Impact of vascular endothelial growth factor receptor 1, 2, and 3 expression on the outcome of patients with gastric cancer

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Tumor angiogenesis is a multistep interactive process in which vascular endothelial growth factor (VEGF) and its receptors have a major role. However, the clinical significance of these molecules in gastric cancer (GC) remains unclear. Our study group comprised 86 patients who underwent gastrectomy and subsequently received chemotherapy for recurrent or residual tumor. Using immunohistochemical techniques, we analyzed the expression of VEGF receptors (VEGF-R) 1, 2, and 3. VEGF-R1 expression (defined as >5% staining) was found in the tumor cells of 65 tumors (76%) and in the stromal vessels of 36 tumors (42%). VEGF-R2 expression was found in tumor cells and stromal vessels of 0 and 46 tumors (0 and 53%), respectively, and VEGF-R3 expression was found in tumor cells and stromal vessels of 0 and 75 tumors (0 and 87%), respectively. Univariate analysis revealed that VEGF-R expression correlated with shorter survival (VEGF-R1 in stromal vessels, $P = 0.001$; VEGF-R2 in stromal vessels, $P = 0.009$; VEGF-R3 in stromal vessels, $P = 0.005$) and lower response to S-1 (VEGF-R1 in stromal vessels, $P = 0.039$). Multivariate analysis of potential prognostic factors showed that VEGF-R1 and VEGF-R2 in stromal vessels were independent predictors of poor outcome. Our data suggest that VEGF-R expression can be a predictor of unfavorable clinical outcome in GC. VEGF-R are promising candidates as therapeutic targets. (*Cancer Sci* 2009; 100: 310–315)

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide, accounting for over 20 deaths per 100 000 population annually in East Asia (China, Japan), Eastern Europe, and parts of Central and South America.[1] Recently, many chemotherapy regimens using new agents have been developed that show high response rates for advanced GC, and progress in basic research has revealed many factors and mechanisms implicated in sensitivity and resistance to chemotherapy.

Angiogenesis reportedly plays an important role in cancer invasion and metastasis. Vascular endothelial growth factor (VEGF) and VEGF receptor (VEGF-R) represent important regulators of angiogenesis, and increased expression of this family of molecules has been documented in various cancer cell lines[2] and tissues.[3,4] Previous clinical studies have demonstrated that increased expression of VEGF or its family is associated with the grade of angiogenesis and the prognosis for various human cancers.[5–9]

In GC, several studies have found that expression of VEGF ligands and subtypes correlates with prognosis,[10–12] and expression of soluble VEGF-R1 is also a predictor of prognosis.[13] However, the distribution, frequency, and prognostic value of VEGF-R expression in GC have not been clarified. The present study investigated relationships between VEGF-R expression and prognosis in patients with advanced GC.

Materials and Methods

Patients. Subjects were 86 patients who underwent surgery for primary GC and received chemotherapy for the treatment of recurrent or residual tumors at the National Cancer Center Hospital (NCCH). Inclusion criteria were as follows: histologically proven advanced GC; unresectable, locally advanced, or metastatic disease; no prior chemotherapy and no prior adjuvant or neoadjuvant chemotherapy; specimens of primary GC were obtained before the start of chemotherapy by surgical resection or biopsy at NCCH; radiographically measurable disease; first-line chemotherapy was received from January 1995 to December 2004; tumor response and survival times were confirmed; adequate bone marrow, liver, and renal function; and written informed consent. The tissue samples were collected retrospectively from patients who met these criteria. Measurable disease was assessed by computed tomography. Response was evaluated according to the standard International Union against Cancer (UICC) guidelines as complete response (CR), partial response (PR), no change (NC), or progressive disease (PD). The response rate was calculated as the ratio of CR + PR to CR + PR + NC + PD.[14] Written informed consent was obtained before treatment and evaluation of tumor samples.

Immunohistochemical staining. Serial 4-μm sections were made from formalin-fixed paraffin-embedded tissue. Sections were dewaxed in xylene and rehydrated through a graded alcohol series. Antigen retrieval was carried out by incubating sections in target-retrieval solution (Dako Japan, Tokyo, Japan) for 40 min in a 95°C water bath and cooling for at least 20 min.

After quenching endogenous peroxidase with peroxidase-blocking reagent (Dako Japan) for 5 min and washing with Tris-buffered saline containing Tween 20, sections were incubated with the primary antibody (Table 1).

Immunoreaction was detected using the following secondary antibody systems: CSA-II (Dako Japan) for VEGF-R1, VEGF-R2, and VEGF-R3; and the Envison + kit (Dako Japan) for CD34, D2-40, CD31, and factor VIII, according to the instructions of the manufacturer. Sections were counterstained using Mayer's hematoxylin.

Evaluation of immunostaining. The entire specimen was examined at low magnification (×40), and positive cells were counted in areas with strong immunoreactivities at high magnification (×200). The number of immunoreactive cells was counted in three fields of view that exhibited the most positive staining, and the average ratio of immunoreactive cells to the

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Table 1. Antibodies used for immunohistochemistry

Antigen	Antibody	Manufacturer	Dilution	Incubation time (min)
CD34	M 7165	Dako Japan	1:100	30
D2-40	M 3619	Dako Japan	1:50	30
CD31	M 0823	Dako Japan	1:50	Overnight
Factor XIII	N 1505	Dako Japan	1:2	30
VEGF-R1	AF 321	R&D	1:150	15
VEGF-R2	AF 357	R&D	1:50	15
VEGF-R3	AF 349	R&D	1:50	15

total number of cancer cells per field was calculated. The number of immunoreactive vessels was counted in three fields of view that demonstrated the most positive staining, and the average ratio of immunoreactive vessels to the total number of CD34-positive and D2-40-positive vessels per field was calculated. Staining results for VEGF-R1, VEGF-R2, and VEGF-R3 were classified by estimating the percentage of epithelial cells and vessels showing specific immunoreactivity: negative (defined as <5% staining) or positive (defined as >5% staining).[7] Two researchers evaluated the immunostaining results without being informed of the clinical data.

Statistical analysis. We examined objective tumor response to chemotherapy overall survival. Overall survival were calculated as the period from the start of first-line chemotherapy until disease progression or death from any cause, respectively. If patients were lost to follow up, data were censored at the date of the last evaluation. Statistical analysis was carried out using Stat View version 5 software (SAS Institute, Cary, NC, USA). Pearson's correlations were used to assess VEGF and VEGF-R expression, and a χ^2-test was used to assess relationships between VEGF and VEGF-R expression and therapeutic effect. Each factor and overall survival were determined by Kaplan–Meier methods and analyzed using a log-rank test. Multivariate analysis was carried out using a Cox proportional hazard model.

Results

Clinicopathological characteristics. The clinicopathological characteristics of the patients are shown in Table 2. Patients comprised 69 (80%) men and 17 (20%) women, with a median age of 61 years. Tumor stage (assessed according to TNM classification at the time of surgery) was I, II, or III in 35 patients, and distant metastasis was confirmed at the time of surgery (stage IV) in 51 patients. Histopathologically, 39 patients had intestinal-type adenocarcinoma and 47 displayed diffuse-type adenocarcinoma. All patients received chemotherapy; first-line chemotherapy comprised S-1 in 29 patients, 5-fluorouracil (5-FU) in 24 patients, cisplatin (CDDP) and irinotecan (CPT-11) in 28 patients, and other agents in the remaining five patients. The median follow-up time was 13.3 months (range 1.0–71.7 months).

Expression of VEGF-R1, VEGF-R2, and VEGF-R3. VEGF-R1 was immunoreactive in tumor cells (not only in the membrane, but also in the cytoplasm) and tumor stromal vessels (Fig. 1a). VEGF-R1 expression was found in tumor cells of 65 tumors (76%) and in stromal vessels of 36 tumors (42%) (Table 3).

VEGF-R2 and VEGF-R3 were immunoreactive mainly in tumor stromal vessels (Fig. 1b–d). VEGF-R2 expression was found in tumor cells and stromal vessels of 0 and 46 tumors (0 and 53%), respectively, and VEGF-R3 expression was found in tumor cells and stromal vessels of 0 and 75 tumors (0 and 87%), respectively. The three types of VEGF-R were not markedly correlated with each other in terms of expression.

Table 2. Patient characteristics (n = 86)

Characteristic	n
Sex	
Male	69
Female	17
Median age (years)	61 (range 39–84)
Tissue type	
Intestinal	39
Diffuse	47
pStage†	
I	2
II	11
III	22
IV	51
ECOG performance status	
0	42
1	41
2	3
Metastases	
Liver	25
Abdominal lymph node	43
Peritoneum	23
Lung	4
Other	4
First-line chemotherapy	
S-1	29
5-Fluorouracil	24
Cisplatin + irinotecan	28
Other	5

†Japanese classification. ECOG, Eastern Cooperative Oncology Group.

Table 3. Distribution of vascular endothelial growth factor receptor (VEGF-R) 1, VEGF-R2, and VEGF-R3 expression

Status	VEGF-R1				VEGF-R2		VEGF-R3	
	Cytoplasm		Vessel		Vessel		Vessel	
	n	%	n	%	n	%	n	%
Negative (<5%)	21	24	50	58	40	47	11	13
Positive (>5%)	65	76	36	42	46	53	75	87

Relationship of VEGF-R expression with response to chemotherapy and survival. The response rate was 38% (11/29) in the S-1 group, 4% (1/24) in the 5-FU group, and 43% (12/28) in the CDDP and CPT-11 group (Table 4). In the S-1 group, the response rate was lower in the 15 patients in whom stromal vessels stained positive for VEGF-R1 than in the 14 patients in whom stromal vessels did not (20 vs 57%, χ^2-test $P = 0.039$). In the other groups, the response rates were not markedly affected by expression of VEGF-R.

To clarify the relevance of marker positivity in prediction of disease outcome, staining results for VEGF-R1, VEGF-R2, and VEGF-R3 were correlated with patient survival according to the log-rank test. A univariate analysis revealed that VEGF-R expression correlated with shorter survival (VEGF-R1 in stromal vessels, 11.2 vs 15.9 months, $P = 0.001$, Fig. 2a; VEGF-R2 in stromal vessels, 11.0 vs 15.6 months, $P = 0.009$, Fig. 2b; VEGF-R3 in stromal vessels, 12.8 vs 24.3 months, $P = 0.005$, Fig. 2c). Moreover, multivariate analysis of potential prognostic factors showed that VEGF-R1 and VEGF-R2 expression by stromal vessels were independent predictors of poor outcome in advanced GC (Table 5).

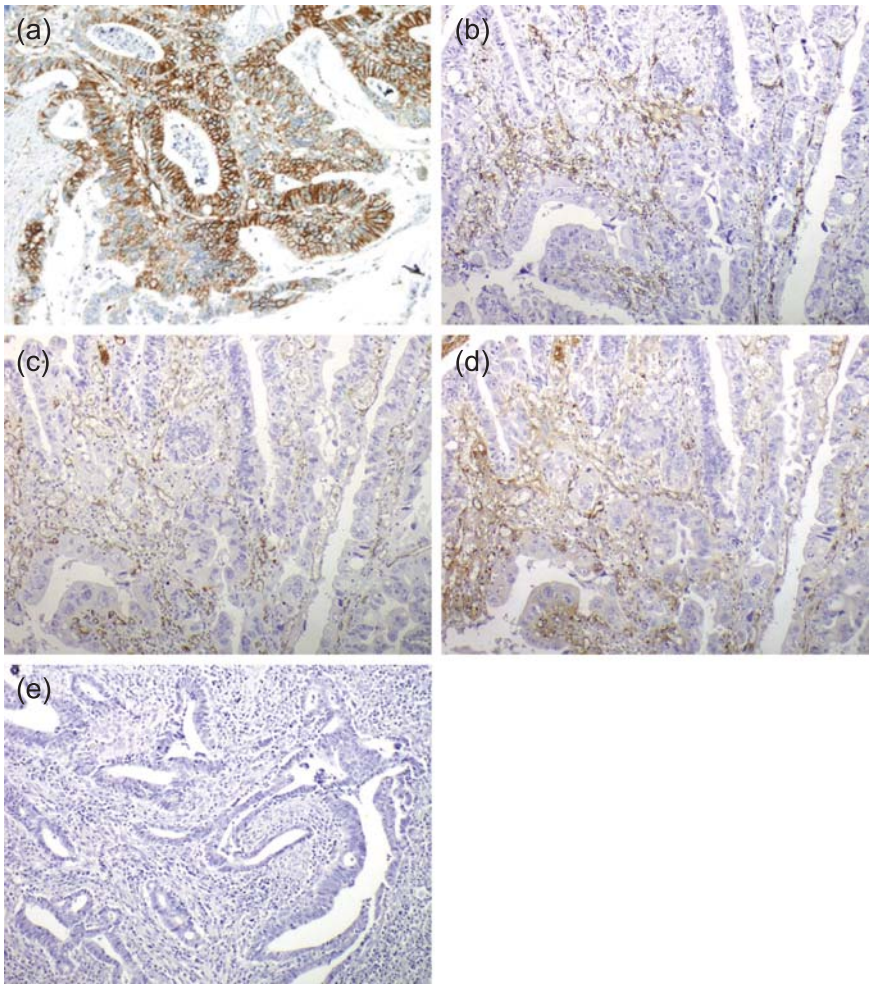

Fig. 1. Typical examples of (a) CD34 staining, (b) D2-40 staining, (c) CD31 staining, (d) factor VIII staining, and (e) negative controls. (a) Vascular endothelial growth factor receptor (VEGF-R) 1 is mainly expressed in tumor cells, secondarily on stromal vessels. (b–d) VEGF-R2 and VEGF-R3 are mainly expressed on stromal vessels. Original magnification, ×200.

Table 4. Relationship between vascular endothelial growth factor receptor (VEGF-R) expression and response to chemotherapy

First-line regimen	*n*	Total response (%)	VEGF-R1				VEGF-R2		VEGF-R3	
			Cytoplasm		Stromal vessels		Stromal vessels		Stromal vessels	
			Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
S-1	29	38	32	57	20	57	31	44	37	50
			$P = 0.234$		$P = 0.039$		$P = 0.474$		$P = 0.715$	
Cisplatin and irinotecan	28	43	33	47	45	41	47	38	46	25
			$P = 0.255$		$P = 0.570$		$P = 0.445$		$P = 0.887$	
5-Flurouracil	24	4	0	4	0	4	4	0	4	0
			–		–		–		–	

Discussion

In the present study, we analyzed VEGF-R expression levels in primary tumors from 86 patients with advanced GC. Our goal was to determine whether such expression levels are related to treatment outcomes such as survival and response. We found that expression of VEGF-R1 and VEGF-R2 in stromal vessels in GC specimens were significant predictors of poor survival in advanced GC. Recently, several studies have reported that the genetic profile of patients is related to the outcome of cancer therapy. In colorectal cancer, VEGF-R2 expression for metastatic tumors was higher when compared to non-metastatic tumors,[5] and in head and neck cancer[15] and breast cancer,[16] some studies have documented that VEGF-R3 expression correlates with lymph node metastasis and malignancy,[7,9,14,17] whereas others have not observed this relationship.[18–20] Further investigations are needed to clarify interactions among VEGF-R subtypes and the effects of VEGF expression in stroma on angiogenesis and lymphangiogenesis. In GC, several studies have reported correlations between the expression of VEGF and poor prognosis, or lymphatic metastasis. However, most studies examined survival from the date of surgery to the time of event. In the present study, we examined the expression of VEGF-R, objective tumor response to chemotherapy, and overall survival; the latter two being calculated as the period from the start of first-line chemotherapy until disease progression or death from any cause, respectively.

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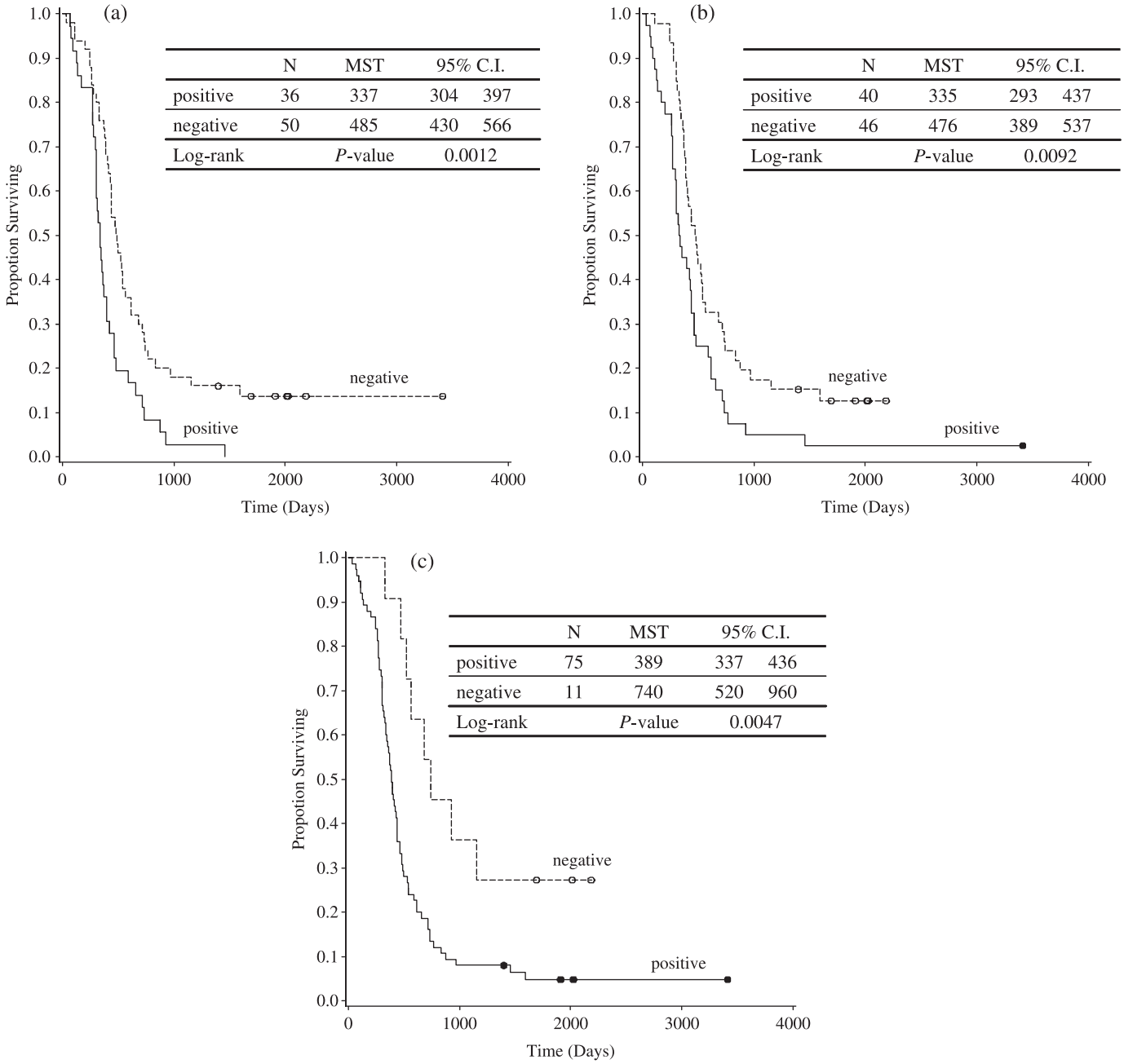

Fig. 2. Impact of (a) vascular endothelial growth factor receptor (VEGF-R) 1, (b) VEGF-R2, and (c) VEGF-R3 expression in stromal vessels on patient survival.

Table 5. Impact of vascular endothelial growth factor receptor (VEGF-R) expression on patient survival from first-line chemotherapy (multivariate analysis)

	Parameter	Hazard ratio	95% confidence interval.		*P*-value
VEGF-R1 (vessel)		**1.75**	**1.09**	**2.80**	**0.020**
PS	1, 2 versus 0	1.45	0.62	2.27	0.109
Tissue type	Diffuse vs intestinal	0.64	0.64	1.00	0.052
Metastasis site	2≥ versus 1	1.5	0.89	2.55	0.132
VEGF-R2 (vessel)		**1.76**	**1.12**	**2.75**	**0.014**
PS	1, 2 versus 0	1.56	1.00	2.46	0.052
Tissue type	Diffuse versus intestinal	0.64	0.41	1.01	0.055
Metastasis site	2≥ versus 1	1.69	1.01	2.81	0.045

PS, Performance Status.

After treatment with S-1, patients with positive staining for VEGF-R1 in stromal vessels showed a lower response rate (20 vs 57%, $P = 0.039$) and shorter survival (10.2 vs 20.2 months, hazard ratio = 3.62: data not shown) than those with negative staining, whereas there was no difference with CDDP and CPT-11. The number of patients treated with S-1 was small, but Boku *et al*. have reported the relationship between VEGF status and the effects of S-1 and 5-FU; patients expressing VEGF showed a slightly lower response rate and relatively shorter survival than those who did not.[21,22] The mechanisms behind this relationship are unclear,[23] but expression of VEGF-R may become a prognostic marker relevant in deciding on a treatment strategy of 5-FU-based drugs.

Our analysis revealed that VEGF-R expression was correlated with shorter survival (VEGF-R1 in stromal vessels, $P = 0.001$; VEGF-R2 in stromal vessels, $P = 0.009$; and VEGF-R3 in stromal vessels, $P = 0.005$), and multivariate analysis of potential prognostic factors showed that VEGF-R1 and VEGF-R2 in stromal vessels were independent predictors of poor outcome. VEGF-R2 is a potent regulator of vascular endothelial cells and has been directly linked to tumor angiogenesis and blood vessel-dependent metastasis. VEGF-R1 may contribute to pathological vascularization directly by stimulating endothelial cell function and indirectly by mediating recruitment of bone marrow progenitor cells.[24] Furthermore, Carmeliet and coworkers demonstrated synergy between the VEGF-R1- and VEGF-R2-specific ligands, indicative of cross-talk between the receptors, allowing modulation of a variety of VEGF-R-dependent signals.[25] In GC, the expression of VEGF or VEGF-C, which are intimately involved in regulation of the lymphangiogenic process, has been reported to be correlated with a poor prognosis.[10,11,26] Juttner *et al*. found that the presence of VEGF-D and its receptor VEGF-R3 was associated with lymphatic metastasis.[12] Given these results, expression of the VEGF family appears to affect the prognosis of GC.

Our immunostaining evaluation revealed that VEGF-R is expressed in tumor cells and tumor stromal vessels.VEGF-R2, which is expressed primarily in vascular endothelial cells, is believed to be the major mediator of angiogenesis in human malignancy, as it regulates activation of downstream effector molecules such as the phosphoinositide 3-kinase plus AKT and mitogen-activated protein kinase pathways. It also potentiates endothelial differentiation, DNA synthesis, and proliferation.[27,28] On the other hand, VEGF-R3 is expressed primarily in lymphatic endothelial cells and regulates lymphangiogenesis.[29] Recently, some studies have documented that the expression of VEGF-R has been observed in tumor cells in several cancers,[30–35] and in the autocrine VEGF–VEGFR loop in cancer cells. Fan *et al*. demonstrated that incubation with VEGF-A or VEGF-B significantly increased colorectal cancer cell migration; however, treatment with a VEGF-R1 antibody blocked this effect.[30] Giatromanolaki *et al*. demonstrated that phosphorylated VEGF-R2 plus KDR receptors are largely expressed in colon cancer cells and intratumoral vasculature, and their expression is associated with tumor diameter and poor histological differentiation.[31] In GC, Tian *et al*. demonstrated that VEGF-R2-positive tumor cells could be stimulated by exogenously added VEGF.[32] In our study, patients with strong positive staining (defined as >50% staining) for VEGF-R1 in the cytoplasm of tumor cells showed shorter survival (12.6 vs 14.2 m, $P = 0.044$; data not shown) than others. Thus, these results suggest that the autocrine VEGF–VEGF-R loop function may contribute to cancer cell proliferation.

In conclusion, our study provides evidence that VEGF-R expression in GC specimens is a risk factor for poor survival in patients with advanced GC. The results of our analysis can help to identify patient subgroups at higher risk for poor disease outcome in GC.

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