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ORIGINAL ARTICLE

Circulating tumor cells as an independent prognostic factor in advanced colorectal cancer: a retrospective study in 121 patients

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Abstract

Purpose This study aimed to evaluate the prognostic value of circulating tumor cells (CTCs) in advanced colorectal cancer (CRC) patients during chemotherapy course.

Methods From January 2016 to September 2017, the clinicopathological variables, such as gender, age, tumor location, tumor de-differentiation, depth of invasion, lymphatic invasion, distant metastasis, TNM stage, CTCs enumeration during 2-6 cycles of chemotherapy, and serum carcinoembryonic antigen (CEA) level during the same period, of 121 newly acquired and histopathologically confirmed CRC patients were collected from the Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine. All patients were followed up for survival until the end of November 2018. Statistical analysis focused on the associations between CTCs counts and clinicopathological variables. Overall survival (OS) and progression-free survival (PFS) among different prognostic factors were calculated using the Kaplan–Meier method, and the differences between the survival curves were compared by using the log-rank test. Factors of prognostic significance were investigated with the multivariate Cox regression analysis.

Results Here, 71 of 121 patients were CTC-positive, in which CTC-positive rate was positively correlated with the depth of invasion, lymphatic invasion, distant metastasis, TNM stage, and serum CEA level ($P < 0.05$ for all). However, no significant difference was found between CTC-positive and other clinicopathological variables ($P > 0.05$ for all), such as gender, age, tumor location, and tumor de-differentiation. CTCs counts gradually increased with the advancement of depth of invasion ($P = 0.002$), lymphatic invasion ($P = 0.004$), distant metastasis ($P = 0.007$), TNM stage ($P = 0.001$), serum CEA level ($P = 0.001$), and decreased tumor de-differentiation ($P = 0.011$). Furthermore, the Kaplan–Meier survival curves showed that patients with CTC-positive had a significantly unfavorable PFS (14 vs. 23 months, $P = 0.001$) and OS (18 vs. 25 months, $P = 0.003$). The multivariate Cox regression analyses revealed that the presence of CTCs during chemotherapy was an independent factor for unfavorable PFS (hazard ratio (HR) 2.682, $P = 0.017$, 95% confidence interval (CI) 1.193–6.029) and OS (HR 2.790, $P = 0.048$, 95% CI 1.010–7.705) in advanced CRC patients.

Conclusions This study provided an evidence that the presence of CTCs may be valuable for predicting survival outcome, and CTCs was associated with unfavorable survival in advanced CRC patients during chemotherapy.

Keywords Circulating tumor cells · Colorectal cancer · Chemotherapy · Predictive value · Survival

Introduction

Colorectal cancer (CRC) is the third most common tumor in men and women worldwide [1]. Due to the lack of effective methods for early diagnosis, in which 15–20% of patients with CRC were diagnosed with liver metastasis at the first time of diagnosis, and the remaining 60% would have recurrence or metastasis during subsequent treatment [2]. These poor outcomes are associated with the fact that the majority of patients are diagnosed at advanced stage (III/IV stage), with distant

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metastasis, lymphatic dissemination, and recurrence [3]. Although tremendous efforts at diagnosis and treatment have been made for advanced CRC patients, several patients benefit from chemotherapy to some extent. Overall survival (OS) is currently approaching 30 months for the patients treated with the combination of cytotoxic chemotherapy and biologic agents, targeting angiogenesis and the epidermal growth factor receptor (EGFR), which inhibit signal transduction pathways [4–7]. However, some patients who did not respond to these systemic treatments still have a dismal outcome. Indeed, approximately 28–44% of patients are ineffective to the first-line treatment with doublet chemotherapy plus either cetuximab or bevacizumab, and about 50% of these patients have postoperative recurrence or distant metastasis [8]. In addition, excessive chemotherapy leads to various side effects that seriously decrease the life quality of patients [9]. Therefore, there is of great importance to develop a more accurate method to identify patients with poor prognosis or with rapid disease progression, in which identification of more reliable predictive biomarkers to chemotherapy make them to be directed towards the most effective treatment for clinical precise management of CRC [10].

Circulating tumor cells (CTCs) are heterogeneous tumor cells that have escaped from the primary or metastatic tumor site and gained access to the vasculature through intravasation [11]. In recent years, several retrospective and prospective studies have indicated CTCs counts could be used as a strong and independent prognostic or predictive biomarker for efficient chemotherapy in breast, prostate, and CRC patients [12–15]. Romiti et al. detected CTCs of CRC patients 1 month after the first cycle of chemotherapy, and reported that CTCs can predict the prognosis [16]. Another clinical trial demonstrated that CTCs could be as a feasible and non-invasive approach to assess the current molecular characteristics of prostate cancer. Similar data were reported, and also demonstrated that CTCs could be used as predictive biomarkers for designing individually tailored therapy against CRC [17]. In general, CTCs have been widely taken as a non-invasive "liquid biopsy" of cancer into account. The counts and characteristic analysis of CTCs have shown their great application potential in both prognostication and treatment of CRC patients.

Materials and methods

Patients and data collection

From January 2016 to September 2017, 121 patients with advanced CRC were recruited in this retrospective study from the Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China).

The inclusion criteria were as follows: (1) age > 18 years old; (2) ALL patients obtained confirmed pathological diagnosis and complete clinicopathological variables, including gender, age, tumor location, tumor de-differentiation, depth of invasion, lymphatic invasion, distant metastasis, TNM stage, CTCs counts, and CEA level during the same period; (3) The peripheral blood samples for detection of CTCs from patients were collected during two–six cycles of chemotherapy; (4) patients received 5-fluorouracil (5-FU) or capecitabine-based chemotherapy (with or without targeted therapy); and (5) patients voluntarily joined the study and signed the informed consent form, with a good compliance and follow-up.

The exclusion criteria were as follows: (1) evidence of the second primary tumor; (2) previous radiotherapy and biological immunotherapy; and (3) with other serious diseases which may affect the prognosis.

The TNM stage of CRC was classified according to American Joint Committee on Cancer (AJCC, the eighth edition, 2017). All patients signed the informed consent form. The study was approved by the Ethics Committee of the Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

OS is defined as the time from blood collection for detection of CTCs to death, and PFS is defined as the period from blood collection for detection of CTCs until disease progression during or after chemotherapy course. Tumor response was evaluated by computed tomography (CT) scanning or magnetic resonance imaging (MRI) according to the response evaluation criteria in solid tumors (RECIST 1.1) every 8–12 weeks during or after chemotherapy course. All patients were followed up until death or the end of November 2018.

CTCs detection

Here, 5 ml peripheral blood sample of advanced CRC patients was collected during treatment. The CTCs detection was performed by the Cyttel method, and immunomagnetic bead-negative enrichment technique combined with immunofluorescence in situ hybridization technologies (imFISH) method was adopted to find out CTCs counts. The protocol of CTCs detection was as follows: First, leukocytes were specifically removed by lysing red blood cells and immunomagnetic beads, and the rarest cells in peripheral blood samples were retained. Secondly, the procedure was performed by using CD45-negative and chromosome polyploidy characteristics in tumor cells, in addition to CD45 staining of the remaining rare cells and imFISH of chromosomes 8 and 17 H1 fluorescent probes, combined with DAPI staining. It can avoid the epithelial-mesenchymal transition on epithelial cell adhesion molecules and effectively improve the detection rate of CTCs. In this study, all 121 patients underwent CTC test. Besides, CTCs ≥ 3 were considered to be CTC-positive, and correspondingly CTC < 3 were considered to be CTC-negative. According to the criteria for CTC interpretation, among 121

CRC patients, 71 cases were classified as CTC-positive group, while 50 cases were classified as CTC-negative group. The positive detection rate was 58.7% (71/121) as well.

Statistical analysis

All the statistical analyses were performed using SPSS 22.0 software (IBM, Armonk, NY, USA). Pearson's chi-square test was used to analyze the differences in the CTC-positive and CTC-negative groups. The Mann–Whitney *U* test or Kruskal–Wallis *H* test was used to compare the CTCs counts among different groups, as appropriate. OS and PFS among different prognostic categories were calculated using the Kaplan–Meier method, and significant differences between the survival curves were compared by using the log-rank test. Based on the univariate analyses of all variables, only variables with a *P* value < 0.05 were included for analysis in the multivariate Cox regression model to investigate the influence of independent factors on OS and PFS. All statistical analyses were 2-sided, and a *P* value < 0.05 was statistically considered significant. Graphical plots were generated using GraphPad prism 7.0 software (GraphPad Software, La Jolla, CA, USA).

Data availability The datasets used during the present study are available from the corresponding author upon reasonable request.

Results

Patients' characteristics

The clinical characteristics of 121 advanced CRC patients is shown in Table 1. Of all the cases, the median age was 65 years (range 34–89 years), 78 (64.5%) were males, and 43 (35.5%) were females. The primary tumors were found from colon and rectum in 76 and 45 patients, respectively. In terms of histological differentiation subtypes, 58 patients were poorly differentiated adenocarcinoma, followed by 63 patients with moderately and well-differentiated subtypes. According to the TNM stage, there were 13 cases with stage IIIa, 29 cases with stage IIIb, 29 cases with stage IIIc, 26 cases with stage IVa, and 24 cases with stage IVb. All the patients received 5-FU or capecitabine-based chemotherapy (with or without targeted therapy). None of the patients with TNM stage IV underwent metastases resection before chemotherapy.

Table 1 The clinicopathologic variables of 121 advanced CRC patients

Variables	No.	%
Total	121	
Age, years		
≤ 60	40	33.1
> 60	81	66.9
Gender		
Male	78	64.5
Female	43	35.5
Primary tumor site		
Colon	76	62.8
Rectum	45	37.2
Tumor de-differentiation		
Poor	58	47.9
Well to moderate	63	52.1
Depth of invasion		
T1	5	4.1
T2	20	16.5
T3	32	26.4
T4a	34	28.1
T4b	30	24.8
Lymphatic invasion		
N1	43	35.5
N2a	27	22.3
N2b	51	42.2
Distant metastasis		
M0	71	58.7
M1a	26	21.5
M1b	24	19.8
TNM stage		
III a	13	10.7
III b	29	24.0
III c	29	24.0
IV a	26	21.5
IV b	24	19.8
CEA (ng/ml)		
< 5	79	65.3
≥ 5	42	34.7
CTC, number/5 mL		
< 3	50	41.3
≥ 3	71	58.7

The relationship between CTCs and clinicopathological variables in advanced CRC patients with treatment

The association of CTC-positive rate with the clinicopathological variables is shown in Table 2. CTC-positive rate was positively correlated with depth of invasion, lymphatic invasion, distant metastasis, TNM stage, and serum CEA level ($P < 0.05$ for all). By contrast, no significant association was found between CTC-positive rate and other clinicopathological variables ($P > 0.05$ for all), such as gender, age, tumor location, and tumor de-differentiation.

Furthermore, CTC counts for all 121 advanced CRC patients ranged from 0 to 30 (mean ± standard error (SE) 5.19 ±

Table 2 The association of CTC-positive rate with the clinicopathological variables

Variables	No.	CTCs Positive	Negative	X^2 value	P
Age, years					
≤ 60	43	26	17	0.088	0.767
> 60	78	45	33		
Gender					
Male	78	47	31	0.226	0.635
Female	43	24	19		
Primary tumor site					
Colon	76	45	31	0.024	0.877
Rectum	45	26	19		
Tumor de-differentiation					
Poor	58	39	19	3.369	0.066
Well to moderate	63	32	31		
Depth of invasion					
T1	5	2	3	18.512	*0.001
T2	20	5	15		
T3	32	17	15		
T4a	34	22	12		
T4b	30	25	5		
Lymphatic invasion					
N1	43	19	24	6.712	*0.035
N2a	27	16	11		
N2b	51	36	15		
Distant metastasis					
M0	71	36	35	4.505	*0.034
M1	50	35	15		
TNM stage					
III a	13	3	10	11.306	*0.023
III b	29	14	15		
III c	29	19	10		
IV a	26	18	8		
IV b	24	17	7		
CEA (ng/ml)					
< 5	79	39	40	8.137	*0.004
≥ 5	42	32	10		

* $P < 0.05$

5.50). In addition, there was a significant increase in CTCs counts with the decrease of tumor de-differentiation, high serum CEA level, depth of invasion, lymphatic invasion, distant metastasis, and TNM stage (Fig. 1a–f).

Univariate and multivariate analyses

During the follow-up period, 40 (33.1%) patients were evaluated for disease progression, 28 (23.1%) patients died, and all died of colorectal cancer. The median follow-up time was 14 months (range 8–27 months).

The results of the univariate analysis for OS and PFS that were associated with the clinical variables are presented in Table 3. Based on the univariate analysis, 6 of the 11 preselected variables, including high serum CEA level, tumor de-differentiation, lymphatic invasion, distant metastasis, TNM stage, and CTC-positive were closely associated with unfavorable PFS (Table 3). However, the multivariate Cox regression analysis further revealed that the CTC-positive, lymphatic invasion, and distant metastasis were independent prognostic factors for unfavorable PFS (Table 4). Besides, the Kaplan–Meier survival curves showed that advanced CRC patients with CTC-positive had a significantly unfavorable PFS (14 vs. 23 months, $P = 0.001$) (Fig. 2a).

Regarding the OS of all the patients, 7 of the 11 preselected variables, including high serum CEA level, lymphatic invasion, distant metastasis, TNM stage, tumor de-differentiation, tumor location, and CTC-positive were significant variables in the univariate analysis (Table 3). However, the multivariate Cox regression analysis further revealed that CTCs, lymphatic invasion, and distant metastasis were independent prognostic factors for unfavorable OS (Table 4). Additionally, the Kaplan–Meier survival curves showed that advanced CRC patients with CTC-positive had a significantly unfavorable OS (18 vs. 25 months, $P = 0.003$) (Fig. 2b).

Discussion

CRC is one of the most common malignant tumors. Recurrence and metastasis are important reasons, affecting the prognosis of CRC. Micrometastasis is an early event of distant metastasis of the tumor. How to detect the recurrence and metastasis of CRC earlier, how to quickly assess the need for adjuvant chemotherapy and chemotherapeutic drug sensitivity for CRC patients, and how to improve the treatment plan in time before the disease progression have become the main challenges during CRC treatment. In recent years, CTCs have been considered as a liquid biopsy for all solid tumors. The technique of isolation and analyzation of CTCs has been shown to be a highly repeatable, and minimally invasive technique for diagnosis, prognosis, and real-time monitoring of drug resistance for cancer treatment [18, 19]. Previous studies have indicated that CTCs are closely associated with the biological behavior of tumor. The existence of CTCs may be a basis for distant metastasis [20, 21]. Besides, there is growing evidence that CTCs might become as a reliable clinical marker for diagnosis and prognosis [22–24].

Since CTCs are derived from tumor tissue, the CTCs counts can reflect the tumor burden in patients to some extent. A number of studies have indicated that CTCs can indicate clinical features, such as tumor invasion and lymph node metastasis in patients with CRC. Our study revealed the relationship between CTCs and the clinicopathological characteristics of

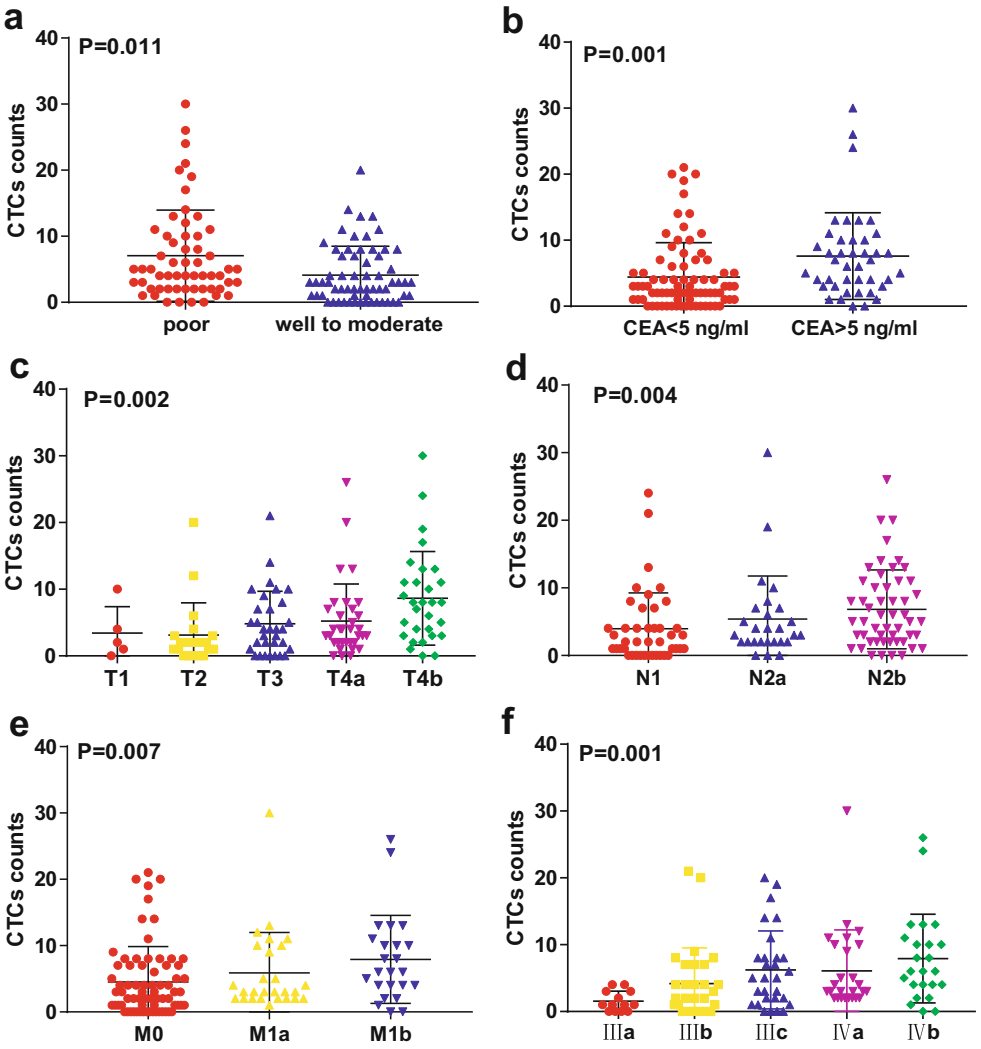

Fig. 1 The relationship between CTCs counts and clinicopathological characteristics in advanced CRC patients. **a** The correlation of CTCs counts with tumor de-differentiation (poor vs. well to moderate, $P = 0.011$). **b** The correlation of CTCs counts with serum CEA level ($P = 0.001$). **c** The correlation of CTCs counts with depth of invasion (T1 vs. T4b, $P = 0.053$; T2 vs. T4b, $P < 0.001$; T3 vs. T4b, $P = 0.013$; T4a vs. T4b, $P = 0.026$). **d** The correlation of CTCs counts with lymphatic invasion (N1 vs. N2a, $P = 0.395$; N1 vs. N2b, $P = 0.010$). **e** The correlation of CTCs counts with distant metastasis (M0 vs. M1a, $P = 0.242$; M0 vs. M1b, $P = 0.014$). **f** The correlation of CTCs counts with TNM stage (IIIa vs. IVb, $P < 0.001$; IIIb vs. IVb, $P = 0.005$; IIIc vs. IVb, $P = 0.225$; IVa vs. IVb, $P = 0.319$)

CRC. It was disclosed that CTC-positive rate was positively and dependently correlated with the pathological stage of tumor. Further refinement of the TNM stage revealed that CTCs were also associated with primary tumor depth of invasion, lymphatic invasion, and distant metastasis. In addition, CTC counts increased along with tumor pathological stage, primary tumor depth of invasion, lymphatic invasion, and distant metastasis. These results suggest that both CTC-positive rates and CTCs counts have positively correlated with lymphatic invasion and the appearance of distant metastases, which make the use of CTCs feasible to evaluate lymphatic invasion and distant metastasis. CTCs are the root cause of tumor metastasis, and their relationship with lymphatic invasion may be similar to tumor angiogenesis and lymphangiogenesis, and are associated with the regulation of vascular endothelial growth factor (VEGF) and its receptors [25, 26]. Moreover, serum CEA values were higher in CTC-positive patients than those in CTC-negative patients. To our knowledge, serum CEA level was used as a specific marker of intestinal tumors in several prognostic scoring systems. A positive correlation between CTC-positive rate and serum CEA level indicates that CTCs could be associated with a higher risk of tumor progression. In addition, the study found determination of CTCs is more significant than post-chemotherapy CEA level in predicting survival outcome. The above-mentioned results are consistent with the results of previous studies [27, 28]. In brief, CTCs could play an important role in predicting the processes of tumor progression and metastasis development, which could be universally applied in cancer screening.

Recurrence and metastasis are important factors, affecting the prognosis of CRC patients. In addition to reflecting the aggressiveness of the disease, CTC counts represent as a potential complementary method to predict the outcome of CRC patients. The study also found that the presence of CTCs was associated with poor survival in advanced CRC patients during chemotherapy course, and the multivariate Cox regression analysis further identified that CTCs was an independent prognostic factor for OS and PFS, which was consistent with

Table 3 The univariate analyses for OS and PFS

Variables	PFS		OS	
	Mean (months)	Log-rank P value	Mean (months)	Log-rank *P* value
Age, year				
≤60	16.58	0.094	19.63	0.091
>60	20.22		22.67	
Gender				
male	19.50	0.526	22.26	0.372
female	17.61		19.91	
Primary tumour site				
colon	20.04	0.211	23.13	0.036
rectum	16.78		18.60	
Histology differentiation				
poor	16.76	*0.032	19.58	*0.017
well to moderate	21.16		23.61	
Depth of invasion				
T1	20.71	0.076	22.48	0.209
T2	22.09		23.91	
T3	18.03		20.65	
T4	14.00		17.72	
Lymphatic invasion				
N1	23.05	***<0.001	24.45	***<0.001
N2a	20.97		24.75	
N2b	13.88		16.96	
Distant metastasis				
M0	23.40	***<0.001	25.27	***<0.001
M1a	14.42		19.48	
M1b	9.58		11.51	
TNM stage				
III stage	23.40	***<0.001	25.27	***<0.001
IV stage	12.25		15.99	
CEA (ng/ml)				
<5	22.08	***<0.001	24.10	***<0.001
≥5	10.83		16.02	
CTC, number/5mL				
<3	23.16	*0.001	24.88	*0.003
≥3	13.99		18.02	

* $P < 0.05$, *** $P < 0.001$

previous studies [29–31]. Galizia et al. recently reported that 75% of 69 CRC patients undergoing radical surgery with more postoperative CTCs counts experienced tumor recurrence. Thus, postoperative CTCs counts could be used as an early indicator for undetectable metastasis [32].

According to previous studies, the relationship between CTCs detection and prognosis during treatment was more significant and convincing compared with the CTCs at baseline [13, 14, 33]. That was the reason why we recruited CTCs counts of advanced CRC patients with two–six cycles of chemotherapy course to predict outcomes in the present study. In addition, CTCs were divided into three phenotypes based on differences in cell-surface biomarker expression levels: epithelial tumor cells, tumor cells undergoing epithelial-to-mesenchymal transition (EMT), and tumor stem cells. The current standard method is on the basis of CellSearch System approved by the United States Food and Drug Administration (FDA), utilizing ferrofluids loaded with an EpCAM antibody to capture the epithelial CTCs, but not CTCs that have undergone EMT [34, 35]. Notably, it was suggested that not only epithelial, but also mesenchymal markers in cell-surface of CTCs could offer valuable assistance for metastasis evaluation

Table 4 The multivariate analyses for PFS and OS

Variables	PFS			OS		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Lymphatic invasion			*0.015			*0.041
N1		reference			reference	
N2a	1.704	0.517-5.611	0.381	1.222	0.223-6.708	0.817
N2b	3.652	1.381-9.654	*0.009	3.943	1.046-14.859	*0.043
Distant metastasis			*0.003			*0.003
M0		reference			reference	
M1a	2.598	1.082-6.236	0.033	2.177	0.673-7.044	0.194
M1b	5.200	2.010-13.452	*0.001	7.295	2.237-23.787	*0.001
CTC, number/5mL			*0.017			*0.048
<3		Reference			Reference	
≥3	2.682	1.193-6.029	*0.017	2.790	1.010-7.705	*0.048

* $P < 0.05$

and tumor staging in CRC patients. In this study, phenotype of CTCs was measured by immunomagnetic bead-negative enrichment technique combined with imFISH. It avoids the EMT through the epithelial cell adhesion molecules and effectively improves the detection rate of CTCs.

To the best of our knowledge, this is a much larger retrospective study using a negative selection method for CTCs isolation to evaluate its prognostic value in advanced CRC patients. Based on our results, CTCs might be widely available to be used as a prognostic and predictive factor in advanced CRC patients who underwent chemotherapy. However, there are still several inherent limitations. First of all, as our results are based on a single-center retrospective study, with a relatively small sample size, the statistical impact of clinical variables on survival might be the modest. More samples with large size are required for stratified analyses to improve the treatment, in addition to provide more accurate information for evidence-based medicine. Another limitation is the lack of dynamic enumeration of CTCs. The dynamic enumeration of CTCs could better reflect the aggressiveness and prognosis of the disease than the isolated enumeration of CTCs [36]. However, it was difficult for us to compare the baseline (pretreatment) level of CTCs with the level at follow-up. Cause treatment interventions (including surgery and chemotherapy), as we know, can reduce CTCs counts, even to negative.

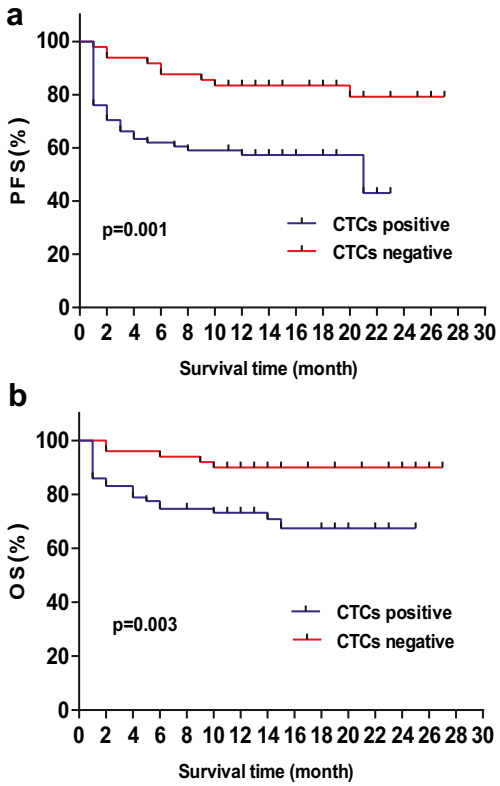

Fig. 2 The Kaplan–Meier progression-free survival (**a**) and overall survival (**b**) curves for stratified patients according to the CTCs counts

In conclusion, CTCs may serve as an independent prognostic factor in terms of PFS and OS in advanced CRC patients during chemotherapy course. Therefore, including the detection of CTCs in the blood in the future treatment guidelines is highly recommended. However, the significant role of CTCs for CRC patients should be further validated in large-scale prospective trials.

Authors' contributions Bin Jiang and Yanjie Zhang designed the study. Haihua Yuan, Lili Wang, and Feng Liu collected the samples. Wenying Zhang, Jiongyi Wang, Meiling Wang, and Xiaohua Hu followed up the OS and PFS of the patients. Haihua Yuan and Lili Wang analyzed the data, and Lili Wang performed the statistical analyses. Lili Wang wrote the manuscript with Haihua Yuan's assistance, and final version of the manuscript was approved by all the authors.

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Compliance with ethical standards

Ethics approval and consent to participate The study was approved by the Ethics Committee of the Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Patient consent for publication The authors declare that the patients have provided written informed consent for the purpose of publication.

Competing interest The authors declare that they have no conflict of interest.

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