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ORIGINAL RESEARCH

VEGF is a target molecule for peritoneal metastasis and malignant ascites in gastric cancer: prognostic significance of VEGF in ascites and efficacy of anti-VEGF monoclonal antibody

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Sachio Fushida
Katsunobu Oyama
Jun Kinoshita
Yasumichi Yagi
Kouichi Okamoto
Hidehiro Tajima
Itasu Ninomiya
Takashi Fujimura
Tetsuo Ohta

Department of Gastroenterological Surgery, Kanazawa University Hospital, Kanazawa, Japan

Background: In gastric cancer, poor prognosis is associated with peritoneal dissemination, which often accompanies malignant ascites. We searched for a target molecule in peritoneal metastasis and investigated its clinical utility as a biomarker.

Methods: Biopsy specimens from both primary lesions and peritoneal metastasis, and if possible, malignant ascites, were obtained from 40 patients with gastric cancer. Vascular endothelial growth factor (VEGF) expression was analyzed by immunohistochemical staining and enzyme-linked immunosorbent assay.

Results: VEGF expression was seen in 70% of peritoneal samples. Of the 40 patients, 35 had malignant ascites. These 35 patients were divided into two groups: 15 with ascites found beyond the pelvic cavity (large group) and 20 whose ascites were within the pelvic cavity (small group). The two groups did not significantly differ by serum VEGF levels, but ascites VEGF levels in the large group were significantly higher than in the small group ($P < 0.0001$). Serum VEGF and ascites VEGF levels were highly correlated in the large group ($r = 0.686$). A high ascites VEGF level was found to be a risk factor for survival ($P = 0.045$). We include a report of a patient with chemoresistant refractory gastric cancer and symptomatic ascites who obtained 8 months of palliation from systemic bevacizumab.

Conclusion: Anti-VEGF therapies are promising, and the ascites VEGF level is an important marker in managing patients with gastric cancer and peritoneal metastasis.

Keywords: vascular endothelial growth factor, malignant ascites, peritoneal metastasis, gastric cancer, bevacizumab

Introduction

Peritoneal metastasis is the most life-threatening type of metastasis in gastric cancer.[1,2] Recent advances in systemic chemotherapy regimens that combine novel antineoplastic agents have shown encouraging tumor response rates and survival for patients with unresectable or metastatic gastric cancer.[3–5] However, the prognosis of patients with peritoneal metastasis includes a median survival time of only 3–6 months.[6,7]

Several processes are associated with formation of peritoneal metastasis in gastric cancer, including cancer cell attachment, invasion, and proliferation in the peritoneum,[8] which are mediated by cytokines, proteases, growth factors, and angiogenic factors.

Vascular endothelial growth factor (VEGF) is a multifunctional cellular factor which can induce neovascularization and increase capillary permeability.[9,10] Accordingly, VEGF is implicated in peritoneal metastasis of gastric cancer and

Correspondence: Sachio Fushida
Gastroenterological Surgery,
Kanazawa University Hospital,
Kanazawa 920-8641, Japan
Tel +81 7 6265 2362
Fax +81 7 6234 4260
Email fushida@staff.kanazawa-u.ac.jp

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subsequent development of malignant ascites, as well as ovarian cancer.[11,12] By increasing abdominal pressure due to malignant ascites, severe symptoms, such as abdominal pain, nausea, anorexia, dyspnea, and cachexia decrease quality of life, and even reduce survival.[13]

Aoyagi et al, using primary tumor specimens, found that VEGF expression correlated with peritoneal metastasis in gastric cancer and appeared to be an essential element in development of peritoneal metastasis.[14] However, no reports provide evidence based on peritoneal metastatic specimens themselves. In the present study, we evaluated VEGF expression immunohistochemically, with paired primary gastric cancer and peritoneal metastasis specimens.

Conversely, increased serum VEGF levels are associated with advanced tumor stage and can be used as a prognostic biomarker in a variety of malignancies.[15–17] In gastric cancer, serum VEGF levels were also significantly higher in patients with advanced-stage cancer, higher lymph node ratio, and perineural invasion,[18] whereas such correlations with ascites VEGF levels were unclear. To understand better the relationship between VEGF levels and malignant ascites, we analyzed and compared VEGF concentrations using an enzyme-linked immunosorbent assay for serum and malignant ascites of individual gastric cancer patients with peritoneal metastasis. VEGF concentration was also considered to assess the effects of VEGF levels on patient survival.

Materials and methods

Patients

Between 2002 and 2010, 64 patients with gastric cancer and peritoneal dissemination were treated at Kanazawa University Hospital. Forty patients underwent both gastroendoscopy and laparoscopy, and biopsy specimens were taken from both primary tumor and peritoneal metastases. Each biopsy specimen was fixed in 10% neutral buffered formalin and embedded in paraffin; if possible, ascetic effusions and serum samples were centrifuged at 1,000× g for 10 minutes and stored at –80°C until analysis.

Immunohistochemical staining

Human epidermal growth factor receptor 2 (HER2), and VEGF were analyzed using immunohistochemical staining with an EnVision™ system (Dako, Carpinteria, CA, USA), which uses dextran polymers conjugated with horseradish peroxidase to avoid any endogenous biotin contamination. Sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase was blocked by immersing the sections in 3% H_2O_2 in 100% methanol for 20 minutes at room temperature. Sections were then incubated with primary antibody in a humidified chamber at 4°C overnight. As the primary antibody, we used mouse monoclonal antibody CB11 (Invitrogen, Carlsbad, CA, USA) for HER2, diluted at 1:50, mouse monoclonal antibody pY992 (Invitrogen) for epidermal growth factor receptor, diluted at 1:50, and rabbit polyclonal antibody A-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for VEGF, diluted at 1:200. Peroxidase activity was detected with enzyme substrate 3-amino-9-ethyl carbazole. For negative controls, sections were incubated with Tris-buffered saline without primary antibodies. Samples in which ≥10% of tumor cells were slightly counterstained with Mayer's hematoxylin were defined as positive.

Enzyme-linked immunosorbent assay

A quantitative sandwich enzyme-linked immunosorbent assay with a Quantikine human VEGF immunoassay kit (R&D Systems, Minneapolis, MN, USA) was used in accordance with the manufacturer's instructions, to measure VEGF in ascites and serum from patients with peritoneal metastasis. The assay quantity limit was 9.0 pg/mL. All experiments were performed in triplicate.

Clinical trial

Patients with refractory peritoneal metastases of gastric cancer and symptomatic ascites resistant to systemic chemotherapy and intraperitoneal docetaxel were treated with single-agent bevacizumab. Bevacizumab was initiated at 5 mg/kg intravenously, every 2 weeks if necessary, for palliation of symptoms. The local committee approved the study protocol (Kanazawa University Hospital, acceptance number 5608) and written informed consent was obtained from the patient.

Statistical analysis

The Mann–Whitney *U* test was used to compare different groups for continuous and categorical variables. Correlations were analyzed by Pearson and Spearman coefficient analysis. Survival rates were calculated with the Kaplan–Meier method and differences were evaluated by log-rank test. $P < 0.05$ was considered to be statistically significant. All statistics were carried out using Statistical Package for the Social Sciences version 10 software (SPSS Inc., Chicago, IL, USA).

Results

VEGF expression in gastric cancer

Forty pairs of primary tumor and peritoneal metastases were immunohistochemically examined for HER2,

epidermal growth factor receptor, and VEGF expression. Immunoreactivity for HER2 was recognized in cell membranes; positive staining was found in 15% (6/40) of primary tumors and 3% (1/40) of peritoneal metastases. Epidermal growth factor receptor was also observed in cell membranes; positive staining was seen in 18% (7/40) of primary tumors and 3% (1/40) of peritoneal metastases. In contrast, VEGF diffusely stained the cytoplasm of cancer cells (Figure 1); positive tumor staining was seen in 85% (34/40) of primary tumors and 70% (28/40) of peritoneal metastases.

Association between VEGF levels and clinicopathological characteristics

Of the 40 patients, 35 had malignant ascites; therefore, 35 pairs of ascites and serum samples were analyzed to quantify VEGF levels. Relationships between VEGF levels and clinicopathological variables are shown in Table 1. When patients were grouped as P1–2 and P3 according to the criteria of the Japanese Research Society for Gastric Cancer,[19] no significant association between peritoneal metastatic grade and VEGF level was seen in either serum or ascites. The 35 patients were divided into two groups based on whether ascites was found beyond the pelvic cavity or not. The 15 patients with ascites beyond the pelvic cavity were classified as the large group and other 20 patients were classified as the small group. The two groups did not significantly differ for serum VEGF levels, but did significantly vary for ascites VEGF levels ($P < 0.0001$). The median ascites VEGF level in the small group was 504 (range 82–7,261) pg/mL; for the large group, it was 700 (range 231–7,113) pg/mL. VEGF levels in both serum and ascites showed no association with gender, age, or prior gastrectomy. Ascites VEGF levels and serum VEGF levels correlated in the large group ($r = 0.686$, $P = 0.0034$) but not in the small group (Figure 2).

Prognostic factors for overall survival

In the present study, we established cutoff values for serum VEGF (472 pg/mL) and ascites VEGF (660 pg/mL) using median values. The relationship between VEGF levels, volume of ascites, and peritoneal metastatic grade was evaluated using the Kaplan–Meier method. Univariate log-rank analysis showed that high ascites VEGF levels reduced overall survival ($P = 0.041$, Figure 3). Neither ascites volume nor peritoneal metastatic grade showed an association with overall survival. Serum VEGF levels also provided no significant evidence with regard to overall survival. Cox regression analysis showed ascites VEGF levels as a risk factor for survival (hazards ratio 2.21, 95% confidence interval 1.015–4.794, $P = 0.045$, Table 2).

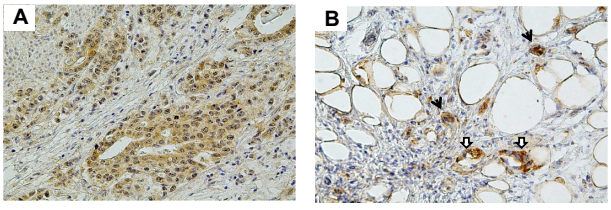

Figure 1 Representative images of vascular endothelial growth factor immunostaining in gastric cancer tissues. (**A**) Diffusely stained cytoplasm of cancer cells in primary tumor. (**B**) Strongly stained cytoplasm of cancer cells (black arrow) and fibroblasts (white arrow) in peritoneal tumor.

Case report

Our patient was a 62-year-old female diagnosed with type 4 gastric cancer, whose clinical course has been partially reported previously by our colleague.[20] Exploratory laparoscopy revealed severe peritoneal metastasis with malignant ascites. The patient received two cycles of S-1 plus intraperitoneal docetaxel; disappearance of her peritoneal metastasis was confirmed by second-look laparoscopy. Subsequently, the patient underwent total gastrectomy with D2 lymph node dissection which completed an R0 resection. Eighteen months after surgery, the patient's cancer recurred and was treated with four cycles of weekly paclitaxel, while the patient suffered from symptomatic ascites requiring frequent paracenteses. Because of lack of response, the patient was administered bevacizumab monotherapy (5 mg/kg) intravenously. Despite only one administration, the patient noted an improvement in abdominal distention and required no paracenteses. After bevacizumab therapy, the patient received eight cycles of weekly paclitaxel. She died of aspiration pneumonia due to ileus (Figure 4).

Discussion

Previous reports have implied that VEGF is associated with tumor progression including peritoneal metastasis; however, most of these reports are based in a xenograft model and the status of the primary tumor.[14,21–23] In the present study, expression of VEGF was found not only in primary gastric tumors but also in peritoneal metastases, and more frequently than either HER2 or epidermal growth factor receptor. Establishment of peritoneal metastasis needs a multistep process involving detachment of cancer cells from their primary tumor, their attachment to the peritoneal surface, infiltration into the subperitoneal space, and proliferation with angiogenesis.[8] VEGF secreted from cancer cells might enhance tumor growth by inducing an angiogenic response in the peritoneal microenvironment. We showed that VEGF

Table 1 Relationship between vascular endothelial growth factor levels and clinicopathological variables

	Patients (n)	s-VEGF (pg/mL)*	*P*-value	a-VEGF (pg/mL)*	*P*-value
Age (years)					
<62	14	408 (90–1191)	0.108	572 (82–7261)	0.538
≥62	21	440 (23–1337)		616 (84–3216)	
Gender					
Male	15	440 (90–1337)	0.752	616 (148–3216)	0.333
Female	20	408 (23–1191)		571 (82–7113)	
P grade‡					
P1, P2	16	405 (90–1337)	0.043	504 (82–7261)	0.298
P3	19	473 (183–1337)		660 (84–7113)	
Ascitic					
Volume†					
Small	20	405 (90–1337)	0.331	504 (82–7261)	<0.0001
Large	15	440 (23–7113)		660 (84–7113)	
Gastrectomy					
Yes	6	382 (90–675)	0.064	504 (82–571)	0.023
No	29	472 (183–1337)		660 (84–7261)	

Notes: *Values are median (range); ‡according to the Japanese Gastric Cancer Association; †according to whether ascites is found beyond the pelvic cavity or not.
Abbreviations: s-VEGF, serum vascular endothelial growth factor; a-VEGF, ascitic vascular endothelial growth factor.

is a convincing molecular target for peritoneal metastases. This is the first report to have formed the basis of clinical specimens from peritoneal tumors.

Malignant ascites with peritoneal metastasis seriously affects patients' quality of life. VEGF mediates formation of malignant ascites by increasing the permeability of blood vessels.[6] In this study, levels of circulating VEGF were not correlated with volume of ascites, because circulating VEGF can derive from both a primary tumor and peritoneal metastases, and may depend on total tumor volume. Conversely, VEGF concentration in the ascites statistically correlated with ascites volume. VEGF may be produced by human peritoneal mesothelial cells when stimulated by basic fibroblast growth factor secreted from cancer cells and other human peritoneal mesothelial cells in the microenvironment.[24] Thus, human peritoneal mesothelial cells are critical to accumulation of malignant ascites through production of diffusible VEGF. In addition to human peritoneal mesothelial cells, intraperitoneal VEGF may come from various sources, such as subperitoneal capillaries, peritoneal metastatic tumor, fibroblasts,[25] and macrophages,[26] whereas intraperitoneal VEGF cannot transfer into the systemic circulation due to capillary hyperpermeability. Accordingly, ascites volume correlates with ascites VEGF concentration. This result agrees with a previous report by Rudlowski et al, who showed the same findings in patients with ovarian cancer.[27]

In the present study, the prognostic value of VEGF levels was also assessed. Although several studies have shown that tumor VEGF is an independent prognostic factor in gastric cancer, measurement of VEGF levels both in serum and ascites is technically simple, does not require a tumor specimen, and is more objective in its evaluation than semiquantitative immunohistochemistry.[28–30] Increased serum VEGF levels have been associated with advanced stage, higher lymph node metastasis, and perineural invasion in gastric cancer.[31,32] These data suggest that anti-VEGF therapy might have an effect on gastric cancer. Although Shah et al reported that anti-VEGF therapy using bevacizumab

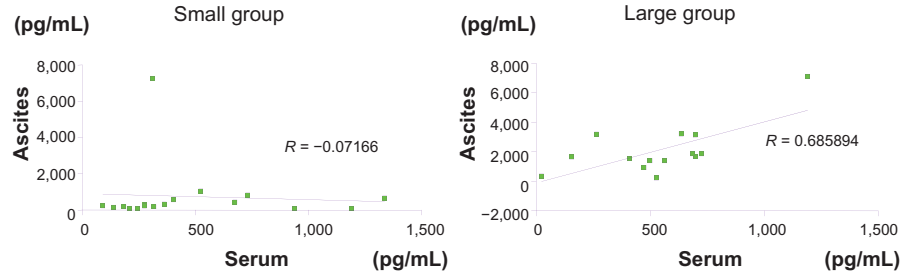

Figure 2 Correlation of vascular endothelial growth factor levels between serum and ascites. There was a good correlation in the large group.
Notes: Patients were divided into two groups. Large group, ascites found beyond pelvic cavity; small group, ascites within pelvic cavity.

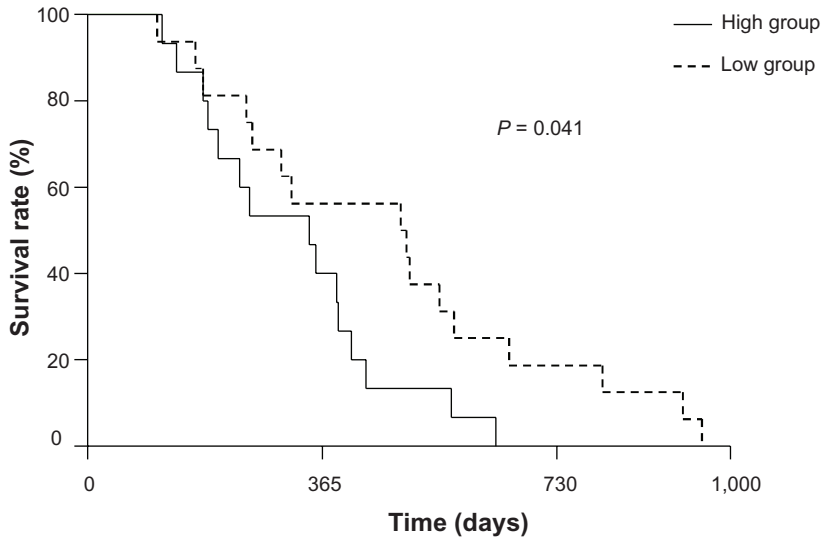

Figure 3 Kaplan–Meier survival curves for overall survival rate according to vascular endothelial growth factor concentration in ascites.
Note: The high ascites vascular endothelial growth factor concentration subgroup showed a shorter overall survival than the low concentration subgroup ($P = 0.041$).

combined with chemotherapy might be a promising therapy for patients with metastatic or unresectable gastric and gastroesophageal junction adenocarcinoma,[33] a randomized, double-blind, placebo-controlled, Phase III study (Avastin in Gastric Cancer) failed to establish evidence about the usefulness of bevacizumab in gastric cancer.[34] Moreover, Vidal et al found that a high preoperative serum VEGF level was an independent prognostic factor for recurrence and survival after R0 resection of gastric cancer.[18] However, we could not find a significant association between serum VEGF level and overall survival. This might be the reason why that all the patients in our study were in stage IV with peritoneal metastasis and did not receive R0 resection. In contrast, the prognostic significance of ascites VEGF level has not been adequately studied. To our knowledge, this is the first study to report that elevated ascites VEGF levels are significantly associated with shorter overall survival in gastric cancer. The principal mechanisms explaining the prognostic significance of VEGF are tumor expansion and massive ascites, which cause severe symptoms, such as bowel obstruction, dyspnea, and cachexia. Additionally, VEGF in malignant ascites may induce immune suppression in cancer by inhibiting dendritic cell maturation[35] and increasing tumor-infiltrating regulatory T cells.[36] Furthermore, high ascites VEGF levels may be associated with upregulation of multidrug resistance-associated protein, leading to resistance to platinum-based treatment, which is often used in unresectable gastric cancer.[37,38] In any case, anti-VEGF therapies should be considered for patients with malignant ascites in gastric cancer.

Table 2 Cox regression analysis

Event	Patients (n)	MST	HR	95% CI	*P*-value
P grade*					
P1, P2	16	433	1.671	0.796–3.509	0.175
P3	19	301			
Volume of ascites‡					
Small	20	387	1.615	0.766–3.408	0.208
Large	15	317			
s-VEGF levels					
Low	18	390	1.609	0.778–3.327	0.199
High	17	252			
a-VEGF levels					
Low	18	487	2.206	1.015–4.794	0.045
High	17	345			

Note: *According to the Japanese Gastric Cancer Association; ‡according to whether ascites is found beyond the pelvic cavity or not.
Abbreviations: s-VEGF, serum vascular endothelial growth factor; a-VEGF, ascitic vascular endothelial growth factor; MST, median survival time; HR, hazards ratio; CI, confidence interval.

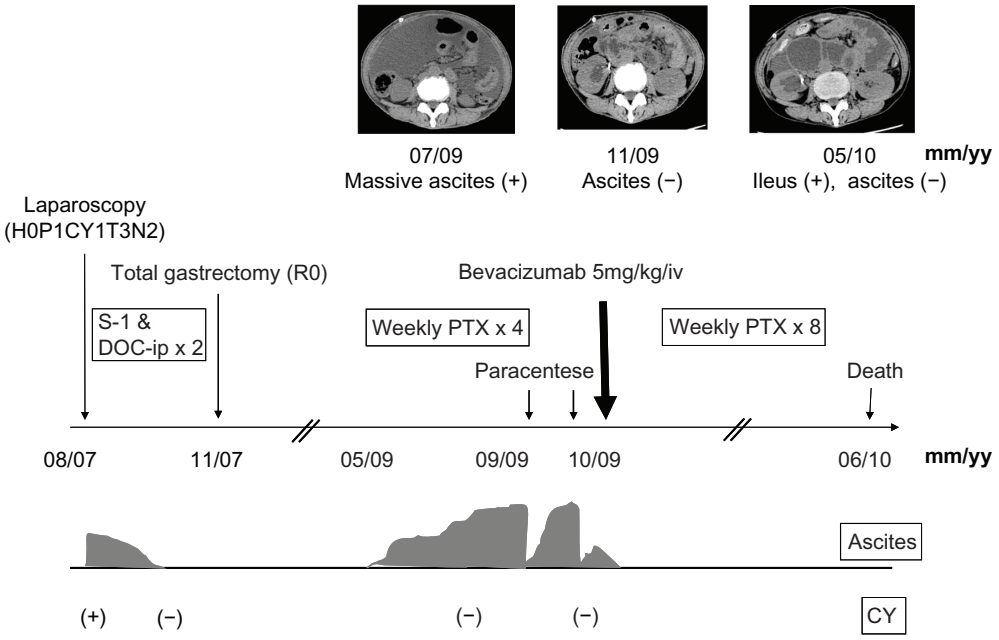

Figure 4 Treatment and disease progression for the presented case.
Abbreviations: PTX, paclitaxel; CY, cytology (peritoneal); DOC-ip, intraperitoneal docetaxel; iv, intravenously; mm/yy, month/year.

Our patient experienced successful palliation of symptomatic ascites using intravenous (systemic) bevacizumab. Several studies using mouse models indicate that intraperitoneal (regional) bevacizumab could be useful for peritoneal metastasis.[23,39] In clinical case reports, bevacizumab was also administrated regionally for patients with malignant ascites.[40,41] Yagi et al reported that bevacizumab had a more pronounced effect on malignant ascites and peritoneal nodules when administered systemically rather than regionally.[42] If bevacizumab is administered regionally, most of the antibody will be neutralized in malignant ascites, which contains large amounts of VEGF, resulting in a low blood concentration. These results support the use of systemically administered bevacizumab, with ascites removed before treatment for more efficacy.

In conclusion, VEGF might be correlated with the development of peritoneal metastasis and malignant ascites. The ascites VEGF level appears to be an important prognostic indicator in gastric cancer with peritoneal metastasis. Further prospective studies will be necessary to validate both ascites VEGF as a predictive marker of poor outcome and the efficacy of bevacizumab for chemoresistant malignant ascites.

Disclosure

The authors report no conflicts of interest in this work.

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