

## Detection of Bone Marrow Micrometastasis in Gastric Cancer Patients by Immunomagnetic Separation

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**Background:** Micrometastasis to the bone marrow can predict widespread disease and a poor prognosis of cancer patients after surgery. The purpose of this study was to evaluate the clinical significance of detecting micrometastasis in the bone marrow of gastric cancer patients.

**Methods:** Bone marrow and peripheral blood samples were obtained from 53 gastric cancer patients at the time of surgery. These samples were enriched by immunomagnetic separation and immunostained with an anti-cytokeratin antibody. Expression of vascular endothelial growth factor and erbB-2/HER2 was examined in the primary tumors.

**Results:** Cytokeratin-positive cancer cells were observed in the bone marrow of 16 (30%) of 53 patients. Among them, two patients also had cancer cells in the peripheral blood. The presence of bone marrow micrometastasis was correlated with the depth of invasion and lymph node metastasis but was not associated with peritoneal dissemination. Detection of bone marrow micrometastasis was not correlated with vascular endothelial growth factor or HER2 expression in the primary tumors. Four patients with micrometastasis had recurrence in the liver or lungs, but this did not occur in patients without micrometastasis.

**Conclusions:** Detection of cancer cells in the bone marrow might be an indicator of postoperative hematogenous metastasis in gastric cancer patients.

**Key Words:** Micrometastasis—Cytokeratin—Bone marrow—Immunomagnetic separation—Gastric cancer—Hematogenous metastasis.

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Almost half of the patients with gastric cancer who undergo surgery have metastatic disease.<sup>1,2</sup> Detection of metastasis by imaging and at operation is limited to patients with a large number of tumor cells. It seems that tumor cells have already seeded in patients at the time of surgery, but systemic adjuvant chemotherapy produces a small survival benefit after curative resection, and its indication may be limited.<sup>3,4</sup> Detection of micrometastasis may allow a more accurate assessment of the prognosis and aid in selecting candidates for intensive chemotherapy among gastric cancer patients. Detection of bone marrow micrometastasis has been shown to influ-

ence the prognosis of patients with breast,<sup>5–7</sup> esophageal,<sup>8</sup> gastric,<sup>9–11</sup> colorectal,<sup>12</sup> and non-small-cell lung cancer.<sup>13</sup>

Immunocytochemistry with anti-cytokeratin (CK) antibody can detect cancer cells, but it can be difficult to detect a few tumor cells within the large population of mononuclear cells found in bone marrow aspirates. Immunomagnetic separation (IMS) has been developed for enrichment of epithelial cells from large samples of mononuclear cells.<sup>14–16</sup> For positive IMS, superparamagnetic polystyrene beads are coated with a monoclonal antibody (Ber-EP4) that is specific for two glycopolypeptide membrane antigens expressed on most normal and neoplastic epithelial cells, including gastric cancer cells.<sup>17</sup> A new kit (CELLlection™; Dynal, Oslo, Norway) allows cancer cells to be separated from the beads and easily observed when compared with the older kit (Dynabeads™ antiepithelial cell, Dynal, Oslo, Norway).<sup>18</sup>

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Received June 4, 2002; accepted September 17, 2002.

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In this study, we used the new kit to examine micrometastasis in the bone marrow and peripheral blood of patients with gastric cancer. To evaluate the clinical implications of detecting micrometastasis, we analyzed the pathologic findings and the outcome after surgery. We also analyzed the association of bone marrow micrometastasis with HER2 and vascular endothelial growth factor (VEGF) expression by the primary tumors.

## MATERIALS AND METHODS

### Patients

We studied 53 patients with primary gastric cancer who underwent gastric resection between September 1999 and March 2001 at the Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University. Fifteen patients were women, and 38 patients were men; their ages ranged from 35 to 78 years (median age, 64 years). Serum levels of carcinoembryonic antigen (CEA) and carcinoma antigen 19-9 (CA 19-9) were measured before surgery. The operation was distal gastrectomy in 29 patients, total gastrectomy in 17 patients, proximal gastrectomy in 5 patients, and lower esophago-gastric resection via a left thoracoabdominal approach in 2 patients. Pathologic diagnosis of the resected specimens was performed according to the *Japanese Classification of Gastric Carcinoma*.<sup>19</sup> Tumor stage and grade were assessed according to the tumor-node-metastasis classification of the International Union Against Cancer.<sup>20</sup> All patients were evaluated after surgery at 2-week intervals for 3 months, at monthly intervals for 1 year, and at 4-month intervals thereafter. The mean follow-up period was 17.5 months (range, 3–29.7 months). Recurrence was diagnosed on the basis of imaging findings, including chest x-ray, computed tomography, and ultrasonography data. The patients who had residual tumors or disease recurrence received chemotherapy.

### Immunomagnetic Separation

Written, informed consent to bone marrow examination was obtained from all patients before enrollment onto this study. Bone marrow (2 mL) was aspirated from the iliac crest, and blood (10 mL) was obtained from a catheter inserted into the peripheral artery under general anesthesia at the beginning of surgery. Mononuclear cells were isolated by density gradient centrifugation by using Ficoll<sup>™</sup> (Amersham Biosciences, Piscataway, NJ) for 15 minutes. After being washed twice by centrifugation through .01 M of phosphate-buffered saline, the mononuclear cells were resuspended in 5 mL of phosphate-buffered saline and incubated with 250  $\mu$ l ( $1 \times 10^7$ ) of Ber-EP4-coated polystyrene beads (CELLec-

tion, Epithelial Enrich<sup>™</sup>; Dynal, Oslo, Norway) for 30 minutes at 4°C. After being placed in a magnetic particle concentrator (MPC-1<sup>™</sup>; Dynal), the supernatant was repeatedly removed. The beads that rosetted with epithelial cells were collected and incubated with DNase Releasing Buffer<sup>™</sup> (Dynal) for 15 minutes at room temperature. The tube was placed in MPC-1, and the supernatant with the free epithelial cells was collected. Resuspended cells were smeared onto precoated slides with a Cytospin 3<sup>™</sup> centrifuge (Shandon Scientific, Cheshire, UK) by using a double Cytofunnel<sup>™</sup> (Shandon Scientific). After overnight drying in air, the slides were fixed in 100% acetone for 10 minutes. The primary antibody was a polyclonal rabbit anti-pancytokeratin antibody (Nichirei, Tokyo, Japan). Immunostaining was performed by the alkaline phosphatase and anti-alkaline phosphatase technique (Dako, Glostrup, Denmark).<sup>21</sup> Alkaline phosphatase activity was monitored by using the new fuchsin (Dako, Carpinteria, CA) stain after endogenous phosphatase activity was blocked by preincubation with levamisole (Dako). After counterstaining with hematoxylin and mounting, the slides were examined by light microscopy. The negative control was peripheral blood samples from healthy volunteers, and the positive control was HT-29 cells resuspended in the blood. CK-positive epithelial cells that lacked any hematopoietic characteristics were recorded.

### Immunohistochemistry

Specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (3  $\mu$ m thick) cut from paraffin blocks of the primary tumors were dewaxed in xylene and rehydrated. Endogenous peroxidase activity was blocked by incubating the sections in .3% hydrogen peroxidase in absolute methanol. Then, immunostaining was performed with the labeled streptavidin-biotin-peroxidase technique (LSAB2 kit<sup>™</sup>; Dako, Glostrup, Denmark). Briefly, sections were incubated overnight in a humidified chamber with the primary antibodies, which were a polyclonal rabbit anti-pancytokeratin antibody (Nichirei), an anti-human VEGF antibody (sc-172; Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-human polyclonal erbB-2/HER2 antibody (Dako). Negative controls used normal rabbit serum at 1/400 instead of the primary antibody. The reaction products were visualized by using 3,3'-diaminobenzidine tetrahydrochloride (Dako) at .5 mg/mL in .03% hydrogen peroxide, and the intensity of cytoplasmic (VEGF) or membrane (HER2) staining was scored by three blinded investigators (K.M., T.N., and H.O.).

### Statistical Analysis

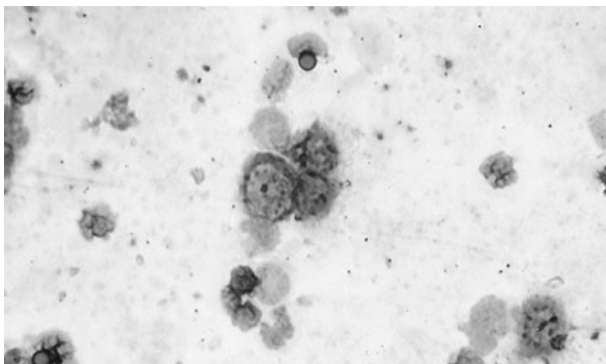
Differences between subgroups with respect to detection of bone marrow micrometastasis and clinicopathologic findings were evaluated by the  $\chi^2$  test or Fisher's exact test. Serum concentrations of CEA and CA 19-9 were analyzed by Student's *t*-test. Differences of  $P < .05$  were regarded as statistically significant.

### RESULTS

After IMS, CK-reactive cells indicating bone marrow micrometastasis were easily distinguished from lymphocytes and myelocytes (Fig. 1). Micrometastasis was detected in the bone marrow of 16 (30%) of 53 patients with gastric cancer. Among them, only two patients also had cancer cells in the peripheral blood. The presence of bone marrow micrometastasis was correlated with the depth of tumor invasion (pT) and the involvement of lymph nodes, but it was not associated with peritoneal dissemination (Table 1). Lymphatic invasion and vascular invasion in the primary tumors were also correlated with bone marrow micrometastasis. Serum levels of CEA and CA 19-9 were not correlated with bone marrow micrometastasis. The mean levels of CEA and CA 19-9 were, respectively, 2.9 ng/mL and 150.4 IU/mL in the patients with micrometastasis versus 3.3 ng/mL and 363 IU/mL in the patients without micrometastasis.

The primary tumors of all patients were stained positive for the anti-pancytokeratin antibody. VEGF and HER2 expression in the primary lesion were not significantly correlated with the detection of micrometastasis in bone marrow (Table 2).

Ten (63%) of 16 patients with bone marrow micrometastasis and 7 (19%) of 37 patients without micrometas-



**FIG. 1.** Cytokeratin-positive micrometastatic cells were collected by immunomagnetic separation from the bone marrow in gastric cancer patients. The slide was immunostained by using the alkaline phosphatase and anti-alkaline phosphatase technique with pan-cytokeratin antibody visualizing by new fuchsin. The original magnification was  $\times 400$ , and the bead shown was 4.5  $\mu\text{m}$ .

**TABLE 1.** Clinicopathologic characteristics of gastric cancer patients with and without bone marrow micrometastasis

Variable	With micrometastasis, n = 16 (30%)	Without micrometastasis, n = 37 (70%)	P value
Sex (male:female)	13:3	25:12	NS
Age (y)	60.7 $\pm$ 10.5	64.9 $\pm$ 10.4	NS
Tumor maximal diameter (cm)	9.76 $\pm$ 3.51	7.23 $\pm$ 5.32	NS
Histology			
Differentiated	7 (25%)	21 (75%)	NS
Undifferentiated	9 (36%)	16 (64%)	
Depth of penetration			
pT1	1 (6.3%)	15 (94%)	.0412
pT2	1 (16.7%)	5 (83.3%)	
pT3	12 (46.2%)	14 (53.8%)	
pT4	2 (40%)	3 (60%)	
Lymph node metastasis			
Positive	14 (45.2%)	17 (54.8%)	.0061
Negative	2 (9.1%)	20 (91%)	
Peritoneal dissemination			
Positive	5 (41.7%)	7 (58.3%)	NS
Negative	11 (26.8%)	30 (73.1%)	
Lymphatic involvement			
Positive	16 (41%)	23 (59%)	.0048
Negative		14 (100%)	
Vascular involvement			
Positive	11 (45.8%)	13 (54.2%)	.0358
Negative	5 (17.2%)	24 (82.8%)	

NS, not significant.

tasis experienced recurrence of cancer ( $P = .0034$ ) (Table 3). Four patients with micrometastasis had recurrence in the liver, lungs, or both, whereas no patient without micrometastasis experienced involvement of these organs ( $P = .0062$ ). There were no differences of peritoneal dissemination or distant lymph node metastasis between the patients with and without micrometastasis. Both patients with cancer cells in the peripheral blood had disease recurrence.

**TABLE 2.** Vascular endothelial growth factor (VEGF) and *erbB-2/HER2* expression in the primary tumors of gastric cancer patients with or without bone marrow micrometastasis

Variable	With micrometastasis, n = 16	Without micrometastasis, n = 37
VEGF expression		
3+	1 (50%)	1 (50%)
2+	2 (22%)	7 (78%)
+	4 (36%)	7 (64%)
-	9 (29%)	22 (71%)
<i>erbB-2/HER2</i> expression		
3+	1 (25%)	3 (75%)
2+	4 (44%)	5 (56%)
+	2 (67%)	1 (33%)
-	9 (24%)	28 (76%)

**TABLE 3.** Site of recurrence after surgery in gastric cancer patients with or without bone marrow micrometastasis

Variable	With micrometastasis	Without micrometastasis	P value
Total	10/16 (62.5%)	7/37 (18.9%)	.0034
Site of recurrence			
Liver and/or lung	4 (100%)		.0062
Peritoneum	3 (43%)	4 (57%)	NS
Lymph node/local	6 (60%)	4 (40%)	NS

NS, not significant.

## DISCUSSION

Although aggressive surgery is still performed for gastric cancer patients, approximately half of them develop distant metastasis. These patients have systemic diseases that cannot be detected by preoperative imaging studies. Detection of micrometastasis in the bone marrow is an established indicator of poor prognosis in breast cancer patients<sup>5-7</sup>; however, this does not necessarily apply to gastric cancer patients.

Detection rates of bone marrow micrometastasis were reported to range from 32.6%<sup>11</sup> to 53%<sup>10</sup> in gastric cancer patients. Although we used IMS to improve the detection sensitivity in this study, only 30% of our patients had bone marrow micrometastasis. The positive rate was lower than those in Western studies<sup>9,10</sup> but was similar to that in another Japanese study.<sup>11</sup> Differences in the tumor stage and survival of gastric cancer patients who underwent surgery in Germany and Japan<sup>22</sup> might have influenced these results. A recent study showed a low detection rate of 8% in patients with adenocarcinoma of the cardia or squamous cell carcinoma of the esophagus.<sup>23</sup> Also, there is no established method for detecting micrometastasis to the bone marrow, and such a method may be necessary to clarify the clinical significance of the findings.

Previous studies have shown that IMS was useful to detect epithelial cells in the bone marrow among a large number of myelocytes or lymphocytes.<sup>15,16</sup> IMS can detect 10 cancer cells per 10<sup>7</sup> mononuclear cells, which is a similar sensitivity to reverse transcriptase-polymerase chain reaction. The new IMS kit (CELLection, Epithelial Enrich) used in this study allowed the nuclei of epithelial cells to be observed and cancer cells to be diagnosed because the beads could be detached and removed. However, it is also possible for sensitivity to be decreased by cell loss during the detachment process. A recent IMS study using beads coated with an epithelial cell adhesion molecule (MOC31) showed bone marrow micrometastasis in 17% of colorectal cancer patients.<sup>24</sup> These results

indicate that IMS might contribute to improving the specificity, but not the sensitivity, of micrometastasis detection.

Although our follow-up period was short, the recurrence rate of patients with bone marrow micrometastasis was higher than that of those without micrometastasis in this study. The detection rate of bone marrow micrometastasis was correlated with tumor stage; however, it was not associated with peritoneal dissemination at the time of or after surgery. Peritoneal dissemination is one of the most frequent forms of recurrence after surgery in patients with advanced gastric cancer. Therefore, detection of bone marrow micrometastasis might be useful for predicting the outcome of early gastric cancer, as reported previously,<sup>25</sup> whereas detection of micrometastasis in the peritoneal cavity might be more useful for predicting the risk of peritoneal dissemination.<sup>26</sup>

In this study, micrometastasis was detected in the bone marrow of all four patients who had recurrence in the liver or lungs after curative surgery. In contrast, no patient without micrometastasis developed hematogenous metastasis but instead had recurrence or peritoneal dissemination. Several studies have shown that detection of bone marrow micrometastasis is a useful prognostic indicator in breast cancer patients.<sup>5-7</sup> In contrast, the detection of tumor cells in the bone marrow was not associated with survival in patients with cancer of the cardia<sup>23</sup> or pancreatic cancer.<sup>27</sup> Most recurrences were due to hematogenous metastasis in the former group of patients, but half of the recurrences in the latter were due to local disease or peritoneal dissemination.

Both VEGF and HER2 expression are correlated with the survival of breast cancer patients.<sup>28,29</sup> VEGF expression was a contributory prognostic indicator,<sup>30</sup> but HER2 expression was not such a significant one for gastric cancer patients.<sup>31</sup> In this study, VEGF and HER2 expression were not correlated with the detection of bone marrow micrometastasis. These negative correlations might be influenced by the small number of the patients and the short follow-up in this study. A previous study showed that the tumor microvessel density was correlated with bone marrow micrometastasis.<sup>25</sup> In this study, lymphatic and vascular invasion in the primary tumors were also correlated with bone marrow micrometastasis. These results suggest that the detection of micrometastasis in the bone marrow might be useful for predicting hematogenous metastasis in gastric cancer patients.

In conclusion, detection of bone marrow micrometastasis in gastric cancer patients was correlated with the depth of tumor invasion and lymph node metastasis, but not with peritoneal dissemination. Micrometastasis to the bone marrow might be a significant indicator of liver or

lung metastasis after surgery, and intensive chemotherapy seems to be indicated for these patients. However, further prospective multicenter studies in a large number of gastric cancer patients are required to fully establish the clinical significance of bone marrow micrometastasis.

### ACKNOWLEDGMENT

The acknowledgment is available online at [www.annalssurgicaloncology.org](http://www.annalssurgicaloncology.org).

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