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RAPID COMMUNICATION

Expression of vascular endothelial growth factor and its receptors VEGFR-1 and 2 in gastrointestinal stromal tumors, leiomyomas and schwannomas

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Abstract

AIM: To investigate the role of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and 2 in the growth and differentiation of gastrointestinal stromal tumors (GISTs).

METHODS: Thirty-three GISTs, 15 leiomyomas and 6 schwannomas were examined by immunohistochemistry in this study.

RESULTS: VEGF protein was expressed in the cytoplasm of tumor cells, and VEGFR-1 and 2 were expressed both in the cytoplasm and on the membrane of all tumors. Immunohistochemical staining revealed that 26 GISTs (78.8%), 9 leiomyomas (60.0%) and 3 schwannomas (50.0%) were positive for VEGF; 24 GISTs (72.7%), 12 leiomyomas (80.0%) and 4 schwannomas (66.7%) were positive for VEGFR-1; 30 GISTs (90.9%), 5 leiomyomas (33.3%) and 4 schwannomas (66.7%) were positive for VEGFR-2. VEGFR-2 expression was statistically different between GISTs and leiomyomas ($P < 0.0001$). However, there was no correlation between the expression of VEGF pathway componenets and the clinical risk categories.

CONCLUSION: Our results suggest that the VEGF pathway may play an important role in the differentiation of GISTs, leiomyomas and schwannomas.

Key words: Gastrointestinal stromal tumor; Leiomyoma; Schwannoma; Vascular endothelial growth factor; Vascular endothelial growth factor receptors

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal (GI) tract that may occur from the oesophagus to the anus, including the omentum[1,2]. Despite their rarity, GISTs are the most common primary mesenchymal tumors of the GI tract[1-3]. The mechanisms of tumourigenesis, progression and differentiation of GISTs are unknown. Traditionally, all primary mesenchymal spindle cell tumors of the GI tract are uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas or leiomyosarcomas). Tumors with epithelioid cytologic features are designated leiomyoblastomas or epithelioid leiomyosarcomas[4]. Recently, Sircar *et al*[5] postulated that GISTs originate from Cajal cells in the GI tract and differ from leiomyomas and schwannomas, which are of mesenchymal cell origin. Cajal cells are thought to be gastrointestinal pacemaker cells that regulate intestinal motility[6]. GISTs are characterized by frequent expression of the bone marrow leukocytic progenitor cell antigen CD34[7] and the c-kit proto-oncogene[2,3,5].

Some GISTs have mutations in the genes encoding c-kit and platelet-derived growth factor alpha (PDGFR-α) that cause constitutive tyrosine kinase activation[3,8-10]. Tumors expressing c-kit or PDGFR-α oncoproteins are indistinguishable with respect to activation of downstream signaling intermediates and cytogenetic changes associated with tumor progression. C-kit and PDGFR-α mutations appear to be alternative and mutually exclusive oncogenic mechanisms in GISTs[9,10].

Vascular endothelial growth factor (VEGF) has been identified as a key regulator of tumor angiogenesis, and VEGF receptors (VEGFR) are the major mediators of the mitogenic and permeability-enhancing effects of VEGF in endothelial cells[11,12]. In addition, VEGF is a survival factor for endothelial cells, and a marked dependence on VEGF has been shown in newly formed but not established

tumor vessels[13]. Although the field of tumor angiogenesis is an area of extensive research, the consequences of enhanced angiogenesis and its reversion on tumor growth and progression are only partially elucidated[14-16]. Recently, coexpression of VEGF and its receptor, either VEGFR-1 (Flt-1) or VEGFR-2 (Flk-1/KDR), has been reported in tumor cells, suggesting the presence of an autocrine and/or a paracrine VEGF/VEGFR growth pathway in solid tumors[17-19]. Further, the expression levels of VEGF and its receptors have been shown to correlate with progressive tumor growth and development of metastasis by many carcinomas[20].

These studies suggest that the VEGF pathway is involved in tumor growth and differentiation. However, there are no data detailing VEGFR expression in GISTs, leiomyomas or schwannomas, or the role of VEGF in the etiology of these tumors. The purpose of this study was to investigate the expression of VEGF and VEGFRs in GISTs.

MATERIALS AND METHODS

Samples

Thirty-three specimens of GISTs (28 from the stomach and 5 from the small intestine), 15 specimens of leiomyomas (4 from the oesophagus, 4 from the stomach and 7 from the large intestine), 6 specimens of schwannomas (5 from the stomach and 1 from the large intestine) were selected from surgical pathology archival tissues at Nagasaki University Hospital between 2001 and 2006. The specimens of GISTs, leiomyomas and schwannomas were 0.8-12.0 cm, 0.1-4.5 cm and 0.6-5.0 cm, respectively. In this study, GISTs were defined as expressing both c-kit and CD34 surface antigens and classified by risk categories, mitosis counts and tumor size[21]. The number of mitoses was determined by counting 50 high-power fields (HPF, × 400) under a Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined both as expressing α-smooth muscle cell actin (SMA) but not c-kit, CD34 or S100-protein and as expressing S100-protein but not c-kit, CD34 or SMA. Two independent pathologists (T. Nakayama and I. Sekine) determined tumor identification/classification.

Immunohistochemical staining

The subcellular localization of VEGF, VEGFR-1 and 2 was determined in GISTs using polyclonal antibodies directed against unique sequences of VEGF, VEGFR-1 and 2. These antibodies were devoid of any cross-reaction with other proteins in the VEGF family. Formalin-fixed and paraffin-embedded specimens were cut into 4 μm thick sections, deparaffinized and preincubated with normal bovine serum to prevent non-specific binding. The sections were incubated overnight at 4℃ with primary polyclonal antibody to human VEGF [(147), 1 mg/L; Santa Cruz Biotechnology Inc., Santa Cruz, CA], VEGFR-1 [Flt-1(C-17), 1 mg/L; Santa Cruz Biotechnology Inc.] or VEGFR-2 [Flk-1(C-20), 1 mg/L; Santa Cruz Biotechnology Inc.], followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (0.4 μg/mL; Santa Cruz Biotechnology, Inc.). The reaction products were visualized using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; Roche Diagnostic Corp., Indianapolis, IN). Negative controls replaced the primary antibody with non-immunized rabbit serum, and human breast cancer tissue served as the positive control[22]. VEGF, VEGFR-1 and 2 expressions were classified into 3 categories depending upon the percentage of cells stained and/or the intensity of staining (-: 0% to 15% positive tumor cells; +: > 15% positive tumor cells).

Table 1 Immunohistochemistry of VEGF pathway components in intestinal stromal tumours, *n* (%)

	n	VEGF		VEGFR-1		VEGFR-2	
		-	+	-	+	-	+
GISTs	33	7 (21.2)	26 (78.8)	9 (27.3)	24 (72.7)	3 (9.1)	30 (90.9) [b]
Leiomyoma	15	6 (40.0)	9 (60.0)	3 (20.0)	12 (80.0)	10 (66.7)	5 (33.3)
Schwannoma	6	3 (50.0)	3 (50.0)	2 (33.3)	4 (66.7)	2 (33.3)	4 (66.7)

[b]$P < 0.0001$ *vs* leiomyomas.

Statistical analysis

The Stat View Ⅱ program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analysis. Analyses comparing the degree of VEGF, VEGFR-1 or 2 expressions in GISTs, leiomyomas and schwannomas were performed using the Mann-Whitney's test.

RESULTS

The results of immunohistochemical stainings for VEGF, VEGFR-1 or 2 are summarized in Table 1. VEGF, VEGFR-1 and 2 expression was heterogeneous in GISTs, leiomyomas and schwannomas and localized to the cytoplasm and/or membrane of tumor cells (Figure 1). Immunohistochemical staining revealed VEGF expression in the cytoplasm of GIST (Figure 1A), leiomyoma (Figure 1B) and schwannoma (Figure 1C) cells. VEGFR-1 expression was shown in the membrane and cytoplasm of GIST (Figure 1E), leiomyoma (Figure 1F) and schwannoma (Figure 1G) cells. VEGFR-2 was expressed in the membrane and cytoplasm of GIST (Figure 1I), leiomyoma (Figure 1J) and schwannoma (Figure 1K) cells. Immunohistochemical staining was positive for VEGF in 26 (78.8%) of 33 GISTs, 9 (60.0%) of 15 leiomyomas and 3 (50.0%) of 6 schwannomas, respectively. Twenty-four (72.7%) of GISTs, 12 (80.0%) of leiomyomas and 4 (66.7%) of schwannomas showed positive staining for VEGFR-1. There was no statistical difference in VEGF or VEGFR-1 expression between GISTs and leiomyomas or schwannomas. Immunohistochemical staining was positive for VEGFR-2 in 30 (90.9%) of GISTs, 5 (33.3%) of 15 leiomyomas an 4 (66.7%) of schwannomas. There was a statistical difference in VEGFR-2 expression between GISTs and leiomyomas ($P < 0.0001$).

The classification of GISTs by risk category, mitosis counts and tumor size is shown in Table 2. All 4 cases within the high risk category expressed VEGF, VEGFR-1 and 2. All 4 cases with over 10 mitoses per 50 HPFs strongly expressed VEGF, VEGFR-1 and 2. Finally, only one tumor that measured over 10 cm strongly

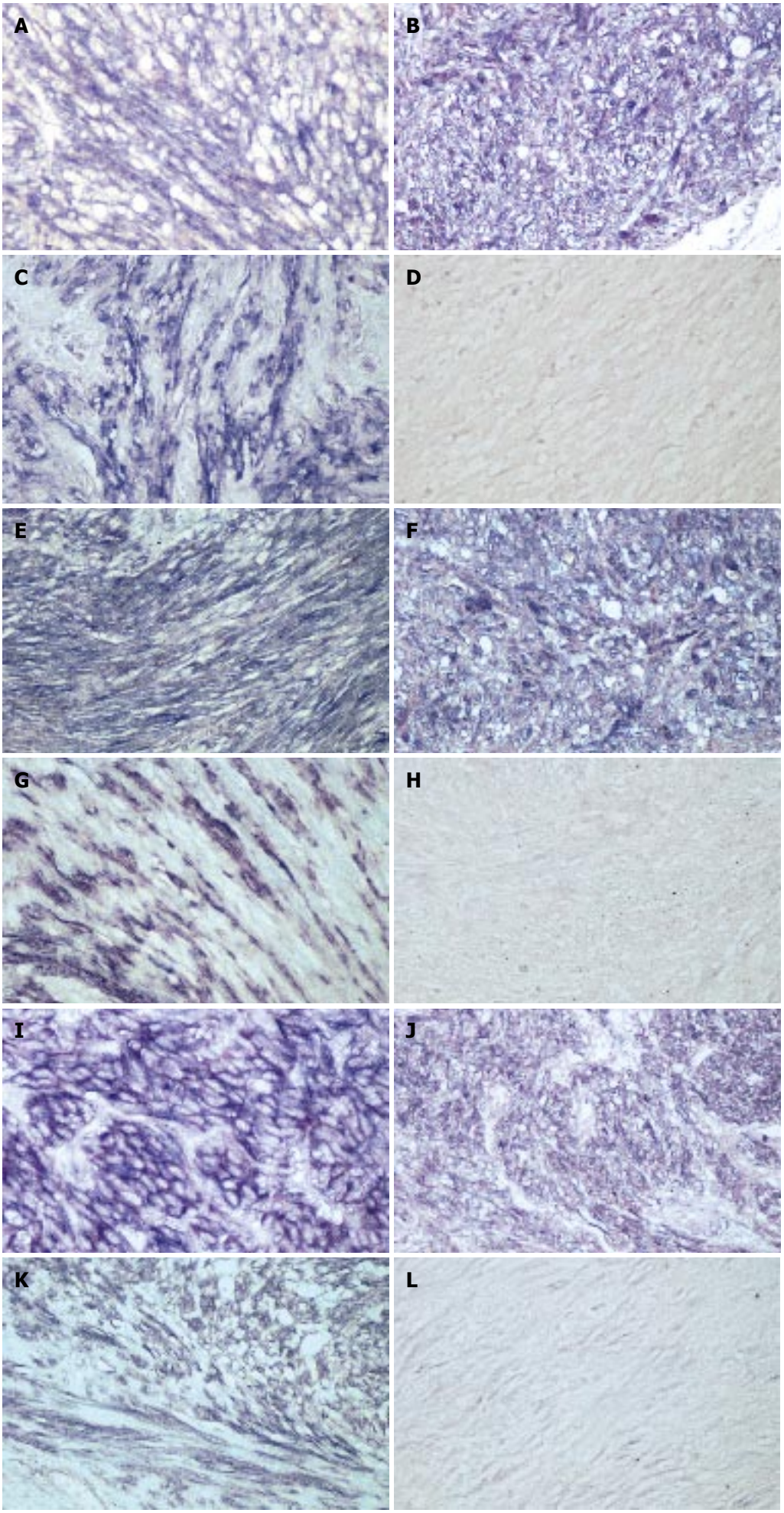

Figure 1 Immunohistochemical staining reveals VEGF expression in the cytoplasm of GIST (**A**), leiomyoma (**B**) and schwannoma (**C**) cells; VEGFR-1 expression in the membrane and cytoplasm of GIST (**E**), leiomyoma (**F**) and schwannoma (**G**) cells; VEGFR-2 expression in the membrane and cytoplasm of GIST (**I**), leiomyoma (**J**) and schwannoma (**K**) cells; negative staining of GIST for VEGF, VEGFR-1 or VEGFR-2 in Figure **D**, **H** or **L**, respectively. BCIP/NBT reaction product demonstrating VEGF, VEGFR-1 and 2 levels. (magnification: x 200).

Table 2 VEGF, VEGFR-1 and 2 expression and categories for GISTs (n = 33) n (%)

		VEGF		VEGFR-1		VEGFR-2	
	n	-	+	-	+	-	+
Total	**33**	**7**	**26**	**9**	**24**	**3**	**30**
Risk categories		NS		NS		NS	
High	4	0 (0.0)	4 (100)	0 (0.0)	4 (100)	0 (0.0)	4 (100)
Intermediate	5	1 (20.0)	4 (80.0)	2 (40.0)	3 (60.0)	0 (0.0)	5 (100)
Low	17	3 (17.6)	14 (82.4)	5 (29.4)	12 (70.6)	2 (11.8)	15 (88.2)
Very low	7	3 (42.9)	4 (57.1)	2 (28.6)	5 (71.4)	1 (14.3)	6 (85.7)
Mitosis counts (per 50 fields, HPF)		NS		NS		NS	
< 2	17	4 (23.5)	13 (76.5)	6 (35.3)	11 (64.7)	2 (11.8)	15 (88.2)
2-5	8	2 (25.0)	6 (75.0)	3 (37.5)	5 (62.5)	1 (12.5)	7 (87.5)
6-10	4	1 (25.0)	3 (75.0)	0 (0.0)	4 (100)	0 (0.0)	4 (100)
>10	4	0 (0.0)	4 (100)	0 (0.0)	4 (100)	0 (0.0)	4 (100)
Tumour size (cm in length)		NS		NS		NS	
< 2	6	3 (50.0)	3 (50.0)	2 (33.3)	4 (66.7)	1 (16.7)	5 (83.3)
2-< 5	20	3 (15.0)	17 (85.0)	5 (25.0)	15 (75.0)	2 (10.0)	18 (90.0)
5-< 10	6	1 (16.7)	5 (83.3)	2 (33.3)	4 (66.7)	0 (0.0)	6 (100)
> 10	1	0 (0.0)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	1 (100)

NS: Not significant.

expressed VEGF, VEGFR-1 and 2. However, there was no correlation between VEGF, VEGFR-1 or VEGFR-2 expression and each classification.

DISCUSSION

The coexpression of VEGF and VEGFR-1 and 2 has been reported in tumor cells, suggesting the presence of an autocrine and/or a paracrine VEGF/VEGFR growth pathway in solid tumors[17-19]. VEGF also has been shown to play a role in the proliferation and/or differentiation of stromal tumors and normal mesenchymal cells[23-26]. VEGF expression in GISTs has been already reported[27,28]. However, there are no studies on VEGF receptor expression in GISTs, leiomyomas and schwannomas, or on the potential roles of VEGF and its receptors in the growth of these tumors. This is the first study to determine the expression of VEGF receptors in GIST and stromal tumors, demonstrating substantial levels of VEGF and its receptors in the cytoplasm of GIST, leiomyoma and schwannoma cells. Therefore, we suggest that VEGF and its receptors may play an important role in the growth and/or differentiation of intestinal stromal tumors via autocrine and/or paracrine pathways.

We did not find any statistical correlation between risk grade and the expression of VEGF or VEGFRs for GISTs. However, all 4 GISTs in the high risk category expressed VEGF and VEGFRs (Table 2). Furthermore, all 4 GISTs that had higher mitosis counts (over ten per 50 HPFs) were positive for VEGF and VEGFRs. Our data suggest that high risk GIST- expressed VEGF and VEGFR level is higher than normal. We thought that the number of high risk GISTs should be less. Further studies are needed to examine the VEGF/VEGFR pathway components in high risk GISTs.

VEGF induces a variety of enzymes and proteins important in the degradation process, including matrix-degrading metalloproteinase, interstitial collagenase, and serine proteases such as urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (TTPA)[29,30]. In this study, we did not evaluate the invasive activities of GIST cells, because all the GISTs were solitary and showed clear margins. However, the activation of these factors by VEGFRs provides for the possibility of conduction to a prodegradative environment that facilitates migration and invasion of tumor cells.

Solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Hypoxia in the tumor microenvironment is sufficient to activate hypoxia-inducible factor (HIF)-dependent gene expression[31]. HIF-1 alpha (HIF-1α) is overexpressed in most human malignancies[32]. HIF-1 binds to HIF responsive elements in the promoter region of certain genes, such as VEGF, to increase transcription[33]. HIF-1 expression in GIST has been already reported and suggested to contribute to tumor angiogenesis in GIST[28]. HIF-1 might play a role in the growth of GIST, because VEGF was expressed greater in larger GISTs in this study. However, we do not have any data about hypoxia, angiogenesis or HIF-1 expression in GIST, because the purpose of this study was to clarify the role of VEGF/VEGFR pathway in GIST cells. We hope that the relationship between hypoxia and GIST growth can be clarified in next study.

Joensuu *et al*[34] have reported a patient in whom Imatinib (STI-571, Gleevec), a tyrosine kinase inhibitor, is effective against GIST. Imatinib has proven to be remarkably efficacious in heavily pretreated GIST patients with advanced disease in phase Ⅲ clinical trials[35]. It was reported that Imatinib down-regulates VEGF expression in the GIST cell line GIST-T1[24]. Furthermore, anti-VEGF reagents are used in clinical trials for the therapy of colorectal, lung and breast cancer[36]. Stimulation of VEGFR upregulates the mitogen-activated protein kinase pathway through the activation of tyrosine kinases[37,38], the same pathway utilized by c-kit activation. These anti-VEGF reagents might be useful for the therapy of GISTs *via* the down-regulation of the VEGF/VEGFR pathway.

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