Abstract. The identification of circulating tumor cells (CTCs) in peripheral blood is a useful approach to estimate prognosis, monitor disease progression and measure treatment effects in several types of malignancies. We have previously used OBP-401, a telomerase-specific, replication-selective, oncolytic adenoviral agent carrying the green fluorescent protein (GFP) gene. GFP-positive cells (GFP\(^+\) cells) were counted under a fluorescence microscope. Our results showed that the number of at least 7.735 \(\mu m\) in diameter GFP\(^+\) cells (L-GFP\(^+\) cells) in the peripheral blood was a significant marker of prognosis in gastric cancer patients. However, tumor cells undergoing epithelial-mesenchymal transition (EMT) have been reported to be smaller in size than cells without EMT features; thus, CTCs undergoing EMT may escape detection with this technique. Therefore, in this study, we analyzed the relationship between patient outcome and the number of GFP\(^+\) cells of any size. We obtained peripheral blood samples from 65 patients with gastric cancer. After infection of OBP-401, GFP\(^+\) cells were counted and measured. The relationship between the number of GFP\(^+\) cells and surgical outcome was analyzed. The median follow-up period of the surviving patients was 36 months. A significant difference in overall survival was found between patients with 0-5 and patients with \(\geq 6\) L-GFP\(^+\) cells. No clear relationship was established between the number of small-sized GFP\(^+\) cells and patient prognosis. The number of L-GFP\(^+\) cells was significantly related to overall survival in patients with gastric cancer. The detection of L-GFP\(^+\) cells using OBP-401 may be a useful prognostic marker in gastric cancer.

Introduction

Distant metastasis is a strong prognostic factor in patients with solid tumors (1-3), and the presence of circulating tumor cells (CTCs) in peripheral blood indicates a systemic disease stage (4). The detection of CTCs in peripheral blood is useful for estimation of prognosis and monitoring of disease progression in breast, prostate, skin, colon and gastrointestinal malignancies. Although various methods have been developed to detect CTCs, the common techniques for the enrichment and detection of CTCs are density gradient separation (5,6), direct enrichment by filtration (7), immunomagnetic separation (8), flow cytometry (9), real-time reverse transcriptase polymerase chain reaction (RT-PCR) (10,11), and microchip technology (12). The CellSearch System (Veridex, LLC, Raritan, NJ, USA) (13) is based on immunomagnetic cell separation and is one of the most widely used automated techniques to enrich and detect CTCs (14-16). The advantage of immunomagnetic cell separation is that CTCs can be visualized with a fluorescence microscope. Cells detected with antibodies against epithelial markers [epithelial cell adhesion molecules (EpCAMs)] are determined to be CTCs. During epithelial-mesenchymal transition (EMT), an important developmental process in CTCs (17), epithelial surface markers are suggested to decrease (18). Thus, CTCs undergoing EMT may escape detection by systems using epithelial markers.

Increased telomerase activity is a common characteristic of malignant tumors, and telomerase plays important roles in carcinogenesis and disease progression (19,20). Therefore, we have developed a novel detection system to enrich cells with high telomerase activity in peripheral blood samples from cancer patients. We used OBP-401 (TelomeScan, Oncolys BioPharma, Tokyo, Japan), which is a telomerase-specific, replication-selective modified viral agent in which the human telomerase reverse transcriptase (TERT) gene promoter is
were also recruited to act as controls. All healthy volunteers in our hospital after surgery. The patients also underwent
cient lymphadenectomy were surgically diagnosed.
the tumor invasion in four patients without gastrectomy and

Cancer (UICC) TNM classification system (28). The depth of
determined according to the seventh edition American Joint

hypertension. The pathologic stage of the disease was

vi) systemic administration of corticosteroids; and vii) unstable

breast-feeding women; iii) active or chronic viral hepatitis;

i) synchronous or metachronous malignancy; ii) pregnant or

vii) written informed consent. The exclusion criteria were:

endoscopic resection, chemotherapy, or radiotherapy; iv) ages,

endoscopic biopsy; ii) clinical solitary tumor; iii) no prior

i) histologically proven adenocarcinoma of the stomach by

(25,26). Thus, CTCs undergoing EMT possibly escape
detection using our technique. Therefore, we analyzed the
relationship between the number of GFP+ cells of any size and
patient outcome at a median-follow up of three years.

Materials and methods

Patients and healthy volunteers. This study is an interim
analysis of our prospective preliminary study on CTCs from
65 patients with treatment-negative gastric adenocarcinoma,
who underwent surgery at the Digestive Disease Center of the
Showa University Northern Yokohama Hospital between April
2010 and May 2011, and from whom we extracted peripheral
blood samples before treatment. The inclusion criteria were:

a) 7.5-ml peripheral vein blood sample was

preparation and assay have been described in our previous
study (22). A 7.5-ml peripheral vein blood sample was

Virus. OBP-401, a telomerase-specific, replication-selective
adenoviral agent in which the E1A promoter element drives
the expression of the EIA and EIB genes and into which the
GFP gene is integrated, was used. The sensitivity and specificity
of the assay using OBP-401 have been reported previously by
Kim et al (29). The test was repeated five times. In the sample
containing one MDA-MB-468 (breast carcinoma) cell and
7.5-ml blood, the numbers of GFP+ cells were one, one, one,
two, and three; in the sample containing 20 MDA-MB-468
(breast carcinoma) cells, the numbers of GFP+ cells were 15,
17, 19, 22, and 24. Viral samples were stored at -80°C.

Sample preparation and immunostaining. Details of sample
preparation and assay have been described in our previous
study (22). A 7.5-ml peripheral vein blood sample was
obtained from each patient before surgery and from each
volunteer. The samples were drawn into tubes containing
citrlic acid, phosphoric acid, and dextrose and stored at 4°C.
The assay was started within 48 h of sample collection. The
samples were centrifuged for 5 min at 540 x g, and the plasma
phase was removed. The cells were then washed four times
with phosphate-buffered saline (PBS) and twice with Roswell
Park Memorial Institute medium. The samples were infected
with 4x10⁸ plaque-forming units (PFU) of OBP-401 virus
by incubation in the medium for 24 h at 37°C. Dead cells
were stained with the red-fluorescent reactive dye L23102 (Life
Technologies, Carlsbad, CA, USA). OBP-401 was inactivated,
and cells were fixed with 2% paraformaldehyde for 20 min at
room temperature. The samples were treated with a surface-
active agent (Emalgen 2025G; Kao Chemicals, Tokyo, Japan)
for 10 min at 40˚C to degrade red blood cells. Phycocyanin-
conjugated anti-human CD45 antibody (BioLegend, San Diego,
CA, USA) was diluted 1:5, and Pacific Blue-labeled anti-human
CD326 (EpCAM) antibody (BioLegend) was diluted 1:10 in
PBS containing 2% fetal bovine serum. Cells were incubated
with the diluted antibodies for 30 min at 25°C. After being
washed with PBS containing 2% fetal bovine serum, the cells
were mounted on two glass slides for microscopic analysis.

Determination of GFP fluorescence intensity threshold. The
threshold for GFP fluorescence intensity was determined as
previously reported (22). Briefly, ~30,000 cultured cells were
added into 7.5-ml blood samples from healthy volunteers,
which were mixed with various cancer cell lines: 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-MB468 (breast carcinoma), and OVCAR-3 (ovarian carcinoma); the cell lines were cultured according to the vendor’s specifications. The blood samples were assayed using CTC detection assay, and the detectable cells were counted by fluorescence microscopy. More than 100 cells were analyzed in each sample. The GFP signal intensity threshold was determined to be $2.85 \times 10^7$ mean equivalent fluorochrome on the basis of the minimal GFP intensity level observed in the blood samples mixed with the cell lines. In addition, there was no significant difference of cell size between the cell before and after OBP-401 infection.

**Determination of cell size threshold.** In our previous study (22), various sizes of GFP$^+$ cells were observed in each sample, making it difficult to identify representative GFP$^+$ cells for comparison between patients and healthy volunteers. Therefore, to establish a constant value, we used the optimum threshold derived from the ROC analysis based on cell size, that is, 7.735 µm, as the threshold to define GFP-positive CTCs. In this study, we categorized GFP$^+$ cells into two groups: smaller (S-GFP$^+$ cells) or larger (L-GFP$^+$ cells) than 7.735 µm in diameter (Fig. 1).

**Cell counting and analysis.** All GFP$^+$ cells on the two slides were analyzed using a computer-controlled fluorescence microscope (IX71, Olympus, Tokyo, Japan); the observer was blinded to the sample detail. S-GFP$^+$ cells with fluorescent emissions $\geq 2.85 \times 10^7$ mean equivalent fluorochrome were counted as GFP$^+$ cells. GFP$^+$ cells included epithelial marker-positive and epithelial marker-negative cells because tumor cells undergoing EMT have been reported to be epithelial marker, such as EpCAM and cytokeratin, negative (18). CD45$^+$ cells were excluded from the analysis.

**Statistical analysis.** All statistical analysis was performed using JMP Pro 10.0.0.2 (SAS Institute, Cary, NC, USA). Parametric comparisons were done using analysis of variance, and nonparametric comparisons were done using the Wilcoxon and Kruskal-Wallis tests. ROC curve analysis was performed to examine the relationship between patient outcome and the number of GFP$^+$ cells. The log-rank test was also used to calculate overall and relapse-free survival rates. Cox proportional hazards analysis was used to investigate risk factor for survival; $P \leq 0.05$ was considered statistically significant.

**Results**

**Participant characteristics.** The clinicopathological characteristics of 65 patients (46 men and 19 women; mean age 60.7 years; range 33-76 years) are summarized in Table I. The median follow-up period of surviving patients was 36 months. Fifty-seven of the 65 patients underwent pathological curative surgery, and of these patients, nine experienced disease recurrence. Fourteen patients died. Twenty-nine patients had distal gastrectomy, 32 had total gastrectomy, and four had exploratory laparotomy. Twenty-eight of the 65 patients received

<table>
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<tr>
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<th>No. of patients</th>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
<td>19</td>
</tr>
<tr>
<td>Age (years; mean, range)</td>
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<td>Gastrectomy</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>III</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
</tr>
<tr>
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</tr>
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<td>T2</td>
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<td>T3</td>
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<td>T4</td>
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<td>39</td>
</tr>
<tr>
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<td>5</td>
</tr>
<tr>
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<td>6</td>
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<td>N3</td>
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<tr>
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<td>56</td>
</tr>
<tr>
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<td>35</td>
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<td>L1</td>
<td>26</td>
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<td>9</td>
</tr>
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</table>

$^a$Well-differentiated or moderately differentiated adenocarcinoma and papillary adenocarcinoma were categorized as differentiated type. Signet-ring cell carcinoma, poorly differentiated adenocarcinoma, and mucinous adenocarcinoma were categorized as undifferentiated type.
chemotherapy after surgery, 19 patients received oral chemotherapy (S-1), and 9 received oral chemotherapy combined with infusion (S-1/cisplatin and S-1/docetaxel).

Association of GFP-positive cells with pathological indices. Comparison of GFP$^+$ cells between healthy volunteers and patients are shown in Fig. 2. The numbers of GFP$^+$ cells (any size) and S-GFP$^+$ cells in the samples from the healthy volunteers were significantly higher than the ones of the patients (P=0.038 and 0.006). There was no significant difference in L-GFP$^+$ cells between the samples from healthy volunteers and the ones from the patients (P=0.760).

There was no significant relationship between the number of GFP$^+$ cells (any size, P=0.329), S-GFP$^+$ cells (P=0.424) and L-GFP$^+$ cells (P=0.213), and cancer stage (Fig. 3A). Although no statistical significance was observed, the number of GFP$^+$ cells (any size) and S-GFP$^+$ cells tended to increase with the progression of the primary tumor (Fig. 3B). However, the number of GFP$^+$ cells in the samples from the node-positive patients was greater than that in the node-negative patients, there was no significant difference (Fig. 3C). Compared with the patients without distant metastasis, those with distant metastases had relatively higher numbers of GFP$^+$ cells (Fig. 3D). The numbers of GFP$^+$ cells were similar in the samples from patients with and without lymphatic invasion (Fig. 3E). For venous invasion, the number of L-GFP$^+$ cells in the samples from the patients with invasion was significantly higher than that in patients without invasion (P=0.031) (Fig. 3F).

Relationship between the patient outcome and the number and size of GFP-positive cells. The numbers of the detected GFP$^+$ cells in the peripheral blood samples are shown in Fig. 4. The mean value of GFP$^+$ cells with any size, <7.735 µm and >7.735 µm were 23.8, 19.0 and 4.8 in the samples from healthy volunteers, and 24, 19 and 5 were prescribed cutoff values of GFP$^+$ cells with any size, <7.735 µm and >7.735 µm. The

Figure 1. Examples of microscopic images. Representative images from gastric cancer samples of GFP-positive cells were counted using a computer-controlled fluorescence microscope by an examiner blinded to the sample status. Scale bar, 10 µm.

Figure 2. Number of GFP-positive (GFP$^+$) cells. The dots indicate the numbers of GFP$^+$ cells in a 7.5-ml blood sample (A) any size, (B) <7.735 µm in diameter, (C) >7.735 µm in diameter. The bottom and top of the box represent the lower and upper quartiles, and the band across the box shows the median. The lower and upper bars at the ends of the whiskers show the lowest data point within 1.5 interquartile ranges of the lower quartile and the highest data point within 1.5 interquartile ranges of the upper quartile, respectively. The gray bars indicate mean value.
overall survival rate of patients who had 24 or more GFP\(^+\) cells was lower than that of patients who had <24 GFP\(^+\) cells (P=0.281) (Fig. 4A); however, the difference was not significant. The overall survival rate of patients who had 20 or more GFP-positive S-GFP\(^+\) cells also tended to be lower than that of patients who had <20 GFP-positive S-GFP\(^+\)
cells (P=0.327) (Fig. 4B). Although there was no significant difference, the overall survival rate of patients who had 5 or more L-GFP+ cells was lower than that of patients who had <5 L-GFP+ cells (P=0.148) (Fig. 4C).

We performed ROC analysis to determine another cutoff values. The ROC analysis showed that the numbers of GFP+ cells (P=0.241, AUC 0.546, cutoff 17, sensitivity 55.6%, and specificity 68.8%) and the number of large GFP+ cells (P=0.770, AUC 0.548, cutoff 6, sensitivity 44.4%, and specificity 81.3%) in the samples from the deceased patients were higher than those in the samples from the surviving patients. A prejudiced value was observed in small GFP+ cells (P=0.159, AUC 0.557, cutoff 29, sensitivity 22.2%, and specificity 100%).

Figure 5. Comparison of GFP-positive (GFP+) cell number between surviving patients and deceased patients. To determine novel threshold, we compared the numbers of GFP+ cells from surviving patients and deceased patients with gastric cancer by ROC analysis. (A) GFP+ cells (any size). (B) Small GFP+ cells (<7.735 µm). (C) Large GFP+ large cells. The number of GFP+ cells (P=0.241, AUC 0.546, cutoff 17, sensitivity 55.6%, and specificity 68.8%) and the number of large GFP+ cells (P=0.770, AUC 0.548, cutoff 6, sensitivity 44.4%, and specificity 81.3%) in the samples from the deceased patients were higher than those in the samples from the surviving patients. A prejudiced value was observed in small GFP+ cells (P=0.159, AUC 0.557, cutoff 29, sensitivity 22.2%, and specificity 100%).

Figure 6. Overall survival using cutoff determined by ROC analysis. (A) GFP-positive (GFP+) cells (any size). (B) Large GFP+ cells (>7.735 µm). (C) Combination of GFP+ cells (any size) and large GFP+ cells. Survival was compared according to the number of CTCs using Kaplan-Meier analysis and the log-rank statistics. *P<0.01, **P<0.05.

Discussion

In this study, we analyzed the correlation between CTCs and prognosis in gastric cancer, which is the second leading cause of cancer-related death worldwide. The usefulness of the detection of CTCs in the diagnosis and estimation of prognosis has already been reported for breast (14,30), prostate (31),
lung (32), and digestive tract (11,33) cancers. The results of the present study indicate that detection of CTCs may also be useful in the prognosis of gastric cancer.

This study showed two major findings. One was that the number of L-GFP+ cells is significantly associated with patient prognosis. In our previous study (22), the prognosis of the patients who had 5 or more GFP+ cells was significantly lower than that of the patients who had <5 L-GFP+ cells. In this study, we obtained a similar result showing that the prognosis of patients who had 6 or more L-GFP+ cells was significantly lower than that of patients who had <6 L-GFP+ cells.

Further, we determined whether the number of GFP+ cells of any diameter may be related to patient prognosis. Patients who had 17 or more GFP+ cells showed lower survival rate than those who had <17 GFP+ cells, although the difference was not significant. Since the combination of the number of total GFP+ cells and L-GFP+ cells showed a significant correlation with patient prognosis whereas the number of only L-GFP+ cells did not, we deemed the number of all GFP+ cells to be related to patient prognosis. On the other hand, the relationship between the number of S-GFP+ cells and prognosis was unclear. Although there was a significant difference in the prognosis between patients who had 29 or more S-GFP+ cells (n=2) and those who had <29 S-GFP+ cells (n=63), unequal numbers of patients were enrolled in the two groups. In our previous study (22), S-GFP+ cells were observed in the blood samples from healthy volunteers. Therefore, S-GFP+ cells may be detected as false-positive CTCs. There is possibility that OBP-401 infection caused increased telomerase activity in non-cancer cells.

One limitation of our study was that the metastatic potential of the detected CTCs was not determined. Our results suggested L-GFP+ cells to be a predictive and prognostic marker; however, further study is needed to determine the metastatic potential of L-GFP+ cells. On the other hand, S-GFP+ cells may contain a small population of CTCs with metastatic potential including tumor cells with EMT. It was suggested that the CTCs with EMT were included in both of S-GFP+ cells and L-GFP+ cells in this study. Clearly, more studies in a larger population of patients, and with different cancer types, are needed to clarify the clinical applicability of CTC detection. Thus, further studies should analyze the functions of viable CTCs after cell sorting, and identify CTCs with metastatic potential using additional tools such as DNA ploidy analysis (34,35). Furthermore, gene expression profiling of viable CTCs, dead cells, primary tumors, and metastatic tumors will also provide important insight into the mechanisms of cancer metastasis. Finally, the results of the present study indicate that CTCs are useful as predictors of disease progression in gastric cancer patients, but they do not constitute an independent prognostic factor.

The number of detected L-GFP+ cells showed a significant relationship with prognosis in gastric cancer. However, the study used a short follow-up period and only a small number of participants. In addition, whether all GFP+ cells have true metastatic potential was unclear. Further studies are warranted to confirm the findings of this study.

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References


