Long-term prognostic impact of circulating tumour cells in gastric cancer patients

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Abstract

AIM
To analyse the long-term prognostic impact of circulating tumour cells (CTCs) in gastric cancer patients who underwent surgery.

METHODS
A 7.5-mL peripheral vein blood sample was obtained from each patient with treatment-negative gastric adenocarcinoma before surgery. OBP-401, a telomerase-specific, replication-selective, oncolytic adenoviral agent carrying the green fluorescent protein gene, was used to label CTCs. Correlations between the number of CTCs and clinical end points were evaluated.

RESULTS
The median follow-up period of the surviving patients with gastric cancer was 60 mo. The CTC number tended to increase concomitantly with disease progression. The overall survival of patients with more than five CTCs in 7.5-mL peripheral blood was lower than that of patients with five or less CTCs, although the difference was not significant ($P = 0.183$). A significant difference in relapse-free survival was found between patients with more than five and those with five or less CTCs ($P = 0.034$).

CONCLUSION
A lower number of CTCs was correlated with higher relapse-free survival rates in patients. Detection of CTCs using OBP-401 may be useful for predicting prognosis in gastric cancer.

Key words: Circulating tumour cells; Gastric cancer; Surgery; Telomerase; Prognosis

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Core tip: We show the long-term prognostic impact of circulating tumour cells (CTCs) in 65 patients with gastric cancer in this report. OBP-401, a telomerase-specific, replication-selective, oncolytic adenoviral agent carrying the green fluorescent protein gene, was used to label CTCs. A lower number of CTCs was correlated with higher relapse-free survival rates in patients with gastric cancer.

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INTRODUCTION
The presence of circulating tumour cells (CTCs) in peripheral blood indicates a systemic disease stage in various malignancies, as CTCs are thought to be the source of haematogenous metastasis[1]. Detection of CTCs in peripheral blood is useful for prognosis, monitoring of disease progression, and evaluation of treatment efficacy in breast[2], lung[3], prostate[4], skin[5], colon[6], gastric[7], and esophageal cancer[8,9]. Although various methods have been developed to detect CTCs, the most commonly used techniques for their enrichment and characterisation are density gradient separation[10], immunomagnetic separation[11], flow cytometry[12], direct enrichment by filtration[13], and microchip technology[14]. The CellSearch System (Veridex, LLC, Raritan, NJ, United States), which is based on immunomagnetic cell enrichment, is one of the most widely used techniques for automated enrichment and detection of CTCs[15,16]. The advantage of immunomagnetic cell separation is that CTCs can be directly visualised under a microscope. In the CellSearch assay, cells detected with antibodies against epithelial markers (e.g., epithelial cell adhesion molecules, or EpCAMs) are classified as CTCs. During the epithelial-mesenchymal transition (EMT), an important process that occurs in CTCs[17], expression of epithelial surface markers is reduced. Thus, systems that rely on epithelial markers may fail to detect CTCs undergoing EMT[18]. Methodologies based on direct enrichment by filtration may circumvent this issue to some extent, although cells detected in this manner often lack tumourigencity.

Increased telomerase activity is a common characteristic of malignant tumours, and telomerase plays important roles in carcinogenesis and disease progression[19]. OBP-401 (TelomeScan, Oncolys BioPharma, Tokyo, Japan) is a telomerase-specific, replication-selective modified viral agent in which the human telomerase reverse transcriptase (TERT) gene promoter is inserted into the E1 region, and the green fluorescent protein (GFP) gene is placed under the control of the cytomegalovirus promoter in the E3 region as a marker of viral replication[20]. Thus, OBP-401 only proliferates in viable cells with high telomerase activity and provides a fluorescent label that allows tumour cells to be labelled, regardless of their epithelial marker expression profiles. We previously used OBP-401 to detect cells with high telomerase activity in blood samples of healthy and treatment-negative gastric cancer patients before surgery. We took 7.5-mL peripheral blood samples from cancer patients before surgery and healthy volunteers. We detected viable GFP-positive CTCs in the blood samples after incubation with OBP-401. This revealed that in patients with gastric cancer, a greater proportion of "high telomerase activity" cells was associated with a significantly poorer prognosis[21]. In this report, we describe the final long-term results (median follow-up time of five years) of this initial study, which demonstrate that the OBP-401-dependent CTC assay has clinical utility in patients with gastric cancer.
MATERIALS AND METHODS

Patients and healthy volunteers
This report was the final 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: (1) histologically proven adenocarcinoma of the stomach by endoscopic biopsy; (2) clinical solitary tumour; (3) no prior endoscopic resection, chemotherapy, or radiotherapy; (4) aged 20-80 years; (5) Eastern Cooperative Oncology Group performance status (Oken et al.22) of 0 or 1; (6) sufficient organ function; and (7) written informed consent. The exclusion criteria were: (1) synchronous or metachronous malignancy; (2) pregnant or breastfeeding women; (3) active or chronic viral hepatitis; (4) active bacterial or fungal infection; (5) diabetes mellitus; (6) systemic administration of corticosteroids; and (7) unstable hypertension. The pathologic stage of the disease was determined according to the seventh edition of the American Joint Committee on Cancer/International Union Against Cancer TNM classification system23. The depth of the tumour invasion in four patients without gastrectomy and the regional lymph node status of seven patients without sufficient lymphadenectomy were surgically diagnosed.

All of the patients were checked regularly in our hospital every 3 mo for the first 3 years post-operation, and every 6 mo for the following two post-operative years. The patients also underwent endoscopy and computed tomography at least once a year; according to their disease stage and course. Healthy volunteers were also recruited as controls. All healthy volunteers were employees of Sysmex Corporation, which included seven men (mean age, 31.4 years; range, 24-39 years) and three women (mean age, 33.7 years; range, 26-48 years). All volunteers underwent medical check-ups upon employment and annually; check-ups included medical interviews, auscultation, chest radiography, and blood and urine analyses. In addition, individual interviews were conducted before sample collection; any volunteer who was currently receiving medical treatment, pregnant, or breastfeeding or who had donated blood within the past month was excluded.

Telomerase-specific viral agent
OBP-401, a telomerase-specific, replication-selective adenoviral agent in which the TERT 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 previously24. Viral samples were stored at -80 °C.

Sample preparation and immunostaining
Details of sample preparation and assay were described in our previous report21. A 7.5-mL peripheral vein blood sample was obtained from each patient before surgery and from each healthy volunteer. The samples were drawn into tubes containing citric 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 × 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 4 × 10⁸ plaque-forming units 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, United States), OBP-401 was inactivated, and the 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. Phycoerythrin-labelled anti-human CD45 antibody (BioLegend, San Diego, CA, United States) was diluted 1:5, and Pacific Blue-labelled anti-human CD326 (EpCAM) antibody (BioLegend) was diluted 1:10 in PBS containing 2% foetal bovine serum. Cells were incubated with the diluted antibodies for 30 min at 25 °C. After being washed with PBS containing 2% foetal bovine serum, the cells were mounted on two glass slides for microscopic analysis.

GFP fluorescence intensity of cultured cancer cell lines
Approximately 30000 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-468 (breast carcinoma), and OVCAR-3 (ovarian carcinoma). The cell lines were cultured according to the vendor’s specifications.

Determination of GFP fluorescence intensity and cell size threshold
The threshold for GFP fluorescence intensity and cell size (diameter) were set based on the values from samples of healthy volunteers and the patients with gastric cancer by using receiver operating characteristic (ROC) analysis. The blood samples were subjected to the CTC detection assay, and GFP-positive cells were scored by fluorescence microscopy.

Cell counting and analysis
All detectable GFP-positive cells on the two slides were analysed under a computer-controlled fluorescence microscope (IX71, Olympus, Tokyo, Japan); the observer was blinded to the sample details. Cells with fluorescence intensities and diameters exceeding the threshold were scored as GFP-positive. Both EpCAM-positive and EpCAM-negative subpopulations...
Informed consent statement
The study protocol was explained to the patients and volunteers before written informed consent was obtained.

Statistical analysis
All statistical analysis was performed using JMP Pro 12.2.0 (SAS Institute, Cary, NC, United States). Non-parametric comparisons were performed using the Wilcoxon signed-rank test, with a normal approximation. ROC analysis was performed to examine the difference between GFP fluorescence intensity and cell size in the blood samples of patients versus those in healthy volunteers. Cox proportional hazards analysis was used to investigate risk factors for survival, and to calculate overall and relapse-free survival rates. $P \leq 0.05$ was considered statistically significant.

RESULTS
Patient characteristics and pathological findings
The clinicopathological characteristics of 65 patients (46 men and 19 women; mean age 60.7 years; range 33-76 years) are summarised in Table 1. The median follow-up period for the surviving patients was 60 mo. Fifty-seven of the 65 patients underwent pathological curative surgery, and of these patients, 10 experienced disease recurrence. Fifteen patients died. Twenty-nine patients had distal gastrectomy, 32 had total gastrectomy, and four had exploratory laparotomy. Twenty of the 57 patients that underwent curative surgery also received adjuvant chemotherapy, and nine of these 20 patients received therapeutic chemotherapy after disease recurrence.

Gallery of GFP-positive cancer cell lines after OBP-401 infection
After OBP-401 infection, GFP-positive cancer cell lines were detected (Figure 1A).

Comparison of GFP fluorescence intensity between cell lines and blood cells
The GFP fluorescence intensity [mean equivalent fluorochrome (MEFL)] of the cell lines and the GFP-positive cells detected in the peripheral blood samples are shown in Figure 1B. MEFL was higher in cell lines than in the GFP-positive cells in the peripheral blood samples from either healthy volunteers or patients with gastric cancer. In turn, MEFL was higher in GFP-positive cells from patients with gastric cancer than in the corresponding cells from healthy volunteers.

Comparison of GFP fluorescence intensity and cell diameter between patients and volunteers
The GFP fluorescence intensity and diameter of cells isolated from the peripheral blood samples are

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Table 1 Patient characteristics and clinical findings

| Variable                        | Number of patients |
|---------------------------------|--------------------|
| Sex                             |                    |
| Male                            | 46                 |
| Female                          | 19                 |
| Age (yr; mean, range)           | 58.8; 33-76        |
| Gastrectomy                     |                    |
| Distal                          | 29                 |
| Total                           | 32                 |
| None                            | 4                  |
| Surgical curability             |                    |
| R0                              | 57                 |
| R1                              | 0                  |
| R2                              | 8                  |
| Clinical course                 |                    |
| Survival without relapse        | 47                 |
| Survival after relapse          | 2                  |
| Survival after non-curative     | 1                  |
| surgery                         |                    |
| Disease                         | 15                 |
| Recurrence site (including overlap) |          |
| Remnant stomach                 | 1                  |
| Hematogenous                    | 5                  |
| Lymphatic                       | 4                  |
| Peritoneal dissemination        | 5                  |
| Non-curative surgery            | 8                  |
| Postoperative chemotherapy      |                    |
| Adjuvant chemotherapy           | 11                 |
| Adjuvant and therapeutic        | 9                  |
| chemotherapy                    |                    |
| Therapeutic chemotherapy after  | 8                  |
| non-curative surgery            |                    |
| TNM stage                       |                    |
| I                               | 40                 |
| II                              | 6                  |
| III                             | 10                 |
| IV                              | 9                  |
| Depth of tumour invasion        |                    |
| T1                              | 36                 |
| T2                              | 8                  |
| T3                              | 9                  |
| T4                              | 12                 |
| Lymph node metastasis           |                    |
| N0                              | 39                 |
| N1                              | 5                  |
| N2                              | 6                  |
| N3                              | 15                 |
| M0                              | 56                 |
| M1                              | 9                  |
| Distant metastasis              |                    |
| Differentiated                  | 25                 |
| Undifferentiated                | 40                 |
| Main histological type          |                    |
| Differentiated                  | 25                 |
| Undifferentiated                | 40                 |
| Lymphatic invasion              |                    |
| L0                              | 35                 |
| L1                              | 26                 |
| L2                              | 2                  |
| V0                              | 35                 |
| V1-2                            | 26                 |
| VX                              | 4                  |

were found in these cells, consistent with the finding that tumour cells undergoing EMT can be EpCAM-negative\cite{18}.

Institutional review board statement and clinical trial registration
The study was approved by the Institutional Review Board of the Showa University, Northern Yokohama Hospital (No. 0903-03). This study was registered with the University Hospital Medical Information Network in Japan, UMIN000004026.

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shown in Figure 1C and D. Based on ROC analyses, we defined cells with 78600927 MEFL or higher GFP fluorescence intensity and 7.7418 µm or larger diameter as the CTCs.

**Association of CTCs with pathological findings**

An increased number of CTCs was associated with disease progression. There was statistically significant difference in the number of CTCs between samples from patients with Stage 1 and those from patients with Stage III disease ($P = 0.0460$, Figure 2A). The number of CTCs also tended to increase concomitantly with progression of the primary tumour, as there was a statistically significant difference in the number of CTCs between samples from patients with T1 and those from patients with T4 tumours ($P = 0.0335$, Figure 2B). There was also a statistically significant difference in the number of CTCs between samples from patients with N0 and those with N2 lymph node spread status ($P = 0.0381$, Figure 2C). However, there was no significant difference in the number of CTCs between samples from patients with distant metastases and those in which distant metastasis was absent ($P = 0.4667$, Figure 2D). The number of CTCs was also higher in samples from patients with lymphatic invasion, although there was no significant difference compared to patients without this clinical feature ($P = 0.1297$, Figure 2E). Similarly, although the number of CTCs in samples from the patients with venous invasion was higher than those in samples without this complication, the difference was not significant ($P = 0.0558$, Figure 2F). Finally, we observed no significant difference in the number of CTCs in samples from patients with differentiated tumours when compared to
those with undifferentiated malignancies ($P = 0.7752$, Figure 2G).

**Overall and relapse-free survival**

The overall survival rate of patients who had more than five CTCs (66.2%) was lower than that of patients who had five or less CTCs (80.5%); however, this difference was not significant ($P = 0.183$, Figure 3A). The relapse-free survival rate of patients who had more than five CTCs (64.3%) was significantly lower than that of patients who had five or less CTCs (88.3%) ($P = 0.034$, Figure 3B).

**Prognostic factor for survival**

We investigated prognostic factors related to patient survival by using Cox proportional hazards analysis. Univariate analysis showed that fStage was, in some cases, a significant factor (fStage II, $P = 0.196$; fStage III, $P = 0.0003$; fStage IV, $P < 0.0001$). In contrast, the presence of more than five CTCs was not a significant factor ($P = 0.183$). Multivariate analysis including these two factors showed fStage to be the
Multivariate analysis

Hazard ratio

P value

95%CI

0.196 66.2%

< 0.0001

7.106

0.182

4.053 482.6

< 0.0001

1.0

0.0003

95%CI

0.281-179.3

2.069

0.847

1.0

A: The overall survival rate of 65 patients was compared using Cox proportional hazards analysis. Although there was no significant difference, the overall survival rate of the patients with more than 5 CTCs was lower than that of patients with 5 or less CTCs (hazard ratio, 2.28; 95% CI: 0.068-4.698; P = 0.034). CTCs: Circulating tumour cells.

Table 2 Risk factors for prognosis of patients (n = 65)

| Variable | Univariate analysis | Multivariate analysis |
|----------|---------------------|-----------------------|
| Number of high telomerase activity cells | | |
| ≤ 5 (n = 47) | 1.00 | 1.00 |
| > 5 (n = 18) | 2.069 | 1.00 |
| fStage | | |
| fStage I | 1.0 | 1.0 |
| fStage II | 7.097 | 7.106 |
| fStage III | 25.18 | 26.17 |
| fStage IV | 85.57 | 85.76 |

| Value | 95%CI | P value |
|-------|-------|--------|
| ≤ 5 (n = 47) | 0.693-5.744 | 0.183 |
| > 5 (n = 18) | 0.068-4.698 | 0.034 |

only significant factor (fStage II, P = 0.182; fStage III, P = 0.0004; fStage IV, P = 0.0001), and the number of CTCs (more than five) to be non-significant (P = 0.847) (Table 2).

Risk factor for relapse after curative surgery

We also investigated factors for increased risk of relapse by Cox proportional hazards analysis. Univariate analysis showed that certain fStages were significant risk factors (fStage II, P = 0.337; fStage III, P = 0.0001; fStage IV, P = 0.005). However, the presence of more than 5 CTCs had no significant influence on relapse rates (P = 0.052). Multivariate analysis including these two factors showed fStage to be the only significant factor (fStage II, P = 0.343; fStage III, P = 0.001; fStage IV, P = 0.004), whereas the number of CTCs was non-significant (P = 0.350, Table 3).

DISCUSSION

Here, we used a telomerase-specific adenoviral agent to detect CTCs to avoid relying on the heterogeneous expression of epithelial markers in CTCs undergoing EMT. The enumeration of CTCs is particularly important in gastric cancer, which is the second leading cause of cancer-related death worldwide. Our current data indicate that detection of CTCs may indeed be a useful prognostic indicator for use in patients with gastric cancer, and are consistent with previous reports

Our previous preliminary study showed that the number of CTCs isolated from cancer patients was related to surgical and pathological disease progression. Specifically, there were more CTCs in samples from patients with Stage III than in those with Stage I disease. The CTC count was also higher in patients with tumour depth T4 than in those with T1, and in individuals with lymph node metastasis status N2 versus those with N0. In addition, we found that the number of CTCs was associated with disease stage and relapse after curative surgery in gastric cancer patients. In the current study, the relapse-free survival rate of patients who had more than five CTCs was significantly lower than that of patients who had five or less. The overall survival rate of patients with more than five CTCs tended to be lower than that of the patients with five or less; in this case, however, the difference was not statistically significant. The number of CTCs was not an independent risk factor for either
Table 3  Risk factors for relapse of patients who underwent curative surgery (n = 57)

| Variable                      | Univariate analysis | Multivariate analysis |
|-------------------------------|---------------------|-----------------------|
|                               | Hazard ratio  | 95%CI   | P value | Hazard ratio  | 95%CI   | P value |
| Number of high telomerase    |          |         |         |        |         |         |
| activity cells ≤ 5           | 1.0       |         | 0.052   | 1.0     | 0.471-8.861 | 0.350   |
| > 5                          | 3.566     | 0.988-12.88 | 0.169-37.96 | 1.971 |             |         |
| fStage I                      | 1.0       | 1.0     |         |         |         |         |
| fStage II                     | 3.635     | 0.169-37.96 | 0.337 | 3.576 | 0.166-37.35 | 0.343   |
| fStage III                    | 17.78     | 4.065-121.8 | 0.0001* | 13.75 | 2.806-100.7 | 0.001*  |
| fStage IV                     | 239.6     | 7.943-7785 | 0.0005* | 289.4 | 9.331-9733 | 0.004*  |

*P < 0.01.

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