Hematogenous Metastasis in Gastric Cancer Requires Isolated Tumor Cells and Expression of Vascular Endothelial Growth Factor Receptor-1

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Abstract

Purpose: Recent studies of cancer metastasis have focused on the role of premetastatic gene expression and circulating tumor cells. We did a blind prospective study in gastric cancer to assess the significance of isolated tumor cells (ITC) and to test the hypothesis that vascular endothelial growth factor receptor-1 (*VEGFR-1*) is expressed within the bone marrow at tumor-specific, premetastatic sites.

Experimental Design: Both bone marrow and peripheral blood samples from 810 gastric cancer patients were collected at the Central Hospital, National Cancer Center (Tokyo, Japan). The samples were transferred to Kyushu University Hospital (Beppu, Japan) where they were analyzed by quantitative real-time reverse transcription-PCR for three epithelial cell markers, carcinoembryonic antigen, cytokeratin-19, and cytokeratin-7, as well as *VEGFR-1*.

Results: ITCs were observed in peripheral blood and bone marrow even in early stages of gastric cancer. The frequency of ITC in bone marrow was significantly associated with the stage of disease by ANOVA ($P < 0.01$). Gastric cancer metastasized when ITCs were observed in the presence of *VEGFR-1*. In the 380 patients who were ITC negative and showed low *VEGFR-1* expression, synchronous (at the time of surgery) and heterochronous (recurrent) metastases were not observed.

Conclusions: ITCs circulate even in early stages of disease. Furthermore, elevated expression of VEGFR-1 facilitates the establishment of hematogenous metastases in gastric cancer. This study indicates that the simultaneous presence of ITC and *VEGFR-1* expression at premetastatic sites is clinically significant for disease progression.

Recent studies of patients with cancer of the gastrointestinal tract have used immunohistochemical approaches and PCR to show the presence of isolated tumor cells (ITC) in peripheral blood and/or bone marrow (1–11). Using immunohistochemistry, some investigators have concluded that the presence of ITC was not related to either recurrence or prognosis of gastric or colorectal cancer (1, 12–14). It is not known whether the presence of ITC detected by quantitative reverse transcription-PCR (RT-PCR) can predict disease-free survival and overall survival in gastrointestinal tract cancer.

The interpretation of published data has been rather uncertain due to small patient populations and to the diversity of analytic variables used by different institutes, such as target organs, target molecules, and assay systems. Because gastric cancer is recognized as one of the major intractable malignancies in Japan, we organized a prospective blind study of a large number of gastric cancer cases to establish the clinical significance of ITC. Two independent groups conducted the current study. One team at the Central Hospital, National Cancer Center (Tokyo Japan) collected samples from both bone marrow and peripheral blood from 810 patients with gastric cancer. For molecular analysis, the samples were transferred to a second group of collaborators at Kyushu University Hospital (Beppu, Japan). No clinical or pathologic information was given to the Kyushu University group until the analyses were completed. Similarly, the expression data analyzed at the Kyushu University were not sent to surgeons in the Central Hospital, National Cancer Center.

Recent studies of cancer metastasis have also examined the role of host responses in hematogenous metastasis and peritoneal dissemination. It is believed that the mechanisms that guide tumor cells to a specific tissue may involve molecular properties inherent in the tumor cells themselves, as well as modulation by host immune cells and host progenitor cells. Kaplan et al. (15) reported that bone marrow–derived hematopoietic progenitor cells that express vascular endothelial

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growth factor receptor-1 (*VEGFR-1*) home to tumor-specific premetastatic sites and form cellular clusters before the arrival of tumor cells both *in vitro* and *in vivo*. Therefore, we hypothesized that metastasis of gastric cancer requires both the presence of ITC and high expression of *VEGFR-1* [VEGFR-1 (high)]. The current study was designed to test this hypothesis. Before this study, we reported a significant association between the up-regulation of *VEGFR-1* and distant metastasis in a few gastric cancer cases (16). However, due to the limited number of cases, we were unable to define the significance of both circulating ITC and elevated expression of *VEGFR-1*.

In the current analysis, we aimed to resolve the controversy surrounding the presence of ITC in peripheral blood and in bone marrow by using quantitative RT-PCR in >800 cases of gastric cancer. Moreover, we sought to determine the clinical significance of ITC numbers and elevated expression of *VEGFR-1* in hematogenous metastasis of gastric cancer.

Materials and Methods

Gastric cancer cases. Physicians (T.F., K.I., T.E., and M.S.) collected bone marrow and peripheral blood samples from 810 Japanese gastric cancer patients who underwent surgery from 2001 to 2004 at the Central Hospital, National Cancer Center. Documented informed consent was obtained from all patients. There were 546 male and 264 female patients with an average age of 61.5 years and a range of 24 to 89 years (Table 1). With regard to hematogenous progression of gastric cancer, there were six cases of lung metastasis and five cases of liver metastasis observed at the time of surgery. During 2 years of observation, follow-up postoperative computed tomography found 13 cases of recurrent lesions. With regard to peritoneal dissemination, 26 cases showed at the time of operation or at postoperative follow-up. Among the 810 cases, 501, 107, 74, and 128 were classified as stages I, II, III, and IV, respectively, according to the Treaty for Japanese Gastric Cancer Association (17).

Normal controls. Preliminary trials were undertaken to assure that results were accurate and reliable. We used highly specific hybridization probes and primers to maintain high specificity for target genes and thereby reduce false-positive outcomes. For normal negative controls, both peripheral blood and bone marrow were collected from 29 patients having no malignancy from April 2000 to March 2003. This group included 20 cases of gallstone, 3 cases of common bile duct stone, and 6 cases of hernia.

RNA extraction. Samples transferred from Tokyo to Beppu remained frozen while in transit. Total RNA was extracted from bone marrow and peripheral blood as described elsewhere (7). In brief, bone marrow was aspirated from the sternum of patients before surgery under general anesthesia. The first 1.0 mL of bone marrow and peripheral blood was discarded to avoid contamination by the skin. The second collected 1.0 mL of bone marrow and peripheral blood was put into 4.0 mL of Isogen-LS (Nippon Gene), and total RNA was extracted according to the manufacturer's protocol.

Primers and probes for quantitative real-time RT-PCR. Quantitative RT-PCR methodology was designed to optimize the specificity and fidelity of the assay. The Kyushu University group had previously investigated the expression of seven representative molecular markers [*carcinoembryonic antigen* (*CEA*), cytokeratin (*CK*)-*7*, *CK-18*, *CK-19*, *CK-20*, *mammaglobin*, and *mucin-1*] in 27 cancers and eight non-epithelial cell lines using quantitative RT-PCR. The expression levels of *CK-7* and *CK-19* showed high sensitivity and specificity for the identification of gastric cancer cells in comparison with the other markers (18). Those epithelial cell markers have been widely used as target genes for the detection of ITC (1, 19–22). Thus, *CEA*, *CK-7*, and *CK-19* were studied by quantitative RT-PCR in all 810 patients. ITCs were considered present when any single marker was positive. The reverse transcription reaction was done as previously described (18). In brief, the first-strand cDNA was synthesized from 2.7 μg of total RNA in 30 μL reaction mixtures containing 5 μL of 5× reverse transcriptase buffer (Life Technologies), 200 μmol/L deoxynucleotide triphosphate, a 100 μmol/L solution of random hexadeoxynucleotides, 50 units of RNasin (Promega), 2 μL of 0.1 mol/L DTT, and 100 units of Moloney leukemia virus reverse transcriptase (Life Technologies). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min, and then chilled on ice.

We did real-time quantitative RT-PCR using a LightCycler instrument (Roche Diagnostics) to detect ITC in peripheral blood and/or bone marrow. The following target genes were used. For *CEA*, primers were as follows: sense, 5′-GACGCAAGAGCCTATGTATG-3′; antisense, 5′-GGCATAGGTCCCGTTATTA-3′; and probes, 5′-CCCAGACTCGTCTTACCTTTCGG-3′-fluorescein (donor) and 5′-LCRed640-AGCGAACCTCAACCTCTCCTGC-3′-phosphorylated (acceptor). For *CK-19*, primers were as follows (23, 24): sense, 5′-AGGTGGATTCCGCTCCGGGCA-3′; antisense, 5′-ATCTTCCTGTCCCTCGAGCA-3′; and probes, 5′-TGAGCGTGAAGCTGGCCCTGGACATCGA-3′-fluorescein (donor) and 5′-LCRed640-GAACCAGGCTTCAGCATCCTTC-3′-phosphorylated (acceptor). For *CK-7*, primers were as follows: sense, 5′-ACATCAAGAACCAGCGTGCC-3′; antisense, 5′-TCACGGCTCCCACTCCATCT-3′; and probes, 5′-TGAGCGTGAAGCTGGCCCTGGACATCGA-3′-fluorescein (donor) and 5′-LCRed640-ATCGCCACCTACCGCAAGCTGCTGGAGG-3′-phosphorylated (acceptor). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The primer was as follows: sense, 5′-TGAACGGGAAGCTCACTGG-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′; and probes, 5′-GAGTGGGTGTCGCTGTTGAAGTCA-3′-fluorescein (donor) and 5′-LCRed640-AGGAGACCACCTGGTGCTCAGTGTA-3′-phosphorylated (acceptor).

VEGFR-1 expression and ribosomal protein S27a internal control. The sense primer of *VEGFR-1* mRNA was 5′-TCATGAATGTTTCCCTGCAA-3′ and antisense primer was 5′-GGAGGTATGGTGCTTCCTGA-3′. We used the ribosomal protein S27a as an internal control. The sequence of ribosomal protein S27a primers was as follows: 5′-TCGTGGTGGTGCTAAGAAAA-3′ (sense) and 5′-TCTCGACGAAGGCGACTAAT-3′ (antisense). Real-time monitoring of PCRs was done using the LightCycler system (Roche Applied Science) and SYBR Green I dye (Roche Diagnostics).

RT-PCR conditions and analysis. For amplification of *GAPDH*, an initial denaturation at 95°C for 10 min was followed by 15 s at 95°C, 15 s at 60°C, and 13 s at 72°C. For CEA amplification, an initial denaturation was followed by 15 s at 95°C, 15 s at 56°C, and 11 s at 72°C. All experiments were done twice to confirm reproducibility. If two runs differed by >2-fold, a third trial was undertaken. Results were calculated from the average of the two closest runs. The results of RT-PCR were sent from Beppu to Tokyo for analyses.

Culture of bone marrow from gastric cancer cases. We extracted 10 mL of bone marrow from the sternum of gastric cancer patients just before surgery (with documented informed consent) in the Department of Surgery, Kyushu University Hospital. Then, mononuclear cells were isolated after centrifugation of whole bone marrow. Marrow mononuclear cells were deposited on slides by centrifugation followed by immunostaining with A45-B/B3 (CK8/18 8/19). The remaining mononuclear cells were grown in RPMI 1640 (which included 10% fetal bovine serum, 1% ITS-A, 2% penicillin/streptomycin, and 1% L-glutamine) with addition of 50 ng/mL epidermal growth factor and 10 ng/mL basic fibroblast growth factor at 37°C 10% O_2 and 5% CO_2. After 4 weeks, cells underwent immunostaining. One cytospin was done with at least 1×10^5 cells with A45-B/B3 antibody, whereas a negative control was done with MOPC21 antibody (IgG1 isotype control). The immunostaining assay was done as described by Solakoglu et al. (25).

Statistical analysis. Expression of *CEA*, *CK-7*, and *CK-19* was adjusted in each case for *GAPDH* expression. We set cutoff values for those expression ratios (*CEA*/*GAPDH*, *CK-7*/*GAPDH*, and *CK-19*/*GAPDH*) as the highest value for each marker in 29 normal controls. As we show in Table 1, the number of *CEA*-positive, *CK-7*–positive,

Table 1. Clinical significance of the presence of ITCs in bone marrow or peripheral blood in gastric cancer cases

Markers	ITC in bone marrow			ITC in peripheral blood		
	Present, *n* (%)	Absent, *n* (%)		Present, *n* (%)	Absent, *n* (%)	
CEA, *CK-7*, or *CK-19* (*N* = 810)	394 (48.6)	416 (51.4)		247 (30.5)	563 (69.5)	
CEA (+)	107 (13.2)	703 (86.8)		62 (7.7)	748 (92.3)	
CK-7 (+)	152 (18.8)	658 (81.2)		72 (8.9)	738 (91.1)	
CK-19 (+)	247 (30.5)	563 (69.5)		145 (17.9)	665 (82.1)	
Case	**Present**	**Absent**	***P***	**Present**	**Absent**	***P***
	394 (100%)	**416 (100%)**		**247 (100%)**	**563 (100%)**	
	***n* (%)**	***n* (%)**		***n* (%)**	***n* (%)**	
Age (y)			NS			NS
(mean ± SD)	61.6 ± 11.4	61.4 ± 11.7		61.5 ± 10.9	61.5 ± 11.9	
Sex			NS			NS
Male (*n* = 546)	262 (48.0)	284 (52.0)		169 (31.0)	377 (69.0)	
Female (*n* = 264)	132 (50.0)	132 (50.0)		78 (29.5)	186 (70.5)	
Histology			NS			NS
Differentiated (*n* = 181)	79 (43.6)	102 (56.4)		56 (30.9)	125 (69.1)	
Undifferentiated (*n* = 629)	315 (50.1)	314 (49.9)		191 (30.4)	438 (69.6)	
Tumor size			0.03			NS
Max diameter (mm), mean ± SD	60.0 ± 44.2	53.6 ± 39.5		57.0 ± 41.9	56.5 ± 42.0	
Tumor stage			NS			NS
pT1 (*n* = 428)	195 (45.6)	233 (54.4)		132 (30.8)	296 (69.2)	
pT2-pT4 (*n* = 382)	199 (52.1)	183 (47.9)		115 (30.1)	267 (69.9)	
Lymph node metastasis			NS			NS
Negative (*n* = 473)	226 (47.8)	247 (52.2)		155 (32.8)	318 (67.2)	
Positive (*n* = 337)	168 (49.9)	169 (50.1)		92 (27.3)	245 (72.7)	
Lymphatic involvement			NS			NS
Negative (*n* = 465)	211 (45.4)	254 (54.6)		149 (32.0)	316 (68.0)	
Positive (*n* = 345)	183 (53.0)	162 (47.0)		98 (28.4)	247 (71.6)	
Vascular involvement			0.005			NS
Negative (*n* = 671)	311 (46.3)	360 (53.7)		203 (30.3)	468 (69.7)	
Positive (*n* = 139)	83 (59.7)	56 (40.3)		44 (31.7)	95 (68.3)	
Lung metastasis at operation			NS			NS
Negative (*n* = 804)	389 (48.4)	415 (51.6)		243 (30.2)	561 (69.8)	
Positive (*n* = 6)	5 (83.3)	1 (16.7)		4 (66.7)	2 (33.3)	
Liver metastasis at operation			NS			NS
Negative (*n* = 805)	391 (48.6)	414 (51.4)		246 (30.6)	559 (69.4)	
Positive (*n* = 5)	3 (60.0)	2 (40.0)		1 (20.0)	4 (80.0)	
Postoperative recurrence*			NS			NS
Negative (*n* = 797)	386 (48.4)	411 (51.6)		244 (30.6)	553 (69.4)	
Positive (*n* = 13)	8 (61.5)	5 (38.5)		3 (23.1)	10 (76.9)	
Peritoneal dissemination			NS			NS
Negative (*n* = 784)	378 (48.2)	406 (51.8)		238 (30.4)	546 (69.6)	
Positive (*n* = 26)	16 (61.5)	10 (38.5)		9 (34.6)	17 (65.4)	
Stage			0.007 †			NS
I (*n* = 492)	230 (46.7)	262 (53.3)		157 (31.9)	335 (68.1)	
II (*n* = 100)	43 (43.0)	57 (57.0)		37 (37.0)	63 (63.0)	
III (*n* = 106)	53 (50.0)	53 (50.0)		26 (24.5)	80 (75.5)	
IV (*n* = 112)	68 (60.7)	44 (39.3)		27 (24.1)	85 (75.9)	

NOTE: The presence of ITC: a case with positive expression for either *CEA*, *CK-19*, or *CK-7*.
Abbreviation: NS, not significant.
*Recurrence of liver metastases and/or lung metastases within 2 y after the operation.
† A significant difference was observed among clinical stages by two-way ANOVA test.

and *CK-19* – positive cases was 107, 152, and 247, respectively. The incidence of *CEA*-positive, *CK-19* – positive, and *CK-7* – positive cases in peripheral blood and those in bone marrow were compared by Fisher's exact test. The relationship between clinicopathologic variables and marker expression was compared by the same analysis. The relationship between the presence of ITC and the stage of disease was calculated by ANOVA test. The expression ratio of *VEGFR-1*/*GAPDH* was calculated and expressed as "*VEGFR-1* expression" in Table 2. The cutoff value was set as the highest value of the 95% confidence interval of *VEGFR-1* expression in normal control cases (16). As depicted in Fig. 1, the clinical significance of the presence of ITC and *VEGFR-1* expression was compared by Fisher's exact test and calculated odds ratio.

Results

Expression of CEA, CK-7, and CK-19 in bone marrow and peripheral blood cells. We identified 107 of 810 (13.2%) cases

Table 2. Clinicopathologic significance of *VEGFR-1* mRNA in bone marrow and peripheral blood from gastric cancer cases

	VEGFR-1 in bone marrow		*P*	*VEGFR-1* in peripheral blood		*P*
	Present	Absent		Present	Absent	
	50 (100%) *n* (%)	760 (100%) *n* (%)		81 (100%) *n* (%)	729 (100%) *n* (%)	
N = 810	50 (6.2)	760 (93.8)		81 (10.0)	729 (90.0)	
Age (y)			0.03			NS
(mean ± SD)	64.9 ± 12.3	61.3 ± 11.5		61.7 ± 13.0	61.5 ± 11.4	
Sex			NS			NS
Male (*n* = 546)	31 (5.7)	515 (94.3)		60 (11.0)	486 (89.0)	
Female (*n* = 264)	19 (7.2)	245 (92.8)		21 (8.0)	243 (92.0)	
Histology			NS			NS
Differentiated (*n* = 181)	14 (7.7)	167 (92.3)		27 (14.9)	154 (85.1)	
Undifferentiated (*n* = 629)	36 (5.7)	593 (94.3)		54 (8.6)	575 (91.4)	
Tumor size			0.006			NS
Max diameter (mm), mean ± SD	72.5 ± 55.6	55.6 ± 1.51		56.6 ± 4.60	56.5 ± 1.51	
Tumor stage			NS			NS
pT1 (*n* = 428)	20 (4.7)	408 (95.3)		42 (9.8)	386 (90.2)	
pT2-pT4 (*n* = 382)	30 (7.9)	352 (92.1)		39 (10.2)	343 (89.8)	
Lymph node metastasis			NS			NS
Negative (*n* = 473)	22 (4.7)	451 (95.3)		44 (9.3)	429 (90.7)	
Positive (*n* = 337)	28 (8.3)	309 (91.7)		37 (11.0)	300 (89.0)	
Lymphatic involvement			NS			NS
Negative (*n* = 465)	24 (5.2)	441 (94.8)		43 (9.2)	422 (90.8)	
Positive (*n* = 345)	26 (7.5)	319 (92.5)		38 (11.0)	307 (89.0)	
Vascular involvement			NS			NS
Negative (*n* = 671)	43 (6.4)	628 (93.6)		60 (8.9)	611 (91.1)	
Positive (*n* = 139)	7 (5.0)	132 (95.0)		21 (15.1)	118 (84.9)	
Lung metastasis at operation			0.048			<0.0001
Negative (*n* = 804)	48 (6.0)	756 (94.0)		76 (9.5)	721 (89.7)	
Positive (*n* = 6)	2 (33.3)	4 (66.7)		5 (83.3)	1 (16.7)	
Liver metastasis at operation			0.033			0.01
Negative (*n* = 805)	48 (6.0)	757 (94.0)		78 (9.7)	727 (90.3)	
Positive (*n* = 5)	2 (40.0)	3 (60.0)		3 (60.0)	2 (40.0)	
Postoperative recurrence*			<0.0001			<0.0001
Negative (*n* = 797)	43 (5.4)	754 (94.6)		72 (9.0)	725 (91.0)	
Positive (*n* = 13)	7 (53.8)	6 (46.2)		9 (69.2)	4 (30.8)	
Peritoneal dissemination			NS			NS
Negative (*n* = 784)	48 (6.1)	736 (93.9)		77 (9.8)	707 (90.2)	
Positive (*n* = 26)	2 (7.7)	24 (92.3)		4 (15.4)	22 (84.6)	
Stage			0.02			NS
I (*n* = 492)	21 (4.3)	471 (95.7)		44 (8.9)	448 (91.1)	
II (*n* = 100)	8 (8.0)	92 (92.0)		12 (12.0)	88 (88.0)	
III (*n* = 106)	7 (6.6)	99 (93.4)		8 (7.5)	98 (92.5)	
IV (*n* = 112)	14 (12.5)	98 (87.5)		17 (15.2)	95 (84.8)	

*Recurrence through the hematogenous pathway; liver metastasis, and lung metastasis within 2 y after the operation.

that were positive for expression of CEA in bone marrow, whereas 62 (7.7%) were positive in peripheral blood (Table 1). As for *CK-7*, 152 patients (18.8%) and 72 cases (8.9%) were positive in bone marrow and in peripheral blood, respectively. *CK-19* expression was detected in bone marrow in 247 cases (30.5%) and in 145 cases (30.5%) in peripheral blood. A significant correlation between peripheral blood and bone marrow was observed for each marker ($P < 0.0001$). Gastric cancer cases with positive expression of any one of the three markers were defined as ITC positive in bone marrow or peripheral blood. As outlined above, ITCs were detected in 394 (48.6%) cases in bone marrow and 247 (30.5%) cases in peripheral blood.

Clinical significance of ITC in bone marrow and peripheral blood in gastric cancer. The frequency of ITC in bone marrow increased with the progression of disease from stage I to stage IV ($P = 0.007$, ANOVA; Table. 1). In addition, there were significant associations between the number of marrow ITC and the size of the tumors ($P = 0.03$, Student's t test) and between frequency of ITC and the incidence of vascular invasion ($P = 0.005$, Fisher's exact test). Therefore, the presence of ITC in bone marrow correlated with the progression of gastric cancer. Interestingly, the ITC identified in 230 of 501 cases (58.4%) were found in stage I patients. Note that the presence of ITC in the peripheral blood was not predictive of the progression of gastric cancer (Table 1).

Clinical magnitude of VEGFR-1 expression in gastric cancer. Our previous study of 88 patients revealed that cases positive for peripheral blood *VEGFR-1* mRNA were associated with advanced clinical stage, deep invasion beyond the muscularis

propria, lymphatic involvement, vascular involvement, lymph node metastasis, positive peritoneal lavage cytology, preoperative metastasis, and postoperative recurrence (16). The current study based on 810 cases (which included the original 88 patients) confirmed those data. As shown in Table 2, high *VEGFR-1* expression was observed in bone marrow in 50 cases (6.2%) and in peripheral blood in 81 cases (10%).

In bone marrow, the average age of patients in the high *VEGFR-1* expression group was significantly greater than that of the low *VEGFR-1* expression group ($P = 0.03$). Tumor size was significantly larger in the high *VEGFR-1* cases than the low *VEGFR-1* cases ($P = 0.006$). As for hematogenous progression of gastric cancer, the frequencies of simultaneous lung metastases and liver metastasis and synchronous metastases (recurrence) were significantly greater in the high *VEGFR-1* cases than those in the low *VEGFR-1* cases ($P = 0.048$, $P = 0.033$, and $P < 0.0001$, respectively). It is intriguing that there was no significant association between *VEGFR-1* status and peritoneal dissemination.

In peripheral blood, a significant association was observed in the simultaneous and synchronous hematogenous metastases as described on the right side of Table 2. As suggested by our preliminary study, the incidence of vascular involvement was

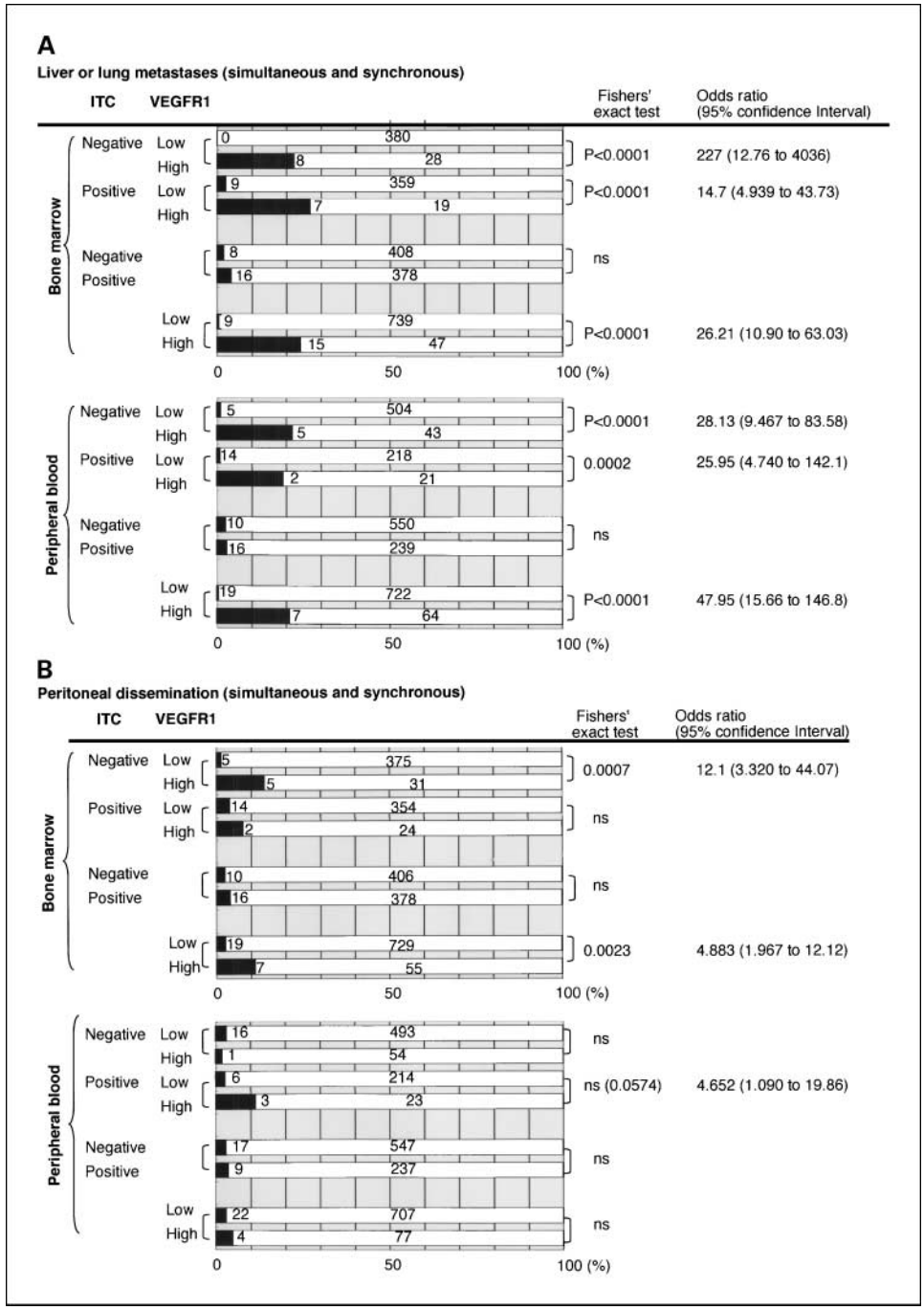

Fig. 1. Comparison of ITC and *VEGFR-1* status, individually and combined. Hematogenous metastasis (*A*) and peritoneal dissemination (*B*) in gastric cancer. Top row, data from bone marrow; bottom row, data from peripheral blood. Black bars, number of positive cases; white bars, number of negative cases. The 380 cases that were ITC negative and low in *VEGFR-1* expression did not form either synchronous (at the time of surgery) or heterochronous (recurrent) metastases.

Table 3. Univariate and multivariate analysis for perioperative and/or postoperative lung and liver metastasis in ITC bone marrow–positive patients

Factors	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P*	RR (95% CI)	*P*
Histologic grade (differentiated/undifferentiated)	0.74 (0.25-2.71)	0.62	—	—
Tumor size (<60 mm/≥60 mm)	3.50 (1.24-11.3)	0.02	1.44 (0.35-6.42)	0.61
Lymph node metastasis (-/+)	10.2 (2.79-65.4)	0.002	17.7 (2.41-203.0)	0.009
Lymphatic involvement (-/+)	3.63 (1.24-13.2)	0.03	0.67 (0.12-4.20)	0.66
Vascular involvement (-/+)	3.09 (1.07-8.56)	0.03	1.54 (0.38-6.11)	0.54
VEGFR-1 mRNA in BM (-/+)	14.7 (4.80-43.9)	<0.0001	10.1 (2.37-46.2)	0.002
VEGFR-1 mRNA in PB (-/+)	20.9 (7.19-69.5)	<0.0001	25.1 (7.13-102.4)	<0.0001

Abbreviations: RR, relative risk; 95% CI, 95% confidence interval; BM, bone marrow; PB, peripheral blood.

significantly greater in the high *VEGFR-1* cases than the low cases ($P = 0.03$).

Hematogenous metastasis of gastric cancer requires both ITC and high VEGFR-1 expression. In Fig. 1A (bone marrow), 380 patients who were ITC negative and *VEGFR-1* (low) showed no lung or liver metastases, whereas 8 of 36 ITC-negative and *VEGFR-1* (high) cases (22.2%) showed metastases. We found an extremely high odds ratio for hematogenous metastasis in bone marrow when combining the presence of ITC and *VEGFR-1* status (227 + 14.7) in predicting hematogenous metastasis compared with the evaluation of *VEGFR-1* alone (26, 21) or ITC status alone (not statistically significant). In Fig. 1A (peripheral blood), predicting hematogenous metastasis by the odds ratio of *VEGFR-1* status alone (47.95) is almost equal to the combined data for ITC and *VEGFR-1* (28.13 + 22.95). In Fig. 1B (bone marrow), there are significant associations in the combined data for ITC negativity and low *VEGFR-1* expression ($P = 0.0007$) and *VEGFR-1* status alone ($P = 0.0023$). However, the odds ratio of ITC and *VEGFR-1* status to the incidence of peritoneal dissemination was lower than data for hematogenous metastases. Moreover, it is interesting that the presence of ITC and the expression of *VEGFR-1* in peripheral blood did not predict peritoneal dissemination.

When analysis is limited to 206 cases of advanced gastric cancer without metastasis at the time of surgery (stage II and III), there were 8 cases with postoperative recurrence and 198 cases without it. Three of 8 (37.5%) cases with recurrence were ITC positive and *VEGFR-1* (high), whereas 192 of 198 (97.0%) cases without recurrence were either ITC negative or *VEGFR-1* (low) ($P = 0.0029$, Fisher's exact test). Thus, *VEGFR-1* status indicated a significant association with the incidence of postoperative hematogenous metastasis in stage II and III cases ($P = 0.0002$). The odds ratio and the relative risk of the combined data were 19.20 and 13.13, respectively, whereas those for *VEGFR-1* alone were 21.90 and 16.40, respectively.

Table 3 shows multivariate analysis for perioperative and/or postoperative lung and liver metastasis in ITC bone marrow–positive patients. High expression of *VEGFR-1* is the strongest independent factor predicting lung metastases as well as liver metastases compared with other clinicopathologic variables. Moreover, the relative risk ratio for metastasis in 26 cases that were ITC positive and *VEGFR-1* (high) was greater than other groups, such as 36 cases of ITC negative *VEGFR-1* (high), 62 cases of *VEGFR-1* (high), and 394 cases of ITC positive (data not shown).

Presence of CK-positive cells in bone marrow. One of four early gastric cancer cases showed an increased number of CK-positive cells 4 weeks after cultivation under the specific conditions described here (Table 4; Fig. 2). Two years have elapsed since the extraction of bone marrow cells before operation. However, there is no evidence for recurrence in this patient (case 214).

Table 4. Increased number of cultured cells with CK-positive expression

Case no.	No. BM cells	Number of CK-positive* cells		Stage of disease †
		Before culture	After culture ‡	
210	1.40E+07	0	10	IB
213	7.50E+06	0	0	IB
214	1.30E+07	<10	1,257	IA
218	2.10E+07	44	65	IV
222	2.40E+06	<10	323	IIIB
223	1.80E+06	<10	127	IIIA
225	1.12E+07	0	11	IB
226	1.40E+07	<10	391	IV

*A45-B/B3 (CK8/18 8/19) antibody.
† According to ref. 17.
‡ Cultivated cells for 4 wk.

Discussion

Presence of ITC and high expression of VEGFR-1 in metastasis. The fraction of ITC-positive cases correlated significantly with the stage of disease by ANOVA (Table 1). However, the differences between ITC-positive rates at different stages of disease did not by themselves suggest a mechanism for gastric cancer metastasis. That result led us to pursue novel factors in metastasis of gastric cancer. In Fig. 1A, hematogenous metastases were significantly associated with ITC status when combined with the level of *VEGFR-1* expression. The odds ratio and relative risk were higher for the combined variables than for ITC alone or *VEGFR-1* alone. This finding was supported by the previous study *in vivo* by Kaplan et al. (15, 26), who found that bone marrow–derived hematopoietic progenitor cells that expressed *VEGFR-1* home to tumor-specific, premetastatic sites and form cellular clusters before the arrival of tumor cells. Thus, our current study provides strong support for the hypothesis that metastases are promoted when ITC circulate in the presence of elevated levels of *VEGFR-1*. Kaplan et al. reported that *VGFR-1* originated from hematopoietic progenitor cells in bone marrow and functions as a cancer niche to facilitate metastasis. Calabrese et al. (27) and Folkins et al. (28) reported that perivascular niches play an important role in the growth of brain tumors originating from cancer stem cells. Those studies were conducted both *in vitro* and *in vivo*. Our study provides clinical data showing the importance of cancer cells and the simultaneous presence of niche-expressing *VEGFR-1* cells.

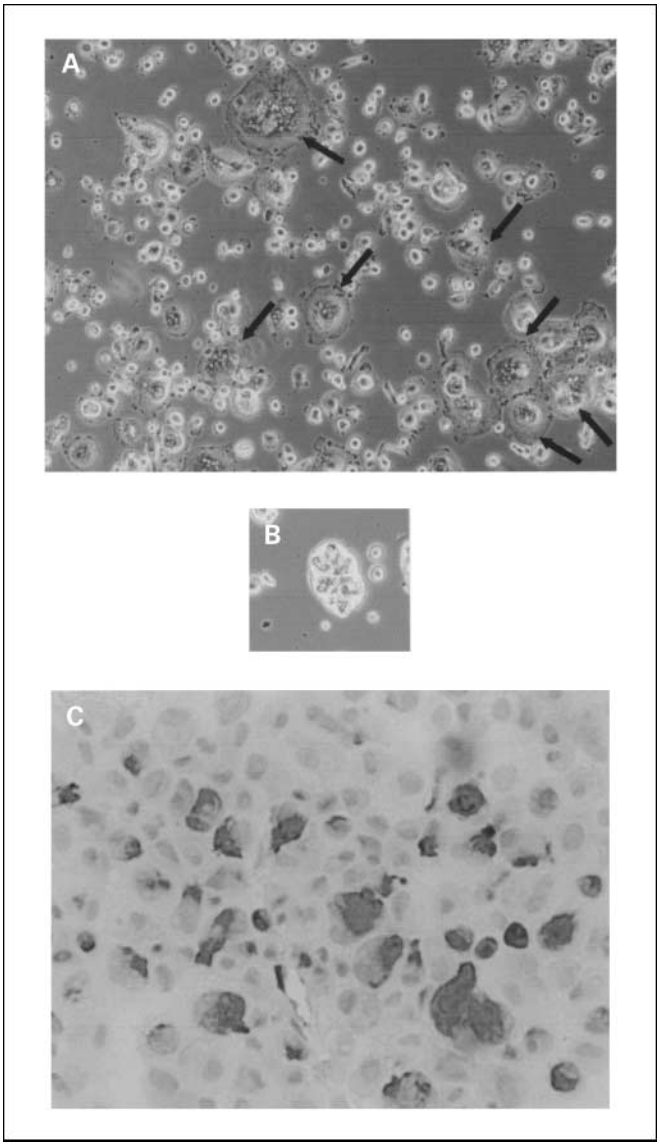

Fig. 2. Cultivation of bone marrow mononuclear cells from a representative case of early gastric cancer (case 214 in Table 4). *A,* proliferating cells (*arrows*) in bone marrow were observed after 4 weeks of culture. *B,* several clusters show a duct-like shape, and the spin preparations were immunostaining with A45-B/B3 was done, and found many positive cells (*C*).

With regard to advanced cases without metastases at the time of surgery (stages II and III), recurrence of disease was more frequently observed in cases in which both ITC and high expression of VEGFR-1 were observed than in other cases of stages II and III. In other words, the development of metastatic lesions requires ITC in the presence of *VEGFR-1*–expressing cells. In further support of this conclusion, Fig. 1A showed that when *VEGFR-1* was low in bone marrow, only 9 of 368 ITC-positive cases developed metastases, whereas none of the 380 cases lacking ITC formed metastatic lesions. Therefore, both ITC and high levels of *VEGFR-1* are necessary to support metastasis.

Presence of ITC in early gastric cancer. It is extraordinary that >50% of early gastric cancer cases expressed at least one ITC marker in early stages of the disease. It is possible that expression of those genes in bone marrow or peripheral blood may not occur in cancer cells themselves but in normal cells that have been stimulated by cytokines produced in the setting of carcinoma. In fact, Jung et al. (29) reported that *CEA* messages could be easily detected in cultured marrow cells after γ-IFN stimulation. In cancer patients, the regulation of cytokines may be altered, and CEA/CK might well be expressed by normal cells subjected to cytokine stimulation, a possibility that should be studied in the near future.

Using the same methodology for detection of CEA, we previously found that CEA expression was associated with lower disease-free survival and overall survival in cases of colorectal cancer (7, 30). In addition, we previously reported that 100 of 206 patients (48.5%) with breast cancer were positive for *CK-7* in peripheral blood or bone marrow. The group expressing *CK-7* in peripheral blood had poorer disease-free survival than did the negative group ($P < 0.01$). The same methodology showed a positive association between gene expression and recurrence or poor prognosis in colon and breast cancers but no statistically significant correlation in gastric cancer. We speculate that gastric cancer cells are circulating or disseminating even from an early stage of disease, and the disease differs from colorectal cancer and breast cancer. As a matter of fact, according to our results with cultured bone marrow mononuclear cells, CK-positive cells were detectable in bone marrow even in early gastric cancer cases (Table 4; Fig. 2). Note, however, that CK expression alone is not clinically relevant. Rather, metastasis and/or recurrence are more likely to occur when CK-positive cells in bone marrow encounter other factors that facilitate development of gastric cancer.

Considering the finding that ITCs were readily detected even at early stages of gastric cancer, the hypotheses of Gray et al. (31) are important for interpreting our current data. They

presented two opposite paradigms describing tumor progression to metastasis. In general, it is believed that metastasis usually occurs through clonal genomic and epigenetic evolution; that is, after growth of the primary tumor, a metastatic cell population is subsequently formed. On the other hand, a parallel evolution model proposes that tumor cells disseminate at a very early period and evolve to metastatic disease independent of the primary tumor (31). The data presented here suggest that gastric cancer is best described by the latter paradigm.

In conclusion, we have shown the presence of ITC in peripheral blood and bone marrow in both early and late stages of gastric cancer. Formation of metastases is most likely when ITCs circulate in the presence of high levels of *VEGFR-1*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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