Vascular endothelial growth factor receptor-3 is a favorable prognostic factor in advanced gastric carcinoma

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Abstract. Vascular endothelial growth factor receptor (VEGFR)-3 is a receptor for VEGF-C and D and is implicated in the development of lymphatic vessels and metastasis. The purpose of this study was to investigate the expression of VEGFR-3 and its clinicopathological significance in primary gastric carcinoma (GC). Pathological and clinical findings from 109 GC cases were reviewed and VEGFR-3 expression was examined using immunohistochemistry. The clinicopathological implications of VEGFR-3 expression were analyzed statistically. VEGFR-3 expression was evaluated for intensity (0-3) and proportion (0-100%). A total score was obtained by multiplying the intensity by the proportion (0-300). A total score of 82 or more was considered positive for VEGFR-3. Of the 109 patients with GC, 19 (17.4%) were positive for VEGFR-3. VEGFR-3 expression was associated with Lauren classification (68.4% for intestinal type, 31.6% for diffuse type, p=0.058). It was more frequent in early gastric cancer (EGC), 28.0% in EGC, 16.2% in advanced gastric carcinoma (AGC), though this difference was not statistically significant. In 78 patients with AGC, VEGFR-3 expression was associated with good overall survival (p=0.052). In a multivariate analysis, the pTNM stage and VEGFR-3 were independent prognostic factors (OR=3.35, p=0.002 for pTNM stage; OR=0.23, p=0.044 for VEGFR-3). However, the expression of VEGFR-3 in EGC was not correlated with overall survival. In conclusion, the expression of VEGFR-3 was associated with the intestinal type (based on Lauren classification) and may be a favorable prognostic factor in AGC.

Introduction

Stomach cancer is the fourth most common cancer. Age-standardized incidence rates are the highest in Japan (69.2 per 100,000 in men, 28.6 per 100,000 in women) (1). High rates are also present in both sexes in eastern Asia, eastern Europe, and Central and South America. Regional lymph node metastasis is an important indicator of tumor aggressiveness as well as a known prognostic factor (2). Therefore, it is important to estimate the degree of lymphatic system invasion and lymphangiogenesis in the evaluation of biological tumor aggressiveness and patient outcome. Vascular endothelial growth factor receptors (VEGFR)-1, 2 and 3 are endothelial-specific receptor tyrosine kinases regulated by members of the vascular endothelial growth factor (VEGF) family. VEGFR-3 expression is predominantly restricted to lymphatic tissue, though it has also been detected in blood vessels of malignant tumors, tumor cells and during wound healing (3,4). In addition to its expression in lymphatic endothelial cells, VEGFR-3 expression has been demonstrated in a variety of human malignancies (5). VEGF-C and VEGF-D, as ligands for VEGFR-3, are also capable of stimulating lymphangiogenesis (3). The role of the VEGF-C, D and/or VEGFR-3 axis in various types of cancers has been investigated by many research groups (5). In clinical studies, a negative correlation between VEGF-C, D and/or VEGFR-3 and patient survival time has been reported in non-small cell lung cancer, colorectal carcinoma, endometrial carcinoma, epithelial ovarian carcinoma and primary breast cancer (6-10). Similarly, higher grade tumors of the prostate and uterine cervix show a higher expression of VEGF-C, D and/or VEGFR-3 in certain studies (11,12). However, other studies have failed to prove the significant value of VEGF-C, D and/or VEGFR-3 expression and the reverse results have been found in breast cancer, head and neck squamous cell carcinoma and lung adenocarcinoma (13-16). In this study, we investigated VEGFR-3 expression and its clinicopathological significance in primary gastric carcinoma.

Materials and methods

Patients and tumor samples. This study comprises 109 patients with primary gastric cancer who were diagnosed and underwent surgery at Kyung Hee University Medical Center between 1998-1999 and 2004-2005. Tumor blocks were selected after an initial review of haematoxylin-eosin-stained slides to confirm representative tumor lesions. Clinical data was collected by retrospective investigation. Patients included 80 males and 29 females, aged from 23 to 84 years (mean, 57

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Key words: vascular endothelial growth factor receptor-3, gastric carcinoma, immunohistochemistry, microarray, prognosis

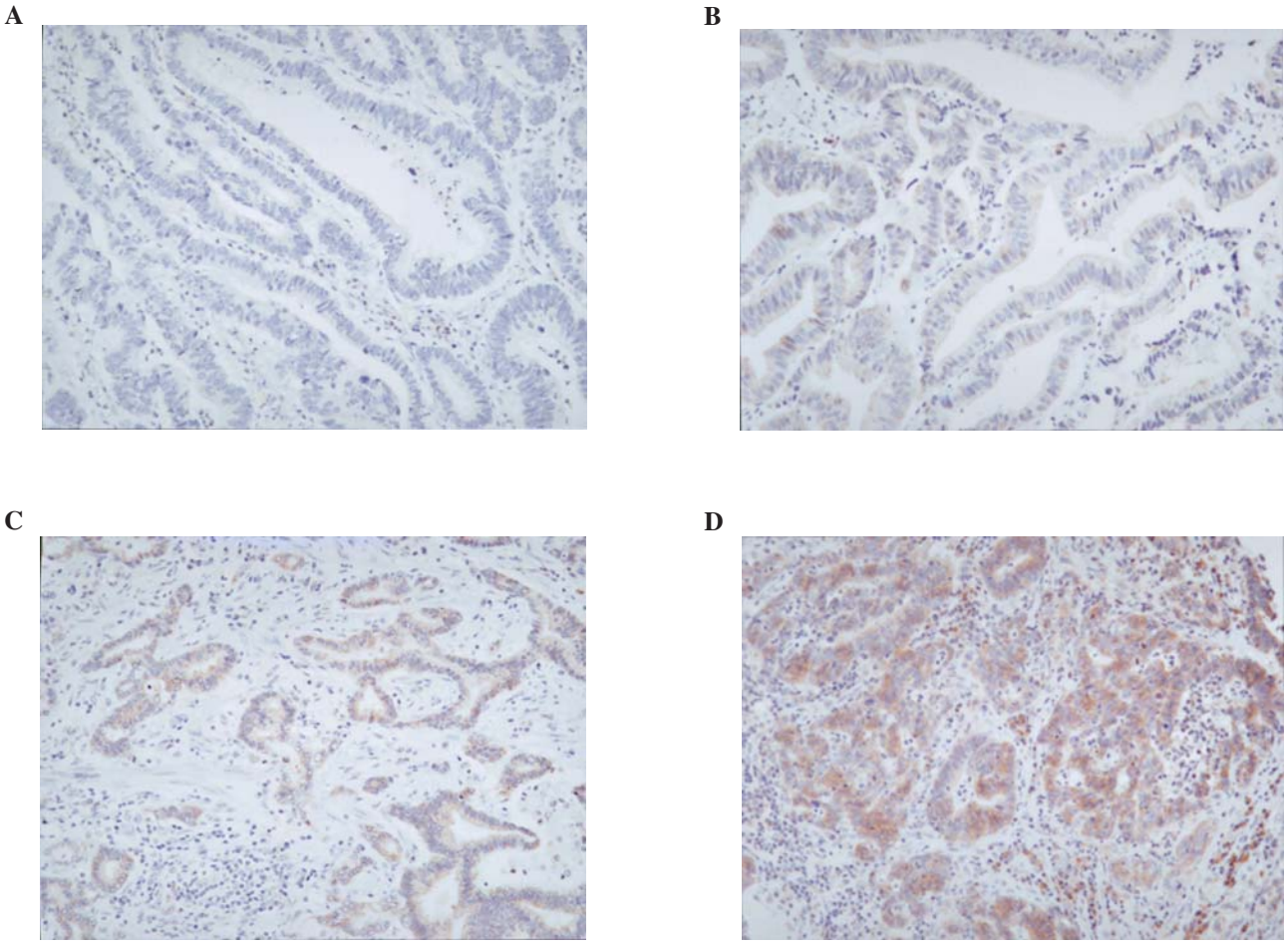

Figure 1. Intensity of VEGFR-3 immunoreactivity (A-D). Intensity 0 (H&E, x200) (A). Intensity 1 (H&E, x200) (B). Intensity 2 (H&E, x200) (C). Intensity 3 (H&E, x200) (D).

years; SD, 11.5 years). There were 25 early gastric carcinomas (EGCs) and 74 advanced gastric carcinomas (AGCs), including 72 adenocarcinomas and 13 signet ring cell carcinomas. Based on Lauren classification, 47 were of the intestinal type and 49 were of the diffuse type. The median overall survival was 81.5 months (range, 0.1-103.8 months). Thirty-eight patients died as a result of gastric cancer. The grading of postoperative gastric carcinoma was undertaken using the TNM classification system set out by the American Joint Committee on Cancer (17). This study was evaluated and approved by the Internal Review Board Committee of Kyung Hee University Medical Center.

Construction of tissue microarrays. Representative paraffin blocks, selected by a primary evaluation of haematoxylin-eosin-stained slides, were chosen for tissue microarray (TMA) preparation. With a sample punch (0.2 cm in diameter), two tissue cores were taken from each tumor and placed in two new recipient paraffin blocks. Each recipient block contained 60 individual tissue cores and was prepared in our laboratory of agar.

Immunohistochemistry. Immunohistochemistry was carried out on 4-μm tissue sections using the Bond Polymer Intense Detection System (VisionBioSystems, VIC, Australia) according to the manufacturer's instructions with minor modifications. In brief, 4-μm sections of formalin-fixed, paraffin-embedded tissues were deparaffinized with Bond Dewax Solution (VisionBioSystems) and an antigen retrieval procedure was performed using Bond ER Solution (Vision BioSystems) for 30 min at 100˚C. Endogenous peroxidase was quenched by incubation with hydrogen peroxide for 5 min. Sections were incubated in a Bond-max automatic slide stainer (VisionBioSystems) for 15 min at an ambient temperature with primary mouse monoclonal antibodies for VEGFR-3 (1:50; KLT9, Novocastra) labeled using a biotin-free polymeric horseradish peroxidase (HRP)-linker antibody conjugate system. Bound peroxidase was visualized using a solution of diaminobenzidine as the chromogen and nuclei were counterstained with haematoxylin.

Slide scoring and analysis. Each section was examined by two independent investigators (S. Lee, J.-Y. Sung) who were blinded to the clinical data. Staining intensity was defined as follows: 0, no staining; 1^+, weak; 2^+ moderate and 3^+ strong. The quantification of positivity (0-100%) was based on an estimate of the percentage of stained tumor cells in the core of the tissue microarray. The final score was obtained by multiplying the staining intensity by the percent positivity, giving immunoscores ranging from 0 to 300. Samples scoring 82 or higher were considered positive.

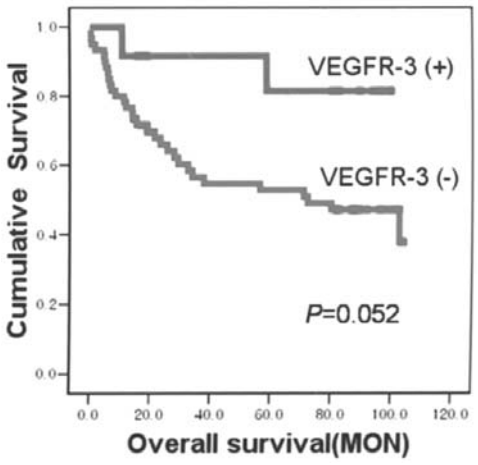

Figure 2. Overall survival time and VEGFR-3 positivity of AGC (Kaplan-Meier survival curve).

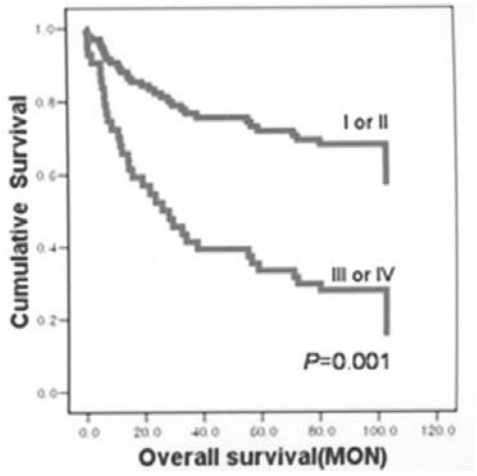

Figure 3. Univariate overall survival analysis of the TNM stage in AGC (Cox regression analysis).

Table I. VEGFR-3 expression scores in gastric carcinoma samples.

VEGFR-3 score	Number of cases (%)
0-80	80 (80.8)
81-160	13 (13.1)
161-240	4 (4.0)
241-300	2 (2.0)

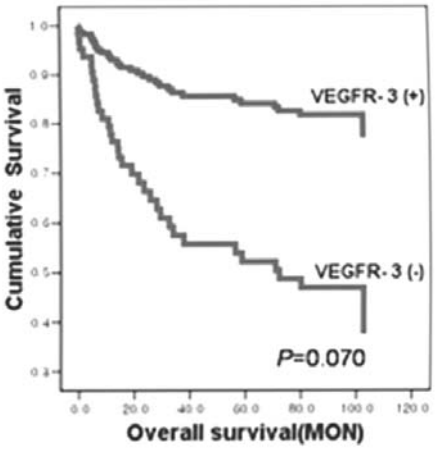

Figure 4. Univariate overall survival analysis of VEGFR-3 expression in AGC (Cox regression analysis).

Data analysis. All statistical calculations were carried out using Statistical Product and Services Solutions (SPSS, version 12.0) statistical software. The Pearson χ^2 test, Fisher exact test, Kaplan-Meier survival curve, log-rank test and Cox regression analysis were used to analyse the data.

Results

VEGFR-3 expression in gastric carcinoma. VEGFR-3 was observed almost exclusively in the cytoplasm of gastric carcinoma cells and certain inflammatory cells including plasma cells (Fig. 1). Normal gastric epithelium and a few vascular endothelial cells also stained weakly. Of the 109 cases of gastric carcinoma, 19 (17.4%) showed positive immunoreactivity (Table I).

Correlation between clinicopathological factors and VEGFR-3 expression. Table II summarizes the relationship between VEGFR-3 expression and clinicopathological factors. VEGFR-3 was more frequently seen in early gastric cancer (EGC), well or moderately differentiated tumors and intestinal type by Lauren classification, though the differences were not statistically significant. VEGFR-3 expression was significantly correlated with patient survival (p=0.034). Although not statistically significant, VEGFR-3 expression correlated with differentiation (61.1% for well to moderately differentiated, 38.9% for poorly differentiated, p=0.059, Pearson χ^2 test) and Lauren classification (68.4% for intestinal type, 31.6% for diffuse type, p=0.058, Pearson χ^2 test). No significant correlations with gender, tumor type, histological type, depth of invasion, lymph node metastasis or disease recurrence were observed.

Survival and prognosis analysis. Analyses of the prognostic effect of VEGFR-3 expression using Kaplan-Meier survival curves and log rank test showed no significant differences (p=0.076) in overall survival time between groups with positive or negative VEGFR-3 expression (mean: 68 and 88.2 months, respectively). Although in the AGC cases, those positive for VEGFR-3 expression had significantly longer overall survival times (mean: 88 months, 95% confidence interval: 49.5-72.1) than those that were negative (mean: 60.8 months, 95% confidence interval 72.8-103.3) (Fig. 2). Cox regression analysis was used to identify the independent prognostic factors. There were no independent prognostic factors before dividing the data into AGC and EGC. A univariate analysis of the TNM stage in the AGC group showed prognostic impacts (p=0.001), with VEGFR-3 expression showing a tendency for a positive correlation with prognosis (p=0.070) (Table III, Figs. 3 and 4). In a multivariate analysis of the AGC group, VEGFR-3 positivity was

Table II. Correlation of clinicopathological features and expression of VEGFR-3 in patients with gastric carcinoma.

Factors	VEGFR-3 score		P-value
	Negative (%)	Positive (%)	
Gender (n=99)			
Male (n=70)	54 (77.1)	16 (22.9)	0.174[b]
Female (n=29)	26 (89.7)	3 (10.3)	
Tumor type (n=99)			
EGC (n=25)	18 (72.0)	7 (28.0)	0.196[a]
AGC (n=74)	62 (83.8)	12 (16.2)	
Histological type (n=85)			
Adenocarcinoma (n=72)	55 (76.4)	17 (23.6)	0.283[b]
Signet ring cell carcinoma (n=13)	12 (92.3)	1 (7.7)	
Differentiation (n=84)			
Well to moderately (n=35)	24 (68.6)	11 (31.4)	0.059[a]
Poor (n=49)	42 (85.7)	7 (14.3)	
Lauren classification (n=96)			
Intestinal (n=47)	34 (72.3)	13 (27.7)	0.058[a]
Diffuse (n=49)	43 (87.8)	6 (12.2)	
Tumor depth (n=99)			
T1-T2a (n=46)	36 (78.3)	10 (21.7)	0.549[a]
T2b-T4 (n=53)	44 (83.0)	9 (17.0)	
N status (n=99)			
N0 (n=36)	27 (75.0)	9 (25.0)	0.267[a]
N1-3 (n=63)	53 (84.1)	10 (15.9)	
TNM stage (n=99)			
I-II (n=72)	56 (77.8)	16 (22.2)	0.263[b]
III-IV (n=27)	24 (88.9)	3 (11.1)	
Death (n=97)			
No (n=59)	43 (72.9)	16 (27.1)	0.034[bc]
Yes (n=38)	35 (92.1)	3 (7.9)	
Disease (n=75)			
No (n=59)	44 (74.6)	15 (25.4)	0.336[b]
Yes (n=16)	14 (87.5)	2 (12.5)	

VEGFR-3, vascular endothelial growth factor receptor-3. [a]Pearson χ^2 test. [b]Fisher exact probability test. [c]Statistically significant.

demostrated as an independent prognostic factor (p=0.044), along with TNM stage (p=0.002) (Table IV). However, in the EGC group VEGFR-3 and TNM stage showed no significance. Other factors such as gender, histology, differentiation and Lauren classification were not significantly correlated with prognosis.

Discussion

VEGFR-3 is essential for embryonic cardiovascular development, although thereafter becomes almost exclusively confined to the lymphatic endothelium of adult tissues (4). Previously, VEGFR-3 was shown to also be expressed in blood capillaries of normal breast tissue, neuroendocrine organs, chronic wounds and in malignant tumor cells (4,18). In addition, monocytes, macrophages, certain dendritic cells and plasma cells express this receptor (19,20).

In this study, VEGFR-3 expression is a potent and independent prognostic indicator in AGC. Unlike previously reported results, high levels of VEGFR-3 were associated with a favorable prognosis in AGC. Although VEGFR-3 expression was higher in EGC than in AGC, it was not correlated with survival in EGC. Intestinal type, well differentiated and early gastric cancer, which are generally

Table III. Univariate prognostic analysis in AGC.

Factor	Relative risk ratio	95% Confidence limit	P-value
Gender			
Male vs female	0.723	0.337-1.550	0.404
Histology			
Adenocarcinoma vs signet ring cell carcinoma	0.758	0.220-2.610	0.660
Differentiation			
Well or moderately vs poorly	1.440	0.622-3.149	0.416
Lauren classification			
Intestinal vs diffuse	1.410	0.689-2.886	0.347
TNM stage			
I or II vs III or IV	3.334	1.685-6.597	0.001[a]
VEGFR-3 score			
Negative vs positive	0.266	0.064-1.115	0.070

Cox regression analysis, [a]statistically significant.

Table IV. Multivariate prognostic analysis in AGC.

Factor	Relative risk ratio	95% Confidence limit	P-value
Gender	0.719	0.300-1.723	0.459
Histology	0.002	0.000-3.290	0.985
Differentiation	0.922	0.296-2.878	0.889
Lauren classification	1.237	0.841-1.819	0.281
TNM stage	3.338	1.541-7.229	0.002[a]
VEGFR-3 score	0.226	0.053-0.962	0.044[a]

Cox regression analysis, [a]statistically significant.

accepted as better prognostic factors, tended to correlate positively with the VEGFR-3 score. Nodal metastasis and depth of invasion were not related to VEGFR-3 expression.

Several hypotheses might explain why a high level of VEGFR-3 is a potent favorable prognostic factor. One is that VEGFR-3 is a potential target in inhibiting immune disorders, including inflammatory bowel disease and the rejection of corneal transplants (21,22). Chen *et al* found that the blockade of VEGFR-3 signaling significantly suppresses corneal antigen-presenting (dendritic) cells, the induction of delayed-type hypersensitivity and rejection of corneal transplants (22), suggesting that VEGFR-3 contributes to adaptive immunity which plays a role in antitumor immunity.

In other studies, the overexpression of VEGF-C and D by tumor cells induced lymphangiogenesis and increased metastasis to regional lymph nodes. However, a soluble form of VEGFR-3 inhibited lymphangiogenesis and metastasis dose-dependently by disrupting VEGF-C, D/VEGFR-3 signaling (23,24). In our study, VEGFR-3 might have acted as the soluble blocking antibody for VEGF-C and D, although, unfortunately, we could not directly investigate ligands of VEGFR-3 such as VEGF-C and D. This needs further investigation and analysis. Many previous studies of VEGF-C, D and VEGFR-3 show their potential as a poor prognostic factor, though VEGFR-3 expression was counted in endothelial cells around the tumor, not in the tumor itself (8,9,25-27). A few studies using cancer cell lines failed to prove that the level of VEGFR-3 expression in tumor cells has a significant prognostic value (13,28). Our study also failed to prove the prognostic value of VEGFR-3 expression in the cytoplasm of primary gastric tumor cells, though high levels of VEGFR-3 in AGC showed a positive correlation to prognosis. This heterogeneity could be interpreted as different bioactivity of VEGFR-3 in different expression sites such as endothelium and malignant tumor cells.

The presence of two isoforms of VEGFR-3 supports this second hypothesis. VEGFR-3 is expressed as transcripts of 4.5 and 5.8 kb in several human fetal and adult tissues. Pajusola *et al* showed that these transcripts encode two polypeptides, VEGFR-3s (short) and VEGFR-3l (long). They have different carboxy terminal tails and are proteolytically processed in transfected leukemia cells (29,30). This finding

shows that different structures in the carboxy terminal tail of VEGFR-3 can make the receptor function differently.

In conclusion, the intratumoral cytoplasmic expression of VEGFR-3 might be a favorable prognostic indicator in our 74 cases of AGC among 109 cases of primary gastric cancer. This result is not totally consistent with previous clinical studies. Further investigation is needed to clarify the mechanism and functional diversity of VEGFR-3 in cancer tissues.

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