\]. Therefore, it is assumed that VEGF-A is one of the biomarkers for prognosis \[31\]. VEGF-A expression in tumor cells was examined to evaluate the degree of risk in many studies. However, there have been few reports focusing on stromal cells surrounding tumor cells. Concerning VEGF-A expression in stromal cells, stromal VEGF-A positivity generally results in a better prognosis than VEGF-A negativity \[20\].

In this report, IHC staining was performed in 165 consecutive patients with colorectal cancer to detect VEGF-A expression in tumor and stromal cells. Our results showed that s-VEGF-A expression might be a factor indicating a better prognosis. These results were consistent with a previous report \[28\] and implied that the functions of VEGF-A expressed in stromal cells might be different from those in tumor cells. Since VEGF has 6 splicing isoforms \[2-6\], we focused on one of them, VEGF165b, which was reported to inhibit neoangiogenesis. Our report demonstrated that s-VEGF-A, including VEGF165 and VEGF165b expressed in stromal cells, might inhibit
angiogenesis and reduce MVD. However, we could not conclude that VEGF165b expression improved the prognosis of colorectal cancer patients because the association between VEGF165b expression and the prognosis has not been investigated in a large series.

In this study, we clarified that s-VEGF-A, including VEGF165b, had a function to inhibit neoangiogenesis. However, it remains unexplained what kinds of cells secrete VEGF165b and what factors induce VEGF165b expression. A previous report showed that a subset of macrophages expressed VEGF-A resulting from CD68 (a macrophage-specific immunostain) macrofH staining \(^{[22]}\). In our series, 76% of CD68-positive cases were s-VEGF-A positive and most of the s-VEGF-A(+) cells were identical to CD68(+) cells under light microscope (data not shown). CD68(+ ) stromal cells, and tumor-associated macrophages (TAMs) have been reported to have dual potential to improve and worsen the prognosis\(^{[19]}\). We speculate that CD68(+) stromal cells may secrete VEGF165b and inhibit the angiogenesis induced by VEGF165 from tumor cells to interfere with tumor progression. In the future, we will study TAMs in colorectal cancer, especially those expressing VEGF165b, which may be a key to developing a novel therapeutic strategy.

In summary, the s-VEGF-A appears to be a good prognostic factor for colorectal cancer and includes VEGF165 and VEGF165b.

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**Research frontiers**

It has been reported that combined chemotherapy and an anti-VEGF-A antibody improves the response ratio of the tumor and extends the length of survival. Tumor cells are the predominant source of VEGF-A; however, stromal cells surrounding the tumor have also been shown to produce VEGF-A. In many reports, VEGF-A expression in tumor cells was examined to evaluate the degree of risk. However, there have been few reports focusing on stromal cells surrounding tumor cells.

**Innovations and breakthroughs**

In this report, immunohistochemical staining was performed in 165 consecutive patients with colorectal cancer to detect VEGF-A expression in tumor and stromal cells. The results showed that s-VEGF-A expression might be a factor indicating a better prognosis. These results implied that the functions of VEGF-A expressed in stromal cells might be different from those in tumor cells. This report demonstrated that s-VEGF-A, including VEGF165 and VEGF165b, expressed in stromal cells, might inhibit angiogenesis and reduce microvessel density.

**Applications**

The authors clarified that s-VEGF-A, including VEGF165b, had a function to inhibit neoangiogenesis. However, it remains unexplained what kinds of cells secrete VEGF165b and what factors induce VEGF165b expression. Studies of TAMs in colorectal cancer, especially those expressing VEGF165b, may be a key to developing a novel therapeutic strategy.

**Peer review**

This is an excellent manuscript, with a well done methodological approach, and showing a correlation with stromal VEGF expression and colorectal cancer prognosis.

**COMMENTS**

**Background**

Neoangiogenesis plays an important role in the progression and metastasis of colorectal cancer and vascular endothelial growth factor (VEGF)-A, among many molecules, is known to be highly important because VEGF-A secreted from tumor cells chiefly binds to VEGFR-2 and induces angiogenesis. In colorectal cancer, it is well known that VEGF-A is highly expressed in cases with hematogenous metastasis. Therefore, VEGF-A is assumed to have value as a prognostic factor. VEGF-A and its receptor system are deeply involved in tumor angiogenesis. Thus, they are important molecular targets in the therapeutic strategy against colorectal cancer.

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**Peer review**

This is an excellent manuscript, with a well done methodological approach, and showing a correlation with stromal VEGF expression and colorectal cancer prognosis.

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Tayama M et al. VEGF165b expression in colorectal cancer

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