

# Significant diagnostic value of circulating tumour cells in colorectal cancer

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**Abstract.** Circulating tumour cells (CTCs) have potential utility in various clinical applications for cancer management. The present study focused on evaluating the diagnostic role of CTCs in colorectal cancer (CRC). A total of 89 blood samples from 59 patients diagnosed with CRC and 30 healthy individuals were collected for CTC detection. The Cytel method is an improved CTC detection strategy, which combines negative enrichment with immunofluorescence and fluorescence *in situ* hybridization. This method effectively detected a significant increase in total CTCs in patients with CRC (49/59) compared with those in healthy controls (3/30). A cut-off value of 2 CTCs/3.2 ml blood yielded a sensitivity of 83.05% and a specificity of 100%. Additionally, three traditional serum tumour markers, namely carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA19-9) and CA72-4, were examined by immunoassays. The diagnostic sensitivity of CTCs was much higher than that of CEA, CA19-9 and CA72-4 alone or in combination, particularly in patients with early stage CRC. The combined sensitivity of CTCs and CEA reached 91.53%, which was only slightly lower than the sensitivity of all four markers combined (CTCs + CEA + CA19-9 + CA72-4). CTCs with aneuploidy of chromosome 7 or 8 were carefully distinguished, and the associations among different types of CTCs, clinicopathological characteristics and overall survival were statistically analysed. Total CTCs were revealed to be significantly associated with tumour differentiation and nerve invasion. CTCs were more likely to be detected in poorly differentiated CRC tumours than in well- and

moderately-differentiated tumours ( $P=0.026$ ). Furthermore, to the best of our knowledge, the present study was the first to report that CTCs with multiploidy of chromosome 7 were significantly associated with TNM stage. These CTCs exhibited a high chance of being identified in the peripheral blood of patients with late-stage CRC (stage III-IV;  $P=0.031$ ). The present study suggests that the combination of CTCs and CEA may serve as an effective potential diagnostic and prognostic indicator in patients with CRC. Detection of CTCs with aneuploidy may have increased specificity in predicting highly malignant and invasive tumours in CRC management.

## Introduction

Colorectal cancer (CRC) is the third most common type of cancer in both men and women, and it is the fourth most common cause of cancer-associated mortality in the United States (1). In 2018, Siegel (2) reported that clinical outcomes of American patients with CRC vary depending on the stage of cancer at diagnosis, with a 5-year survival rate of ~90% for localized disease, 71% for regional disease and only 14% for distantly metastatic CRC (mCRC). Therefore, detecting CRC at an early stage is crucial to reduce the cancer-specific mortality rate. Currently, diagnostic imaging examinations, including CT and MRI, have the ability to depict the size and location of CRC, but have limited potential in detecting small primary tumours and metastatic lesions (3). Colonoscopy and biopsy are considered the gold standard for confirming the diagnosis of CRC. However, biopsy specimens may fail to provide a definitive diagnosis if the viable tumour tissue is difficult to obtain or if the tissue samples are extensively ulcerated or necrotic (4). Additionally, several serum tumour markers, including carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA19-9) and cancer antigen 72-4 (CA72-4), are widely used for the screening, diagnosis and postoperative surveillance of gastrointestinal cancer, but have insufficient sensitivity and specificity (5). In addition, patients with CRC diagnosed in the early stages often receive surgery to remove the original tumour and the nearby lymph nodes, yet 20-30% of these patients suffer from recurrence or metastasis within 5 years of radical resection, indicating the presence of minimal residual disease (6). Consequently, developing a less invasive and reliable method for the early diagnosis and dynamic management of CRC is urgently required.

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Circulating tumour cells (CTCs) are tumour cells that are shed from the primary tumour and metastatic foci, and enter the peripheral bloodstream (7); they can be classified into signal CTCs and CTC clusters (8). Unlike the conventional theory that the metastatic dissemination of cancer cells represents the final stage of a deteriorating process, CTCs often disseminate at the beginning during the process of tumorigenesis, with some of them invading distant organs and eventually developing into overt metastatic lesions (9). Therefore, the detection of CTCs in the circulation may represent a feasible way to improve the early diagnosis and treatment of patients with CRC prior to metastasis. Clinical studies have suggested that CTCs are significantly associated with poor progression-free survival (PFS) and overall survival (OS) time in patients with CRC. For example, Bork *et al* (10) revealed that >1 CTC per 7.5 ml blood in the blood was significantly associated with worse OS time (38.4 months vs. 49.8 months;  $P < 0.001$ ) in non-metastatic patients with CRC (UICC I-III), as well as in the complete cohort (33.6 months vs. 48.4 months;  $P < 0.001$ ), compared with non-detected group. Furthermore, Cohen *et al* (11) detected CTCs from 7.5 ml blood of 430 patients with metastatic CRC. It suggested that patients with >3 CTCs had shorter PFS time (4.4 months vs. 7.8 months,  $P = 0.004$ ) and OS time (9.4 months vs. 20.6 months,  $P < 0.0001$ ) compared with those whose CTCs was <3.

Since there may only be 1 CTC in  $1 \times 10^7$  leukocytes per ml of blood, it is challenging to isolate CTCs from peripheral blood. The principles of CTC isolation involve CTC enrichment followed by detection. The former is achieved by means of physical properties of the cells, such as size, density or specific biological features, whereas the latter is commonly achieved by immunostaining and microscopy, or by PCR-based methods (12). The most frequently used CTC detection technology reported in these studies is the CellSearch<sup>®</sup> system (Janssen Diagnostics). This system enriches CTCs using ferromagnetic beads coated with antibodies that target epithelial cell adhesion molecule (EpCAM), and defines CTCs according to their morphological characteristics, positive expression of cytokeratin and absence of CD45 (also known as leukocyte common antigen). However, certain CTCs may lose epithelial cell markers during the process of epithelial-to-mesenchymal transition, resulting in a reduced positive rate and accuracy of the CellSearch<sup>®</sup> system (13). The advantage of the Cytel method (14) is that the enrichment of CTCs does not rely on the expression of EpCAM, and the enriched cells can be used for subsequent experiments, including cell culture and other tests. The Cytel method involves a leukocyte depletion mechanism. After collecting a peripheral blood sample, erythrocytes can be removed by hypotonic haemolysis. Since all leukocytes express CD45, these can be removed using anti-CD45 antibody-conjugated magnetic beads.

Abnormal chromosome numbers (aneuploidy) are invariably found in the pleomorphic cells of malignant tumours and have been recognized as a common feature of cancer. This type of somatic copy number alteration has been proposed to drive tumorigenesis (15). Aneuploidy of chromosomes 7 and 8 is commonly observed in patients with CRC, with a high frequency of numerical abnormalities of the entire chromosome 7, as well as loss, gain or amplification of specific regions of chromosome 8 in primary CRCs with associated

metastases (16). Detecting aneuploidy in peripheral blood cells may represent a novel approach for CTC detection, and assessing aneuploidy of chromosomes 7 and 8 at diagnosis may be of great clinical significance in patients with CRC. Therefore, the Cytel method uses immunofluorescence and fluorescence *in situ* hybridization (imFISH) on the remaining cells, using DNA probes for chromosome 7 (CEP7), chromosome 8 (CEP8) and human CD45. Only cells that are CD45-negative and that emit signals (>2) of CEP7 or CEP8 are recognized as CTCs. The Cytel method may allow the preservation of the rare karyocytes in the peripheral blood. By combining the detection of multiple molecular markers and abnormal chromosome alterations in cancer cells, the detection rate and accuracy of diagnostic tests may be improved.

In the present study, a total of 89 blood samples from 59 patients diagnosed with CRC and 30 healthy individuals were collected for single CTC identification using the Cytel method. Subsequently, the diagnostic sensitivities of CTCs and serum tumour markers (CEA, CA19-9 and CA72-4) were compared to assess the efficacy of CTCs in detecting CRC. Furthermore, the associations between total and aneuploid CTCs and the clinicopathological characteristics of patients with CRC were explored.

## Materials and methods

**Subjects and blood sample collection.** A total of 59 patients with newly diagnosed CRC were enrolled in the present study at Beijing Shijitan Hospital (Beijing, China) between July 2016 and August 2017. These patients had no history of any other malignancies during the previous 5 years. Blood samples were collected at the diagnosis of CRC. Patients received standard surgical resection of tumours and other adjuvant therapy according to the National Comprehensive Cancer Network (NCCN) guidelines for CRC (17). Patients with stage IV CRC also received surgery due to bowel obstruction or intestinal haemorrhage. The 59 patients with CRC were followed up until December 2018 (primary endpoint). The medical records of these patients were carefully reviewed to obtain clinicopathological data and follow-up information. A total of 30 healthy donors, including 17 males and 13 females with an average age of 62.7 years (range, 44-84 years), were included as controls. None of the controls had tumours, a family history of cancer or other systemic diseases. Written informed consent was obtained from all subjects. The present study was approved by the Ethics Review Committee of Beijing Shijitan Hospital.

Peripheral blood (3.2 ml) was collected into acid citrate dextrose (ACD) anticoagulant tubes (Becton, Dickinson and Company) and stored at 4°C. The blood sample had to be processed for CTC detection within 24 h. To avoid bias, CTC detection and clinicopathological data collection were blindly and independently performed by different researchers.

**CTC detection using the Cytel method.** The collected blood (4 ml, including 0.8 ml of ACD) was added with PBS to 45 ml in a centrifuge tube and then centrifuged at 600 x g for 5 min at 4°C to separate cells from plasma. The supernatant was discarded, and the remaining cells were resuspended in 45 ml erythrocyte lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). The centrifuge tube was placed in a

vertical mixer to ensure the red blood cells would be fully lysed by hypotonic haemolysis, at room temperature. The aforementioned centrifugation process was repeated, and the supernatant was discarded. Residual cell particles were resuspended in 300  $\mu$ l PBS and incubated with 20  $\mu$ l anti-CD45 antibody-conjugated magnetic beads (Catalog no. 11153D, Thermo Fisher Scientific, Inc.) for 30 min at room temperature, with gentle tilting and rotation. The suspension was subsequently added to 3 ml isolation buffer (Thermo Fisher Scientific, Inc.), prior to centrifugation at 300 x g for 5 min at 4°C, and the supernatant was subsequently discarded. The remaining suspension was added with 100  $\mu$ l PBS and placed in a magnetic stand (Promega Corporation) for 2 min to separate the magnetic beads. Following centrifugation at 2,070 x g for 3 min at 4°C, the supernatant was discarded. The 100  $\mu$ l suspension was mixed with 100  $\mu$ l fixative solution (Thermo Fisher Scientific, Inc.). The solution containing CTCs was transferred onto superfrost plus slides (Thermo Fisher Scientific, Inc.) and dried overnight at 33°C, in the oven. The prepared slides were immersed in saline-sodium citrate (SSC) buffer (Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 15 min, and then dehydrated by increasing concentrations of ethanol (75, 85 and 100%) for 3 min each. A total of 10  $\mu$ l hybridization solution containing centromere DNA probes of chromosome 7 (green) and 8 (orange) (Abbott Pharmaceutical Co. Ltd.) was added to the slides, which were mounted and incubated in a StatSpin ThermoBrite hybridization oven (Abbott Pharmaceutical Co. Ltd.) at 37°C for 90 min. Following hybridization, the slides were put in the working fluid of formamide at 43°C for 15 min to wash the probes, and also washed with 2x SSC twice. The Alexa Fluor 594-conjugated anti-human CD45 antibody (1:100; cat. no. FAB1430T; R&D Systems, Inc.) was added to the slides, incubated at 33°C for 1 h in the oven and subsequently removed. The nucleus was stained with 10  $\mu$ l nuclear dye DAPI (Vector Laboratories, Inc.; Maravai LifeSciences) at room temperature for 5 min. The sealing slides were automatically scanned and analysed using the Cyttel Pathfinder™ system (Cyttel Biosciences Inc.) in the high-power field (10x40).

**Measurement of serum tumour markers.** Peripheral blood (3 ml) was collected into tubes without anticoagulant and centrifuged at 1,500 x g and 4°C for 10 min. The expression levels of CEA, CA19-9 and CA72-4 in serum samples were determined using an automatic immunoassay analyser (Cobas e601; Roche Diagnostics). The normal reference values for the three serum tumour markers were 0-5 ng/ml for CEA, 0-37.0 U/ml for CA19-9 and 0-6.9 U/ml for CA72-4. Exceeding the upper limit of the normal threshold was considered positive. For the combined detection of two or more tumour markers, an elevated result of any of the tumour markers was considered positive. The diagnostic sensitivity was calculated by dividing the number of positive cases by the total number of CRC cases.

**Statistical analysis.** Statistical analysis was performed using the SPSS statistical software (v.19.0; IBM Corp.). Data are presented as the mean  $\pm$  standard deviation. A receiver operating characteristic (ROC) curve was plotted to determine the sensitivity and specificity of CTC detection for diagnosis. Differences between groups were determined using two-tailed

unpaired Student's t-test. The  $\chi^2$  test or Fisher's exact test was performed to explore the associations between total and aneuploid CTCs and patient clinicopathological characteristics. Kaplan-Meier analysis and the log-rank test were employed to evaluate the prognostic significance of CTCs in patient survival.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Detection of CTCs in patients with CRC and in healthy individuals.** Cells enriched from the peripheral blood of patients with CRC and from healthy individuals were examined for the presence of cell nuclei (DAPI-stained) and chromosome ploidy (CEP7- and/or CEP8-stained). In addition, immunofluorescence staining of CD45 was applied to exclude leukocytes. Based on the staining results of DAPI, CD45, CEP7 and CEP8, cells were divided into five groups: Group A, CD45<sup>-</sup>, DAPI<sup>+</sup> and CEP7>2/CEP8=2 (the number of hybridization signals for CEP7>2 or CEP8=2); Group B, CD45<sup>-</sup>, DAPI<sup>+</sup> and CEP7=2/CEP8>2; Group C, CD45<sup>-</sup>, DAPI<sup>+</sup> and CEP7>2/CEP8>2; Group D, CD45<sup>-</sup>, DAPI<sup>+</sup> and CEP7=2/CEP8=2; and Group E, CD45<sup>+</sup>, DAPI<sup>+</sup> and CEP7≥2/CEP8≥2 (Fig. 1). Cells in groups A-C characterized as nucleated cells with aneuploidy of chromosomes 7 and/or 8 but without CD45 expression were defined as CTCs, whereas cells in groups D and E were defined as indeterminate cells (normal leukocytes).

Based on the aforementioned CTC evaluation criteria, CTCs were identified in the peripheral blood of 3/30 (10.00%) healthy individuals, and their CTC count was 1. However, CTCs were identified in 49/59 (83.05%) patients with CRC, with a range of 2-46 cells/3.2 ml, which was significantly higher than in the control group (Fig. 2A). The ROC curve was then plotted to characterize the discriminating power of CTCs for the diagnosis of CRC. According to Youden's index, a cut-off value of 2 cells/3.2 ml yielded a sensitivity of 83.05% and a specificity of 100% (area under the curve, 0.904; 95% CI, 0.841-0.967; Fig. 2B). Although there was no significant association observed between CTC increase and overall survival according to the Kaplan-Meier analysis (Fig. 2C), there was a trend showing that patients with CRC with <2 CTCs may have an improved prognosis compared with those with ≥2 CTCs, since all 10 patients with <2 CTCs were alive at the primary endpoint. The association between CTC numbers and TNM staging, and between CTCs with multiploidy of chromosomes 7 or 8 and prognosis in patients with CRC were also investigated, but there were no significant differences identified (data not shown).

**Diagnostic sensitivity of CTCs and serum biomarkers in the diagnosis of CRC.** Several serum tumour markers, such as CEA, CA19-9 and CA72-4, have been suggested as candidate biomarkers for diagnosis, prognosis, monitoring recurrence and guiding treatment in patients with CRC (4). Therefore, the present study aimed to compare the sensitivities of CTCs and tumour markers to diagnose CRC. The diagnostic sensitivity was used to represent the rate of correctly classified positive cases. Among the three individual serum tumour markers, CEA had the highest diagnostic sensitivity (50.85%)

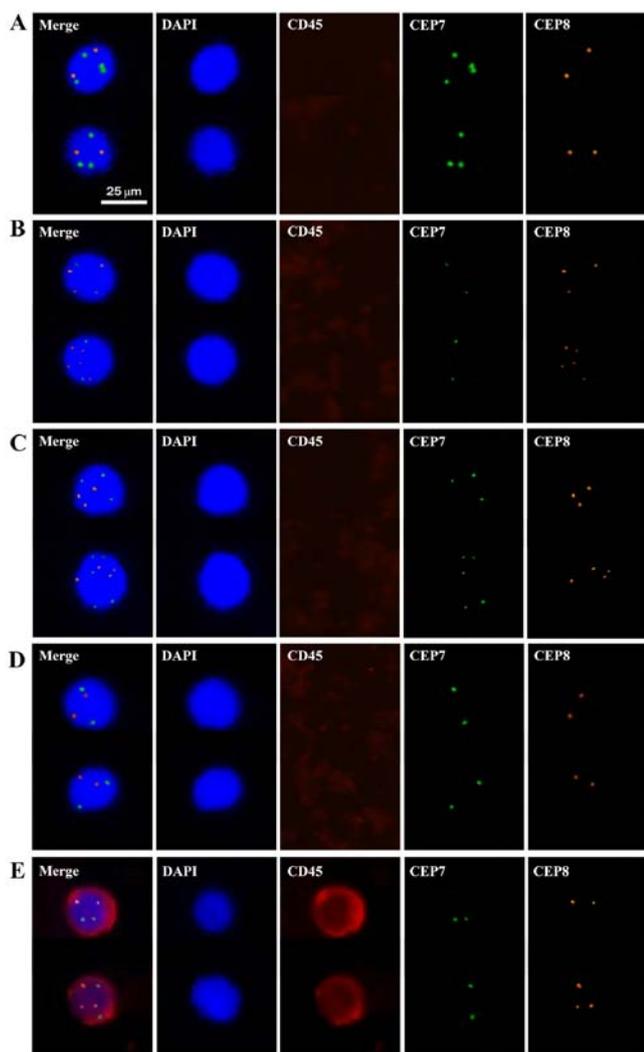


Figure 1. Representative images of cells detected in the peripheral blood of patients with colorectal cancer. Cell nuclei stained with DAPI appear blue. Cytokines stained with CD45 antibody appear red. Chromosomes stained with CEP7 appear green. Chromosomes stained with CEP8 appear orange. (A) CD45<sup>+</sup>, DAPI<sup>+</sup> and CEP7>2/CEP8=2; (B) CD45<sup>+</sup>, DAPI<sup>+</sup> and CEP7=2/CEP8>2; (C) CD45<sup>+</sup>, DAPI<sup>+</sup> and CEP7>2/CEP8>2; (D) CD45<sup>+</sup>, DAPI<sup>+</sup> and CEP7=2/CEP8=2; (E) CD45<sup>+</sup>, DAPI<sup>+</sup> and CEP7≥2/CEP8≥2. Cells in groups A-C were defined as CTCs, whereas cells in groups D and E were defined as normal leukocytes. Magnification, x400. CTC, circulating tumour cell; CEP7, chromosome 7; CEP8, chromosome 8.

in the 59 patients with CRC, followed by CA72-4 (28.81%) and CA19-9 (23.73%; Table I). Combinations of any two or all three of the serum tumour markers produced sensitivities that were markedly improved (range, 40.68-64.41%) compared with those of the single tumour markers. In addition, the diagnostic sensitivity of CTCs was 83.05%, which was notably higher than that of each traditional serum tumour marker and of the combinations. The sensitivity reached 93.22% when combining CTCs and all three biomarkers. Interestingly, the sensitivity of CTCs + CEA (91.53%) was only slightly lower than the combined sensitivity of all four markers, indicating that CTCs + CEA may be an effective combination of serum tumour markers for the diagnosis and prognosis of CRC.

Furthermore, an association between tumour staging and the detection rate of CTCs and tumour markers was observed. As shown in Fig. 3, positive CEA expression (≥5 ng/ml) were

Table I. Diagnostic sensitivity of CTCs and serum tumour markers in patients with colorectal cancer.

Serum tumour markers	Diagnostic sensitivity (positive/total), %
CEA	50.85 (30/59)
CA19-9	23.73 (14/59)
CA72-4	28.81 (17/59)
CEA + CA19-9	59.32 (35/59)
CEA + CA72-4	61.02 (36/59)
CA19-9 + CA72-4	40.68 (24/59)
CEA + CA19-9 + CA72-4	64.41 (38/59)
CTCs	83.05 (49/59)
CTCs + CEA	91.53 (54/59)
CTCs + CA19-9	84.75 (50/59)
CTCs + CA72-4	88.14 (52/59)
CTCs + CEA + CA19-9 + CA72-4	93.22 (55/59)

CEA, carcinoembryonic antigen; CA19-9, cancer antigen 19-9; CA72-4, cancer antigen 72-4; CTC, circulating tumour cell.

detected in 34.38% of stage I-II patients with CRC, 53.33% of stage III patients and 91.67% of stage IV patients, and these rates were higher than those for CA19-9 and CA72-4 at all pathological stages. The combined diagnostic sensitivity of CEA + CA19-9 + CA72-4 was further increased in patients with stage I-II (53.13%), stage III (66.67%) and stage IV (91.67%, same as CEA alone) CRC. Additionally, increased CTC levels (≥2 cells/3.2 ml) were observed in patients with CRC compared with normal individuals, with increased CTC number in 78.13% of patients with stage I-II disease, 86.67% of patients with stage III disease and 91.67% of patients with stage IV disease. This corresponded to the elevated diagnostic sensitivity of different stages of cancer. Furthermore, when combining CTCs and CEA, the detection rate in stage I-II patients reached 84.38%. The increase in the detection sensitivity of CTCs suggested that CTCs may be used as an efficient biomarker in the detection of all stages of CRC, particularly early stage tumours.

*Association of total or aneuploid CTCs with clinicopathological characteristics of patients with CRC.* As shown in Table II, there was a significant difference in CTC status between the well-, moderately and poorly differentiated CRC groups. CTCs were more likely to be detected in poorly differentiated CRC tumours (12/12) than in well- or moderately differentiated tumours. Furthermore, the results suggested that there was a significant association between CTCs and nerve invasion (pathological diagnosis). The detection rate of CTCs could be remarkably increased with cancer progression, such as invasion and metastasis.

As shown in Fig. 1, CTCs detected in the peripheral blood of patients with CRC had triploidy, tetraploidy or multiploidy (≥5 copies of chromosomes 7 or 8), indicating the existence of heterogeneous polysomic chromosomes in CTCs. The association between CTCs with multiploidy of chromosomes 7 or 8 and clinicopathological characteristics was evaluated.

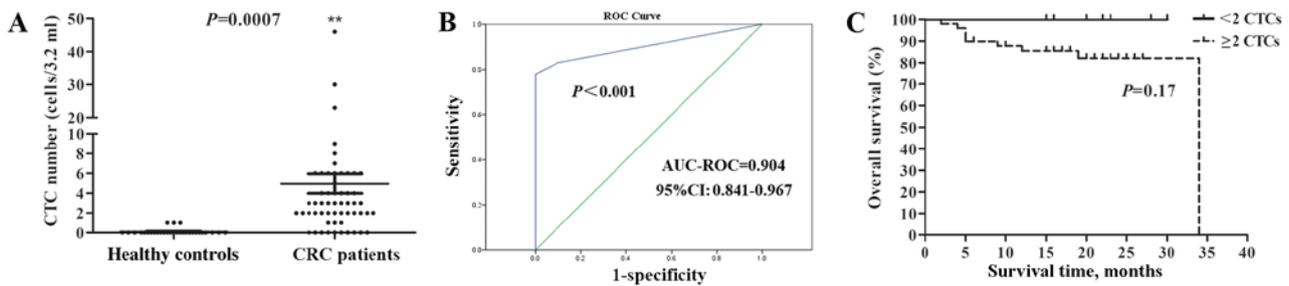


Figure 2. Detection of CTCs in patients with CRC and healthy individuals. (A) Comparison of CTC numbers between healthy individuals and patients with CRC. (B) ROC curves for the ability of CTCs to discriminate between patients with CRC and healthy individuals. (C) Kaplan-Meier survival curves showing overall survival of patients with CRC based on the number of CTCs. CTC, circulating tumour cell; CRC, colorectal cancer; ROC, receiver operating characteristic; AUC, area under the curve.

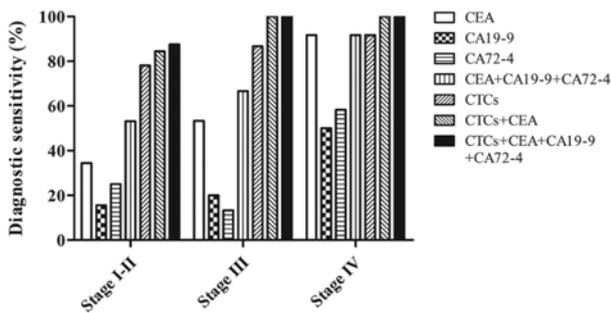


Figure 3. Diagnostic sensitivity of CEA, CA19-9, CA72-4 and CTCs (alone or in combination) in 59 patients with different stages of colorectal cancer. CEA, carcinoembryonic antigen; CA19-9, cancer antigen 19-9; CA72-4, cancer antigen 72-4; CTC, circulating tumour cell.

CTCs with aneuploidy of chromosome 8 were not significantly associated with any of the clinicopathological characteristics presented in Table II (data not shown). However, CTCs with multiploidy of chromosome 7 were significantly associated with TNM stage and serum CA19-9 levels (Table II). This type of CTC was more commonly observed in the peripheral blood of patients with late-stage CRC.

## Discussion

CTC detection is a non-invasive approach that has potential utility for clinical diagnosis, prognosis and evaluation of therapeutic responses for different solid tumours. Therefore, it is important to establish a reliable CTC detection strategy. Various CTC detection methods, including the CellSearch® system, the AdnaTest, isolation by size of epithelial tumour cells (ISET®) and nested reverse transcription-quantitative (RT-q)PCR-based techniques, have been reported in CRC studies (10,12). The CellSearch® system is currently the only method for CTC detection approved by the FDA. However, the CTC-positive rates obtained using the CellSearch® system are low, ranging between 18.8 and 33% in patients with mCRC according to different studies (10,18). Furthermore, CTCs are barely detectable using this system in patients with non-mCRC (10). Gorges *et al* (19) identified CTCs using the CellSearch® technology and/or the AdnaTest in parallel, and demonstrated that a combined analysis with both methods leads to an elevated rate of CTC detection in patients with mCRC (positive rate, 33% for CellSearch®; 30% for the AdnaTest;

50% for both combined). The methods for CTC detection have been recently improved. Chen *et al* (20) introduced a novel isolation method, known as the ISET® system, involving automatic isolation and staining procedures to capture CTCs, and revealed that CTCs were detected in 38/72 (52.8%) patients with CRC. In another study based on nested RT-qPCR, epithelial cell transforming 2 (ECT2) was screened as a candidate marker gene for quantifying CTCs, with a detection rate of ECT2 of ~60% in patients with different stages of CRC (21).

Combining negative enrichment and imFISH, a strategy that is independent of epithelial marker expression and tumour size, has shown great potential for CTC detection, yielding diagnostic sensitivities of 84, 76.2 and 69.4% for detecting CTCs in lung, ovarian and ampullary cancer, respectively (22-24). In the present study, the Cytel method (combining probes for CEP7 and CEP8 with an anti-CD45 antibody) was designed to detect CTCs in patients with CRC and in healthy controls. This strategy achieved a diagnostic sensitivity of 83.05% and a specificity of 100%, when using a cut-off value of 2 CTCs/3.2 ml of peripheral blood. The detection rate of CTCs with this method was higher than that of the CellSearch® system, which has been reported to be 15.33% (10), higher than that of the ISET® method, which has been reported to be 35% (20), and higher than that of the approach using RT-qPCR and ECT2, which has been reported to be 59% (21). Furthermore, the Cytel method requires less peripheral blood (3.2 ml) than the CellSearch® system (7 ml). The present study suggested that the Cytel method may be a sensitive and convenient strategy that could be helpful in improving the CTC detection rate in CRC.

In the present study, CTCs were identified in 3/30 (10.00%) healthy donors. In theory, CTCs should not be present in healthy individuals. However, Miller *et al* (25) demonstrated that CTCs detected by the CellSearch® technique are extremely rare in healthy volunteers (<math><3.5\%</math> for a threshold of  $\geq 1$  CTC per 7.5 ml of blood) and patients with benign disease (<math><7.5\%</math> for a threshold of  $\geq 1$  CTC per 7.5 ml of blood), and Ilie *et al* (26) also successively detected CTCs in patients with chronic obstructive pulmonary disease 1-4 years before they were diagnosed with lung cancer. To explain the above question, it is hypothesised that the epithelial cells isolated using different CTC detection methods may not all be the real CTCs, but instead include few cells with metabolic abnormality. These cells may come from donors with unhealthy lifestyles and eating habits, such as long-term smoking or high fat

Table II. Association of total or aneuploid CTCs with the clinicopathological characteristics of patients (n=59) with colorectal cancer.

Characteristics	Number of patients	Total CTCs, n			CTCs with multiploidy of chromosome 7, n		
		Negative	Positive	P-value	Negative	Positive	P-value
Age, years				0.396			0.157
<60	23	3	20		7	16	
≥60	36	7	29		17	19	
Sex				0.307			0.075
Male	34	7	27		17	17	
Female	25	3	22		7	18	
Tumour size, cm				0.604			0.533
<5	36	6	30		15	21	
≥5	23	4	19		9	14	
Location <sup>a</sup>				0.262			0.115
Left-sided	43	6	37		20	23	
Right-sided	16	4	12		4	12	
Histological differentiation				0.026 <sup>b</sup>			0.163
Well	9	4	5		4	5	
Moderate	38	6	32		18	20	
Poor	12	0	12		2	10	
Primary tumour				0.894			0.841
T1-T2	8	1	7		4	4	
T3	26	5	21		10	16	
T4	25	4	21		10	15	
Lymph node				0.350			0.111
N0	35	7	28		17	18	
N1-N2	24	3	21		7	17	
Distant metastasis				0.343			0.406
M0	47	9	38		20	27	
M1	12	1	11		4	8	
TNM staging				0.229			0.031 <sup>b</sup>
I-II	32	7	25		17	15	
III-IV	27	3	24		7	20	
Venous invasion				0.293			0.311
Absent	46	9	37		20	26	
Present	13	1	12		4	9	
Nerve invasion				0.008 <sup>b</sup>			0.306
Absent	45	4	41		17	28	
Present	14	6	8		7	7	
CEA, ng/ml				0.612			0.438
<5	29	5	24		11	18	
≥5	30	5	25		13	17	
CA19-9, U/ml				0.248			0.020 <sup>b</sup>
<37	45	9	36		22	23	
≥37	14	1	13		2	12	
CA72-4, U/ml				0.600			0.177
<6.9	42	7	35		15	27	
≥6.9	17	3	14		9	8	
Ki-67 index, %				0.602			0.257
<70	11	2	9		3	8	
≥70	48	8	40		21	27	

Table II. Continued.

Characteristics	Number of patients	Total CTCs, n			CTCs with multiploidy of chromosome 7, n		
		Negative	Positive	P-value	Negative	Positive	P-value
PD-L1 expression				0.507			0.493
Negative	21	4	17		8	13	
Positive	38	6	32		16	22	

<sup>a</sup>Right-sided: Cecum, ascending colon and transverse colon; left-sided: Descending colon and sigmoid colon. <sup>b</sup>P<0.05. CEA, carcinoembryonic antigen; CA19-9, cancer antigen 19-9; CA72-4, cancer antigen 72-4; CTC, circulating tumour cell; PD-L1, programmed death-ligand 1.

diet. Thus, it is possible to identify CTC-like cells in healthy people. However, the number of these abnormal cells is very low. Only one CTC was identified in the three healthy donors in the present study, and a cut-off value (2 CTCs/3.2 ml blood) was determined for the accurate distinction of tumour patients from healthy individuals. In conclusion, CTCs may be a useful tool for the early diagnosis and risk assessment of patients with cancer and healthy individuals.

At present, traditional serum tumour markers, such as CEA, CA19-9 and CA72-4, are widely used in the clinical management of CRC. According to the American Society of Clinical Oncology, CEA detection is recommended to monitor recurrence following curative surgery in patients with stage II and III CRC (27). The preoperative serum CA19-9 levels have been reported to be a prognostic indicator in patients with CRC (28). Additionally, a recent study demonstrated that CA72-4 can supplement CEA in evaluating CRC recurrence and metastasis (29). Early detection of tumour markers before distal metastasis occurs may have a significant impact on medical decision making and clinical outcomes of patients, particularly in the early stages. Furthermore, preoperative or postoperative serial CTC measurements may provide a method to predict or monitor early relapse in patients with non-mCRC (10). Based on the aforementioned results, the detection sensitivities of CTCs and three serum tumour markers were analysed simultaneously in patients with different stages of CRC to develop a non-invasive, effective and reliable biomarker detection strategy for the early diagnosis of CRC.

In the present study, the diagnostic sensitivities of single and combined serum tumour markers ranged between 40.68 and 64.41%, respectively, which was consistent with the findings of Gao *et al* (30). However, the sensitivity of the CTC detection method reached 83.05%, which was higher than that of the traditional tumour markers, even when combining CEA, CA19-9 and CA72-4. A similar result was obtained in a previous study using the same CTC detection method in patients with lung cancer (22). For patients with stage I-II CRC, CEA had the highest sensitivity (34.38%) among the three individual serum tumour markers, while the detection rate of the CTC method was 78.13%. It was clear that the CTC method was more sensitive for detecting early stage CRC compared with the use of other serum biomarkers. Furthermore, combining the CTC method with CEA achieved a sensitivity of 91.53%, which was only slightly lower than that for the combination of the four biomarkers (93.22%).

This finding suggests that the combination of CTCs and CEA may be an effective and convenient routine detection strategy for diagnosing CRC, and for predicting and monitoring early recurrence and metastasis in patients with CRC.

The current study revealed novel insights into the potential diagnostic application of a CTC detection method based on aneuploidy detection. Davoli *et al* (31) revealed that an abnormally high number of chromosomes was commonly associated with high-grade tumours and poor prognosis. Papazachariou *et al* (32) have identified that the number of CTCs with chromosome 7 aneuploidy is significantly involved with late stage tumours and poor prognosis. In the present study, the association between total and aneuploid CTCs and patient clinicopathological characteristics was carefully analysed. Total CTCs were found to be significantly associated with tumour differentiation and nerve invasion. This finding indicated that the detection rate of CTCs increased along with tumour progression, which was consistent with the aforementioned results regarding the detection rate of CTCs in different stages of CRC. Additionally, there was a significant difference in CTCs with multiploidy of chromosome 7 between different TNM stages. These CTCs were more likely to be detected in patients with stage III-IV CRC than in patients with stage I-II CRC. The present study suggested that detection of CTCs based on aneuploidy detection could be more specific for predicting highly malignant and invasive tumours in CRC management than detection using current serum markers. A careful classification of aneuploid CTCs may provide novel insights for improved prediction of CRC.

In conclusion, the current data fully support the potential clinical value of total and aneuploid CTCs as identified with the Cytel method in CRC diagnosis. The combination of CTCs and CEA has the potential to become an effective and convenient routine screening strategy for predicting CRC at different stages. However, the present study was performed at a single medical centre with a small sample size, which may limit the power of the statistical analysis. The dynamic change in the number of CTCs at different stages of treatment and the association between CTCs and patient survival should be studied in future research.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

HG and LD designed the study. HY and LM performed the experiments and wrote the initial draft of the manuscript. WL and YZ contributed to the analysis and interpretation of data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Review Committee of Beijing Shijitan Hospital [Beijing, China; approval no. 2016KY(55)]. Research has been conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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