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ORIGINAL ARTICLE

The role of folate receptor-positive circulating tumor cell analysis in the diagnosis of colorectal cancer: a retrospective cohort study

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Abstract

Objective To explore the application value of folate receptor-positive circulating tumor cell analysis (FR^+-CTC analysis) in the diagnosis of colorectal cancer (CRC).

Methods Clinical data of CRC patients and healthy subjects admitted to our hospital from January 2019 to October 2019 were retrospectively collected. CTC result and serological and pathological outcomes of the study patients were collected and analyzed. Receiver operating characteristic curve (ROC curve) was drawn.

Results The CTC levels of cancer patients (9.34 ± 3.53 FU/3 ml) were significantly higher than those of healthy subjects (7.00 ± 2.33 FU/3 ml). CTC levels could be related to cancer stage and metastasis in patients. ROC curves were drawn and the area under the ROC curve (AUC) was 0.702. The cutoff value was determined to be 8.87 FU/3 ml. At this cutoff value, the sensitivity and specificity of FR^+-CTC analysis in the diagnosis of colorectal cancer were 61.8% and 82.6%, respectively. The diagnostic efficiency of FR^+-CTCs in advanced CRC was significantly higher than that in the early stage. And the cutoff value of early and advanced stage CRC was determined to be 9.66 FU/3 ml.

Conclusion FR^+-CTC analysis has high potential in recurrence diagnosis and decision of adjuvant chemotherapy for CRC.

Keywords Circulating tumor cells · Colorectal cancer · Diagnosis · Folate receptor

Research background

Colorectal cancer (CRC) is a common malignant tumor. With the improvement of people's living standards and changes in dietary habits, its incidence has been increasing annually, and it has risen to the third most common malignant tumor [1]. Surgical resection is the main treatment for colorectal cancer, but tumor recurrence and metastasis often lead to the deterioration and death of patients. Early diagnosis and individualized treatment are the key factors in determining the prognosis. Current CRC early screening strategies have many limitations [2]. Colonoscopy is the gold standard for colorectal cancer screening [3]. However, it is expensive and invasive, has poor patient compliance, and is difficult to use for widespread screening. Therefore, it is very important to find a new noninvasive diagnostic method.

Circulating tumor cells (CTCs) is a common term for all types of tumor cells existing in the peripheral blood. CTCs are considered to provide the basis of hematogenous metastasis of malignant tumors. CTCs with high activity and high metastatic potential can survive in the circulatory system and proliferate in a suitable environment, leading to tumor recurrence and metastasis [4, 5]. For reasons such as blood flow shear force, microenvironment changes and immune system killing, CTC content in the circulatory system is extremely low. In the blood of patients with metastatic tumors, there is only 1 CTC in every 10^{5-7} white blood cells. CTC detection is performed using liquid biopsy with real-time monitoring function which is a new non-invasive diagnostic tool [6]. Its precise counting and molecular marker detection play important guiding roles in the prognosis judgment, efficacy evaluation and individualized treatment of tumor patients [7]. CTCs have been widely reported to be an effective prognostic marker for colorectal cancer and they can be used in the monitoring of tumor recurrence and metastasis, and the

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assessment of overall survival [8, 9]. However, CTC detection still faces a series of problems, such as false positive and false negative results, unclear refinement of the guiding role of CRC staging and the quantification of treatment options. Currently, most CTC detection methods are based on epithelial markers (such as EpCAM and CK), and such detection methods tend to miss CTCs with a higher degree of malignancy after EMT. Therefore, an increasing number of scholars have begun to focus on the development of CTC detection techniques that do not rely on epithelial markers [10].

Folate receptor-positive circulating tumor cell analysis (FR^+-CTC analysis) is a novel CTC detection method that is independent of tumor epithelial markers and has high sensitivity. It has been demonstrated to have good diagnostic efficacy in non-small cell lung cancer and solitary pulmonary nodules [11, 12]. In addition, a study also found that FR^+-CTCs could be a potential diagnostic biomarker for pancreatic cancer [13]. However, their diagnostic value in CRC remains unclear.

Materials and methods

Study design

The study was approved by the Ethics Committee of The Renmin Hospital of Wuhan University. Clinical data were collected after informed consent was obtained by telephone follow-up.

Data from CRC patients admitted to the sDepartment of Gastrointestinal Surgery, Renmin Hospital of Wuhan University, from January 2019 to October 2019 were retrospectively collected. The inclusion criteria were as follows: (1) colorectal cancer confirmed by pathological examination; and (2) no surgery, chemotherapy or other antitumor treatments received before CTC detection. The exclusion criteria were as follows: (1) no definite pathological diagnosis; (2) complications with other malignant tumors; and (3) incomplete medical records.

The clinical data collected included the following: (1) basic information, such as the patient's admission number, name, sex, age, etc.; (2) preoperative CTC, CEA, etc.; and (3) patient tumor location, pathological type, vascular and nerve invasion, etc. Patients were divided into early stage (stage I and II) and advanced stage (stage III and IV) according to the TNM Staging System for Colorectal Cancer (7th Edition).

We defined a healthy population as those without colorectal cancer and other malignant tumor. We included subjects who voluntarily underwent a physical examination at the physical examination center during the same period.

FR^+-CTC analysis

Three milliliters of peripheral blood were withdrawn into an EDTA-containing anticoagulant tube from each subject before the surgical operation. FR^+-CTC analysis was performed using the CytoploRare® Detection Kit provided by GenoBiotech (China) Co. Ltd.

Statistic analysis

SPSS 23.0 software was used for data statistical analysis. Graphpad 6.0 software was used to draw statistical graphs. CTC levels, serological and pathological outcomes were compared between patients in different groups. The categorical variables were described using frequency and percentage. Continuous variables were described using mean values ± SD or median with interquartile range (IQR). We used the *t*-test to compare the continuous variables of the normal distribution, and the Mann–Whitney *U* test (two groups) or Kruskal–Wallis test (multiple groups) compare the nonnormally distributed variables. For categorical variables, a chi-square test or Fisher exact test was used. Receiver operating characteristic curve (ROC curve) was drawn. The cutoff value was determined by the maximum point of Youden index, and the diagnostic efficiency of the cutoff value was evaluated.

$p < 0.05$ was considered statistically significant.

Results

Comparison of CTC in CRC patients and healthy subjects with different clinical features

Sixty-six patients diagnosed with colorectal cancer by pathological diagnosis and 172 healthy subjects were enrolled. Comparison of CTC in CRC patients and healthy subjects with different clinical features are shown in Table 1. In CRC patients, the CTC levels in gender, N stage and nerve invasion showed a significant difference ($p < 0.05$), while in other aspects, such as age, TNM stage, vascular invasion, CEA, tumor size and cancer type, there was no statistical significance. In healthy subjects, there was no significant difference in gender and age in CTC levels.

ROC curve determines the cutoff value for CTC diagnosis of CRC

The CTC levels of cancer patients (9.34 ± 3.53 FU/3 ml) were significantly higher than those of healthy subjects

Table 1 Comparison of CTC in CRC patients and healthy subjects with different clinical features

Variables	*n*	CTC (FU/3 ml)	*p*
CRC patients	66	9.34 ± 3.53	
Age (y)			
≥ 65	32	8.96 ± 3.22	0.408
< 65	34	9.69 ± 3.82	
Gender			
Male	31	10.41 ± 3.61	**0.019**
Female	35	8.39 ± 3.23	
N stage			
N0	33	8.56 ± 3.55	**0.043**
N1	30	10.32 ± 3.17	
TNM stage			
Early stage	29	8.48 ± 3.60	0.051
Advanced stage	34	10.18 ± 3.18	
Vascular invasion			
With	15	10.42 ± 4.11	0.24
Without	46	9.20 ± 3.24	
Nerve invasion			
With	16	11.08 ± 3.25	**0.033**
Without	45	8.94 ± 3.41	
CEA			
≥ 5	15	9.22 ± 2.39	0.748
< 5	40	9.57 ± 3.85	
Tumor size (cm)			
≥ 4.5	31	9.06 ± 3.18	0.265
< 4.5	29	10.07 ± 3.75	
Cancer type			
Left-sided colon carcinoma	15	8.92 ± 3.52	0.461
Right-sided colon carcinoma	22	8.81 ± 2.98	
Rectal carcinoma	29	9.95 ± 3.93	
Healthy subjects	172	7.00 ± 2.33	
Age (y)			
≥ 65	53	6.73 ± 2.10	0.314
< 65	119	7.11 ± 2.42	
Gender			
Male	127	7.11 ± 2.38	0.27
Female	44	6.66 ± 2.18	

Bold values indicate $p < 0.05$

(7.00 ± 2.33 FU/3 ml). The ROC curve was plotted according to the CTC levels of CRC patients and healthy subjects, as shown in Fig. 1. The AUC was 0.702 (95%CI: 0.620 to 0.783, $p < 0.0001$), and the cutoff value determined by the maximum Youden index was 8.87. Under this cutoff value, the sensitivity and specificity of FR^+-CTC analysis in the diagnosis of colorectal cancer were 61.8% and 82.6%, indicating a high diagnostic value.

Diagnostic value of CTC in patients with different stages of CRC

We compared the diagnostic value of CTC in different stages of CRC cancer (Fig. 2). The AUC of early and advanced stage were 0.624 (95%CI: 0.500–0.747, $p < 0.05$) and 0.789 (95%CI: 0.696–0.882, $p < 0.0001$), respectively. Furthermore, we plotted the ROC curve according the patients in different stages. The AUC was 0.683 (95%CI: 0.548–0.818, $p < 0.05$), the cutoff value was 9.66 FU/3 ml.

Diagnostic value of CTC in patients with different types of tumors

The ROC curves of different tumor types were compared (Fig. 3). In left-sided colon carcinoma, the AUC was 0.667 (95%CI: 0.498–0.837, $p < 0.05$), while in right-sided colon carcinoma and rectal carcinoma, the AUC were 0.692 (95%CI: 0.559–0.826, $p < 0.01$), and 0.727 (95%CI: 0.611–0.842, $p < 0.0001$), respectively.

Discussion

CTCs are the basis of the distant metastasis of tumor cells. Studies have shown that CTCs can be detected in the peripheral blood of tumor patients at the early stage of tumorigenesis [14]. When entering the circulatory system, CTCs undergo epithelial-mesenchymal transition (EMT), during which the expression of CTC epithelial markers [such as E-cadherin, cytokeratin (CK), and epithelial cell adhesion molecule (EpCAM)] decreases and the expression of interstitial markers [such as N-cadherin and vimentin] increases [15]. During tumor progression, EMT induces neovascularization, induces CTCs to enter the circulatory system, enhances CTC migration, and increases the viability of CTCs by enhancing their antiapoptotic ability and promoting immune escape. EMT is also associated with resistance to chemotherapy and targeted therapies [16].

Currently, the commonly used CTC detection technology can be divided into two categories: immunomagnetic bead separation technology based on cell surface antibodies and physical separation technology based on the physical properties of tumor cells [17]. Immunomagnetic bead separation technology has a relatively high enrichment rate and specificity and is usually divided into a negative selection and positive selection [18]. Negative selection results in CTC enrichment by their consuming other cells in the blood. The advantage of negative selection is that it does not depend on tumor cell surface antigens and avoids false negatives caused by EMT. However, its positive rate is affected by the efficacy of leukocyte clearance. Positive selection captures CTCs through tumor cell surface-specific antigens (such as

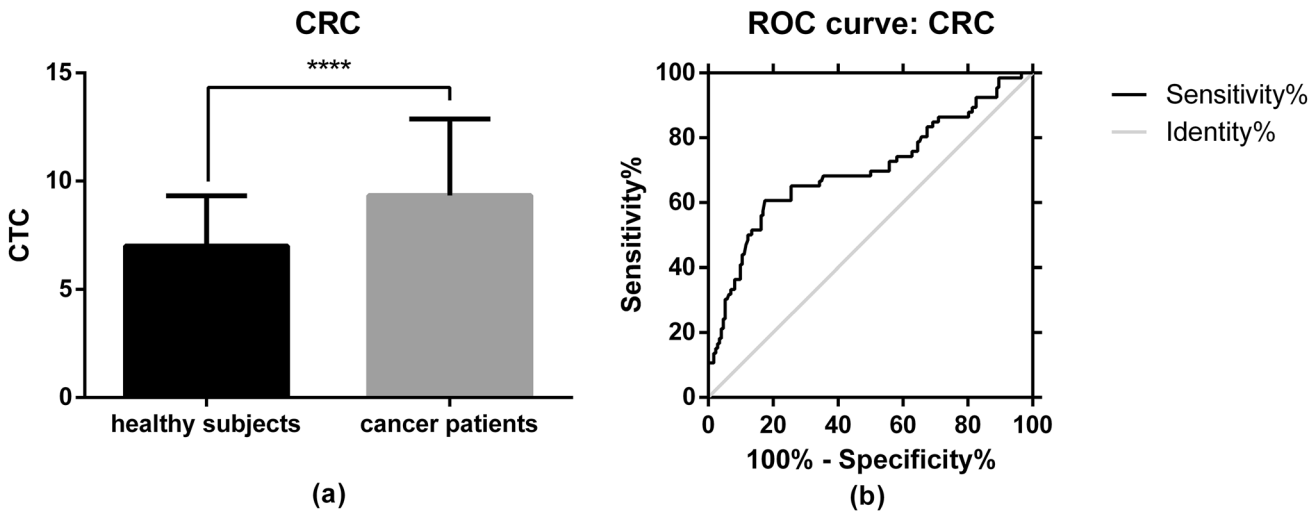

Fig. 1 ROC curve determines the cutoff value for CTC diagnosis of CRC. **a** Comparison of CTC between CRC patients and healthy subjects. The CTC levels of CRC patients (9.34 ± 3.53 FU/3 ml) were significantly higher than those of healthy subjects (7.00 ± 2.33 FU/3 ml, $p < 0.0001$). **b** The ROC curve of CRC. The AUC was 0.702 (95%CI: 0.620 to 0.783, $p < 0.0001$), and the cutoff value was 8.87 FU/3 ml

EpCAM and CK). EpCAM is expressed in almost all cells of epithelial origin, so it is the most commonly used. High-purity CTCs can be obtained by screening epithelial cells from the blood with corresponding antibody-labeled immunomagnetic beads. However, there is no universal tumor cell surface antigen, and false-negative results can occur due to the influence of EMT. Based on the physical characteristics of CTCs, physical methods, such as density gradient centrifugation and filtration platforms, can be used to separate CTCs from other cells [19]. Physical separation technology has a low cost and is not affected by the EMT mechanism, and the selected CTCs can be further used for subsequent molecular biology research. However, it is susceptible to internal and external factors, and isolated CTCs have low purity, so their application in clinical practice requires further verification.

Folate is an essential low-molecular weight vitamin that the body cannot synthesize on its own. Folate is endocytosed into cells by binding to folate receptor (FR) on the cell surface. FR is a glycoprotein expressed on the surface of the cell membrane with strong tumor specificity. It is highly expressed on the surface of tumor cells but barely expressed in the circulating cells of healthy people [20]. Therefore, FR has been widely studied as an ideal target for diagnosis and antitumor therapy [21]. FR^+-CTC analysis utilizes the combination of highly expressed FR on the surfaces of CTCs and the probe and calculates the number of CTCs by real-time quantitative PCR assay using the probe. This method can amplify the scarce CTCs in the peripheral blood into hundreds of thousands of surface receptor molecules, avoiding the influence of EMT, and it has obvious advantages over traditional CTC detection technology.

In this study, 66 patients diagnosed with colorectal cancer by pathological diagnosis and 172 healthy subjects were enrolled. The difference in CTC levels between the two groups of subjects was statistically significant, consistent with the results reported in the literature [22]. Tsai et al. [8] tested 667 blood samples, including from patients with colorectal adenoma and colorectal cancer and from healthy donors, using the CellMax method, and the results showed that CTC count had high sensitivity in the diagnosis of colorectal cancer. A certain level of CTCs was also detected in the blood samples of healthy subjects, which could be a small part of activated monocytes with functional FR expression in the peripheral blood, or it could be circulating cells that have been shed from normal tissues (such as the lung) into the peripheral blood [23]. Studies have shown that the levels of CTCs in patients is closely related to tumor recurrence, metastasis and prognosis. In this study, CTC levels in patients without lymph node metastasis (N0) were significantly lower than those with lymph node metastasis (N1), and similar differences were observed between patients without nerve invasion and those with nerve invasion. Our study confirmed the role of CTCs in the prognosis of CRC. In addition, male patients had higher levels of CTCs, which had not been reported in other types of tumors. Future studies with larger samples are needed to confirm the influence of different genders on FR^+-CTC level. Currently, studies have shown that folic acid plays different roles in CRC patients of different genders [24]. If studies with large sample sizes

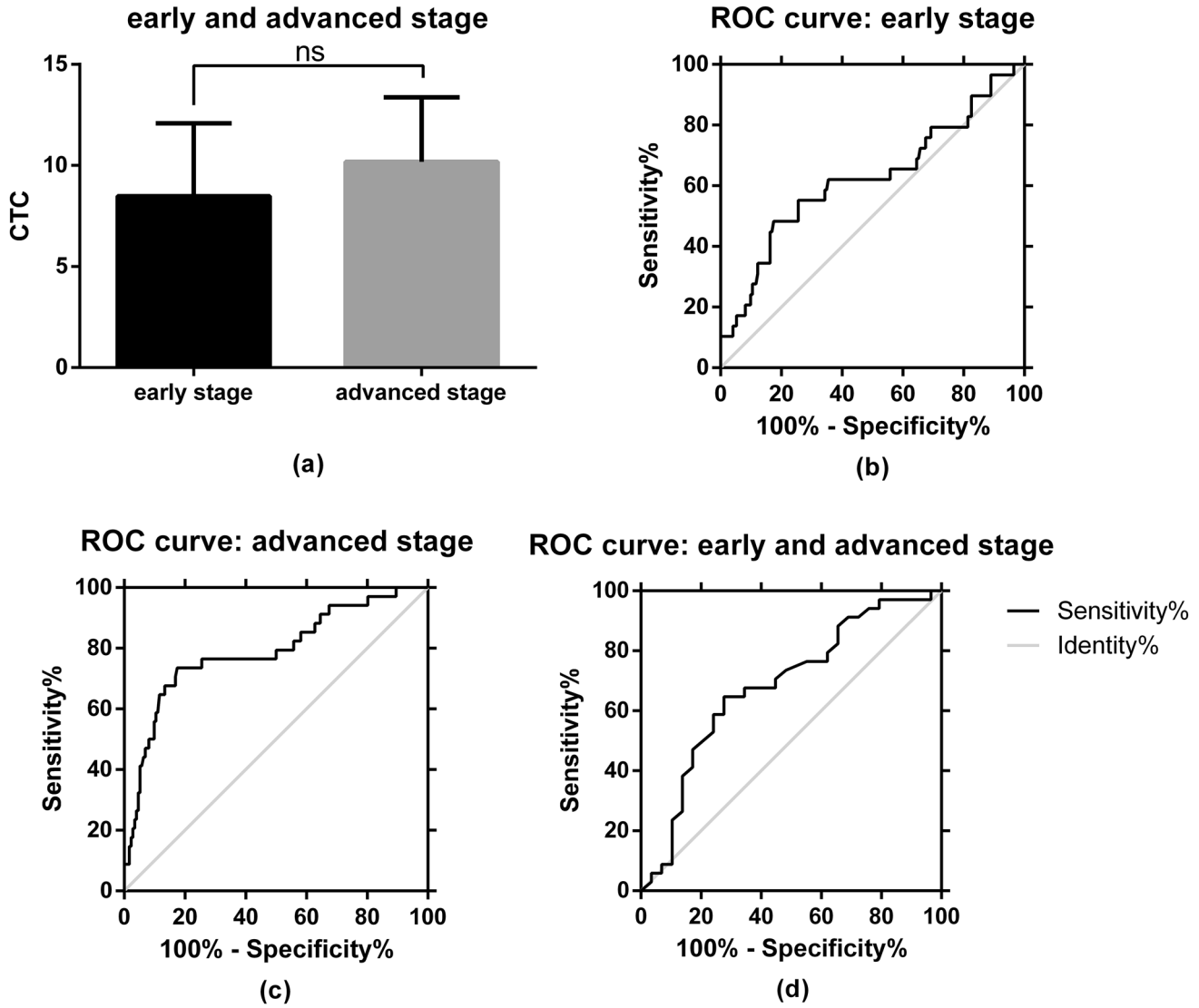

Fig. 2 Diagnostic value of CTC in patients with different stages of CRC. **a** Comparison of CTC between early-stage patients and advanced-stage patients. The CTC levels of early-stage patients were lower than that of advanced-stage patients ($p=0.051$). **b** The ROC curve of early-stage CRC. The AUC was 0.624 (95%CI: 0.500–0.747, $p=0.0336$). **c** The ROC curve of advanced-stage CRC. The AUC was 0.789 (95%CI: 0.696–0.882, $p<0.0001$). **d** The ROC curve of different stages. The AUC was 0.683 (95%CI: 0.548–0.818, $p=0.0128$), the cutoff value was 9.66 FU/3 ml

remain to suggest the gender difference, further studies at the molecular level are needed to explain the differences.

ROC curves were drawn based on the CTC levels of CRC patients and healthy subjects, and the area under the curve (AUC) was 0.702. The cutoff value was determined to be 8.87 FU/3 ml, and the sensitivity and specificity were 60.6% and 82.6%, respectively. Similar results had been reported in the published literature [25, 26]. To our knowledge, no other studies have calculated the cutoff value of FR^+-CTCs in the diagnosis of CRC. Compared with traditional CRC diagnosis methods, such as CT and CEA, CT requires the tumor to have a certain volume, and the diagnostic efficiency is related to the experience levels of the physician. Serum CEA levels are mainly used in the diagnosis of metastatic CRC, and the positive rate is often not high in early-stage patients. The CEA-positive rate of CRC patients enrolled in this study was relatively low. In contrast, FR^+-CTC analysis has higher diagnostic efficiency in non-metastatic CRC.

Stage often determines a patient's prognosis, and this is also true in CRC. In clinical practice, the treatment of early and advanced CRC is different. Surgery is the main treatment for CRC cure, but in advanced stage cases, adjuvant systemic chemotherapy is currently recommended for optimizing the chances of healing [27]. Therefore, it is important to be able to predict the stage of CRC. In this study, the diagnostic efficiency of FR^+-CTC in advanced CRC was

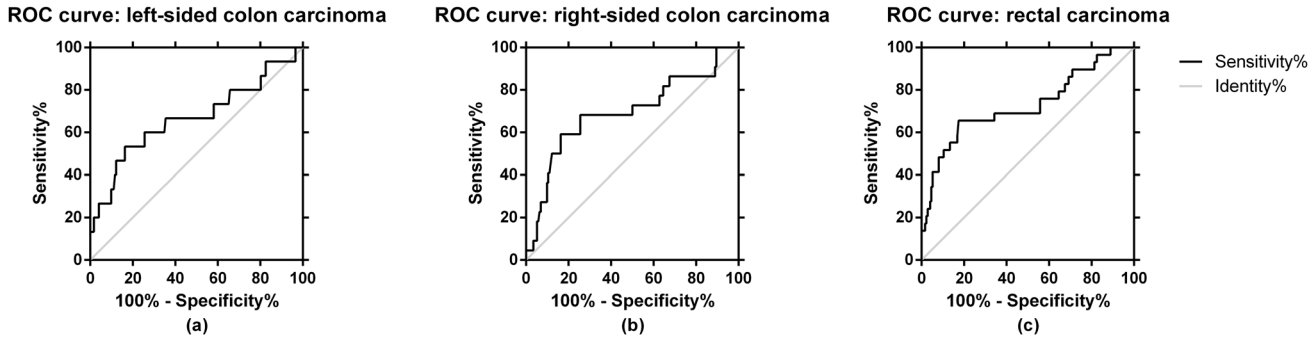

Fig. 3 Diagnostic value of CTC in patients with different types of tumors. **a** The ROC curve of left-sided colon carcinoma. The AUC was 0.667 (95%CI: 0.498–0.837, $p=0.0319$). **b** The ROC curve of right-sided colon carcinoma. The AUC were 0.692 (95%CI: 0.559–0.826, $p=0.0034$). **c** The ROC curve of rectal carcinoma. The AUC was 0.727 (95%CI: 0.611–0.842, $p<0.0001$)

significantly higher than that in early stage. By plotting ROC curve, the cutoff value of early and advanced stage CRC was determined to be 9.66 FU/3 ml. So, we can assess tumor stage in a non-surgical manner, which helps to individualize treatment.

In this study, ROC curves of different types of CRC were compared. The diagnostic value of CTC in right-sided colon carcinoma and rectal carcinoma is relatively high, while that in left-sided colon carcinoma is low. These results could be related to insufficient sample size and the different embryonic development and biological characteristics of different sides in CRC [28].

CTCs have important clinical application prospects in the clinical diagnosis, treatment guidance and recurrence and metastasis monitoring of CRC [6]. Further research on CTCs could be helpful for the early diagnosis of cancer, prediction of patient prognosis, monitoring of treatment response, determination of new therapeutic targets and a better understanding of the progression and metastasis of cancer [29]. FR^+-CTC analysis is a novel CTC detection method. This study showed that its results might be related to cancer stage and metastasis in patients, and its cutoff value in the diagnosis of CRC was determined to be 8.87. However, the number of samples included in this study was small. In future studies, we will require more clinical studies with larger samples to clarify the value of this detection method in the diagnosis and treatment of cancer diseases.

In conclusion, FR^+-CTC analysis has high potential in recurrence diagnosis and decision of adjuvant chemotherapy for CRC.

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Availability of data and material Data and material are available from the corresponding author by request.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

Ethics approval All procedures performed were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication Approved by all authors for publication.

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