# Aneuploidy of chromosome 8 in circulating tumor cells correlates with prognosis in patients with advanced gastric cancer

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#### Abstract

**Objective:** Previous work indicated that an euploidy of chromosome 8 in circulating tumor cells (CTCs) correlated with the rapeutic efficacy for advanced gastric cancer (AGC) patients. In this follow-up study performed on the same population of AGC patients, we investigated whether and how an euploidy of chromosome 8 in CTCs correlates with patients' clinical prognosis.

**Methods:** The prospective study was performed on 31 patients with newly diagnosed AGC. Previously established integrated subtraction enrichment (SE) and immunostaining-fluorescence *in situ* hybridization (iFISH) platform was applied to identify, enumerate and characterize CTCs. Quantification of CTCs and analysis of their aneuploidy of chromosome 8 were performed on patients before and after therapy.

**Results:** CTCs were measured in 93.5% of AGC patients, and two CTC subtypes with diverse threshold values were identified, multiploid CTCs with the threshold of  $\geq$ 2 per 7.5 mL and multiploid plus triploid CTCs with the threshold of  $\geq$ 4, which were found to significantly correlate with poor progression-free survival (PFS) and overall survival (OS). In particular, patients with  $\geq$ 10% increased multiploid CTCs after an initial 6 weeks of therapy had poor PFS and OS, whereas improved PFS and OS were observed on those who had  $\geq$ 10% decreased multiploid CTCs. After adjusting for clinically significant factors,  $\geq$ 10% increased post-therapy multiploid CTCs was the only independent predictor of PFS and OS.

**Conclusions:** Aneuploidy of CTCs correlates with prognosis of AGC patients. Quantitative comparison monitoring multiploid CTCs before and after therapy may help predict improved or inferior prognosis and chemoresistance.

Keywords: Circulating tumor cells; advanced gastric cancer; aneuploidy; iFISH; prognosis

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### Introduction

Circulating tumor cells (CTCs) are shed from primary or metastatic solid tumors into the circulation (1). Several studies suggest that measuring CTCs serves as a "liquid biopsy", enabling non-invasive and frequent monitoring of therapeutic responses in cancer patients in real time (1-6). A study performed on 52 advanced gastric cancer (AGC) patients in Japan confirmed that patients with unfavorable CTC counts 2 or 4 weeks after initiation of chemotherapy had shorter median progression-free survival (PFS) and overall survival (OS) compared with those with favorable CTC counts (7). Similar results were also obtained in our previous work with 136 AGC patients, indicating that

quantification of CTCs may be a promising approach for evaluating chemotherapeutic efficacy and predicting prognosis of AGC patients. Also, a persistently favorable CTC count or an early conversion to a favorable CTC count after therapy may indicate improved prognosis. In contrast, patients with persistently unfavorable CTCs or who converted to an unfavorable CTC count after therapy fared significantly worse, suggesting a limited treatment response. Longitudinal changes of CTCs in response to therapy may help in rapidly indicating acquired resistance in individual patients before they are radiographically evaluated (8).

In addition to counting CTCs, pinpointing specific targeted-therapy-or chemotherapy-resistant CTC subtype classified by tumor biomarker expression and/or chromosome ploidy may be a better approach (3,9-11). Our previous study indicated that aneuploidy of chromosome 8 in CTCs of AGC patients correlated with chemotherapeutic efficacy of paclitaxel (PTX) and cisplatin (DDP) (12). The number of pre-treatment triploid CTCs in patients was inversely correlated with chemotherapeutic efficacy. After administration of PTX or DDP, triploid CTCs had intrinsic resistance to chemotherapeutic reagents, whereas multiploid CTCs (≥4 copies on chromosome 8) developing acquired resistance. Similar observations were made in a DDP-treated gastric neuroendocrine cancer patient-derived xenograft (PDX) model (13). Identification of diverse CTC subtypes with distinct clinical significance could help guide more precise personal therapy and allow more robust analyses (14).

In this study, we extended previous studies to investigate whether and how distinct CTC subtypes with diverse ploidy of chromosome 8 correlate with patients' clinical prognosis in terms of PFS and OS. Subtraction enrichment (SE) integrated with immunostaining-fluorescence *in situ* hybridization (iFISH) was applied to enrich and characterize aneuploidy of chromosome 8 in CTCs (12). Correlations of quantified multiploid CTCs in patients under therapy with clinical prognosis were investigated to evaluate the utility of comparing multiploid CTCs for monitoring therapeutic response in AGC patients.

#### **Materials and methods**

#### Patients and sample collection

Thirty-one newly diagnosed AGC patients (>18 years) were enrolled in the study at Peking University Cancer Hospital from October 2013 to August 2014. Locally advanced, recurrent, metastatic gastric or gastroesophageal junction adenocarcinoma was histopathologically diagnosed and confirmed. Patients who had not received treatment and those who had a Karnofsky performance status >60, with adequate organ function and evaluable tumor lesions were eligible for this study. All enrolled patients were given firstline PTX or DDP-based chemotherapy (15). Those histopathologically diagnosed as human epidermal growth factor receptor 2 (HER2)-positive cancer received anti-HER2 targeted therapy and DDP chemotherapy (16).

Six weeks (2 cycles) after therapy, evaluation of clinical response was performed using computed tomography (CT) according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 criteria (17). Responses were categorized as partial response (PR, at least a 30% decrease in the sum of diameters of target lesions), progressive disease (PD, at least a 20% increase in the sum of diameters of target lesions), or stable disease (SD, neither sufficient shrinkage to qualify as PR nor a sufficient increase to qualify as PD). Written informed consent was obtained from all subjects. The study was approved by the Ethics Committee of Peking University Cancer Hospital and was performed according to the Declaration of Helsinki principles.

#### Enrichment and characterization of CTCs by SE-iFISH

Peripheral blood samples were collected from patients before and 6 weeks (the first evaluation of efficacy) after therapy initiation. CTCs were enriched using SE-iFISH (Cytelligen, San Diego, CA, USA) according to a previously published protocol (12). Briefly, 7.5 mL of patient blood was collected into a tube containing acidcitrate-dextrose (ACD) anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA), followed by thoroughly mixing and overlaying on 3 mL of hCTC separation matrix. The solution was centrifuged at 450x g for 5 min at room temperature. Supernatants above red blood cells were collected and incubated with immunomagnetic particles conjugated to anti-leukocytes monoclonal antibodies at room temperature for 10 min with gentle shaking. Samples were then subjected to magnetic separation to remove leukocytes. The magnetic particle-free solution was spun down at 500x g for 2 min at room temperature. Sedimented cells were thoroughly mixed with cell fixative and applied onto Cytelligen-coated CTC slides. Samples were subsequently subjected to immunostaining with a cocktail of Alexa Fluor 594-conjugated monoclonal anti-CD45 and

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Alexa Fluor 488-conjugated anti-pan-cytokeratin (PanCK) (CK4, 5, 6, 8, 10, 13 and 18) for 1 h in the dark, followed by FISH with Centromere Probe (CEP) 8 Spectrum-Orange (Vysis, Abbott Laboratories, Abbott Park, IL, USA) using a S500 StatSpin ThermoBrite Slide Hybridization/Denaturation System (Abbott Molecular, Des Plaines, IL, USA). CTCs were identified as 4',6diamidino-2-phenylindole (DAPI)+/CD45-/PanCK+ orwith aneuploid chromosome 8.

#### Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics (Version 21.0; IBM Corp., New York, USA). Correlation of CTC positivity with clinicopathologic characteristics and clinical responses was examined using Fisher's exact test. The threshold of CTCs was established with nonparametric receiver operating characteristic (ROC) analysis, and optimal threshold values were determined to maximize the Youden index (sensitivity+ specificity-1), which was determined by selecting a point that maximizes the number of subjects correctly classified, and giving equal weight to sensitivity and specificity (18). PFS was defined as the duration from initial blood collection to the time that clinical progression was confirmed or the participants were censored. OS was defined as the time from initial blood collection to the date that death occurred or the participants were censored. Kaplan-Meier survival plots for PFS or OS were generated based on numbers of total or aneuploid CTCs before and after therapy, and survival curves were compared using logrank tests. Cox proportional hazards regression was applied to determine the univariate and multivariate hazard ratios (HR) for PFS and OS. Pre- and post-therapy total CTC counts, subtypes of multiploid chromosome 8 CTCs, and standard clinical factors including gender, age, liver and peritoneal metastases, Lauren classification, HER2 status and the type of treatment were subjected to univariate analyses for PFS and OS. Significant parameters in the univariate analysis were then included in the multivariate analysis. P<0.05 was considered statistically significant, and all P values were two-sided.

# Results

# Correlation of CTCs in pre-treatment patients with clinicopathological characteristics

Quantification of total and aneuploid CTCs (≥4 copies on

chromosome 8) in pre-treatment patients by SE-iFISH was performed on all of the 31 enrolled AGC patients. The median number of CTCs detected in whole population of patients was 3 (range, 0-720) and the mean number (*x*±*s*) was 32±128. And  $\geq$ 1 CTCs per 7.5 ml were detected in 93.5% of patients before treatment. Examination of correlation between CTC numbers and various clinicopathological characteristics is summarized in *Table 1*. All patients with mixed type carcinoma were positive for CTCs  $(\geq 2 \text{ per } 7.5 \text{ mL})$ , whereas patients with intestinal and diffuse type cancer had a CTC positivity of 83.3% and 33.3%, respectively. Further pairwise comparison revealed that positivity of CTC in intestinal and mixed type patients was significantly higher than that of diffuse type (P=0.038, P=0.021, respectively). No obvious correlation was found in other clinicopathological characteristics, including gender, age, primary tumor sites, metastatic sites, and HER2 status.

#### Post-therapy CTCs correlate with PFS and OS

Of the 31 enrolled AGC patients, 22 had completed two cycles of therapy and a post-therapy CTC evaluation. At the time of analysis, 18 of 22 patients had PD, and 14 died. The median PFS and OS were 8.1 [95% confidence interval (95% CI), 6.7–9.4] months and 18.7 (95% CI, 12.7–24.6) months, respectively. To establish an optimal threshold of CTCs for predicting PFS and OS, ROC curves and Youden index were estimated for both pre- and post-treatment CTCs (*Supplementary Figure S1*) (18). We observed that <4 CTCs per 7.5 mL was a favourable threshold, whereas  $\geq$ 4 was an unfavorable value in this study.

In pre-treatment patients, those with  $\geq$ 4 CTCs had the median PFS and OS as shown in *Figure 1A*, *B* and these were not significantly different from patients with <4 CTCs (P=0.487, P=0.533, respectively). However, 6 weeks after therapy initiation, the median PFS of patients with  $\geq$ 4 post-therapy CTCs was 4.5 (95% CI, 3.8–5.2) months, which was significantly shorter than that of patients with <4 post-therapy CTCs [8.4 (95% CI, 8.0–8.8) months, P=0.001] (*Figure 1C*). For median OS, similar results were obtained. The patients with  $\geq$ 4 post-therapy CTCs had inferior median OS [14.3 (95% CI, 6.7–21.9) months] compared with those with <4 post-therapy CTCs [20.0 (95% CI, 14.9–25.0) months, P=0.044) (*Figure 1D*). Data suggest that a threshold of unfavorable post-therapy CTCs ( $\geq$ 4) may help predict inferior PFS and OS.

Variables	Cases No.	CTC number (per 7.5 mL) [n (%)]				
		≥1	≥2	≥3	≥4	≥5
Total	31	29 (93.5)	24 (77.4)	18 (58.1)	15 (48.4)	14 (45.2)
Gender						
Female	10	9 (90.0)	9 (90.0)	6 (60.0)	6 (60.0)	6 (60.0)
Male	21	20 (95.2)	15 (71.4)	12 (57.1)	9 (42.9)	8 (38.1)
Р		0.548	0.251	0.579	0.306	0.224
Age (year)						
≥60	19	18 (94.7)	14 (73.7)	12 (63.2)	11 (57.9)	10 (52.6)
<60	12	11 (91.7)	10 (83.3)	6 (50.0)	4 (33.3)	4 (33.3)
Р		0.632	0.435	0.362	0.168	0.249
Primary tumor site						
Stomach	18	17 (94.4)	13 (72.2)	9 (50.0)	8 (44.4)	7 (38.9)
Gastroesophageal junction	13	12 (92.3)	11 (84.6)	9 (69.2)	7 (53.8)	7 (53.8)
Р		0.671	0.358	0.242	0.439	0.323
Lauren classification						
Intestinal	18	17 (94.4)	15 (83.3)	12 (66.7)	9 (50.0)	9 (50.0)
Diffuse	6	5 (83.3)	2 (33.3)	2 (33.3)	2 (33.3)	2 (33.3)
Mixed	7	7 (100)	7 (100)	4 (57.1)	4 (57.1)	3 (42.9)
Р		0.427	0.010	0.358	0.673	0.657
Sites of metastasis						
Liver						
Yes	10	9 (90.0)	7 (70.0)	6 (60.0)	4 (40.0)	4 (40.0)
No	21	20 (95.2)	17 (81.0)	12 (57.1)	11 (52.4)	10 (47.6)
Р		0.548	0.401	0.597	0.398	0.497
Peritoneum						
Yes	5	5 (100)	4 (80.0)	2 (40.0)	1 (20.0)	1 (20.0)
No	26	21 (80.8)	20 (76.9)	16 (61.5)	14 (53.8)	13 (50.0)
Р		0.699	0.688	0.341	0.186	0.233
HER2 status*						
Positive	13	12 (92.3)	10 (76.9)	8 (61.5)	6 (46.2)	5 (38.5)
Negative	16	15 (93.8)	12 (75.0)	9 (56.3)	8 (50.0)	8 (50.0)
Р		0.727	0.583	0.714	0.978	0.816

 Table 1 Correlation of CTCs in pre-treatment patients with clinical characteristics (N=31)

CTCs, circulating tumor cells; HER2, human epidermal growth factor receptor 2; \*, two patients had unavailable HER2 status information.

# Correlation of multiploidy of chromosome 8 in CTCs with PFS and OS

and multiploid chromosome 8 CTCs (hereafter referred to triploid and multiploid CTCs respectively) with patient prognosis was investigated.

Our previous work indicated that an euploidy of chromosome 8 in CTCs correlated with therapeutic response in AGC patients. Triploid chromosome 8 CTCs were constantly chemoresistant, whereas multiploid CTCs may develop acquired resistance after chemotherapy (12). In the current study, correlation of enumeration of triploid Based on estimated ROC curves and Youden index,  $\geq 1$ triploid (data not shown) and  $\geq 2$  multiploid (*Supplementary Figure S2*) CTCs were selected as an unfavorable threshold in this study. Although neither pre- nor post-treatment triploid CTCs were correlated with PFS and OS (data not shown), patients with  $\geq 2$  post-therapy multiploid CTCs

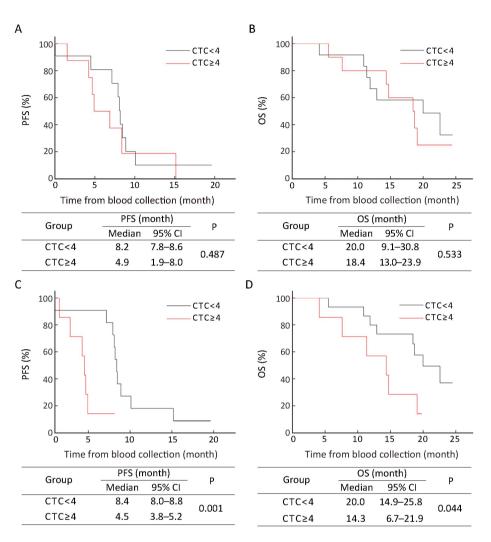


Figure 1 Kaplan-Meier curves for progression-free survival (PFS) (A, C) and overall survival (OS) (B, D) in patients with  $\geq$ 4 and <4 CTCs either pre- (A, B) or post-therapy (C, D).

had significantly shorter median PFS and OS compared with those with <2 post-therapy multiploid CTCs [4.5 (95% CI, 3.8–5.2) months vs. 8.4 (95% CI, 7.8–9.0) months, P=0.011; 11.4 (95% CI, 10.2–12.6) months vs. 22.6 (95% CI, 16.8–28.2) months, P=0.001]. No significant correlation of pre-treatment multiploid CTCs with PFS and OS was found (*Figure 2*). Six weeks after therapy, inferior PFS and OS were observed in patients with  $\geq 2$ multiploid CTCs compared with patients with <2 multiploid. Thus, unfavorable ( $\geq 2$ ) post-therapy multiploid CTCs may predict inferior PFS and OS for AGC patients.

# Variations of multiploid CTCs in response to therapy correlate with PFS and OS

To investigate the significance of multiploid CTCs,

variations of quantified multiploid CTCs before and after treatment in response to therapy were compared. Patients after 6 weeks of therapy were classified into two groups: Group 1 with the number of multiploid CTCs decreased  $\geq 10\%$  following therapy; and Group 2 with the number of multiploid CTCs increased  $\geq 10\%$ . As shown in *Figure 3A*, the median PFS of Group 2 was significantly shorter than that of Group 1 [4.5 (95% CI, 1.2–7.8) months vs. 8.3 (95% CI, 7.7–9.1) months, P=0.003]. Similar results were observed with respect to the median OS. Group 2 had inferior median OS compared to Group 1 [11.4 (95% CI, 3.8–23.0) months vs. 20.0 (95% CI, 18.0–22.0) months, P=0.004] (*Figure 3B*). Statistical analysis showed that quantitative comparison of multiploid CTCs after therapy may help evaluate therapeutic response and patient

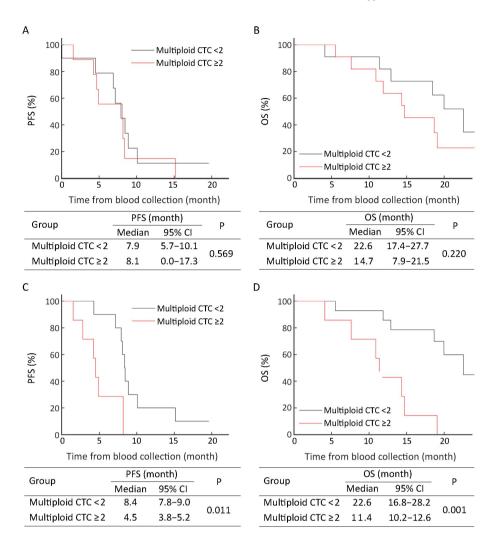


Figure 2 Correlation of multiploid CTCs in pre-treatment (A, B) and post-treatment (C, D) patients with progression-free survival (PFS) (A, C) and overall survival (OS) (B, D).

prognosis. Patients with  $\geq 10\%$  decreased multiploid CTCs after the first 2 cycles of therapy may have improved PFS and OS and those with  $\geq 10\%$  increased multiploid CTCs after the first 2 cycles of therapy may have inferior PFS and OS.

To compare the predictive value of variable multiploid CTCs with the one-site unfavorable CTCs, Cox proportional hazards regression model was used and the results showed that several unfavorable parameters were associated with inferior PFS and OS (*Figure 4*). A  $\geq 10\%$  increase of multiploid CTCs after therapy indicated the highest risk of progression and mortality. Moreover, multivariate analysis demonstrated that after adjusting for clinically significant factors (including post-therapy CTCs, post-therapy multiploid CTCs and increase of multiploid

CTCs which were significant predictors in Cox univariate analysis), only  $\geq 10\%$  increase in multiploid CTCs was an independent predictor of PFS (HR, 9.697; 95% CI, 1.582–59.444; P=0.014) and OS (HR, 6.833; 95% CI, 1.350–34.583; P=0.020). Thus, comparing multiploid chromosome 8 CTCs before and after treatment can be used to evaluate therapeutic efficacy.

#### Discussion

Clinical validity of the CTCs shed from various solid tumors including AGC has been published elsewhere (1,3,4,11). Nevertheless, clinical application of a conventional epithelial cellular adhesion molecule (EpCAM)-dependent strategy for CTC measurement is А

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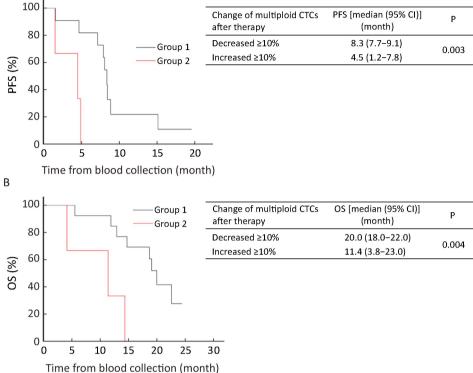


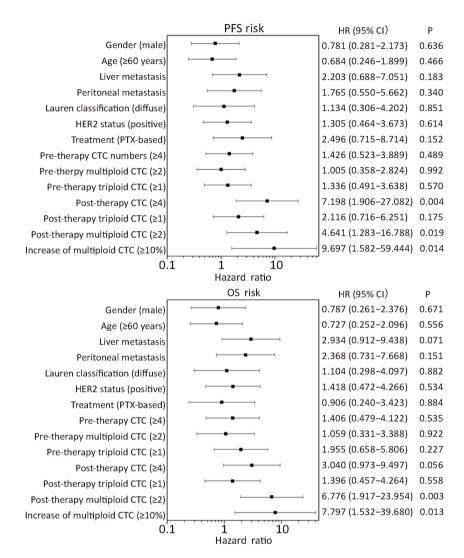
Figure 3 Correlation of variations of multiploid chromosome 8 CTCs after therapy with progression-free survival (PFS) (A) and overall survival (OS) (B). Patients are categorized into two groups according to the trends of changes of multiploid CTCs from pre-treatment to post-treatment. Group 1, multiploid CTCs are decreased  $\geq 10\%$ ; Group 2, multiploid CTCs are increased  $\geq 10\%$ .

limited due to heterogeneous expression of EpCAM or the absence of both EpCAM and cytokeratin (CK) induced by epithelial-mesenchymal transition (EMT) (4,19-21). Our previous work confirmed that EpCAM-independent SE-iFISH was better than an EpCAM-dependent strategy for measuring CTCs in AGC patients (12). Here, we report that most patients had  $\geq$ 1 CTC per 7.5 mL as measured by SE-iFISH. Post-therapy patients with  $\geq$ 4 CTCs had worse PFS and OS compared with those with <4 CTCs. So, post-therapy CTC counts may indicate the prognosis of AGC.

Triploid CTCs measured before or after treatment did not correlate with PFS and OS, perhaps due to the intrinsic chemoresistance of trisomy CTC, causing an unchanged number of triploid CTCs after therapy (12). However, unfavorable ( $\geq 2$  per 7.5 mL) multiploid CTCs in posttherapy patients was significantly associated with inferior PFS and OS. Moreover, patients with  $\geq 10\%$  decrease in multiploid CTCs after the first 6 weeks of therapy may have improved PFS and OS, whereas those with  $\geq 10\%$ increase in multiploid CTCs had inferior PFS and OS.

Compared with one-site unfavorable total CTCs and the subtype of multiploid chromosome 8 CTCs, a 10% increase in post-treatment multiploid CTCs had the greatest highest HR for PFS and OS, and it was the only independent prognostic value according to Cox multivariate analysis. Although the optimal cut-off value for variation of multiploid CTCs needs to be further analyzed in expanded studies, data in this study show a correlation of multiploid CTCs with acquired drug resistance (11). Also, multiploid CTC counts in post-therapy patients might predict the course of AGC as they offer a more rapid method of evaluation compared with radiographic imaging. Karvotypic characterization of enriched CTCs during therapy might replace imaging to assess therapeutic resistance, and guide the clinical treatment of cancer patients.

Molecular mechanisms of aneuploidy of CTCs and its correlation to diverse therapeutic sensitivities warrant the study. On-going next generation sequencing (NGS)



**Figure 4** Forest plots displaying Cox univariate analysis of variables predicting to progression-free survival (PFS) and overall survival (OS). Variables include clinicopathological characteristics, numbers of total CTCs, subtypes of triploid and multiploid chromosome 8 CTCs in pre- and post-treatment patients, as well as variation of multiploid CTCs after therapy.

analysis of a single CTC may reveal genomic landscapes of CTC subtypes possessing distinct clinical significance (22-25), which should facilitate the elucidation of evolutionary mechanisms involved in therapy resistance.

#### Conclusions

Unfavorable  $(\geq 4)$  post-therapy CTCs may predict inferior prognosis in AGC patients. Previous work showed that triploid CTCs in AGC patients were constantly resistant to chemotherapy, and multiploid CTCs developed an acquired chemoresistance (11). The data from this study suggested that unfavorable  $(\geq 2)$  multiploid CTCs in posttherapy patients were significantly associated with inferior PFS and OS. Thus, CTCs may be used to assess therapeutic efficacy and predict inferior prognosis.

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# Footnote

*Conflicts of Interest*: The authors have no conflicts of interest to declare.

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#### Li et al. Subtype of CTC and its correlation to prognosis

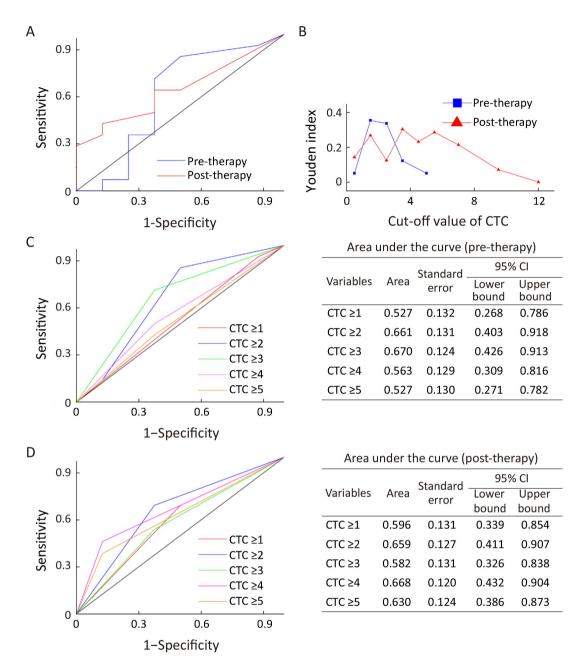
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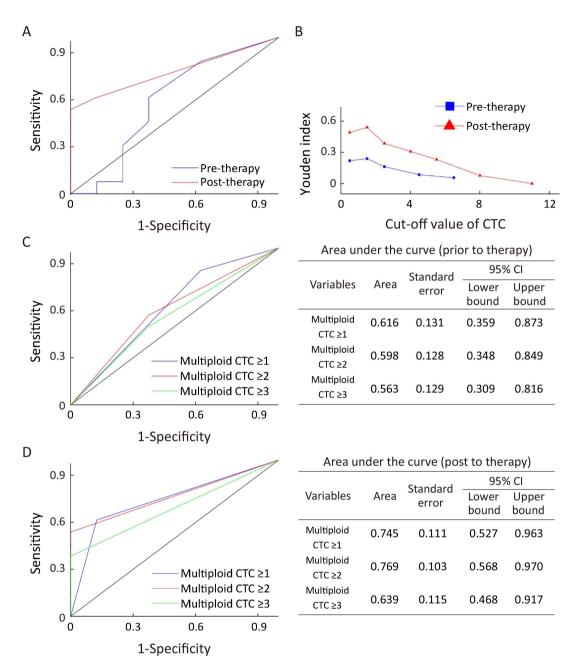
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**Figure S1** Selection of the optimal cut-off value of CTCs for predicting overall survival (OS). (A) Receiver operating characteristic (ROC) curves for pre- and post-therapy CTCs predicting OS; (B) Youden index for different cut-off values of CTCs based on the ROC curves in (A) [Youden index=sensitivity+specificity-1]; (C, D) ROC areas under the curve (AUCs) for CTC thresholds of 1 to 5 in pre- (C) and post-treatment (D) patients. The cut-off points of 1 and 2 for pre-therapy CTCs and 1 to 5 for post-therapy CTCs all exhibit larger Youden index. When the AUCs for CTC thresholds of 1 to 5 are compared, a cut-off value of 4 for post-therapy CTCs maximizes the AUC area (0.668 for post-therapy CTCs). Therefore,  $\geq$ 4 CTCs per 7.5 mL is selected as the unfavorable threshold.



**Figure S2** Selection of the optimal cut-off value of multiploid CTCs for overall survival (OS). (A) Receiver operating characteristic (ROC) curves for pre- and post-therapy multiploid CTCs predicting OS; (B) Youden index for different cut-off values of multiploid CTCs based on the ROC curves in (A); (C, D) ROC areas under the curve (AUCs) for multiploid CTC thresholds of 1 to 3 in pre- (C) and post-treatment patients (D). The threshold of  $\geq$ 2 multiploid CTCs is selected as unfavorable because of the maximal AUC area (0.769 for post-therapy CTCs).