Association of plasma VEGF-A, soluble VEGFR-1 and VEGFR-2 levels and clinical response and survival in advanced colorectal cancer patients receiving bevacizumab with modified FOLFOX6

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Abstract. For individualized bevacizumab-based therapy, non-invasive biomarkers are necessary. This study assessed the predictive value of plasma vascular endothelial growth factor (VEGF)-A, soluble VEGF receptor (sVEGFR)-1 and sVEGFR-2 levels as biomarkers for clinical response and survival in advanced colorectal cancer (CRC) patients treated with bevacizumab and modified FOLFOX6 (mFOLFOX6). Forty-six unresectable advanced CRC patients and 20 healthy controls were included in this study. CRC patients were treated with bevacizumab and mFOLFOX6. Pretreatment plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels were measured using the multiplex immunoassay. Plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels were significantly higher in CRC patients than in the healthy subjects. The plasma sVEGFR-1 levels in the responder patients [complete response (CR)/partial response (PR)] and stable disease (SD) patients were significantly lower than those in the progressive disease (PD) patients (CR/PR vs. PD, p=0.025; SD vs. PD, p=0.032), while the plasma VEGF-A and sVEGFR-2 levels did not show any significant differences between the two groups of patients. Patients with higher sVEGFR-1 levels showed a significantly poorer progression-free survival (PFS) and overall survival (OS) than those with lower VEGFR-1 levels. In contrast, VEGF-A and sVEGFR-2 did not show any significant relationship between PFS and OS according to the status of each level. In the multivariate Cox proportional hazard regression analysis, sVEGFR-1 levels showed a significant relationship between PFS and OS. These results suggest that plasma sVEGFR-1 levels have a predictive value for clinical response and survival in advanced CRC patients treated with bevacizumab and mFOLFOX6. Larger scale studies are needed to further validate our results.

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Abbreviations: VEGF, vascular endothelial growth factor; sVEGFR-1, soluble vascular endothelial growth factor receptor-1; sVEGFR-2, soluble vascular endothelial growth factor receptor-2; CRC, colorectal cancer

Key words: vascular endothelial growth factor-A, vascular endothelial growth factor receptor-1, vascular endothelial growth factor receptor-2, bevacizumab, FOLFOX, colorectal cancer

Introduction

Bevacizumab is a humanized monoclonal antibody against vascular endothelial growth factor (VEGF) (1,2). In Japan, the use of bevacizumab alongside chemotherapy was approved in 2007 for the treatment of unresectable advanced CRC patients. Subsequently, clinical trials of bevacizumab have been undertaken in combination with chemotherapy, such as 5-fluorouracil (5-FU) and leucovorin (LV), 5-FU and LV (5-FU/LV) plus oxaliplatin (FOLFOX), 5-FU/LV plus ilinotecan (FOLFIRI) and capecitabine plus oxaliplatin (XELOX) (3,4). For tailored individualized therapy, many attempts have been made to identify predictive biomarkers to help select those patients that will benefit from targeted agents such as the association between the KRAS mutation status and survival outcomes in patients with metastatic CRC treated with cetuximab (5). For bevacizumab, however, no established predictive biomarkers have been identified which are associated with either treatment response or survival in patients with advanced CRC (3,6).

VEGF and its receptors are essential for the neovascularization of cancer. Numerous studies have indicated that VEGF expression in tumor specimens is correlated with microvessel density, metastasis, tumor growth and poor prognosis in a variety of human solid cancer types including colorectal cancer (CRC) (7,8). High preoperative serum or plasma VEGF concentrations may predict poor prognosis in patients with CRC (9,10). However, the values of VEGF levels as biomarkers of anti-angiogenic therapy have yet to be established and require further evaluation (5,11-13). The VEGF family consists of related homodimeric glycoproteins, including VEGF-A (also called VEGF), -B, -C, -D and -E. It is known that VEGF-A binds to two types of cell membrane receptors: VEGF (VEGFR)-1 and VEGFR-2, located in the endothelium. Moreover, VEGF-A stimulates endothelial migration, proliferation, permeability and survival

(14,15). In addition to these receptors, circulating soluble forms of VEGFR-1 (sVEGFR-1) and VEGFR-2 (sVEGFR-2) have attracted attention as potential biomarkers of various malignancies. The sVEGFR-1 has been examined both as a potential surrogate marker for disease progression and/or as a potential inhibitor of tumor angiogenesis in colon, breast and renal cell carcinoma (16-21). However, the clinical significance of plasma sVEGFR-1 and sVEGFR-2 levels as biomarkers of anti-angiogenic therapy combined with chemotherapy have yet to be sufficiently investigated.

The present study aimed to evaluate the predictive value of plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels as biomarkers for clinical response and survival in unresectable advanced CRC patients treated with bevacizumab and modified FOLFOX 6 (mFOLFOX6) as a first-line therapy.

Materials and methods

Patients and study treatment blood samples. Forty-six unresectable advanced CRC patients (TNM stage IV) and 20 healthy controls were enrolled in this study. The patients were treated with bevacizumab and mFOLFOX6 as a first-line therapy between 2007 and 2009. Bevacizumab was administered at a dosage of 5 mg/kg on day 1 of every two-week period. The regimen of mFOLFOX6 was: oxaliplatin 85 mg/m^2 on day 1 and 5-FU/LV (LV 200 mg/m^2 on day 1, 5-FU 400 mg/m^2 on day 1 and 5-FU 2,400 mg/m^2 continuous infusion on days 1 and 2). The median number of cycles of bevacizumab and mFOLFOX6 were 10. Patients were treated until disease progression, development of unacceptable toxicity or patient refusal. Peripheral blood was obtained from each patient before the treatment of bevacizumab combined with mFOLFOX6. Informed written consent was obtained from patients included in the study.

VEGFR-A, sVEGFR-1 and sVEGFR-2 measurements. Plasma samples were collected from the peripheral blood of each patient by centrifugation and stored at -80˚C until use. VEGF-A, sVEGFR-1 and sVEGFR-2 plasma levels were measured using the multiplex human immunoassay kit and the multiplex human soluble cytokine receptor panel kit (both from Millipore Co., MA, USA). Measurements were performed as follows: each 96-well filter plate was washed with 200 μl wash buffer, followed by filtration under a vacuum. The standard and control were added into appropriate wells, followed by 25 μl assay buffer, 25 μl samples and matrix solution. The bead mix was diluted in wash buffer, and 25 μl of the mix were added to each well. The plates were maintained at 4˚C overnight. The following day, the medium was vacuum-filtered, and 25 μl detection antibody was added to each well. The plates were incubated for 1 h at room temperature (RT). Streptavidin-phycoerythin (25 μl) was added to each well and incubated for 30 min at RT. The wells were washed twice with 200 μl wash buffer, and 150 μl sheath fluid was added. The plates were read on a Luminex 200™ (Millipore Co.), and data were analyzed by xPONENT and Milliplex analyst software. The samples were examined in duplicate.

Assessment of efficacy. Tumor responses were assessed every 4-6 weeks using RECIST criteria and classified into four groups: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Response rates were calculated by the number of patients with CR or PR.

Table I. Patient characteristics.

Characteristics of the patients (n=46)	VEGF-A levels	
	No. of patients	Percent
Median age (range)	63 (49-77)	
Gender		
Male	33	71.7
Female	13	28.3
Primary sites		
Colon	32	69.6
Rectum	14	30.4
Site of metastasis[a]		
Liver	22	47.8
Lung	17	34.1
Lymph node	15	32.6
Omentum	14	30.4
Local	6	13.0
Bone	1	2.2
Serum CEA		
CEA (+)	35	76.1
CEA (-)	11	23.9
Serum CA19-9		
CA19-9 (+)	16	34.8
CA19-9 (-)	30	65.2

[a]Overlapping cases are included.

Statistical analysis. Plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels between the advanced CRC patients and healthy controls were analyzed by the Student's t-test. Differences in the markers between the clinical responses were examined with analysis of variance and multi-comparison tests. The correlation between VEGF-A, sVEGFR-1 and sVEGFR-2 and the clinicopathological parameters was evaluated using Fisher's exact and the Chi-square tests. Progression-free (PFS) and overall survival (OS) curves were analyzed using the Kaplan-Meier method, and the differences were examined using log-rank tests. Univariate and multivariate analyses were performed using Cox proportional hazard regression analysis. The tests were analyzed using JMP software (SAS Institute Inc., Cary, NC, USA). Statistical significance was determined from two-sided tests as $p<0.05$.

Results

Patient characteristics. Table I shows the characteristics of the patients in this study. The median age was 63 years (range 49-77), with 33 male (71.7%) and 13 female (28.3%) patients. The patients had a good performance status (ECOG PS0). As

Table II. VEGF-A, sVEGFR-1 and sVEGFR-2 levels of CRC patients and healthy controls.

	No.	Median (pg/ml)	Average ± SD (pg/ml)	P-value
VEGF-A				0.004
Controls	20	54.4	68.9±69.2	
Patients	46	158.0	194.0±178.6	
sVEGFR-1				0.048
Controls	20	334.9	375.6±200.5	
Patients	46	610.5	668.3±661.5	
sVEGFR-2				<0.001
Controls	20	10938.8	10665.7±3207.4	
Patients	46	17800.5	17296.6±3987.0	

Table III. Plasma VEGF-A, sVEGR-1 and sVEGFR-2 levels and clinical response.

	No. of patients	Median (pg/ml)	Average ± SD (pg/ml)	P-value
VEGF-A				
CR/PR	11[a]	186.0	156.4±71.5	0.300[b]
SD	17	106.0	144.5±140.9	0.073[c]
PD	18	214.2	249.5±214.9	
sVEGFR-1				
CR/PR	11[a]	172.0	197.8±46.8	0.025[b]
SD	17	250.0	470.7±583.9	0.032[c]
PD	18	860.0	906.2±694.7	
sVEGFR-2				
CR/PR	11[a]	16010.0	16148.9±2273.4	0.486[b]
SD	17	17210.0	17276.8±5780.3	0.587[c]
PD	18	18035.5	17549.7±2570.2	

[a]CR, 1; PR, 10. [b]CR/PR vs. PD; [c]SD vs. PD.

primary tumor sites, 32 patients (69.6%) had colon cancer and 14 patients (30.4%) had rectal cancer. The evaluable tumor sites for treatment were the liver (22 patients; 47.8%), lung (17 patients; 34.1%), lymph node (15 patients; 32.6%), omentum (14 patients; 30.4%), local (6 patients; 13.0%) and/or bone metastasis (1 patient; 2.2%). Serum CEA-positive patients numbered 35 (76.1%), and there were 16 serum CA19-9-positive patients (34.8%).

Comparison of VEGF-A, sVEGFR-1 and sVEGFR-2 levels in CRC patients and healthy controls. Table II shows the comparison of plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels in patients with metastatic CRC and healthy controls. Plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels were significantly higher in patients with metastatic CRC than in the healthy controls.

Plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels and clinical response. In this study, the patients received bevacizumab plus mFOLFOX6 as a first-line therapy. After treatment, 1 patient achieved CR, 10 patients had PR, 17 patients had SD and 18 patients had PD. The overall response rate was 23.9% (11/46). Table III shows the association between the plasma

Table IV. Association of patient characteristics and VEGF-A, sVEGFR-1 and sVEGFR-2 levels.

	VEGF-A levels			sVEGFR-1 levels			sVEGFR-2 levels		
Characteristics of the patients (n=46)	High (n=24)	Low (n=22)	P-value	High (n=23)	Low (n=23)	P-value	High (n=23)	Low (n=23)	P-value
Median age (range)	61.0 (46-77)	64.5 (49-76)	0.411	62.0 (46-77)	64.0 (50-74)	0.622	61.0 (46-74)	65.0 (52-76)	0.163
Gender									
Male	20 (83.3)	14 (63.6)	0.129	17 (73.9)	17 (73.9)	1.000	19 (82.6)	15 (65.2)	0.179
Female	4 (16.7)	8 (36.4)		6 (26.1)	6 (26.1)		4 (17.4)	8 (34.8)	
Primary sites									
Colon	19 (79.2)	15 (68.2)	0.397	19 (82.6)	15 (65.2)	0.179	19 (82.6)	15 (65.2)	0.179
Rectum	5 (20.8)	7 (31.8)		4 (17.4)	8 (34.8)		4 (17.4)	8 (34.8)	
Serum CEA									
CEA (+)	19 (79.2)	16 (72.7)	0.609	17 (73.9)	18 (78.3)	0.730	17 (73.9)	18 (78.3)	0.730
CEA (-)	5 (20.8)	6 (27.3)		6 (26.1)	5 (21.7)		6 (26.1)	5 (21.7)	
Serum CA19-9									
CA19-9 (+)	6 (25.0)	10 (45.5)	0.146	7 (30.4)	9 (39.1)	0.536	8 (34.8)	8 (34.8)	1.000
CA19-9 (-)	18 (75.0)	12 (54.5)		16 (69.6)	14 (60.9)		15 (65.2)	15 (65.2)	

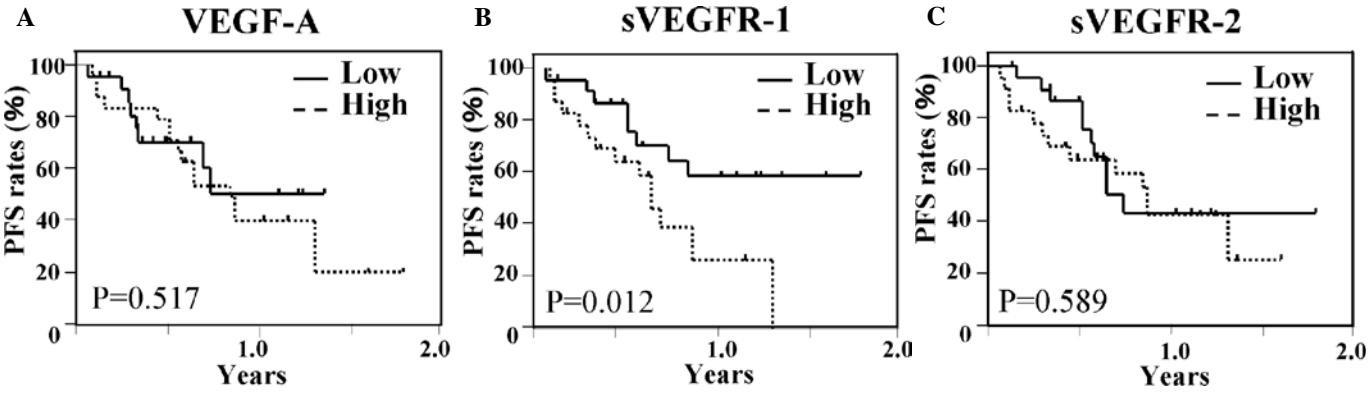

Figure 1. Progression-free survival curves according to the status of plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels. Kaplan-Meier progression-free survival (PFS) curves according to the status of plasma VEGF-A (A), sVEGFR-1 (B) and sVEGFR-2 (C) levels were examined. Each marker was divided into a higher- and lower-group by the medium level. (A) No significant differences were noted between patients with higher levels (n=24) and those with lower levels (n=22). (B) There were significant differences between the sVEGFR-1 in the higher- (n=23) and lower-group (n=23) (P=0.012). (C) No significant differences were noted between the higher- (n=23) and lower-group (n=23).

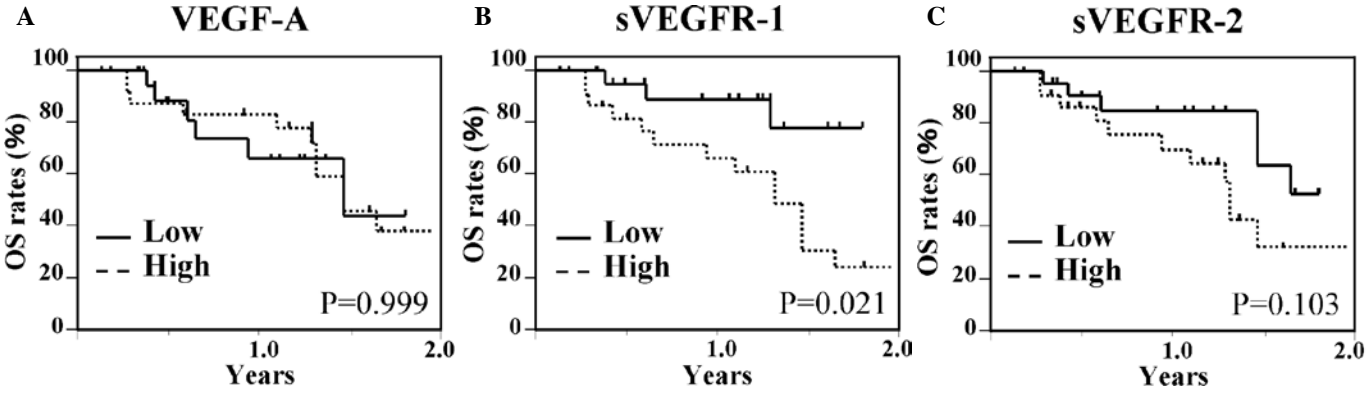

Figure 2. Overall survival curves according to the status of plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels. Kaplan-Meier overall survival (OS) curves according to the status of plasma VEGF-A (A), sVEGFR-1 (B) and sVEGFR-2 (C) levels were examined. Each marker was divided into a higher- and a lower-group according to the medium level. (A) No significant differences were noted between the higher- (n=24) and lower-group (n=22). (B) There were significant differences between sVEGFR-1 in the higher- (n=23) and lower-group (n=23) (P=0.021). (C) No significant differences were noted between the higher- (n=23) and lower-group (n=23).

VEGF-A, sVEGFR-1 and sVEGFR-2 levels and clinical responses. Regarding the VEGF-A levels, there were no significant differences between the responder, SD and PD groups. In contrast, the average level of sVEGFR-1 was 197.8±46.8 pg/ml in the responder patients, 470.7±583.9 pg/ml in the SD patients and 906.2±694.7 pg/ml in the PD patients. Significant differences were noted between the CR/PR vs. PD group (p=0.025), and the SD vs. PD group (p=0.032). No significant differences were noted when sVEGFR-2 levels and clinical responses were compared. These results suggest that sVEGFR-1 levels show a significant relationship with the clinical response.

Association of patient characteristics and VEGF-A, sVEGFR-1 and sVEGFR-2 levels. To examine the association of patient characteristics and VEGF-A, sVEGFR-1 and sVEGFR-2 levels, patients were divided into two groups (a higher level and a lower level group) by the setting of a cut-off based on median levels (Table IV). The median levels of VEGF-A, sVEGFR-1 and sVEGFR-2 were 165.0 pg/ml, 327.5 pg/ml and 17800.5 pg/ml, respectively (data not shown). No statistically significant differences were noted in the patient characteristics and VEGF-A, sVEGFR-1 and sVEGFR-2 levels.

Plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels and survival. Fig. 1 shows the Kaplan-Meier PFS curves according to the status of plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels. In a comparative analysis based on the levels of sVEGFR-1, significant differences were noted between patients with higher and those with lower levels. In contrast, in the analysis of VEGF-A and sVEGR-2, no significant differences were found between patients with higher and those with lower levels. OS curves according to the status of plasma VEGF-A, sVEGFR-1 and sVEGFR-2 levels were then examined (Fig. 2). Patients with higher sVEGFR-1 levels showed a significantly poorer OS than those with lower VEGFR-1 levels. An analysis of VEGF-A and sVEGFR-2 showed no significant differences between patients with higher and those with lower levels of sVEGFR-1. These results suggest that sVEGFR-1 levels are significantly associated with the PFS and OS of patients treated with bevacizumab and mFOLFOX6.

Table V. Multivariate analysis of biological factors for PFS.

Factors	Multivariate analysis for PFS		
	Regression coefficient	Hazard ratio (95% CI)	P-value
VEGF-A	0.364	1.439 (0.505-4.303)	0.496
sVEGFR-1	1.119	3.063 (1.189-8.520)	0.021
sVEGFR-2	-0.143	0.866 (0.343-2.184)	0.758
Serum CEA	-0.222	0.861 (0.277-2.620)	0.695
Serum CA19-9	0.849	2.338 (0.736-8.105)	0.151

PFS, progression-free survival; 95% CI, 95% confidence interval.

Table VI. Multivariate analysis of biological factors for OS.

Factors	Multivariate analysis for OS		
	Regression coefficient	Hazard ratio (95% CI)	P-value
VEGF-A	-0.291	0.747 (0.103-3.341)	0.698
sVEGFR-1	1.216	1.824 (0.654-5.604)	0.040
sVEGFR-2	-0.356	0.701 (0.140-3.171)	0.254
Serum CEA	-0.165	0.848 (0.229-4.018)	0.816
Serum CA19-9	-0.356	0.701 (0.140-3.171)	0.647

OS, overall survival; 95% CI, 95% confidence interval.

Multivariate analysis of biological factors for survival. Table V shows the multivariate analysis of biological factors for PFS. In this analysis, sVEGFR-1 levels showed a significant relationship with PFS. Table VI shows the multivariate analysis of biological factors for OS, and sVEGFR-1 levels showed a significant relationship with OS. In the analysis of clinicopathological factors of patients and survival, there was no significant relationship with PFS and OS (data not shown).

These results suggest that plasma sVEGFR-1 levels are a useful prognostic indicator for PFS and OS for advanced CRC patients treated with bevacizumab and mFOLFOX6.

Discussion

This study showed that plasma sVEGFR-1 levels, but not VEGF-A, and sVEGFR-2, are associated with clinical response and survival in advanced CRC patients treated with bevacizumab and mFOLFOX6.

It has been well established that VEGF is a key mediator of tumor vascularization. There is growing recognition of the central role that the VEGF family plays in angiogenesis, the formation of new blood vessels, which is necessary for the growth and spread of a tumor (22). The VEGF family consists of seven members, VEGF-A (also called VEGF), -B, -C, -D, -E, -F and placental growth factor (PIGF), which share eight cytokine residues in a VEGF homology domain (23). VEGF-A, particularly the VEGF165 and VEGF121 isoforms, plays an integral role in tumor angiogenesis both as an activator and survival factor in endothelial cells. Circulating plasma VEGF levels have been studied as a possible surrogate marker of angiogenesis in numerous malignancies (24). However, the applicability of plasma VEGF levels to predict the response and survival of patients treated with bevacizumab and chemotherapy has yet to be sufficiently proven. Burstein *et al* reported that lower levels of plasma VEGF were associated with longer time to progression in advanced breast cancer patients receiving bevacizumab and vinorelbine chemotherapy (25). In contrast, Denduluri *et al* reported that baseline levels of plasma VEGF in breast cancer patients did not predict clinical response to bevacizumab (12). In our study, pretreatment plasma VEGF-A levels in patients receiving bevacizumab with mFOLFOX6 did not show any significant relationship between clinical response and survival. Notably, Holden *et al* reported that in their retrospective analysis of 398 metastatic CRC patients, the survival benefit associated with bevacizumab was independent of pretreatment plasma VEGF levels (26). Since VEGF is only one of the markers of anti-angiogenesis, its significance in clinical response and survival may be asserted through several different pathways.

It is known that sVEGFR-1 and sVEGFR-2 are generated either via proteolytic cleavage of the ectodomain from the cell surface or via alternative mRNA splicing, which gives rise to a secreted polypeptide lacking a transmembrane region and functioning as a high-affinity receptor of VEGF. Expression of VEGFRs, including VEGFR-1 and VEGFR-2 (both of which are expressed in a number of tumor cell types in addition to endothelial cells), has been correlated with various disease stages (27-29). It has previously been shown that circulating levels of soluble forms of these receptors, which are not capable of signal transduction, bind VEGF in the bloodstream and reduce the levels of free VEGF. This limits the pro-angiogenic effects of VEGF at the endothelial cell level (1,30). In particular, sVEGFR-1 has been studied, not only as a potential surrogate marker for disease progression, but also as a potential inhibitor of tumor angiogenesis in various types of cancers (5,31). Previous studies have shown that sVEGFR-1 and/or sVEGFR-2 levels in plasma were higher in cancer patients than in healthy volunteers (21,32,33). Our present data also demonstrated that plasma sVEGFR-1 and sVEGFR-2 levels of advanced CRC patients are significantly higher than those of healthy volunteers. Toi *et al* (18) and Yamaguchi *et al* (20) reported that sVEGFR-1 levels in tumor tissue were an independent prognostic indicator of disease progression in CRC patients. However, the predictive values of plasma sVEGFR-1 and sVEGFR-2 levels for chemotherapy responses are still controversial. Ustuner *et al* reported that no significant differences were detected between the concentration of serum sVEGFR-1 and sVEGR-2 and chemotherapy response in small cell lung cancer patients (34). In contrast, Wierzbowska *et al* reported correlations between the pretreatment plasma VEGFR-1 concentration, tumor burden and poor prognosis in acute myeloid leukemia (AML) patients (32). Additionally, the serum VEGFR-1/VEGF ratio had a greater prognostic value

than VEGF alone in their study. Hu *et al* also reported that plasma sVEGFR-1, but not sVEGFR-2, was an independent prognostic factor in AML and myelodysplastic syndromes (21). Little is currently known regarding the potential of plasma sVEGFR-1 and sVEGFR-2 levels as a predictive biomarker for treatment response and survival in CRC patients treated with bevacizumab-based therapy. Our results in CRC patients indicated that the pretreatment level of sVGEFR-2 showed no association with clinical response and survival. This is in line with previous reports that failed to detect a predictive marker for bevacizumab with chemotherapy. Nevertheless, our data showed that plasma sVEGFR-1 levels predicted the treatment response and survival in advanced CRC patients treated with bevacizumab and mFOLFOX6. To the best of our knowledge, the present study is the first to suggest the predictive value of sVEGFR-1 for clinical response and survival in advanced CRC patients treated with bevacizumab and mFOLFOX6. It has been documented that the plasma sVEGFR-1 level is related to tumor phenotype or prognosis, suggesting that sVEGFR-1 has a significant biological function in tumor cells (30). Although it is difficult to elucidate the correlation between the plasma sVEGFR-1 levels and clinical response and survival, plasma sVEGFR-1 levels may reflect tumor malignancy and predict tumor progression in the metastatic site. Notably, Willett *et al* reported that pretreatment sVEGFR-1 levels in patients with rectal cancer were correlated with post-treatment tumor stage after combination therapy with bevacizumab, radiation and chemotherapy (11). These results support the possible predictive value of plasma sVEGFR-1 levels in CRC patients treated with bevacizumab-based chemotherapy. We await further studies that may elucidate, in detail, the association between plasma sVEGFR-1 and clinical response and survival.

Although there were limitations to the present study due to the small sample size and the fact that it was a single-arm study, we believe that our findings warrant the further evaluation of plasma sVEGFR-1 as a predictive marker for clinical response and survival in metastatic CRC patients. Larger scale studies are needed to further validate our results.

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