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Title: Circulating tumor cells as an independent predictor of survival in advanced gastric cancer

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A short running head: CTCs as a survival predictor in gastric cancer

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Synopsis:

CTCs were positive in 18.4% of advanced gastric cancer patients. In addition to performance status, and macroscopic distant metastasis, CY and CTC were independent predictors of poor prognosis, and could provide a useful tool to select patients for intensive treatment.

Abstract

Background

When considering the indication of surgery for highly advanced gastric cancer, careful selection of the patients is important. In addition to tumor-node-metastasis factors and peritoneal lavage cytology (CY), which are important predictors of prognosis, detection of circulating tumor cells (CTCs) could be another potential marker.

Patients and methods

We prospectively evaluated CTCs using a semi-automated immuno-magnetic separation system (CellSearch®) in 136 patients with advanced gastric cancer to determine the frequency of CTC positivity. In 123 patients in whom CY was also evaluated, the significance of CTC, as well as CY, was investigated as a potential biomarker to predict progression-free survival (PFS) or monitor therapeutic effect.

Results

CTCs were positive in 25 patients (18.4%). Positive CTC counts were more common in tumors with diffuse histological type, and with distant metastasis. The PFS of CTC-positive patients was significantly shorter than those of CTC-negative patients (HR 2.03; *P*=0.016). A multivariate analysis in 123 patients showed that CTC and CY, as well as performance status and macroscopic distant metastasis were independent factors for PFS. When both CTC and CY were converted to negative by therapeutic interventions, long-term PFS was achieved.

Conclusions

Detection of CTCs was an independent predictor of shorter PFS in advanced gastric cancer. CTCs could be a valuable biomarker to select patients who require intensive treatment. The combined status of CTC and CY would be useful in selecting patients for radical surgery. Further investigation with a larger number of patients is necessary to establish the importance of CTCs.

Introduction

Gastric cancer is one of the leading causes of cancer death in the world. Surgery with postoperative adjuvant chemotherapy is a standard treatment for localized advanced gastric cancer, according to the recent Japanese guidelines[1]. Neoadjuvant chemotherapy followed by surgery is also a promising approach that is often used in Western countries[2]. The prognosis of patients with a good response to chemotherapy is better than those with a poor response[3]. When the tumor is chemo-sensitive, even metastatic patients have the possibility to undergo curative resection and achieve long-term survival[4, 5]. When considering the indication of surgery for highly advanced gastric cancer, careful selection of patients who could benefit from surgery is very important. The clinical decision is usually made based on the diagnosis using the tumor-node-metastasis (TNM) factors. However, even after curative surgery recurrence could happen for tumors that have been diagnosed as early-stage disease. Therefore, we need more dependable biomarkers to predict the prognosis of patients with gastric cancer.

Detection of free cancer cells in the peripheral blood circulation (circulating tumor cells: CTCs) could be a potential prognostic marker. Although the existence of CTCs had been reported in several types of cancer, its significance has not been well established because of its high false positive rate and the non-viability of isolated cancer cells. In recent studies indicating its relevance in breast and colorectal cancer, CTCs were detected using a semi-automated immuno-magnetic separation system (CellSearch®) that provides highly reproducible results[6, 7]. To date, only a few studies have evaluated CTCs in gastric cancer using the CellSearch® system[8, 9].

Evaluation of peritoneal free cancer cells could be another useful selection tool, because it is known to be a strong prognostic factor in gastric cancer[10]. The current Japanese gastric cancer classification and UICC/AJCC cancer staging system both classify gastric cancer with positive peritoneal lavage cytology (CY1 in Japanese classification, and Cyt+ in UICC staging system) as stage IV[11, 12]. However, recent studies have shown that a significant proportion of patients with positive peritoneal lavage cytology achieved long-term survival with the combination of surgery and chemotherapy[5, 13, 14]. Therefore, surgical resection could be considered at least for some patients with positive peritoneal lavage cytology. However, the optimal indication remains controversial.

In this prospective study, we evaluated CTCs and peritoneal lavage cytology (CY) in patients with advanced gastric cancer. The main purpose of this study was to determine the frequency of CTC positivity in advanced gastric cancer, and to determine whether CTC is a significant

independent predictor of progression-free survival (PFS). The secondary aim was to evaluate the relationship between CTC positivity and several clinical factors, and to evaluate the clinical significance of cytological detection of cancer cells in the circulation and/or peritoneal cavity as a biomarker to predict patient survival or monitor therapeutic effect.

Patients and Methods

Study design

This prospective study was conducted at Kyoto University Hospital. Inclusion criteria were histologically proven adenocarcinoma of the stomach, diagnosed as T2 or higher, age 20 years old or older, and the provision of written informed consent. Exclusion criteria were previous treatment of gastric cancer, and multiple primary cancers. The main purpose of this study was to determine the frequency of CTC positivity in advanced gastric cancer, and to determine whether CTC is a significant predictor of progression-free survival (PFS) of patients with advanced gastric cancer. The secondary aim was to evaluate the relationship between CTC positivity and several clinical factors, and to evaluate the clinical significance of cytological detection of cancer cells in the circulation and/or peritoneal cavity as a biomarker to predict patient survival or monitor therapeutic effect.

Sample size considerations were based on the estimated survival difference of patients with negative and positive CTCs. When the three-year PFS rate of each group was estimated as 72% and 44%, and the frequency of CTCs was estimated as 30%, based on previous studies of breast cancer[6], a total of 109 patients were required for a two-tailed log-rank test at 5% significance and 80% power. We determined the sample size as 120 patients over three years including a safety margin, and the accrual started in July 2008. The study protocol was approved by the institutional review board of Kyoto University (E-443). Because of the lower frequency of patients with CTCs and fewer accruals, the study period was extended to 4.75 years and a total of 140 patients were enrolled by March 2013.

Patient evaluation and follow-up

Prior to initial intervention, all patients were evaluated by multi-detector-row computed tomographic (MDCT) scans of the neck, chest, abdomen and pelvis, upper endoscopy, upper gastrointestinal series, serum CEA, and CA19-9. Patients with large tumors or bulky lymph node metastasis underwent staging laparoscopy to evaluate peritoneal lavage cytology and

dissemination. The age, sex, performance status, histological type, clinical TNM factors, and clinical stage according to the 7th version of the TNM classification were documented for each patient[15]. Peritoneal lavage was performed during the staging laparoscopy or initial surgery using 50 mL saline, and lavage fluid was collected from the Douglas' pouch and/or the left subphrenic space for peritoneal lavage cytology (CY).

All patients were followed up regularly at the outpatient unit to check for symptoms suggesting recurrence or progression of disease. MDCT scans were conducted at least every three months for measurement of tumors when the initial treatment was chemotherapy. After patients underwent surgery, MDCT scans were conducted at least every six months for patients with pathological stage II or higher, for the first three years. For patients with pathological stage I, MDCT scan was conducted annually. Other modalities, including ultrasonography, gastrointestinal series, endoscopy, magnetic resonance imaging (MRI), and positron emission tomography with [16]-fluorodeoxyglucose (FDG-PET) were also conducted to determine the diagnosis, if necessary. Relapse or progression of the disease was determined by evaluation of these images according to the Response Evaluation Criteria in Solid Tumors (RECIST) [17]. Histological evaluation of the surgical specimen was done according to the Japanese Classification of Gastric Carcinoma[12]. PFS was defined as the time from the date of registry to the date of disease progression, relapse after surgery, or death from any cause. Overall survival (OS) was defined as the time from the date of registry to the date of death from any cause. Patient follow-up data was locked on March 31, 2014.

Isolation and detection of CTCs

Peripheral blood was collected for the evaluation of CTCs prior to the initial intervention (staging laparoscopy, surgery, or chemotherapy). Blood samples were drawn into 10-mL evacuated CellSave® tubes (Veridex LLC, Raritan, NJ). Samples were sent to the in-hospital laboratory and processed within 72 hours. The CellSearch® System (Veridex LLC, Raritan, NJ) was used for the detection and counting of CTCs in 7.5mL of peripheral blood samples. Among the cells isolated with EpCAM-coated magnetic beads, CTCs were defined as intact cells that stained positive for CK8/18/19 antibody and negative for CD45 antibody, and were counted without prior knowledge of the clinical status of the patient. When the number of CTCs was less than four, independent counting was performed by another investigator, and only cells defined as CTCs by both investigators were included. In patients with positive CTCs, follow-up blood sampling and analysis of CTCs were conducted one to two weeks after the initial treatment

(chemotherapy or surgery).

Statistical analysis

Categorical data were compared with the Chi-square test, and continuous variables were compared using Student's t-test. *P*-values <0.05 were considered to be statistically significant. Multivariate analysis was performed to compare the CTC-negative and -positive groups, using the Logistic regression models to estimate the odds ratio for each factor. For survival analyses, the Kaplan-Meier method was used with the log-rank test for univariate analyses. Cox proportional hazards models were used for multivariate analyses and the hazard ratio (HR) was estimated. All statistical analyses were performed using SPSS Statistic 19 software (IBM, Armonk, NY).

Results

Counts of CTCs and clinical factors

Written consent was obtained from 140 patients. One patient had a history of previous chemotherapy and did not meet the inclusion criteria. Analysis of CTCs failed in three patients because of sampling error. In a total of 136 patients, in whom CTCs were successfully counted, CTCs were positive in 25 patients (18.4%). The number of CTCs per 7.5mL of blood in the CTC-positive patients ranged from 1 to 1123. In CTC-positive patients, the median CTC count was four (**Supplemental Figure 1**).

The relationships between the status of CTCs and the clinical factors were analyzed and summarized in **Table 1**. Positive CTC count was more common in tumors with diffuse histological type (*P*=0.044), and distant metastasis (*P*=0.004). The detection ratio of CTCs was as high as 33% (13/39) in patients with distant metastasis, compared with 12.4% (12/97) in patients without distant metastasis. Multivariate analysis using variables with *P* values less than 0.20 showed that distant metastasis had the highest odds ratio of 2.96 (95% CI=0.97-9.00), although the *P* value did not reach significance.

We also evaluated the relationship between clinicopahological factors and numbers of CTCs to clarify the characteristics of patients with high CTC count. Patients with five or more CTCs per 7.5mL of blood was diagnosed as more advanced clinical stage than patients with low CTC count (**Supplemental Table 1**)

CTCs and patient survival

All but two patients were followed successfully until their death or March 31, 2014. The follow-up rate was 98.5%. The median follow-up period was 26.0 months. To determine the number of CTCs that most clearly distinguish patients with rapid progression of disease from those with slow progression, receiver-operating characteristic (ROC) curve analysis was performed. When the cutoff value was set as one CTC per 7.5mL, the balanced error rate was minimal (**supplemental Figure 2**). At this level, the P value of the COX proportional hazards model signifying the difference between two survival curves of rapid and slow progression of disease was also minimal (*P*=0.018). Thus, a cutoff of one CTC per 7.5 ml of blood was chosen as a significant level to distinguish patients with a poor prognosis from those with a better prognosis. The PFS of patients with a CTC level ≥1 was significantly shorter than those with a CTC=0 (HR 2.03 [95%CI: 1.13-3.66]; *P*=0.016, **Figure 1a**). The OS of patients with CTC≥1 was also significantly worse than patients with negative CTC (HR 2.20 [95%CI: 1.120-4.03]; P=0.009, **Figure 1b**).

Analysis of prognostic factors

Among the 136 patients, chemotherapy was initiated without staging laparoscopy to evaluate CY status in 13 patients. To identify significant prognostic factors among several clinical factors including CY, a total of 123 patients, in whom both CTC and CY were successfully analyzed prior to the initial therapeutic intervention, were included in the subsequent analysis. Among them, 57 patients underwent radical gastrectomy first, without being evaluated by staging laparoscopy. The remaining 66 patients initially underwent staging laparoscopy and/or laparoscopic bypass. After staging laparoscopy/laparoscopic bypass, three underwent surgery, one patient chose best supportive care, and the remaining 62 patients underwent chemotherapy first. Among them, 44 patients eventually had surgery. All patients were followed up regularly to check for recurrence or progression at the outpatient unit for the study period (**Supplemental Figure 3**). Two patients were lost to follow-up at 84 and 839 days: the drop-out rate was 1.5%

The Kaplan-Meier method and log- rank test were used to evaluate the effect of several clinical factors on PFS. PFS was significantly shorter for T4 tumors, lymph node metastasis, diffuse histological type, macroscopic distant metastasis, CTC-positive, and CY-positive. Multivariate analysis using the Cox proportional hazards models with variables that had a P value of less than 0.20 in univariate analysis showed that performance status, macroscopic distant metastasis, CTC-positive, and CY-positive were independent prognostic factors. (**Table**

2).

The univariate and multivariate analyses were also performed to evaluate the effect of clinical factors on OS. The same factors that were correlated to shorter PFS were associated with shorter OS. Multivariate analyses showed that performance status and macroscopic distant metastasis were independent prognostic factors (**Table 3**).

Cytological detection of cancer and prognosis

To evaluate the impact of cytological detection of gastric cancer in either the peripheral blood (CTC) or the peritoneal cavity (CY) on the prognosis of patients, we classified the status of CTC and CY into four categories: negative for both factors (CTC=0 and CY=0, n=80), positive for CTC alone (CTC≥1 and CY=0, n=15), positive for CY alone (CTC=0 and CY=1, n=20), positive for both factors (CTC≥1 and CY=1, n=8). Among 28 CY-positive patients, all but three patients had induction chemotherapy. Reasons for not receiving induction chemotherapy were age of 86 (n=1), gastrectomy without staging laparoscopy (n=2). Kaplan-Meier PFS curves of the four groups are shown in **Figure 2**. When the CY factor was negative, the PFS was similar between patients that were CTC-negative (CTC=0 and CY=0) and those that were CTC-positive (CTC≥1 and CY=0) (P=0.555). By contrast, when the CY factor was positive, the PFS of CTC-negative patients (CTC=0 and CY=1) was significantly better than the double-positive patients (CTC=1 and CY=1, P<0.001).

To characterize patients in each category, the relationship between several clinicopahological factors and status of CTC and/or CY was evaluated (**Supplemental Table 2**). In CY-positive patients, all tumor was T4, and histology was diffuse type in all but three patients. We also analyzed the relationship between chemosensitivity and status of CTC and/or CY in 40 patients who received induction chemotherapy and clinical response was evaluable. Clinical objective response was correlated with the status of CTC and CY. Especially, responses were more often observed in CY-negative patients than in CY-positive patients. (**Supplemental Table 3**).

CTC as a monitoring marker for therapeutic intervention

Of 25 CTC-positive patients, 17 patients underwent induction chemotherapy. Among them, the CTC was recounted after two to three courses of chemotherapy in 12 patients. CTC counts following chemotherapy decreased in all patients compared. In six patients, the CTCs disappeared completely (**Supplemental Figure 4**). Clinical response was evaluated according

to the RECIST criteria in 10 patients who had target lesions; seven patients achieved PR (partial response) and three had SD (stable disease). Disappearance of CTCs was achieved in five out of seven patients with PR, compared with one of three patients with SD.

Among the CTC-positive patients at baseline, CTC and CY status following induction chemotherapy and/or surgery was obtained in 16 patients. Among these, both CTCs and CY were proven to be negative after chemotherapy and/or surgery in seven patients. PFS of the seven patients was much better than those remaining positive for either factor (**Figure 3**, *P*=0.002).

Discussion

In this prospective study, CTCs were detected in 18% of patients with advanced gastric cancer. While the detection of CTCs was as high as 33% in metastatic patients, we also detected CTCs in 11% of patients with resectable tumor. Regarding the histological type, CTCs were detected twice as often in the diffuse type as in the intestinal type. These results are consistent with a recent meta-analysis of CTCs in gastric cancer[18]. It is difficult to speculate the reasons why CTC-positive cases are dominant with diffuse histological type. However, in our series, CTCs were detected more often in patients with peritoneal metastasis (9/25, 36%), rather than in patients with liver metastasis (1/5, 20%). Although the intestinal type is known to be associated with liver metastasis, probably tumor cells are mainly in the portal blood flow in such cases, and not often detected in the peripheral blood.

Survival analyses showed that both OS and PFS were significantly shorter for patients with CTCs compared with those without. Among several clinical factors, multivariate analysis showed that the presence of CTCs and CY, as well as performance status and macroscopic distant metastasis, were independent predictors of shorter PFS. These data suggest that CTCs and CY are more reliable factors than clinical tumor (T) stage, or lymph node (N) stage to select a certain group of patients for intensive treatment. It should be noted that the similar multivariate analysis for OS did not show the significance of CTCs and CY as prognostic factors. One of the reasons of this discrepancy would be that this study was designed to detect the difference of PFS between CTC-negative and –positive patients. To evaluate if CTCs were independent predictor for shorter OS, we might need longer follow-up periods to obtain more mature survival curve.

It is noteworthy that the presence of CTCs was not only a predictor of PFS, but could also be a useful biomarker for monitoring therapeutic effect in gastric cancer. Although the number of

patients in this study was too small to evaluate CTCs as a monitoring marker, changes of the status of CTCs seemed to correlate well with the objective response to chemotherapy. These results are consistent with a previous study of patients with metastatic gastric cancer[9]. Negative conversion of CTCs by therapeutic intervention was also associated with better survival of patients. These data strongly indicate that CTCs could become a valuable biomarker in making a difficult clinical decision, i.e., the indication for radical surgery after induction chemotherapy for marginally resectable gastric cancer.

In highly advanced gastric cancer, the assessment of CY, as well as peritoneal dissemination by staging laparoscopy is currently recommended by the National Comprehensive Cancer Network therapeutic guidelines in United States[19]. Tumors that are positive for CY are classified as stage IV in both the UICC/AJCC and the Japanese classification[1, 11]. However, initial surgical resection is still often done in Japan, where staging laparoscopy is not in routine practice. For patients that are CY positive but without gross distant metastasis, surgical resection followed by chemotherapy is considered as a standard treatment, because around 20% of patients achieve long-term survival[13]. However, the current study clearly indicated that, when the status of CTCs was used in combination with CY, the prognosis of patients that were CTC-negative was significantly better than for patients that were positive for both CY and CTC factors. It would be very useful to be able to select these patients prior to initial resection and to provide more intensive treatment based upon evaluation of the CTC and CY status. This study also demonstrated an improved prognosis of patients in whom both CTC and CY factors were successfully converted to negative status following therapeutic intervention. When patients with highly advanced disease exhibit an excellent response to initial therapy, evaluation of CTCs, as well as CY, could provide valuable information when considering the utility of surgical intervention.

There are several limitations in this study. First, there is no consensus about proper cut-off value to diagnose CTC-positive cases. For breast cancer, five CTCs are often used as a proper cut-off[6]. In gastric cancer patients, number of CTCs in each case tends to be smaller than breast cancer. In previous studies of CTCs in gastric cancer, Hiraiwa et al. used two CTCs, while Uenosono et al. used one CTC as the cut-off[8, 20]. We determined the cut-off as one CTC by ROC curve analysis, which is the standard method to assess the proper cut-off value of a diagnostic test. Our cut-off needs to be evaluated in other data sets to determine if it is generally acceptable. Second, because decision of treatment was wade on doctors' and patients' preference, treatments were heterogenous. It may not be fair to compare survivals

between groups with different treatments. However, CTC-positive patients were more heavily treated than CTC-negative patients, and yet survival time was shorter. This finding strongly suggests that the prognosis of CTC-positive patients were much poorer indeed. Third, because the CellSearch system captures CTCs using EpCAM, some CTCs can escape from the detection due to epithelial-mesenchymal transition. However, we believe that CTCs detected in this study represent important and viable population, because CTC-positivity were closely related to distant metastasis and poorer prognosis of patients.

In conclusion, this study demonstrated that CTCs were detected in a significant proportion of patients with advanced gastric cancer. Detection of CTCs was an independent predictor of shorter PFS, and could be a useful biomarker in the selection of patients who require intensive treatment or would benefit from radical surgery. To establish the usefulness of CTCs as a biomarker to aid in the clinical decision process for advanced gastric cancer, further study with a greater number of patients is necessary.

Acknowledgements

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Disclosure

The authors have declared no conflicts of interest.

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Figure legends

Figure 1

a. Progression-free survival curves of patients with CTC=0 and CTC≥1.
b. Overall survival curves of patients with CTC=0 and CTC≥1.

Figure 2

Progression-free survival curves of patients with four categories: negative for CTC and CY factors (CTC=0 and CY=0), positive only for CTC factor (CTC≥1 and CY=0), positive only for CY factor (CTC=0 and CY=≥1), and positive for both factors (CTC≥1 and CY=1).

Figure 3

Progression-free survival curves of patients in whom with both CTC and CY factors were proven to be negative (CTC=0 and CY=0), and in patients with either factor remaining positive (CTC ≥1 or CY=1) after therapeutic intervention.

Supplemental Figure 1

Scatter plot of CTC counts in patients with advanced gastric cancer.

Supplemental Figure 2

ROC curve analysis to determine a cut-off value of CTCs.

Supplemental Figure 3

Flow diagram of the treatment course for the 123 patients with advanced gastric cancer.

Supplemental Figure 4

CTC counts in patients who received chemotherapy before starting treatment (pre-treatment) and after two to three cycles of treatment (post-treatment).

Fig 1

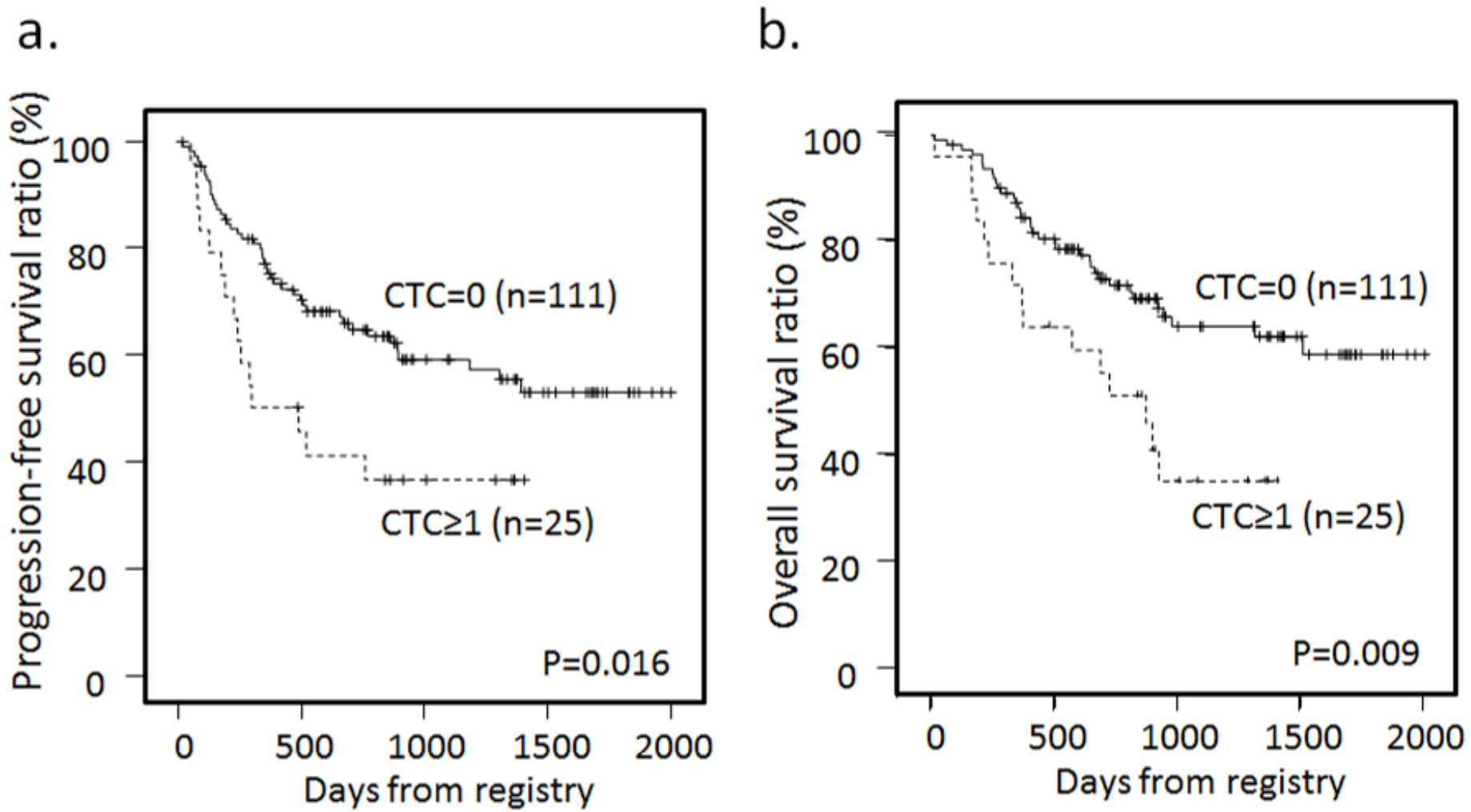
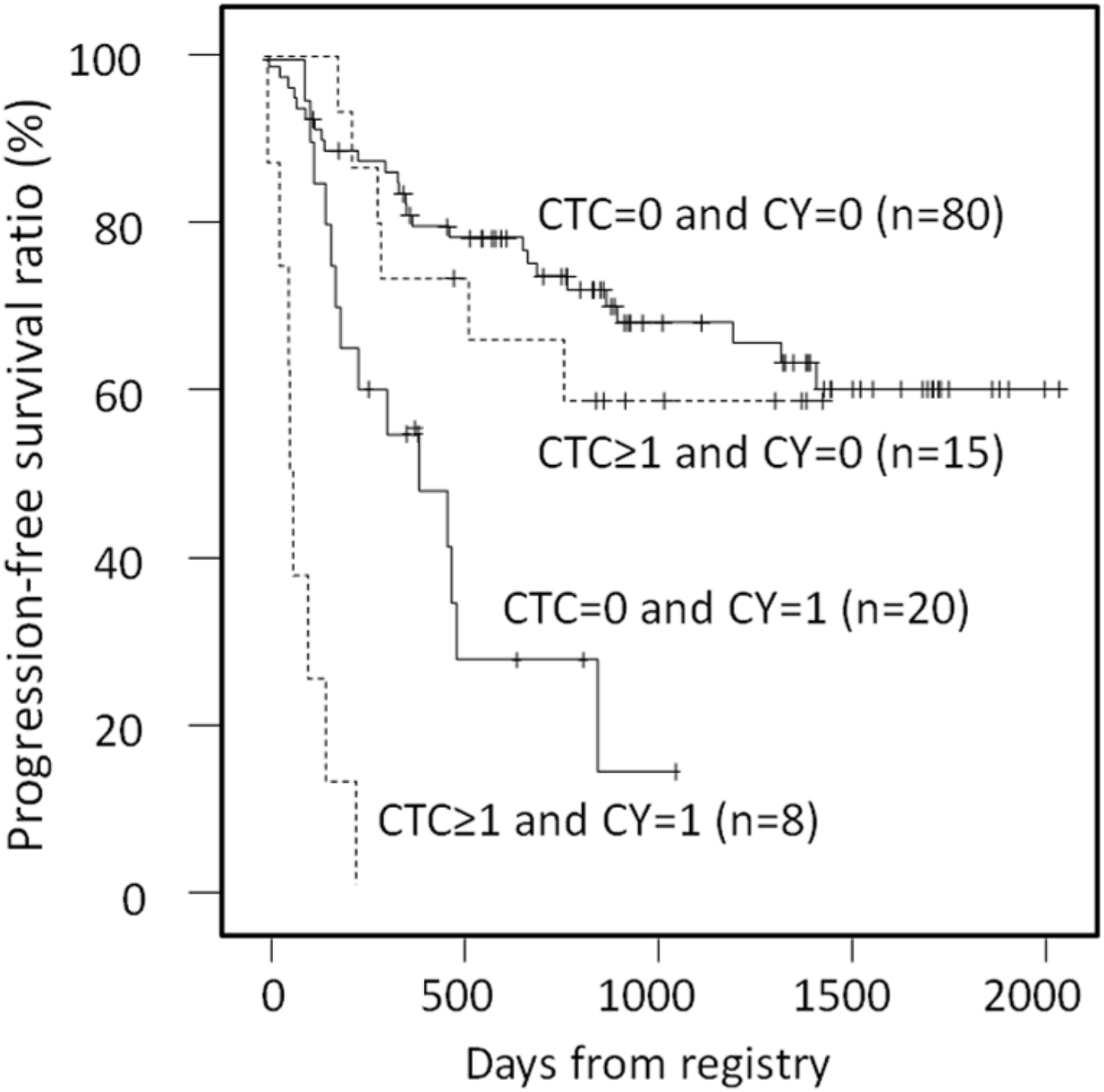

a.
Progression-free survival ratio (%)
100
80
60
40
20
0
CTC=0 (n=111)
CTC≥1 (n=25)
P=0.016
0
500
1000
1500
2000
Days from registry
b.
Overall survival ratio (%)
100
80
60
40
20
0
CTC=0 (n=111)
CTC≥1 (n=25)
P=0.009
0
500
1000
1500
2000
Days from registry

Fig 2


Progression-free survival ratio (%)
100
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Days from registry
CTC=0 and CY=0 (n=80)
CTC≥1 and CY=0 (n=15)
CTC=0 and CY=1 (n=20)
CTC≥1 and CY=1 (n=8)

Fig 3

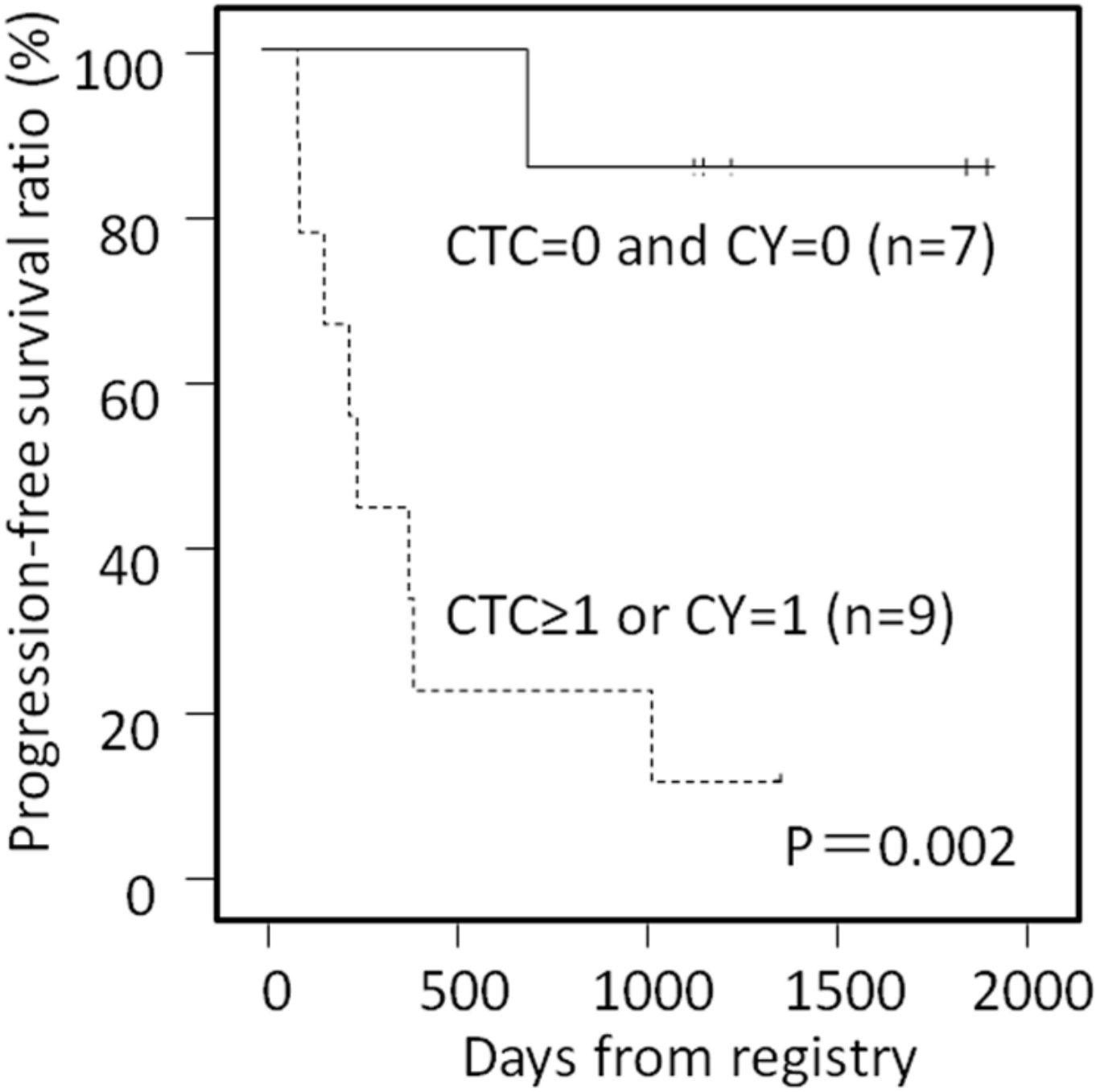

Progression-free survival ratio (%)
100
80
60
40
20
0
CTC=0 and CY=0 (n=7)
CTC≥1 or CY=1 (n=9)
P＝0.002
0
500
1000
1500
2000
Days from registry

Table 1 CTC and clinical factors in advanced gastric cancer

Clinical factors	CTC=0	CTC≥1	Univariate analysis	Multivariate analysis	
	N=111	*N*=25	*P* value[1)]	Odds Ratio (95% CI)	*P* value[2)]
Age (mean±SD)	67.2±11.6	64.7±10.7	0.318	-	-
Sex (Male vs. Female)	70/41	17/8	0.642	-	-
Histological type (Diffuse vs. Intestinal)	70/41	21/4	**0.044**	2.25 (0.66-7.69)	0.195
Performance status (1-2 vs. 0)	12/99	0/25	0.227	-	-
Tumor stage (T4 vs. T2-3)	73/38	19/6	0.327	-	-
Lymph node metastasis (N1-3 vs. N0)	75/36	21/4	0.103	2.19 (0.50-9.69)	0.302
Distant metastasis (M1 vs. M0)	26/85	13/12	**0.004**	2.96 (0.97-9.00)	0.056
Clinical stage (3-4 vs. 1-2)	70/41	20/5	0.106	1.76 (0.37-8.40)	0.479
CEA (≥5 vs. <5)	19/92	8/17	0.092	2.09 (0.72-6.01)	0.174
CA19-9 (≥37 vs. <37)	19/92	8/17	0.092	1.72 (0.59-5.00)	0.321

1) Student's t-test for age, Chi-square test for other factors, 2) Logistic regression models

P value less than 0.05 indicated in bold with underline. Abbreviations: CTC, circulating tumor cell; CI, confidence interval

Table 2 Univariate and multivariate analysis of clinical factors for progression-free survival

Clinical factors	Univariate analysis	Multivariate analysis	
	P value[1)]	Hazard Ratio (95% CI)	*P* value[2)]
Sex (Male vs. Female)	0.682	-	-
Performance status (1 vs. 0)	0.099	3.31 (1.32-8.32)	**0.011**
Tumor stage (T4 vs. T2-3)	**<0.001**	2.04 (0.78-5.32)	0.146
Lymph node metastasis (N1-3 vs. N0)	**0.001**	1.62 (0.72-3.67)	0.247
Histological type (Diffuse vs. Intestinal)	**0.001**	2.23 (1.00-4.98)	0.050
Macroscopic distant metastasis (Positive vs. Negative)	**<0.001**	3.09 (1.44-6.61)	**0.004**
CTC (≥1 vs. 0)	**0.021**	2.14 (1.09-4.20)	**0.027**
CY (1 vs. 0)	**<0.001**	2.31 (1.05-5.08)	**0.038**
CEA (≥5 vs. <5)	0.812	-	-
CA19-9 (≥37 vs. <37)	0.095	1.24 (0.62-2.47)	0.535

1) Log-rank test, 2) Cox proportional hazards models

P value less than 0.05 was shown in bold with underline.

Abbreviations: CI, confidence interval; CTC, circulating tumor cell; CY, peritoneal lavage cytology

Table 3 Univariate and multivariate analysis of clinical factors for overall survival

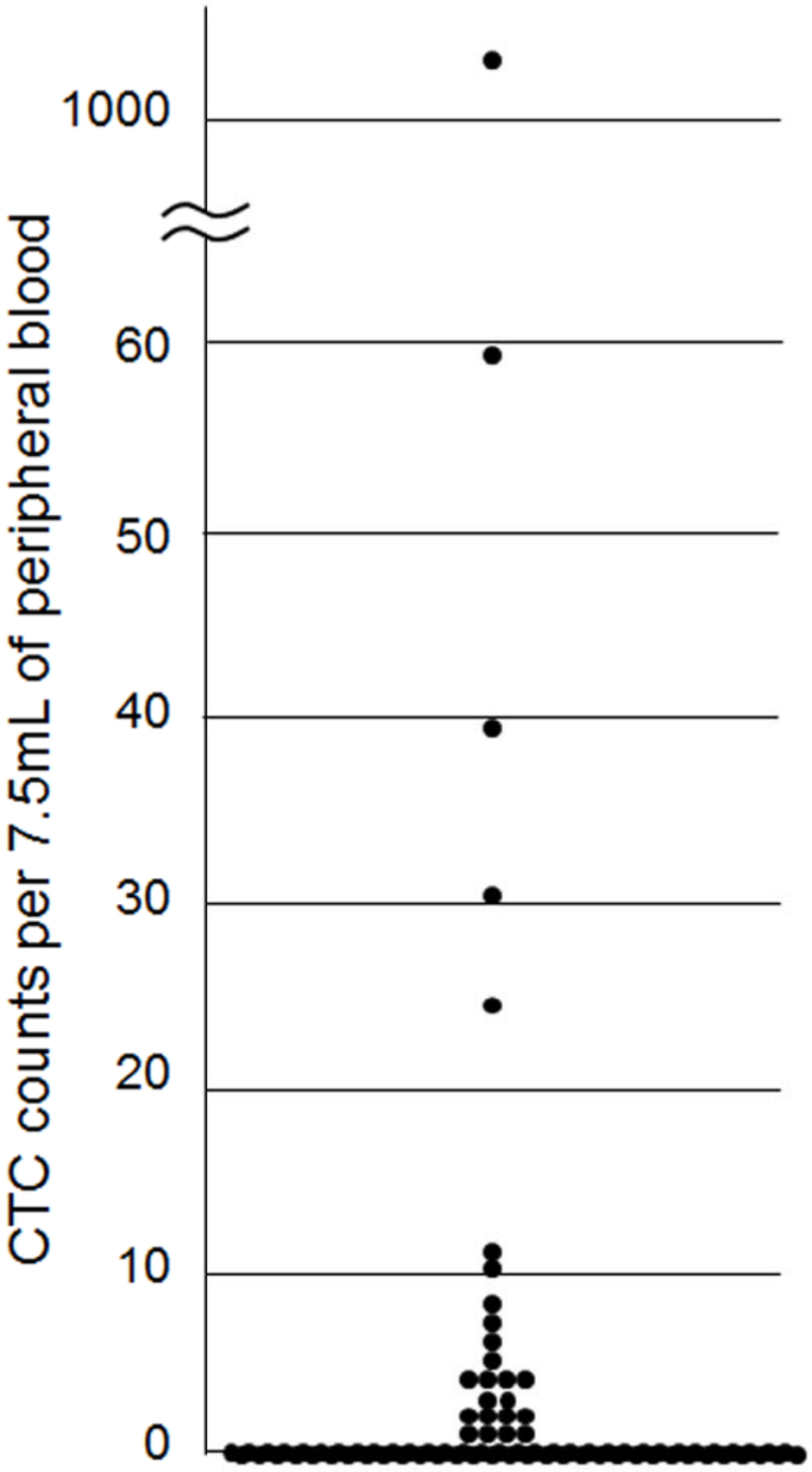
Clinical factors	Univariate analysis	Multivariate analysis	
	P value[1)]	Hazard Ratio (95% CI)	*P* value[2)]
Sex (Male vs. Female)	0.818	-	-
Performance status (1 vs. 0)	0.114	2.73 (1.03-7.29)	**0.044**
Tumor stage (T4 vs. T2-3)	**<0.001**	2.46 (0.77-7.86)	0.129
Lymph node metastasis (N1-3 vs. N0)	**<0.001**	1.61 (0.62-4.12)	0.331
Histological type (Diffuse vs. Intestinal)	**0.001**	2.36 (0.89-6.28)	0.086
Macroscopic distant metastasis (Positive vs. Negative)	**<0.001**	2.56 (1.15-5.70)	**0.021**
CTC (≥1 vs. 0)	**0.042**	1.37 (0.68-2.77)	0.375
CY (1 vs. 0)	**<0.001**	2.08 (0.94-4.63)	0.073
CEA (≥5 vs. <5)	0.762	-	-
CA19-9 (≥37 vs. <37)	0.026	1.45 (0.57-2.29)	0.702

2) Log-rank test, 2) Cox proportional hazards models

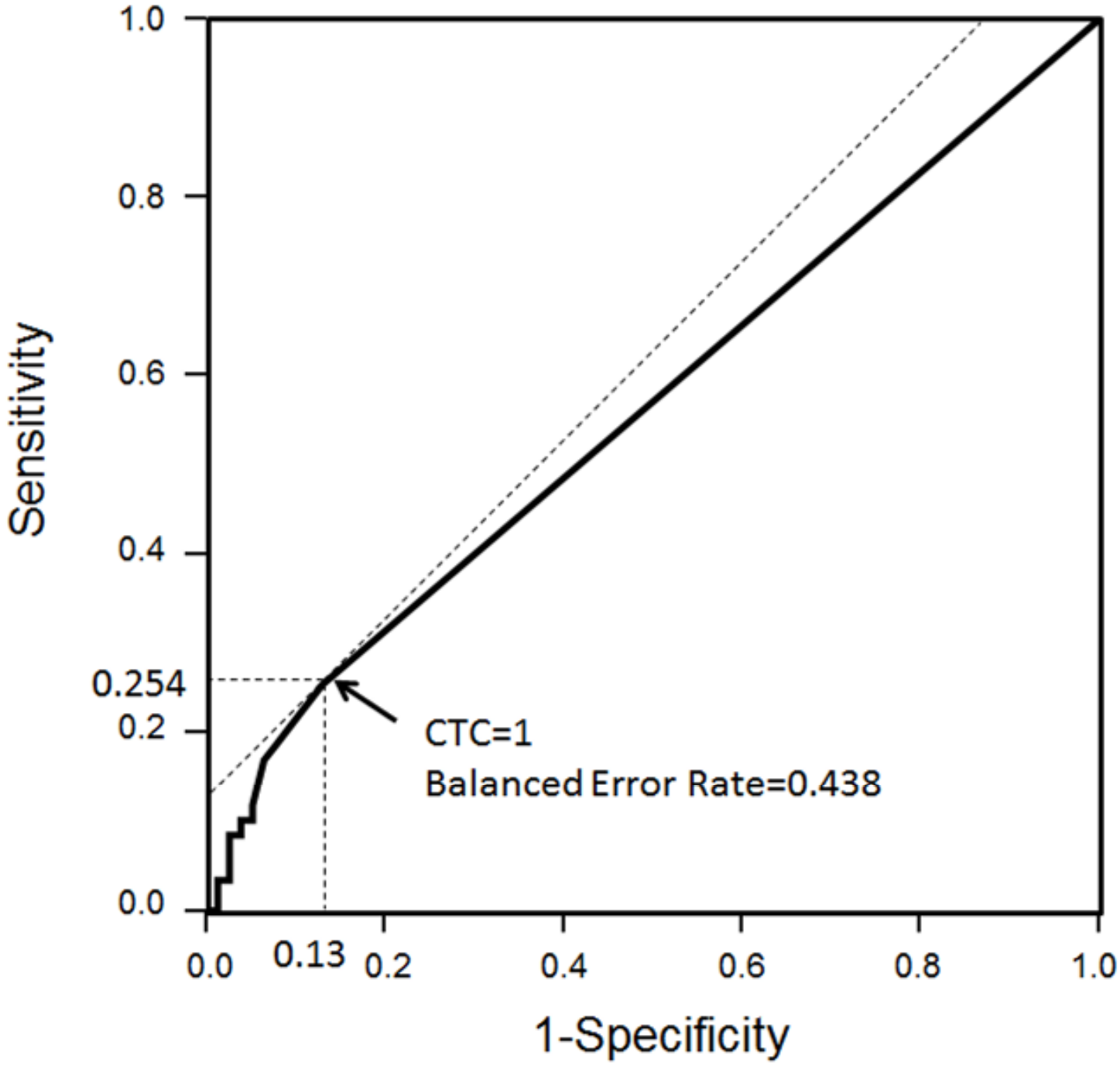
P value less than 0.05 was shown in bold with underline.

Abbreviations: CI, confidence interval; CTC, circulating tumor cell; CY, peritoneal lavage cytology

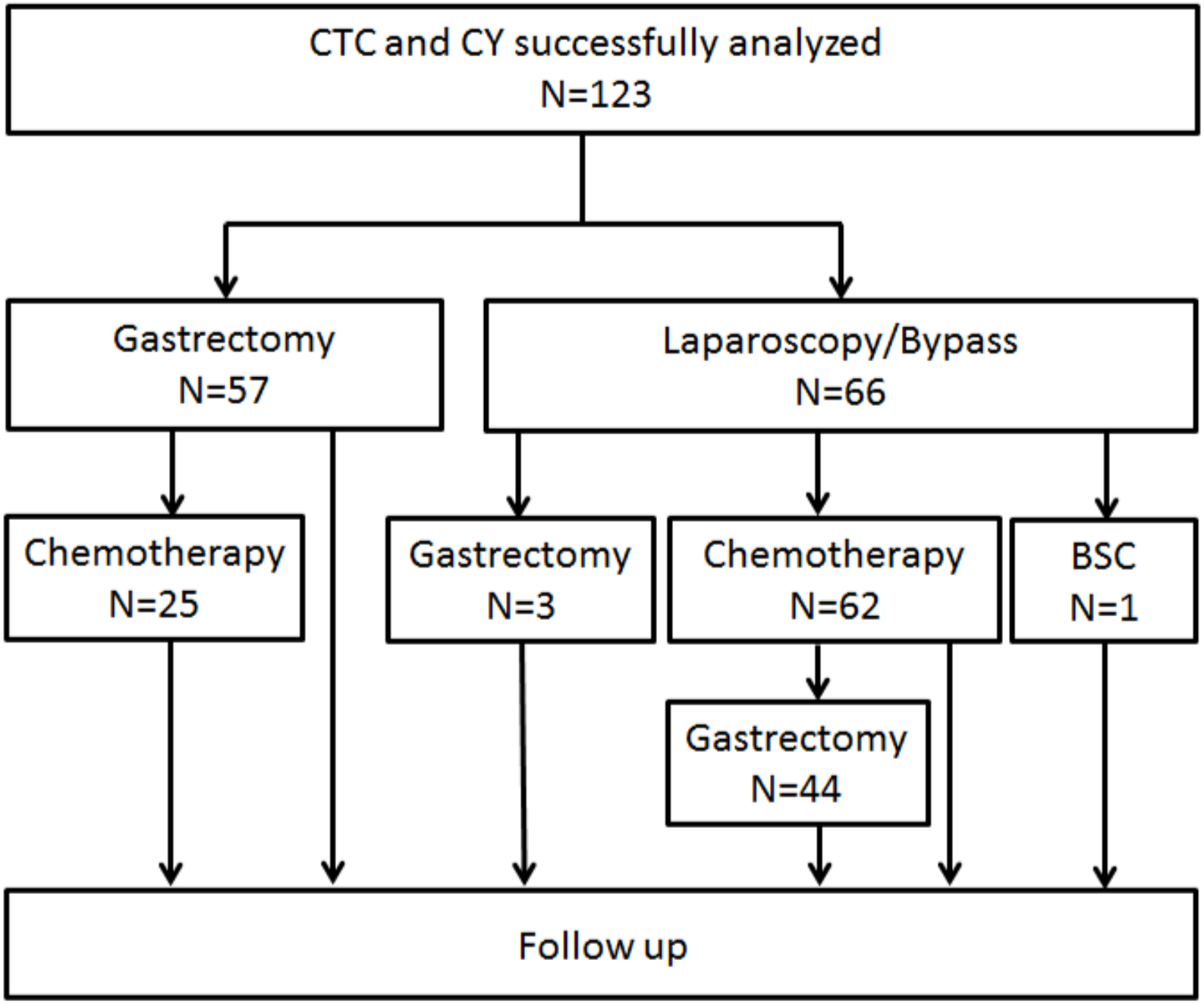
Sup Fig 1


1000
60
50
40
30
20
10
0
CTC counts per 7.5mL of peripheral blood

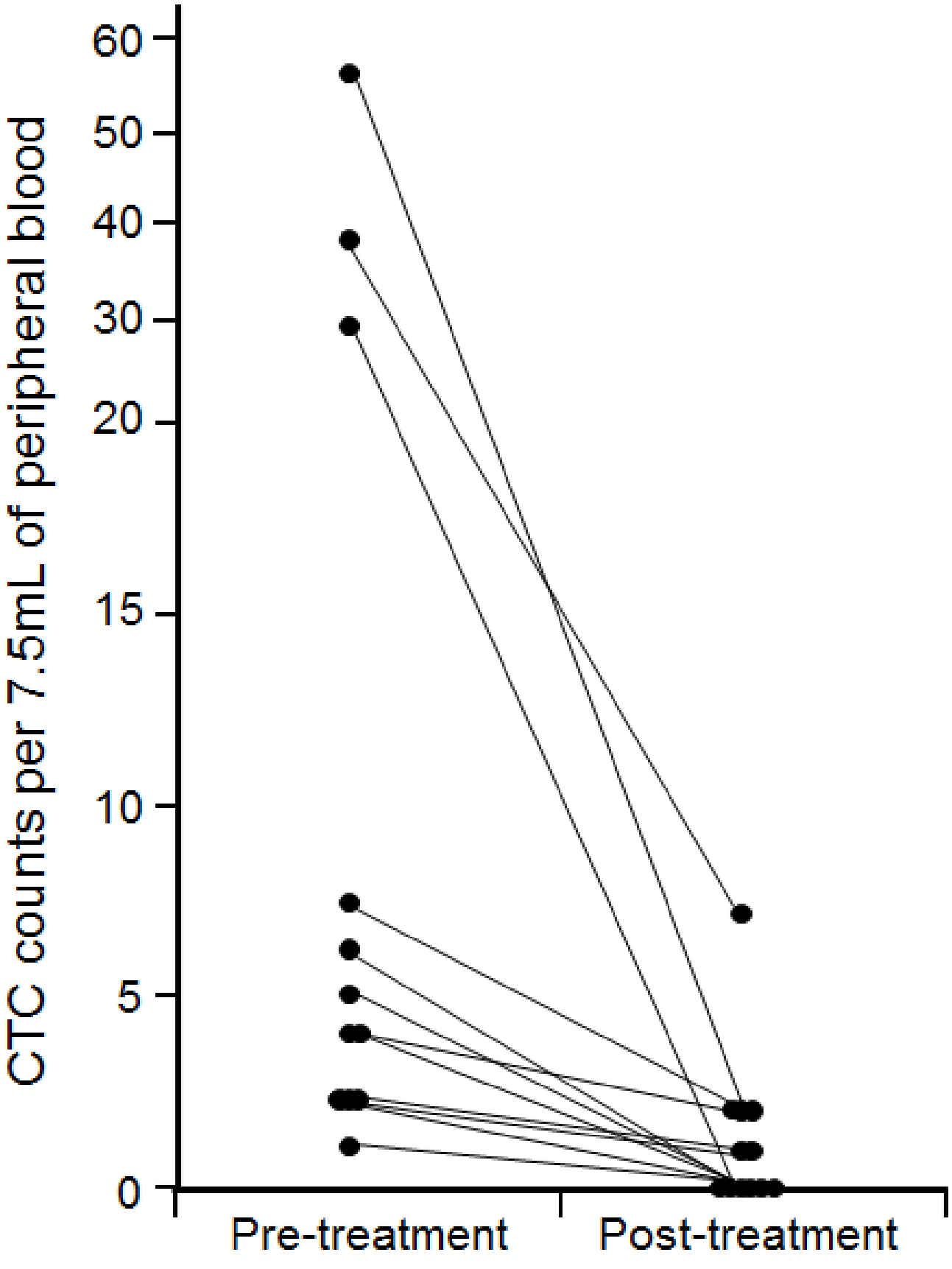
Sup Fig 2


1.0
0.8
0.6
0.4
0.254
0.2
0.0
Sensitivity
0.0
0.13
0.2
0.4
0.6
0.8
1.0
1-Specificity
CTC=1
Balanced Error Rate=0.438

Sup Fig 3


CTC and CY successfully analyzed
N=123
Gastrectomy
N=57
Laparoscopy/Bypass
N=66
Chemotherapy
N=25
Gastrectomy
N=3
Chemotherapy
N=62
BSC
N=1
Gastrectomy
N=44
Follow up

Sup Fig 4


CTC counts per 7.5mL of peripheral blood
60
50
40
30
20
15
10
5
0
Pre-treatment
Post-treatment

Supplemental Table 1 Number of CTC and clinicopathological factors

Clinical factors	Number of CTCs		Univariate analysis
	CTC<5 *N*=14	CTC≥5 *N*=11	*P* value[1)]
Age (mean±SD)	67.4±10.6	60.0±9.4	0.084
Sex (Male vs. Female)	8/6	9/2	0.189
Histological type (Diffuse vs. Intestinal)	10/4	11/0	0.053
Performance status (1-2 vs. 0)	0/14	0/11	NA
Tumor stage (T4 vs. T2-3)	9/5	10/1	0.122
Lymph node metastasis (N1-3 vs. N0)	11/3	10/1	0.404
Distant metastasis (M1 vs. M0)	6/8	7/4	0.302
Clinical stage (3-4 vs. 1-2)	9/5	11/0	**0.027**
CEA (≥5 vs. <5)	3/11	5/6	0.201
CA19-9 (≥37 vs. <37)	3/11	5/6	0.201

1) Student's t-test for age, Chi-square test for other factors, *P* value less than 0.05 indicated in bold with underline.

Abbreviations: CTC, circulating tumor cell

Supplemental Table 2 CTC/CY and clinicopathological factors

Clinical factors	CY=0		CY=1		Univariate analysis
	CTC=0	CTC≥1	CTC=0	CTC≥1	*P* value[1)]
	N=80	*N*=15	*N*=20	*N*=8	
Sex (Male vs. Female)	52/28	9/6	11/9	6/2	0.745
Histological type (Diffuse vs. Intestinal)	49/31	11/4	17/3	8/0	**0.039**
Performance status (1-2 vs. 0)	10/70	0/15	1/19	0/8	0.274
Tumor stage (T4 vs. T2-3)	45/35	9/6	20/0	8/0	<0.001
Lymph node metastasis (N1-3 vs. N0)	49/31	11/4	15/5	8/0	0.111
Distant metastasis (M1 vs. M0)	4/76	4/11	20/0	8/0	<0.001
Clinical stage (3-4 vs. 1-2)	41/39	10/5	20/0	8/0	<0.001
CEA (≥5 vs. <5)	15/65	3/12	2/18	3/5	0.412
CA19-9 (≥37 vs. <37)	10/70	4/11	7/13	3/5	0.053

2) Student's t-test for age, Chi-square test for other factors, 2) Logistic regression models

P value less than 0.05 indicated in bold with underline. Abbreviations: CTC, circulating tumor cell; CI, confidence interval

Supplemental Table 3 CTC/CY and clinical response to chemotherapy

Categories	Clinical Response[1)]		Univariate analysis
	PR *N*=18	SD/PD *N*=22	*P* value[2)]
CTC/CY			0.023
CTC=0 and CY=0	9	9	
CTC≥1 and CY=0	6	1	
CTC=0 and CY=1	3	8	
CTC≥1 and CY=1	0	4	
CTC			0.455
CTC=0	12	17	
CTC≥1	6	5	
CY			0.014
CY0	15	10	
CY1	3	12	

1) According to the RECIST (Response Evaluation Criteria in Solid Tumor), 2) Chi-square test

Abbreviations: PR, partial response; SD, stable disease; PD, progressive disease; CTC, circulating tumor cell; CY, peritoneal lavage cytology