Change in number and size of circulating tumor cells with high telomerase activity during treatment of patients with gastric cancer

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Abstract. Detection of circulating tumor cells (CTCs) in peripheral blood is useful for estimating the prognosis of patients with cancer. We previously reported the detection of CTCs by OBP-401, a telomerase-specific, replication-selective, oncolytic adenoviral agent carrying the green fluorescent protein (GFP) gene. We demonstrated that the number of large (L)-GFP+ cells (≥7.735 μm in diameter) in peripheral blood samples correlated significantly with the prognosis of treatment-naïve gastric cancer patients, whereas the number of small (S)-GFP+ cells (<7.735 μm in diameter) did not. In the present study, we studied the change in the number of GFP+ cells during treatment, and analyzed the association between the number of GFP+ cells in blood samples and the outcome of patients. Peripheral blood samples were obtained from 37 gastric patients prior and subsequent to surgery (three samples per time point). Upon infection of blood cells with OBP-401, GFP+ cells of different sizes were counted and measured. The association between the number of GFP+ cells and surgical outcome was determined by statistical analysis. The median follow-up period after surgery was 39 months. Although the difference was not significant, patients with ≥6 L-GFP+ cells in preoperative blood samples had a lower relapse-free survival rate than patients with 0-5 L-GFP+ cells. There was no significant correlation between the number of L-GFP+ cells in postoperative blood samples and the prognosis of patients receiving adjuvant therapy. Although the difference was not significant, the number of S-GFP+ cells in samples from patients who had received postoperative chemotherapy was higher than in those who had not. The number of L-GFP+ cells was not significantly correlated with the relapse-free survival rate in gastric cancer patients who underwent surgery. The number of S-GFP+ cells was relatively high in samples from patients who had received postoperative chemotherapy.

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Introduction

The presence of circulating tumor cells (CTCs) in peripheral blood indicates a systemic disease stage (1), and detection of CTCs in peripheral blood is useful for estimating prognosis and monitoring disease progression in breast (2), prostate (3), skin (4) and colon (5) malignancies. The commonly used techniques for enrichment and detection of CTCs are density gradient separation (6,7), direct enrichment by filtration (8), immunomagnetic separation (9), flow cytometry (10), reverse transcription-polymerase chain reaction (RT-PCR) (11,12) and microchip technology (13). The CELLSEARCH® System (Veridex LLC, Raritan, NJ, USA) (14) is a widely used automated method of immunomagnetic cell enrichment (15-17) that detects CTCs by detection of epithelial markers such as epithelial cell adhesion molecule (EpCAM) and cytokeratin. The expression of these markers, however, decreases during the epithelial-mesenchymal transition (EMT) (18,19); thus, CTCs undergoing EMT may escape detection.

Increased telomerase activity is a common characteristic of malignant tumors, and telomerase activity strongly correlates with carcinogenesis and disease progression (20-22). As a novel means of detecting cells with high telomerase activity in peripheral blood samples of cancer patients, a telomerase-specific, replication-competent adenovirus variant termed OBP-401 (TelomeScan®; Oncolys BioPharma, Inc., Tokyo, Japan) was used (22). In this variant, the human telomerase reverse transcriptase (TERT) gene promoter drives the expression of the viral genes adenovirus early region 1A (E1A) and E1B, while the cytomegalovirus promoter drives

the expression of the green fluorescent protein (GFP) gene, which serves as a marker of viral replication (22).

In our previous study (23), we used OBP-401 to detect CTCs in patients with gastric cancer. Gastric cancer is the fifth most common cancer worldwide and the third most frequent cause of cancer-associated mortality, affecting ~723,000 people in 2012 (24). In that study, blood samples from 65 treatment-naïve gastric cancer patients were collected prior to surgery (23). CTCs are larger than normal blood cells (25,26), and it was observed that the GFP+ cells in these samples with a diameter ≥7.735 μm (termed L-GTP+ cells) expressed EpCAM and other CTC markers (23). Additional results indicated that the number of L-GFP+ cells was significantly correlated with venous invasion (23) and overall survival after a median follow-up period of 3 years (27).

The number of CTCs following initial treatment correlates significantly with treatment efficacy and prognosis in patients with breast (2), prostate (3) and colorectal cancer (5). However, whether this is the case for patients with gastric cancer, is controversial. In the present study, the number of CTCs (defined as L-GFP+ cells) were determined prior and subsequent to treatment.

Materials and methods

Patients and healthy volunteers. The present report is an interim analysis of part of our prospective study of CTCs as GFP+ cells in patients with treatment-naïve gastric adenocarcinoma who underwent surgery at the Digestive Disease Center of Showa University Northern Yokohama Hospital (Tsuzuki-ku, Japan) between April 2010 and May 2011. Peripheral blood samples (three samples per time point) were obtained from 37 patients before surgery and at 4 and 24 weeks after surgery. The inclusion criteria were as follows: i) Histologically confirmed adenocarcinoma of the stomach by endoscopic biopsy; ii) clinical solitary tumor; iii) no prior endoscopic resection, chemotherapy or radiotherapy; iv) age, 20-80 years; v) Eastern Cooperative Oncology Group performance status (28) of 0 or 1; vi) sufficient organ function; and vii) written informed consent. The exclusion criteria were as follows: i) Synchronous or metachronous malignancy; ii) pregnancy or breast-feeding; iii) active or chronic viral hepatitis; iv) active bacterial or fungal infection; v) diabetes mellitus; vi) systemic administration of corticosteroids; and vii) unstable hypertension. The pathological stage of the disease was determined according to the 7th edition of the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis classification system (29). The depth of the tumor invasion in 4 patients without gastrectomy and the regional lymph node status of 7 patients without sufficient lymphadenectomy were surgically diagnosed.

All patients were followed up at our hospital every 3 months after surgery. The patients underwent endoscopy and computed tomography at least once per year depending on their disease stage and course. Postoperative therapy was allowed if required.

Peripheral blood samples were collected from healthy volunteers to establish the cut-off size of GFP+ cells. All volunteers were employees of Sysmex Corporation (Kobe, Japan), and were interviewed individually prior to sample collection. The volunteer group consisted of 7 men (mean age, 31.4 years; range, 24-39 years) and 3 women (mean age, 33.7 years; range, 26-48 years). All volunteers underwent medical check-ups upon employment and annually thereafter. Check-ups included medical interviews, auscultation, chest radiography, and blood and urine analyses. None of the volunteers was receiving medical treatment, pregnant or breast-feeding, or had donated blood within the month previous to the initiation of the study.

The present study was approved by the Institutional Review Board of Showa University Northern Yokohama Hospital (Tsuzuki-ku, Japan; approval number 0903-03). The study protocol was explained to patients and volunteers prior to obtention of written informed consent. Our study was registered with the University Hospital Medical Information Network in Japan (Tokyo, Japan; registration number 000004026).

Viruses. OBP-401, a telomerase-specific, replication-selective adenovirus variant in which the TERT promoter drives the expression of the viral EIA and EIB genes, and into which the GFP gene is integrated (22), was used. The sensitivity and specificity of the OBP-401 assay have been reported by Kim *et al* (30). Briefly, the assay was evaluated five times using MDA-MB-468 breast carcinoma cells as positive controls. The numbers of GFP+ cells in samples containing 1 MDA-MB-468 cell and 7.5 ml blood were 1, 1, 1, 2 and 3, respectively, while the numbers of GFP+ cells in samples containing 20 MDA-MB-468 cells and 7.5 ml blood were 15, 17, 19, 22 and 24, respectively. Viral samples were stored at -80˚C.

Sample preparation and immunostaining. Details of sample preparation and assay are described in our previous report (23). A 7.5-ml peripheral vein blood sample was obtained from each patient before surgery and at 4 and 24 weeks after surgery. Blood was collected in tubes containing citric acid, phosphoric acid and dextrose, and stored at 4˚C. Samples were assayed within 48 h of collection. Samples were centrifuged for 5 min at 540 x *g*, and the plasma phase was removed. Cell pellets were washed four times with PBS (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and twice with RPMI medium (Sigma-Aldrich; Merck Millipore). Upon suspension in RPMI medium, cells were infected with $4x10^8$ plaque-forming units of OBP-401 for 24 h at 37˚C. Dead cells were stained with the red fluorescent reactive dye L23102 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Upon inactivation of OBP-401, cells were fixed with 2% paraformaldehyde for 20 min at room temperature and treated with a surface-active agent (Emalgen 2025 G; Kao Chemicals, Tokyo, Japan) for 10 min at 40˚C to degrade red blood cells. Cells were incubated with phycoerythrin-labeled anti-human cluster of differentiation (CD) 45 antibody (catalogue number 368510; BioLegend, San Diego, CA, USA; 1:5 dilution) for 30 min at 25˚C, which was diluted into PBS containing 2% fetal bovine serum (FBS; Sigma-Aldrich; Merck Millipore). Cells were washed with PBS containing 2% FBS and mounted on glass slides for microscopic analysis (two slides per sample).

Determination of GFP fluorescence intensity threshold. The threshold for GFP fluorescence intensity was determined as previously reported (23). Briefly, blood samples (7.5 ml) from

healthy volunteers were mixed with ~30,000 A549 (lung carcinoma), HepG2 (hepatocellular carcinoma), HEC-1 (endometrial carcinoma), KATO-III (gastric carcinoma), SBC-3 (small cell lung carcinoma), LNCaP (prostate adenocarcinoma), MDA-MB-468 (breast carcinoma) or OVCAR-3 (ovarian carcinoma) cells. The A549, HepG2, HEC-1, KATO-III, SBC-3 and MDA-MB-468 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan), while the LNCap and OVCAR-3 cell lines were obtained from the RIKEN Cell Bank (Tokyo, Japan). Cell lines were cultured according to the vendor's specifications. The samples were assayed using OBP-401, and GFP+ cells were visualized by fluorescence microscopy and counted. The GFP signal intensity threshold was determined to be $2.85x10^7$ molecules of equivalent fluorochrome on the basis of the minimal GFP intensity level observed in the blood samples mixed with the cells. There was no significant difference in cell size prior and subsequent to OBP-401 infection.

Determination of cell size threshold. In our previous study (23), the various sizes of GFP+ cells in each sample made it difficult to determine which GFP+ cells best represented CTCs. To establish a constant value, the optimum threshold derived from a receiver operating characteristic curve analysis based on cell size was used to define GFP+ CTCs; this threshold was 7.735 μm. Thus, two groups of GFP+ cells were analyzed: Small (S)-GFP+ cells, measuring <7.735 μM in diameter, and large (L)-GFP+ cells, measuring ≥7.735 μm in diameter (27).

Cell counting and analysis. In a blind analysis, GFP+ cells on slides were counted on a computer-controlled fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan). Cells with fluorescent emission counts of $\geq 2.85x10^7$ molecules of equivalent fluorochrome were counted as GFP+ cells. The criteria for a GFP+ cell did not include the presence of epithelial markers such as EpCAM or cytokeratin, since tumor cells undergoing EMT have been reported to lose epithelial markers (31). CD45+ cells were excluded from the analysis.

Statistical analysis. All statistical analyses were performed using JMP Pro 11.1.1 software (SAS Institute, Cary, NC, USA). The Kaplan-Meier analysis and the Wilcoxon test were used to calculate relapse-free survival rates and for non-parametric comparisons of the numbers of GFP+ cells. The relapse-free survival rate of the patients who underwent non-curative surgery declined to 0 months. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Participant characteristics. The clinicopathological characteristics of 37 patients (26 men and 11 women; mean age, 60.7 years; range, 33-76 years) are summarized in Table I. The median follow-up period of the surviving patients was 39 months. A total of 33 patients underwent pathological curative surgery, and 5 of these patients experienced disease recurrence. In total, 6 patients succumbed to disease; 16 patients underwent distal gastrectomy; 18 patients underwent total gastrectomy; and 3 underwent exploratory laparotomy. Of the 37 patients, 17 received chemotherapy

Table I. Patient characteristics and pathological findings.

Variable	Number of patients
Gender	
Male	26
Female	11
Age, years (mean; range)	60.7; 33-76
Gastrectomy	
Distal	16
Total	18
None	3
Curability	
R0	33
R1	0
R2	4
TNM stage	
I	22
II	5
III	6
IV	4
Depth of tumor invasion	
T1	20
T2	5
T3	5
T4	7
Lymph node metastasis	
N0	22
N1	4
N2	3
N3	8
Distant metastasis	
M0	33
M1	4
Main histological type[a]	
Differentiated	13
Undifferentiated	24
Lymphatic invasion	
L0	18
L1	16
LX	3
Venous invasion	
V0	19
V1-2	15
VX	3
Postoperative chemotherapy	
Yes (oral)	13
Yes (oral and infusion)	4
No	20
Outcome	
Relapse free	28
Relapse	5
Non-curative surgery	4

[a]Well differentiated, moderately differentiated and papillary adenocarcinoma were categorized as differentiated. Signet-ring cell carcinoma, poorly differentiated adenocarcinoma and mucinous adenocarcinoma were categorized as undifferentiated. TNM, tumor-node-metastasis.

post-surgery; 13 patients received oral chemotherapy (S-1); and 4 received oral chemotherapy combined with infusion (S-1/cisplatin and S-1/docetaxel).

Association between the number of L-GFP+ cells and patient outcome. The association between the number of L-GFP+ cells and the relapse-free survival rate is shown in Fig. 1. Of the 37 patients enrolled in our study, 33 and 30 were relapse free at 4 and 24 weeks, respectively, after surgery. There were no significant differences in the relapse-free survival rates of patients with ≥6 L-GFP+ cells and patients with <6 L-GFP+ cells, either before (P=0.072; Fig. 1A) or at 4 (P=0.109; Fig. 1B) or 24 (P=0.342; Fig. 1C) weeks after surgery. At 4 weeks after surgery, the relapse-free survival rate was, however, relatively higher in patients with ≥6 GFP+ cells than in patients with <6 GFP+ cells.

Association between patient outcome and changes in the number of GFP+ cells. The numbers of L-GFP+ cells in peripheral blood samples from patients without relapse following curative surgery (R-), patients with relapse following curative surgery (R+) and patients who underwent non-curative surgery (Non-C) were compared at three time points. The mean numbers of L-GFP+ cells at 0, 4 and 24 weeks after surgery were as follows: R-, 5.6, 8.3 and 6.1, respectively; R+, 6.0, 4.0 and 7.2, respectively; and Non-C, 6.8, 0.8 and 4.0, respectively (Fig. 2A). There was a significant difference between the number of L-GFP+ cells in samples from the R- patients and the Non-C patients at 4 weeks after surgery (P=0.034).

The mean numbers of S-GFP+ cells at 0, 4 and 24 weeks after surgery were as follows: R-, 8.7, 11.3 and 11.6, respectively; R+, 11.8, 10.4 and 13.0, respectively; and Non-C, 10.3, 10.8 and 20.0, respectively (Fig. 2B). There were no significant differences between the three groups either prior or subsequent to surgery. The number of S-GFP+ cells in samples from Non-C patients at 24 weeks after surgery was, however, relatively high.

The associations between the number of GFP+ cells, postoperative chemotherapy and outcome are represented in Fig. 3. Of the 37 patients, 6 and 17 were receiving postoperative chemotherapy 4 and 24 weeks after surgery. The number of L-GFP+ cells was similar prior and subsequent to surgery, regardless of chemotherapy (Fig. 3A). Although there was no significant difference, the number of S-GFP+ cells was relatively high in the samples from the patients who were receiving postoperative chemotherapy 24 weeks after surgery (Fig. 3B).

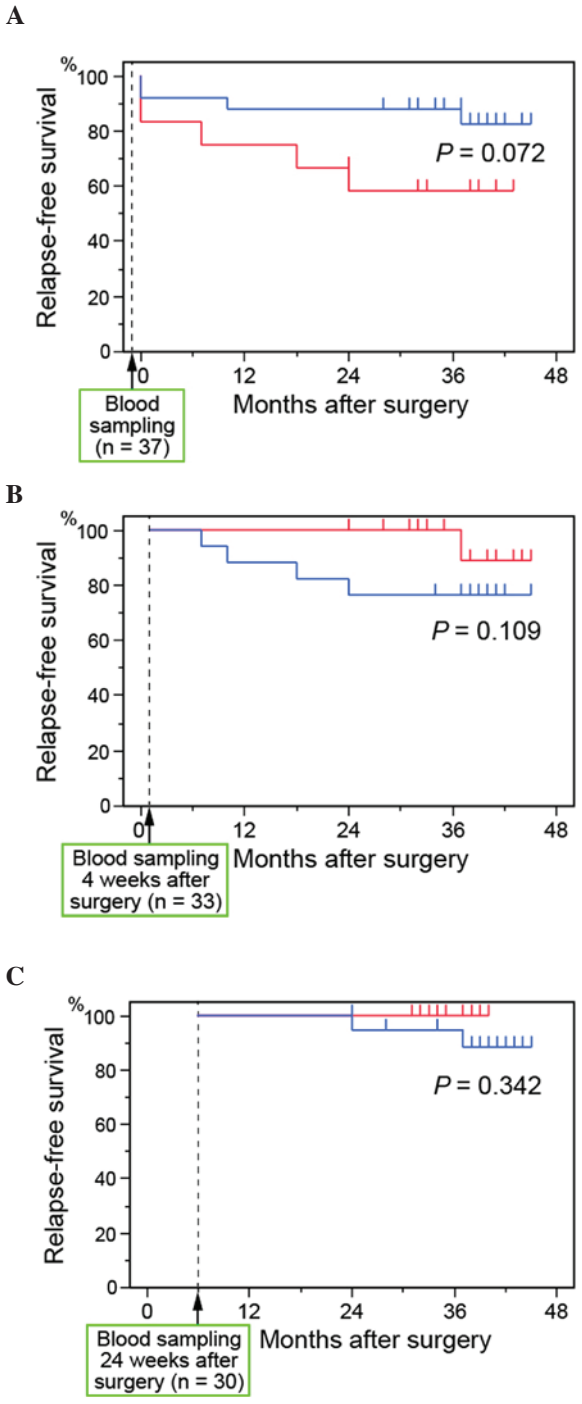

Figure 1. Association between L-GFP+ cell number and relapse-free survival rate. Data were analyzed using Kaplan-Meier analysis and the Wilcoxon test. (A-C) Red line, ≥6 L-GFP+ cells; blue line, <6 L-GFP+ cells. The size cut-off for GFP+ cells was 7.735 μM. (A) Relapse-free survival rate of 37 patients prior to surgery. There was no significant difference in the relapse-free survival rate of patients (n=12) with ≥6 L-GFP+ cells and that of patients (n=25) with <6 L-GFP+ cells (P=0.072). (B) Relapse-free survival rate of 33 patients 4 weeks after surgery. The relapse-free survival rate of patients with ≥6 L-GFP+ cells was relatively higher than that of patients with <6 L-GFP+ cells (P=0.109). (C) Relapse-free survival rate of 30 patients 24 weeks after surgery. There was no significant difference in the relapse-free survival rate of patients with ≥6 L-GFP+ cells and that of patients with <6 L-GFP+ cells (P=0.342). GFP, green fluorescent protein; L, large.

Discussion

In the present study, the association between the number of CTCs (L-GFP+ cells) and treatment outcome was assessed in patients with gastric cancer, which was the third leading cause of cancer-associated mortality worldwide in 2012 (24). The usefulness of CTCs for diagnosis and estimation of prognosis has been reported for breast (15,31), prostate (32), lung (33), esophagus (11,12), colon (34) and gastric cancer (23,27). The results of the present study indicate that the number of CTCs during treatment is unrelated to prognosis in gastric cancer.

A major finding of our study is that there is no significant correlation between patient prognosis and the number of L-GFP+ cells in samples from gastric cancer patients following

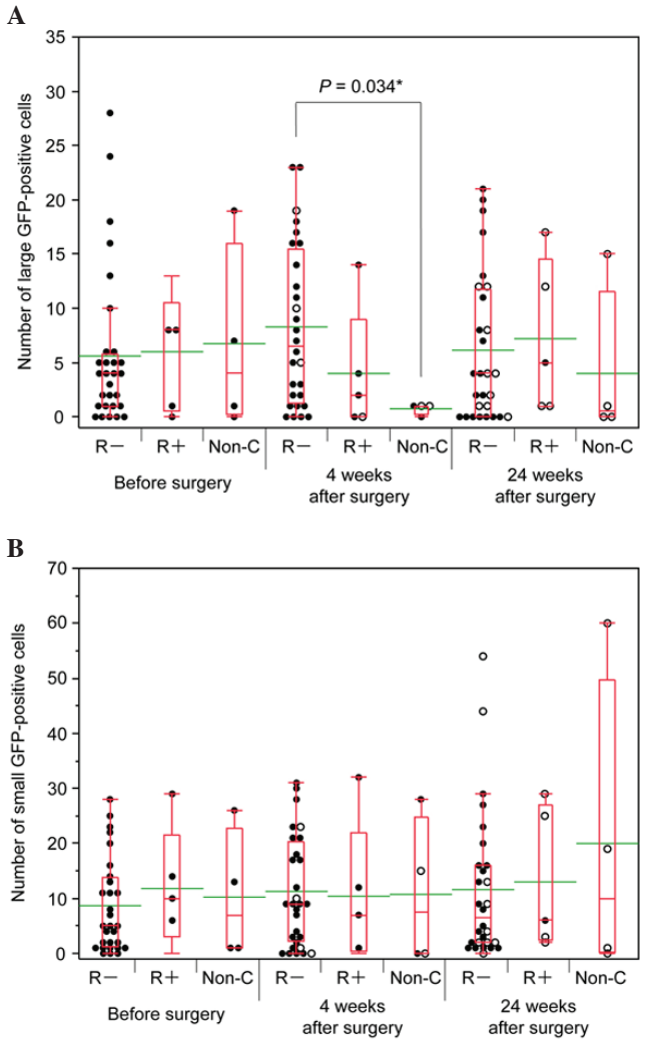

Figure 2. Association of GFP+ cell number with patient outcome. (A and B) The black dots indicate the number of GFP+ cells in 7.5-ml blood samples from chemotherapy-negative patients. The white dots indicate the number of GFP+ cells in samples from patients receiving chemotherapy. The bottom and top of the box represent the lower and upper quartiles, respectively, while the band across the box represents the median. The lower and upper bars at the ends of the whiskers indicate the lowest datum within 1.5 interquartile ranges of the lower quartile and the highest datum within 1.5 interquartile ranges of the upper quartile, respectively. The green bars indicate the mean value. (A) Numbers of L-GFP+ cells (≥7.735 μm in diameter). The mean numbers of L-GFP+ cells before surgery, 4 weeks post-surgery and 24 weeks post-surgery were as follows: R-, 5.6, 8.3 and 6.1; R+, 6.0, 4.0 and 7.2; and Non-C, 6.8, 0.8 and 4.0, respectively. There was a significant difference in the number of L-GFP+ cells in samples from R- patients and Non-C patients (P=0.034). (B) Numbers of S-GFP+ cells (<7.735 μm in diameter). The mean numbers of S-GFP+ cells before surgery, 4 weeks post-surgery and 24 weeks post-surgery were as follows: R-, 8.7, 11.3 and 11.6; R+, 11.8, 10.4 and 13.0; and Non-C, 10.3, 10.8 and 20.0, respectively. Although there were no significant differences, the number of S-GFP+ cells in samples from Non-C patients 24 weeks after surgery was relatively higher than the numbers of S-GFP+ cells in the samples from R- (P=0.932) and R+ patients (P=0.713). R-, patients without relapse; R+, patients with relapse; Non-C, patients who underwent non-curative surgery; GFP, green fluorescent protein; L, large; S, small.

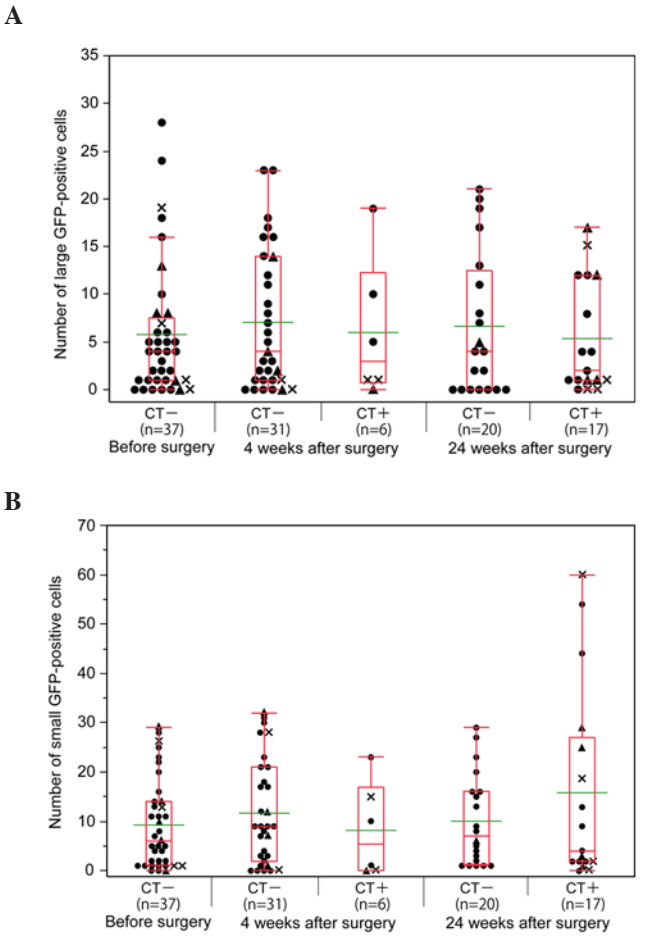

Figure 3. Association of GFP+ cell number with chemotherapy. (A and B) The symbols represent the number of GFP+ cells in a 7.5-ml blood sample. The dots represent samples from patients who did not relapse following curative surgery. The triangles represent samples from patients who relapsed following curative surgery. X symbols represent samples from patients who underwent non-curative surgery. The bottom and top of the box represent the lower and upper quartiles, respectively, and the band across the box represents the median. The lower and upper bars at the ends of the whiskers indicate the lowest datum within 1.5 interquartile ranges of the lower quartile and the highest datum within 1.5 interquartile ranges of the upper quartile, respectively. The green bars indicate the mean value. (A) Numbers of L-GFP+ cells (≥7.735 μm in diameter). The numbers of L-GFP+ cells were similar before and after surgery regardless of chemotherapy. (B) Numbers of S-GFP+ cells (<7.735 μm in diameter). The numbers of S-GFP+ cells were similar before and after surgery. Although there was no significant difference, the number of S-GFP+ cells was relatively high in samples from patients who were receiving postoperative chemotherapy compared with that in the samples from patients without chemotherapy 24 weeks after surgery (P=0.866). GFP, green fluorescent protein; L, large; S, small; CT, chemotherapy.

surgery. This result is consistent with that of our previous study, which used PCR to detect CTCs in patients with esophageal cancer (11). Significant associations between the number of CTCs during treatment and prognosis have been reported for patients with breast (2), prostate (3) and colorectal (5) cancer.

The difference in the results of these studies and the present study may reflect the type of cancer examined. The CTC number in our study may have been affected by the postoperative chemotherapy regimens, which were not the same for all patients. In addition, the median follow-up period of 3 years in our study may be too short to accurately evaluate the association between CTCs and prognosis. It is contrary to our expectation that the number of L-GFP+ cells in samples from the Non-C patients was significantly lower than R- patients 4 weeks after surgery. Since the number of Non-C patients in the present study is small, this significant difference should be confirmed by a large-scale prospective study.

The second major finding of our study is that the number of S-GFP+ cells increases following chemotherapy, which suggests that chemotherapy reduces the size of CTCs. The present authors suggest that the ratio of drug-resistant CTCs (e.g., cancer stem-like cells) to drug-sensitive CTCs increases following chemotherapy. Therefore, drug-resistant CTCs may be smaller than drug-sensitive CTCs. It is also possible that OBP-401 infection increases telomerase activity in non-cancer cells, which are smaller than CTCs.

A limitation of our study is that the metastatic potential of the GFP+ cells was not determined. Additional studies in a larger population of patients with different cancer types are required to clarify the clinical applicability of CTC detection. Future studies should analyze the functions of viable CTCs upon cell sorting and identify CTCs with metastatic potential using additional tools such as DNA ploidy analysis (35,36). Gene expression profiling of CTCs and primary tumors will also provide important insights into the mechanisms of cancer metastasis.

In summary, the results of the present study indicate that CTCs may be useful as predictors of disease progression in treatment-naïve gastric cancer patients; however, CTCs do not constitute a prognostic factor in patients during treatment.

There was no significant association between the change in the number of CTCs, treatment or prognosis in gastric cancer patients who underwent curative surgery. However, the present study used a short follow-up period, and only a small number of participants were included. In addition, whether all GFP+ cells have true metastatic potential was unclear. Therefore, further studies are warranted to confirm the findings of the present study.

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