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Original Article

Clinical characteristics and prognostic analysis of circulating tumor cells in peripheral blood and surgical lavage fluid from patients with colorectal cancer

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Abstract: This study aimed to characterize the distribution of circulating tumor cells (CTCs) in preoperative peripheral blood and intraoperative surgical lavage fluid from patients with colorectal cancer (CRC), and to evaluate the association between CTC levels and clinical prognosis to determine their potential as novel prognostic biomarkers. Preoperative peripheral blood (7.5 ml) was collected from 185 CRC patients and from 50 healthy controls. During laparoscopic surgery, 150 ml of surgical lavage fluid was obtained from each patient at three key time points: before tumor resection, after tumor resection and after mesenteric incision of the tumor. CTCs were detected using the CanPatrol™ enrichment system combined with RNA in situ hybridization. Associations between CTC counts and clinical indicators were analyzed, and their relationship with progression-free survival (PFS) and overall survival (OS) was evaluated. The CTC-positive rate in both peripheral blood and surgical lavage fluid was associated with tumor invasion depth, TNM stage and metastasis. TNM stage and CTC counts in peripheral blood and lavage fluid were identified as independent predictors of mortality. Among patients with CTC-positive lavage fluid after tumor mesangial dissection, both PFS and OS were significantly shorter compared with CTC-negative patients (P_{PFS}=0.0015, P_{OS}=0.0013). These findings indicate that CTC counts in peripheral blood and surgical lavage fluid serve as important biomarkers for predicting CRC prognosis and are closely associated with both PFS and OS.

Keywords: Circulating tumor cells, colorectal cancer, peripheral blood, lavage fluid, clinical characteristics, prognosis

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy worldwide and the second leading cause of tumor-related death. Extensive research on the tumor microenvironment, gene mutations, and disease subtypes has demonstrated that real-time, individualized disease analysis and clinical decision-making, particularly in the postoperative setting, can significantly improve patient outcomes [1]. Liquid biopsy, a non-invasive technique that complements traditional tissue biopsy, has garnered substantial attention in recent years. Its diagnostic, prognostic, and predictive value has led to broad clinical application, especially in metastatic CRC [2]. Furthermore, liquid biopsy provides dynamic insights into tumor biology, offering important clues regarding CRC pathophysiology and metastatic dissemination [3]. Current therapeutic strategies for CRC include surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. Among these, immunotherapy shows marked efficacy in patients with mismatch repair deficiency or high microsatellite instability; however, its overall efficacy remains limited [4].

Surgery remains the cornerstone of treatment for CRC; however, local recurrence and metastasis continue to be the principal causes of treatment failure. Evidence indicates that pa-

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tients with gastric cancer who have negative postoperative peritoneal washing cytology exhibit a significantly lower recurrence rate [5]. Similarly, the intraoperative dissemination of tumor cells poses a substantial challenge in CRC surgeries. The "membrane anatomy" concept highlights that the mesentery and its associated fascial planes envelop hollow organs like an anatomical "envelope". Disruption of this integrity during surgery may facilitate the shedding of tumor cells into the peritoneal cavity, thereby increasing the risk of local recurrence [6].

Research has indicated that the most common mechanisms underlying recurrence and metastasis in CRC include undetected local, peritoneal, lymphatic or hematogenous micrometastases prior to surgical resection, incomplete tumor removal, and intraoperative tumor cell shedding and implantation. These factors collectively contribute to postoperative local recurrence and distant metastasis. Compared with patients with positive cytology findings in peritoneal lavage fluid, those with negative cytology results prior to tumor resection demonstrate a lower overall recurrence rate, and their local recurrence rate is significantly reduced. Similarly, negative tumor cytology findings in postoperative peritoneal lavage fluid are associated with a markedly lower total recurrence rate [7]. Therefore, intraoperative tumor cell dissemination remains a major challenge in CRC surgery. The introduction of the total mesorectal excision (TME) concept emphasized resection of rectal tumors along the same embryological plane [8]. This approach substantially improved surgical outcomes, significantly reducing local recurrence and increasing long-term survival. In some studies conducted in specific regions, the long -term outcomes of TME for rectal cancers were even superior to those achieved for colon tumors [9]. Subsequent research has further revealed that laparoscopic radical CRC resection based on the principles of TME and membrane anatomy is both safe and reliable [10]. Compared with traditional laparoscopic approaches, it provides superior intraoperative bleeding control, reduces surgical trauma, accelerates postoperative recovery, and markedly enhances lymph node dissection rates [11]. However, important questions remain unresolved. It is still unclear whether laparoscopic CRC surgery guided by membrane anatomy can achieve true oncologic resectability, whether intraoperative cancer cell shedding occurs despite membrane dissection, whether complete tumor resection can be consistently attained to reduce intraoperative dissemination, and whether these factors ultimately translate into lower local recurrence and distant metastasis rates and improved long-term prognosis. Whether membrane-based laparoscopic techniques increase the risk of peritoneal tumor dissemination also remains a concern among surgeons. Furthermore, no domestic or international studies have yet verified whether tumor cell dissociation occurs during surgeries performed according to the membrane anatomy model. Similarly, current evidence does not confirm the "membrane anatomy hypothesis" regarding the potential presence of free cancer cells beneath the envelope-like fascial structures. Although significant advances have been made in the diagnosis and treatment of CRC, the long-term survival rate remains below 60%. This outcome is closely associated with CRC molecular subtype and tumor lymph node metastasis (TNM stage) [12].

Recent studies have indicated that circulating tumor cells (CTCs) are closely associated with tumor recurrence and metastasis [13]. Derived from primary tumor cells, CTCs enter the bloodstream and serve as seeds for metastatic colonization in distant organs. This process underlies the development of recurrence and metastasis across multiple malignancies, including breast, lung, gastric, and head-and-neck cancers [14]. CTC-based liquid biopsy offers noninvasiveness and a repeatable method for monitoring tumor metastasis, disease progression, and treatment response. Detection of CTCs in solid tumors can also reduce the need for invasive procedures to evaluate metastasis spread and recurrence [15]. Some studies have reported that mesenchymal circulating tumor cells (MCTCs) possess prognostic relevance in CRC, and epithelial-mesenchymal transition (EMT) has been recognized as a key mechanism driving enhanced survival and invasion capabilities in epithelial tumor cells [16]. Previous studies have confirmed that EMT plays a key role in CTC-mediated metastasis by enabling CTCs to acquire mesenchymal phenotypes that confer metastatic and survival advantages [17]. Clinical evidence has further shown that MCTCs may promote EMT during various stages of anticancer therapy, contributing to increased drug resistance, tumor recurrence, and metastasis, ultimately leading

to poorer patient prognosis [15]. However, the clinical significance and prognostic value of CTCs and MCTCs in CRC remain inadequately defined.

The innovative aspect of this study lies in the application of membrane anatomy theory to laparoscopic CRC surgery. By detecting tumor cells in peritoneal lavage fluid obtained before radical CRC resection, after tumor resection, and after opening the mesocolic membrane of the excised specimen, this study sought to further validate the membrane anatomy framework. The overarching aim was to elucidate the clinical characteristics and prognostic relevance of CTCs in the peripheral blood and surgical lavage fluid of CRC patients, thereby promoting the development and broader clinical application of membrane anatomy-based surgical techniques. To achieve this, peripheral blood samples were collected from CRC patients preoperatively and one week postoperatively. In addition, peritoneal lavage fluid was obtained before and after laparoscopic tumor resection. The mesocolon of the resected tumor specimen was subsequently incised ex vivo, rinsed with saline, and collected for analysis. CTCs were quantified using the immunofluorescence method. By comparing the number of free tumor cells in the peritoneal and mesocolic lavage samples before and after surgery, we aimed to provide further evidence supporting the membrane anatomy theory. Clinicopathological data were concurrently collected to analyze correlations between preoperative tumor cell counts in peritoneal and mesocolic lavage fluid and patient pathological characteristics, as well as to evaluate the predictive value of these measurements for postoperative prognosis, recurrence, and metastasis. Monitoring the changes of CTCs and MCTCs in membrane anatomy-based lavage fluid and peripheral blood offers significant clinical value for assessing disease status, treatment response, recurrence risk, metastasis potential, and overall prognosis in CRC patients.

Materials and methods

Subjects and samples

This study was approved by the Ethics Committee of Tongren People's Hospital (Approval No. 2019) [18]) and conducted using a retrospective cohort design. Sample size estimation was performed using a chi-square test with $\alpha=0.05$ and statistical power =0.8. Considering an anticipated 15% loss to follow-up, the required sample size for the experimental group was calculated to be 197 cases. However, due to incomplete clinical data and 12 patients lost to follow-up, the final sample size included 185 eligible CRC patients. A total of 185 CRC patients who underwent CRC surgery at our hospital between January 2019 and January 2020 were selected as the observation group, while 50 cancer-free patients were selected as controls; 12 cases were excluded based on predefined criteria through the electronic medical record system. Preoperative evaluation was performed in accordance with NCCN guidelines. Hematological tests included routine blood work, blood biochemistry, gastrointestinal tumor markers, infection immunology, coagulation profile, and ABO and RH blood typing. Cardiopulmonary function was assessed via pulmonary function tests, electrocardiography, Holter monitoring, and Doppler ultrasound. Abdominal plain scan and enhanced CT scans were performed to assess tumor location, size, depth of invasion, resectability, and preoperative risk. In addition, patients with rectal cancer underwent pelvic magnetic resonance imaging to evaluate tumor metastasis and local infiltration. A comprehensive assessment, including psychological and family factors, was performed to confirm eligibility, eliminate confounding factors, formulate individualized treatment plans, and determine the surgical approach. CTCs data and clinicopathological characteristics were collected for all included patients. Patients in the observation group were selected according to the following inclusion criteria: 1) aged ≥18 years. 2) Diagnosis of primary colorectal cancer confirmed by electronic colonoscopy (or anoscopy) and pathological biopsy. 3) Postoperative pathological confirmation of CRC. 4) Underwent laparoscopic radical colorectal cancer resection based on membrane dissection. 5) No evidence of distant organ metastasis on preoperative imaging and auxiliary examinations (TNM stage I-IV). 6) Willingness and ability to undergo routine postoperative follow-up and complete scheduled assessments. 7) Availability of complete clinical and follow-up data. Exclusion criteria were as follows: 1) Conversion from laparoscopic to open surgery. 2) Detection of distant metastasis or peritoneal dissemination during the operation. 3) History of other malignancies. 4) Se-

vere dysfunction of vital organs, including the heart, lungs, liver, and kidneys. 5) Hematologic or immune system disorders. 6) Presence of acute complications such as bleeding, intestinal obstruction and perforation. 7) Pregnancy or lactation. 8) Death from non-tumor-related causes. Fifty healthy individuals were selected as the control group based on the following inclusion criteria: 1) aged 18 years, with a sex distribution matched to the CRC group. 2) Voluntary provision of informed consent. 3) No clinically confirmed diseases following comprehensive evaluation. 4) Absence of gastrointestinal disorders confirmed through questionnaires, medical history reviews, and, when applicable, gastroscopy and colonoscopy. 5) Overall good health and ability to comply with all required examinations and data collection. Exclusion criteria for healthy controls included: 1) Clinically diagnosed gastrointestinal diseases. 2) Family history of CRC in first-degree relatives. 3) History of previous malignancies. 4) Severe dysfunction of vital organs (heart, lungs, liver or kidneys). 5) Hematologic or immune system diseases. 6) Pregnancy or lactation.

Development and validation of the cohort characteristics

A preliminary search of the electronic medical system at our hospital identified 197 patients with CRC who underwent surgical treatment between January 2019 and January 2020. Among these, 2 patients with renal failure, 3 with immune system diseases, 7 with long-term bleeding, intestinal obstruction, or intestinal perforation, and 2 with incomplete clinical data were excluded.

The preoperative data of eligible patients encompassed gender, age, body mass index (BMI), TMN stage, and comorbidities such as arrhythmia, coronary artery disease, hypertension, diabetes, moderate-to-severe anemia (hemoglobin ≤90 g/L), hypoalbuminemia (serum albumin ≤30 g/L), liver function impairment (alanine aminotransferase ≥80 U/L), renal dysfunction (serum creatinine ≥133 μmol/L), and digestive tract tumor markers (alpha-fetoprotein, AFP; carcinoembryonic antigen, CEA; carbohydrate antigen, CA19-9; ferritin; carbohydrate antigen 72-4, CA72-4). Intraoperative data included the type and duration of surgery, intraoperative blood pressure and heart rate (recorded at 5-minute intervals), intraoperative blood loss, transfusion volume, and the use of saline irrigation. To assess data accuracy, we manually reviewed and printed the electronic records for 30 selected procedures and compared them with outputs generated by the electronic data extraction algorithm. This comparison confirmed compliance with data protection standards and consistency of data acquisition. Furthermore, two experienced gastroenterologists, Qiao Song and Tian Yong, independently reviewed all medical records to ensure that each diagnosis met the predefined eligibility criteria.

Methods

Study design and patients

This study adopted a retrospective cohort design. All CRC surgeries were performed by the same medical team at Tongren People's Hospital. TNM staging was conducted according to the American Joint Committee on Cancer (AJCC) colorectal cancer staging system. Postoperative follow-up was carried out according to the National Comprehensive Cancer Network (NCCN) guidelines for CRC.

Experimental procedure

Sample collection methods and detection indicators: A total of 7.5 ml of preoperative peripheral blood was collected from each of the 185 CRC patients and 50 healthy controls. During laparoscopic surgery, intraoperative lavage solutions were separately collected from CRC patients at three time points: before tumor resection, after tumor resection, and following incision of the mesentery of the excised tumor. The procedure for collecting intraoperative lavage fluid is presented in **Figure 1**. Samples were divided into five groups: CTCs (peripheral blood), MCTCs (peripheral blood), CTCs (lavage fluid before resection), CTCs (lavage fluid after resection) and CTCs (lavage fluid after tumor mesocolon opening). CTCs were filtered and isolated from peripheral blood and surgical lavage fluid using CanPatrol™ CTCs enrichment technology. The primary indicators of this study were CTC counts in peripheral blood and lavage fluid. Additionally, preoperative peripheral blood was analyzed for digestive tract tumor markers, including AFP, CEA, CA19-9, ferritin, and CA72-4, which served as secondary indicators. These five markers were selected because they constitute the standard panel of digestive tract tumor mark-

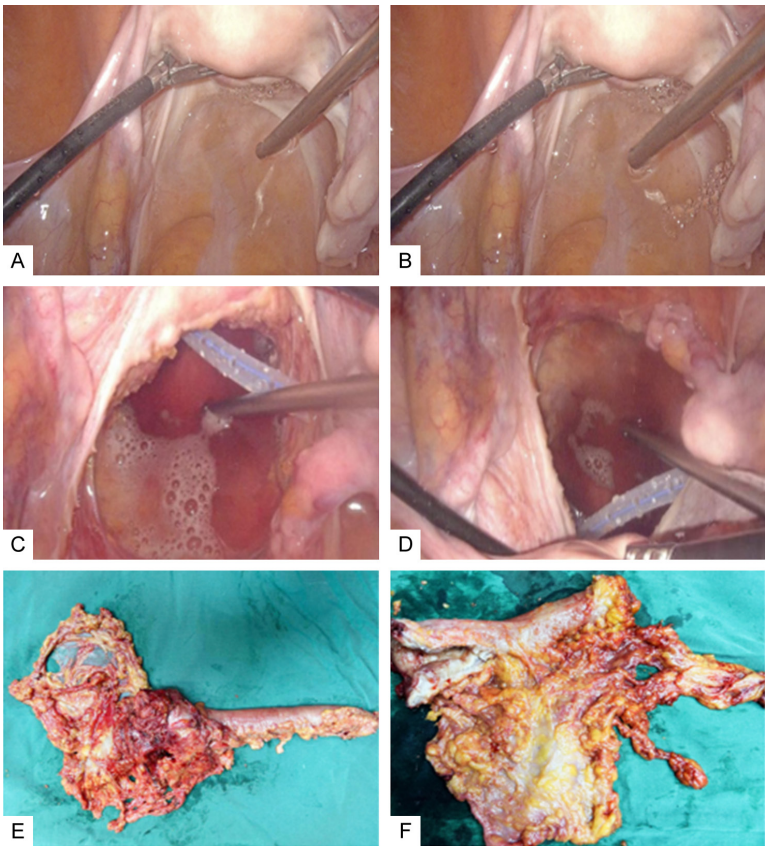


Figure 1. Procedure for collecting lavage fluid at different stages of CRC surgery. A. Tumor tissue is irrigated prior to removal; B. Lavage fluid is collected before tumor excision; C. Tumor bed is re-irrigated after excision of the tissue; D. Lavage fluid is obtained following tumor resection; E. Tumor specimens are examined after resection with the mesentery intact; F. Lavage fluid is collected from the mesenteric tissue after surgical dissection of the mesentery. CRC: Colorectal Cancer. Note: CRC, colorectal cancer.

ers at our hospital and represent the most specific biological tumor markers for gastrointestinal malignancies, thereby providing a consistent basis for subsequent analyses in this study.

Enrichment and identification of CTCs and MCTCs: To identify CTCs and MCTCs, samples were enriched and subjected to fluorescence in situ hybridization (FISH). All procedures were performed in accordance with the manufacturer's instructions, with modifications as required by the researchers. Cell enrichment was achieved via a subtraction-based method. A total of 150 ml of lavage solution, treated with normal saline, was collected at three time points: before, during, and after colorectal cancer surgery. Peripheral blood samples (7.5 mL) were collected 1 day before and 1 week after surgery. Samples were centrifuged at 600×g for 5 minutes, and all cell pellets were immediately resuspended in 3 mL of hematopoietic cell separation matrix (Beijing Lyle Biotechnology Co., LTD., China). The mixture was centrifuged again at 400×g for 5 minutes to remove red blood cells. The resulting supernatant was incubated with anti-leukocyte (CD45) immunomagnetic beads at 25°C for 15 min. The separation substrate was applied, followed by an additional centrifugation at 400×g for 5 min. The supernatant was magnetically separated to remove the beads, and the bead-free solution was centrifuged at 500×g for 2 min. The enriched cells were then thoroughly mixed with 100 mL of cell fixative solution and applied to pre-coated CTC slides. The slides were dried overnight in preparation for subsequent Fish hybridization.

FISH hybridization of CTCs and MCTCs: All acquired samples were processed in accordance with the manufacturer's instructions (Beijing Lyle Biotechnology Co., Ltd., China). Samples were first treated with a centromeric probe (CEP8; Beijing Lyle Biotechnology Co., Ltd., China) for 4 hours. Subsequently, cells were incubated at room temperature for 30 minutes with monoclonal anti-CD45 antibody conjugated with Alexa Fluor 584 and Cy5-conjugated monoclonal anti-CD31 antibody (both from Beijing Lyle Biotechnology Co., Ltd., China) at a 1:200 dilution. Nuclear staining was performed using 4', 6-diamino-2-phenylindole (DAPI; Life Technologies, Carlsbad, California, USA). Stained cells were analyzed under a fluorescence microscope. CTCs and MCTCs were identified and counted by at least two pathologists. Cells were classified as DAPI+, CD45-, and CD31 -/+ cells, and enumeration was performed according to established fluorescence criteria.

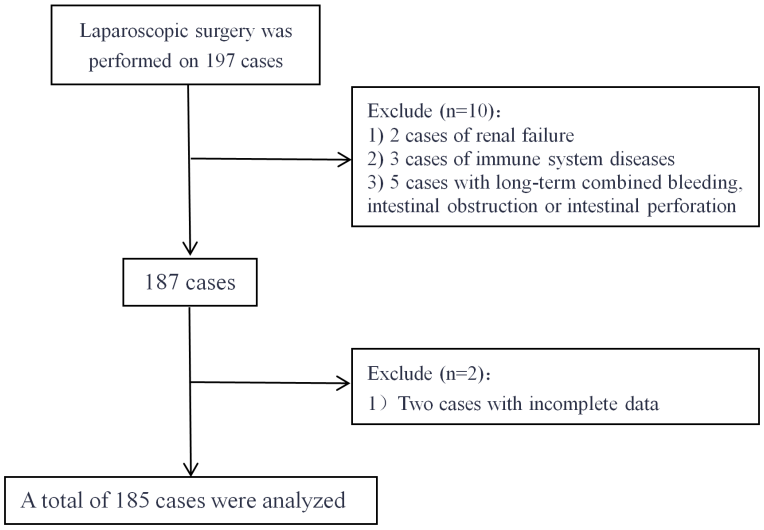


Figure 2. Flow diagram of patient selection.

Determination of CTC and MCTC results

CTCs and MCTCs were defined based on the following criteria:

1) Cells exhibited round, elongated or oval morphology with a diameter ≥8 μm and an intact nucleus. 2) Under a fluorescence microscope, the number of signal points in nuclear fluorescence in-situ hybridization exceeded 2, and the leukocyte surface antigen CD45 was negative. 3) A count of >5 CTCs or MCTCs per 150 ml of surgical lavage fluid or >5 CTCs or MCTCs per 7.5 ml of peripheral blood was considered positive.

Outcome measures

1) We investigated the association between CTC counts and clinicopathological factors, including tumor stage, tumor location, tumor size, metastasis, degree of differentiation, and serum tumor markers (CEA and CA19-9). 2) Long-term follow-up of the 185 CRC patients was conducted to assess progression-free survival (PFS) and overall survival (OS), aiming to validate the prognostic significance of CTCs counts.

Disease follow-up

The follow-up period for all patients spanned 5 years. Medical history assessment and physical examinations were conducted once every three months during the first two years after surgery and every six months during the subsequent three years. Chest, abdominal, and pelvic CT scans were performed at the same intervals: every 3 months for the initial 2 years and once every 6 months for the subsequent 3 years. Colonoscopy was scheduled 1 year after surgery. For patients in whom preoperative colonoscopy was not feasible due to tumor obstruction, the examination was performed within 3 months after surgery. Any detected abnormality warranted a follow-up examination within 1 year. If no abnormality was found, colonoscopy was repeated within 3 years and subsequently at three-year intervals.

Statistical methods

Data were analyzed using SPSS 29.0 statistical software. Categorical variables were expressed as absolute counts and relative frequencies (%), while continuous variables were presented as mean and standard deviation (SD). For continuous variables with a normal distribution and homogeneity of variance, comparisons between groups were conducted using the independent-samples t-test. Otherwise, the Mann-Whitney U test was applied. Categorical variables were compared using the chi-squared (χ^2) test or Fisher's exact test. Survival analyses were performed using the Kaplan-Meier method, with differences between survival curves evaluated using the log-rank test. A two-sided P value<0.05 was considered statistically significant.

Results

Association between CTCs in peripheral blood and surgical lavage fluid and clinical characteristics of CRC patients

A preliminary search of our hospital's electronic medical record system identified 197 CRC patients who underwent surgical treatment between January 2019 and January 2020. After excluding 2 patients with renal failure, 3 with immune system diseases, 5 with long-term complications including bleeding, intestinal obstruction or intestinal perforation, and 2 with incomplete clinical data, a total of 185 patients met the study inclusion criteria (**Figure 2**). For comparison, 50 blood samples were collected from healthy controls. For the

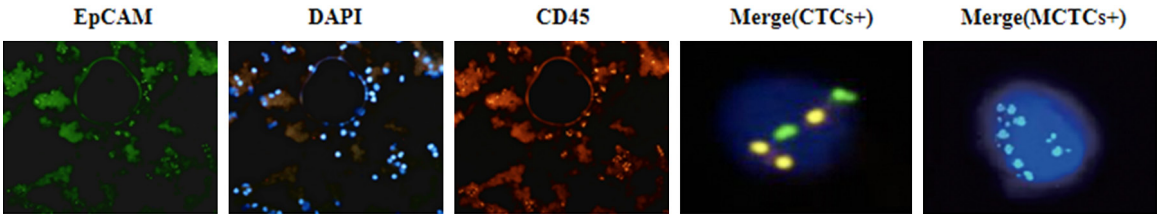


Figure 3. Representative immunofluorescence micrographs of CTCs and MCTCs in CRC patients. CTCs and MCTCs are defined as captured cells with a cell diameter >8 mm. Merge (CTCs+): combined probe labeling of cells positive for CTCs. Merge (MCTCs+): combined probes to labeling of cells positive for MCTCs. Note: CTCs, Circulating Tumor Cells; MCTCs, Micrometastatic Circulating Tumor Cells; CRC, colorectal cancer; EpCAM, epithelial cell adhesion molecules, DAPI, 4, 6-diamidino-2-phenylindole.

185 CRC patients, intraoperative lavage fluid samples were collected at three time points: before tumor resection, after tumor resection, and from the mesenteric opening of the excised tumor tissue. CTCs and MCTCs images are shown in **Figure 3**. The relationship between CTC and MCTC counts and clinicopathological characteristics-including sex, age, primary tumor site, tumor invasion depth, TNM stage, and distant metastasis was analyzed. The results showed that the depth of tumor invasion, TNM stage, and distant metastasis were correlated with preoperative positive detection of CTCs and MCTCs in peripheral blood ($P<0.05$). No significant correlations were observed between the other indicators and the positive detection rates of CTCs and MCTCs in peripheral blood before surgery ($P<0.05$) (**Table 1**). Similarly, analysis of peritoneal lavage fluid demonstrated that in the CTCs-positive groups - before resection, after resection, and after mesocolon opening - the positive detection of CTCs was significantly correlated with tumor invasion depth, TNM stage, and distant metastasis ($P<0.05$). No significant associations were observed between CTC positivity in surgical lavage fluid and other clinical indicators across the three time points ($P<0.05$) (**Table 2**).

Correlation of CTCs and MCTCs in peripheral blood and CTCs in surgical lavage fluid with digestive tract tumor markers

The results indicated a positive correlation between CTCs and CEA levels in peripheral blood (r=0.237, P=0.017). MCTCs in peripheral blood showed a positive correlation with CEA levels (r=0.578, P<0.001). No significant correlations were observed between CTCs or MCTCs and other digestive tract tumor markers, including AFP, CA 19-9, serum ferritin (SF), or CA72-4 levels (P<0.05). For surgical lavage fluid, CEA levels were positively correlated with CTCs-positive groups at all three collection points: before tumor resection, after resection, and after tumor mesocolon opening (P<0.001) (**Table 3**).

Comparison of CTC and CTCs and MCTC levels in peripheral blood

The total counts of CTCs and MCTCs in CRC patients were significantly higher than those in non-tumor controls ($P<0.05$). Their profiles are shown in **Figure 4**.

Independent factors influencing postoperative mortality in CRC patients

During the follow-up period, multiple factors, including gender, age, family history, history of polyps, TNM stage, pathological type, surgical resection method, and counts of CTCs and MCTCs of CRC in peripheral blood - were considered as potential determinants of prognosis. Multivariate analysis was employed to adjust for potential confounding factors. The results showed that TNM stage, CTCs, peripheral blood MCTCs, CTCs in pre-resection lavage fluid, CTCs in post-resection lavage fluid, and CTCs in lavage fluid after tumor mesocolon opening were all independent predictors of postoperative mortality in CRC patients ($P<0.05$) (**Table 4**).

Association between CTCs in peripheral blood, CTCs in surgical lavage fluid, and survival in CRC patients

To evaluated the role of CTCs in the prognosis of CRC patients, this study followed up the prognostic significance of CTCs in CRC patients, all patients were followed for 60 months post-

Table 1. Association between preoperative CTCs and MCTCs in peripheral blood of CRC patients and clinicopathological features

Variants	n (%)	CTCs (peripheral blood)				MCTCs (peripheral blood)			
		CTCs negative [n (%)]	CTCs positive [n (%)]	χ^2	*P*	MCTCs negative [n (%)]	MCTCs positive [n (%)]	χ^2	*P*
Cases	185	88 (47.6)	97 (52.4)			104 (56.2)	81 (43.8)		
Age (years)	58.47±10.02	61.45±12.23	59.91±11.65	4.455	0.108	59.65±12.26	62.33±12.64	0.017	0.896
Gender				1.179	0.555			0.385	0.825
Male	101 (54.6)	49 (55.7)	53 (54.6)			57 (54.8)	45 (55.5)		
Female	84 (45.4)	39 (44.3)	44 (45.4)			50 (45.2)	36 (44.4)		
Tumor location				1.580	0.454			2.000	0.157
Caecum	28 (15.1)	11 (12.5)	17 (17.5)			12 (11.5)	16 (19.8)		
Ascending colon	35 (18.9)	21 (23.9)	14 (14.4)			23 (22.1)	12 (14.8)		
Transverse colon	33 (17.9)	15 (17.0)	18 (18.6)			18 (17.3)	15 (18.5)		
Descending colon	25 (13.5)	10 (11.4)	15 (15.5)			12 (11.6)	13 (16.0)		
Sigmoid colon	64 (34.7)	31 (35.2)	33 (34.0)			39 (37.5)	25 (30.9)		
Depth of tumor invasion				6.490	0.011			8.403	0.003
T1	35 (18.9)	17 (19.3)	18 (18.6)			19 (18.3)	16 (19.7)		
T2	70 (37.8)	33 (37.5)	37 (38.1)			43 (41.3)	27 (33.3)		
T3	55 (29.7)	28 (31.8)	27 (27.8)			30 (28.8)	25 (30.9)		
T4	25 (13.5)	10 (11.4)	15 (15.5)			12 (11.5)	13 (16.0)		
TNM stage				4108	0.041			4.493	0.034
I	35 (18.9)	23 (26.1)	12 (12.4)			24 (23.1)	11 (13.6)		
II	73 (39.5)	36 (40.9)	37 (38.1)			38 (36.5)	35 (43.2)		
III	42 (22.7)	20 (22.7)	22 (22.7)			28 (26.9)	14 (17.3)		
IV	35 (18.9)	9 (10.2)	26 (26.8)			14 (13.5)	21 (25.9)		
Differentiation				2.369	0.124			1.047	0.306
Low	43 (23.2)	26 (29.5)	17 (17.5)			28 (26.9)	15 (18.5)		
Moderate	107 (57.9)	53 (60.3)	54 (55.7)			59 (56.7)	48 (59.3)		
Well	35 (18.9)	9 (10.2)	26 (26.8)			15 (14.4)	20 (24.7)		
Metastasis				4.493	0.034			5.843	0.016
Lymph node	38 (20.5)	11 (12.5)	27 (27.8)			13 (28.8)	25 (30.9)		
Organ	23 (7.0)	7 (8.0)	16 (16.5)			8 (7.7)	15 (18.5)		

Note: CTCs, Circulating Tumor Cells; MCTCs, Micrometastatic Circulating Tumor Cells; TNM, Tumor Node Metastasis; CRC, Colorectal Cancer.

treatment and PFS and OS were recorded. Survival analyses were performed using Kaplan-Meier curves. In peripheral blood, patients with CTC counts >5 and MCTC counts >5 per 7.5 mL had significantly shorter PFS compared to those with CTCs ≤5 and MCTCs ≤5 ($P<0.01$). Similarly, OS was significantly reduced in patients with CTCs >5 and MCTCs >5 in 7.5 mL of peripheral blood ($P<0.01$) (**Figure 5A-D**). For surgical lavage fluid, when CTC counts exceeded 5 per 150 mL at any of the three collection points - before tumor resection, after resection or after tumor mesocolon opening - both PFS and OS were significantly shorter than in patients with CTCs ≤5 ($P<0.01$) (**Figure 5E-J**). These results indicate that monitoring CTCs in peripheral blood and intraoperative lavage fluid is an important biomarker for predicting CRC prognosis. Moreover, CTC counts in lavage fluid at different surgical stages may provide an indirect evaluation of the curative effect of surgical treatment.

Discussion

In this research, we aimed to explore the distribution of CTCs in CRC patients and evaluate their potential as prognostic biomarkers. CTCs are tumor-derived cells that enter the bloodstream and retain genetic and epigenetic characteristics of the primary tumor. Previous studies have reported that the detection rate of CTCs increases significantly with advancing TNM stage. The number of CTCs is considered an independent prognostic factor, correlating with the extent of lymph node involvement and the presence of distant metastases [18]. CTCs levels are strongly associated with PFS and OS, and they can serve as an important indicator for evaluating disease prognosis and predic-

Table 2. Association between positive detection rates of CTCs in surgical lavage fluid and clinicopathological features in CRC patients

Variants	N (%)	CTCs negative (before resection) [n (%)]	CTCs positive (before resection) [n (%)]	χ^2	*P*	CTCs negative (after resection) [n (%)]	CTCs positive (after resection) [n (%)]	χ^2	*P*	CTCs negative (after tumor mesangium opening) [n (%)]	CTCs positive (after tumor mesangium opening) [n (%)]	χ^2	*P*
Cases	185	75 (37.8)	110 (59.5)			96 (51.9)	89 (48.1)			58 (31.4)	127 (68.6)		
Age (years)	58.47±10.02	58.63±9.98	60.34±11.26	3.885	0.051	60.33±10.07	59.36±10.57	2.779	0.066	60.26±10.63	59.65±12.26	0.092	0.955
Gender				0.281	0.596			2.681	0.613			0.448	0.799
Male	101 (54.6)	45 (60.0)	56 (50.9)			54 (56.3)	47 (52.8)			39 (67.2)	62 (48.8)		
Female	84 (45.4)	30 (40.0)	54 (49.1)			42 (43.7)	42 (47.2)			19 (32.8)	65 (51.2)		
Tumor location				1.700	0.427			3.479	0.176			2.858	0.0919
Caecum	28 (15.1)	11 (14.7)	17 (15.5)			10 (10.4)	18 (20.2)			8 (13.8)	20 (15.7)		
Ascending colon	35 (18.9)	10 (13.3)	25 (22.7)			14 (14.6)	21 (23.6)			7 (12.1)	28 (22.0)		
Transverse colon	33 (17.9)	14 (18.7)	19 (17.3)			10 (10.4)	23 (25.8)			8 (13.8)	25 (19.7)		
Descending colon	25 (13.5)	11 (14.7)	14 (12.7)			9 (9.4)	16 (18.0)			4 (6.9)	21 (16.5)		
Sigmoid colon	64 (34.7)	29 (38.6)	35 (31.8)			53 (55.2)	11 (12.4)			31 (53.4)	33 (26.0)		
Depth of tumor invasion				4.353	0.037			3.605	0.043			5.734	0.017
T1	35 (18.9)	19 (25.3)	16 (14.5)			21 (21.9)	14 (11.2)			6 (10.3)	29 (22.8)		
T2	70 (37.8)	34 (45.3)	36 (32.7)			44 (45.8)	26 (29.2)			27 (46.5)	43 (33.9)		
T3	55 (29.7)	15 (20.0)	40 (36.4)			26 (27.1)	29 (32.6)			20 (34.5)	35 (27.6)		
T4	25 (13.5)	7 (9.3)	18 (16.4)			5 (5.2)	20 (22.5)			5 (8.6)	20 (15.7)		
TNM stage				5.452	0.020			5.316	0.021			6.238	0.013
I	35 (18.9)	21 (28.0)	14 (12.7)			20 (20.8)	15 (16.9)			6 (10.3)	29 (22.8)		
II	73 (39.5)	32 (42.7)	41 (37.3)			43 (44.8)	30 (33.7)			25 (43.1)	48 (37.8)		
III	42 (22.7)	10 (13.3)	32 (29.1)			20 (20.8)	22 (24.7)			15 (25.9)	27 (21.3)		
IV	35 (18.9)	12 (16.0)	23 (20.9)			13 (13.5)	22 (24.7)			12 (20.7)	23 (18.1)		
Differentiation				1.778	0.182			1.264	0.261			0.020	0.888
Low	43 (23.2)	27 (40.0)	16 (14.5)			12 (12.5)	31 (34.8)			13 (22.4)	30 (23.6)		
Moderate	107 (57.9)	34 (45.3)	73 (66.4)			70 (72.9)	37 (41.6)			40 (69.0)	67 (52.8)		
Well	35 (18.9)	14 (18.7)	21 (19.1)			14 (14.7)	21 (23.6)			5 (8.6)	30 (23.6)		
Metastasis				5.099	0.024			4.447	0.045			8.339	0.004
Lymph node	38 (20.5)	10 (13.3)	28 (25.5)			20 (20.8)	18 (20.2)			15 (25.9)	33 (26.0)		
Organ	23 (7.0)	6 (8.0)	17 (15.5)			14 (14.6)	9 (10.1)			5 (8.6)	18 (14.2)		

Note: CTCs, Circulating Tumor Cells; TNM, Tumor Node Metastasis; CRC, Colorectal Cancer.

Table 3. Correlation between CTCs and MCTCs in peripheral blood, CTCs in surgical lavage fluid, and biomarkers of digestive tract malignancies (r/P)

Digestive tract tumor markers	CTCs positive (peripheral blood)	MCTCs positive (peripheral blood)	CTCs positive (before resection)	CTCs positive (after resection)	CTCs positive (after tumor mesangium opening)
AFP (ng/ml)	0.024/0.817	0.024/0.884	0.112/0.327	0.175/0.125	0.171/0.095
CEA (ng/ml)	0.578/<0.001	0.237/0.017	0.425/<0.001	0.478/<0.001	0.455/<0.001
CA19-9 (U/ml)	0.031/0.602	0.004/0.969	0.101/0.377	0.176/0.099	0.163/0.171
SF (ng/ml)	0.041/0.412	0.063/0.057	0.170/0.082	0.010/0.914	0.000/0.999
CA72-4 (U/ml)	0.052/0.289	0.136/0.384	0.152/0.107	0.013/0.895	0.174/0.088

Note: CTCs, Circulating Tumor Cells; MCTCs, Micrometastatic Circulating Tumor Cells; AFP, Agence France Presse; CEA, Carcinoembryonic Antigen; CA19-9, Carbohydrate Antigen 19-9; SF, Serum Ferritin; CA72-4,Carbohydrate Antigen 72-4.

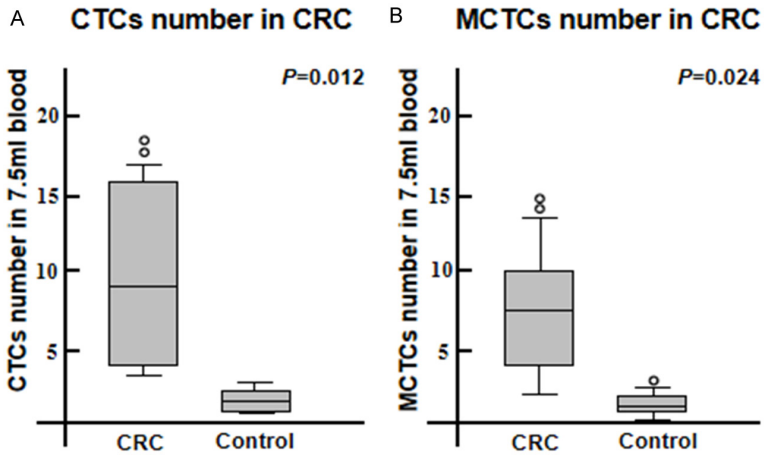


Figure 4. Comparison of CTCs and MCTCs in peripheral blood of CRC patients. A. Total number of CTCs; B. Total number of MCTCs. CTCs, Circulating Tumor Cells; MCTCs, Micrometastatic Circulating Tumor Cells; CRC, Colorectal Cancer.

ting recurrence risk [19]. EMT, characterized by the loss of intercellular adhesion, acquisition of migratory phenotypes, and enhanced cellular motility, plays a vital role in tumor metastasis [20]. Accumulating evidence indicates that EMT is widely involved in the generation of CTCs. Meanwhile, CTCs circulating in the blood may undergo EMT in response to anti-tumor therapies, enhancing their survival and facilitating metastatic colonization [21]. Therefore, the detection of total CTCs and intermediate phenotypic CTCs in peripheral blood may more accurately reflect the malignant characteristics of tumors. This approach can also guide precise risk stratification and individualized treatment for patients, ultimately improving prognostic assessment and treatment planning.

The concept of complete mesocolic excision (CME) was introduced in CRC surgery to achieve optimal oncological outcomes. CME emphasizes the en bloc resection of the tumor within its visceral fascia, along with the associated intestine and mesentery, to minimize tumor cell shedding and prevent metastasis, thereby reducing residual implants and micrometastases. Concurrently, extensive lymph node dissection is performed to maximize radicality and achieve a true curative effect. To date, there has been no domestic or international verification of the "membrane anatomy hypothesis" through the quantification of CTCs in intraoperative peritoneal lavage fluid. However, controversies remain regarding whether laparoscopic CRC surgery under membrane anatomy can achieve complete radical resection, prevent intraoperative cancer cell dissemination, increase the integrity of tumor specimen removal, reduce the risk of local recurrence and distant metastasis, and ultimately improve the long-term prognosis. The potential impact of membrane anatomy-guided laparoscopic surgery on peritoneal dissemination remains a key concern for colorectal surgeons.

This study demonstrated a significant correlation between CTC counts and clinical stage in 185 patients with stage I-IV CRC. The positive detection rates of various CTC-related indicators - including those in peripheral blood and at different intraoperative lavage fluid collection points - were significantly associated with tumor invasion depth, TNM stage and the presence of tumor metastases. These findings imply that elevated CTC levels in both peripheral blood and surgical irrigation fluid reflect more aggressive tumor behavior, including

Table 4. Multivariate analysis of factors influencing prognosis in CRC patients

Factors	β	SE	Wald χ^2	OR	95% CI	*P*
Gender	1.110	1.023	1.175	3.038	0.405-22.667	0.275
Age	1.000	0.873	1.309	2.721	0.488-15.132	0.250
Family history	1.107	1.012	1.250	2.891	0.461-18.132	0.263
Polyp history	1.016	0.898	1.405	2.843	0.473-16.154	0.259
TMN stage	1.251	0.037	1030.124	3.450	3.243-3.774	0.001
Pathological classification	1.266	1.202	1.191	3.551	0.333-37.658	0.290
Surgical resection method	2.043	1.696	1.438	7.727	0.275-213.145	0.226
CTCs (peripheral blood)	2.639	0.029	6891.254	13.987	13.107-14.851	0.001
MCTCs (peripheral blood)	0.935	0.425	4.842	2.550	1.108-5.870	0.028
CTCs (lavage fluid before resection)	0.046	0.013	11.846	0.187	0.014-0.526	*P*<0.001
CTCs (lavage fluid after resection)	0.814	0.568	3.918	0.743	0.382-2.349	0.047
CTCs (lavage fluid after tumor mesangium opening)	0.186	0.016	10.974	1.692	0.093-7.457	*P*<0.001

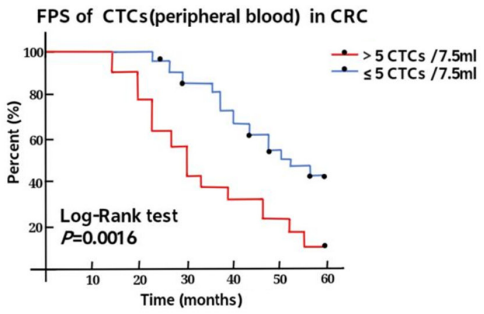
Note: CTCs, Circulating Tumor Cells; MCTCs, Micrometastatic Circulating Tumor Cells; TNM, Tumor Node Metastasis; CRC, colorectal cancer.

deeper invasion, advanced TNM stage and increased metastatic potential. Furthermore, CTC levels in both peripheral blood and surgical lavage fluid were strongly associated with serum CEA levels. The combined assessment of these indices may aid in predicting the risk of lymph node metastasis in CRC patients and support the development of individualized treatment plans. Our results show that CTCs and MCTCs serve as specific biomarkers in the peripheral blood of CRC patients, consistent with previous studies [22]. The TNM stage, peripheral blood CTCs, peripheral blood MCTCs, CTCs in pre-resection lavage fluid, CTCs in post-resection lavage fluid and CTCs in mesangial lavage fluid were identified as independent risk factors for patient death. These results indicate that in patients undergoing laparoscopic radical resection of colorectal tumors following the membrane anatomy approach, the intraoperative dynamics of tumor cells can be effectively evaluated through the detection of free tumor cells in peritoneal fluid. Importantly, this study represents the first application of tumor cell detection in mesangial lavage fluid to verify the "membrane anatomy theory", with our findings providing empirical support for the theoretical framework [6]. The mechanism underlying recurrence and metastasis in CRC is highly complex, with elevated expression of CTC levels playing a particularly critical role in patients with advanced CRC. We hypothesize that high CTC expression may be a major contributor to the increased mortality observed in advanced CRC. Tumor cells within the mesangial tissue and the "envelope" structure of colorectal tumors are correlated with pathological characteristics. Moreover, CTCs detected in peripheral blood and intraoperative lavage fluid provide valuable prognostic predictive value for patients with CRC. Particularly, CTCs in the lavage fluid collected during membrane anatomy-based CRC surgery have higher predictive value for evaluating the long-term prognosis of patients after surgical treatment.

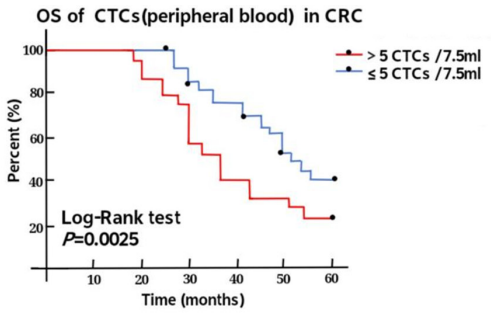
This study demonstrates that CTCs and MCTCs in peripheral blood are highly valuable biomarkers for monitoring disease progression in CRC patients. Laparoscopic surgery was performed based on the concept of membrane anatomy, and CTCs were detected in peritoneal lavage fluid during the procedure. We analyzed both the efficacy and safety of radical CRC resection under the membrane anatomy approach, as well as the predictive value of CTCs for tumor recurrence, metastasis and prognosis. Our research found that the combined detection of CTCs and CEA provides a more effective approach for monitoring CRC patient survival. Unlike previous studies, we quantified CTCs in surgical lavage fluid both during and after the operation, which allows for assessment of surgical quality, detection of intraoperative cancer cell shedding, and indirect evaluation of the prognostic value of the surgical intervention.

This study does have certain limitations. Due to the limited probe specificity of the detection kit, only CTCs and MCTCs were measured in peripheral blood, and only CTCs were assessed in surgical lavage fluid. Moreover, the study cohort was relatively small, including 185 patients with a 60-month follow-up, which restricted the

A


FPS of CTCs(peripheral blood) in CRC
> 5 CTCs /7.5ml
≤ 5 CTCs /7.5ml
Percent (%)
Log-Rank test
P=0.0016
Time (months)

B


OS of CTCs(peripheral blood) in CRC
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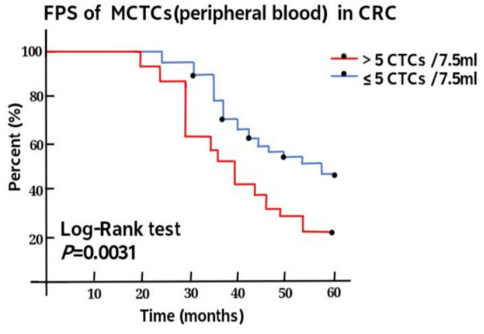
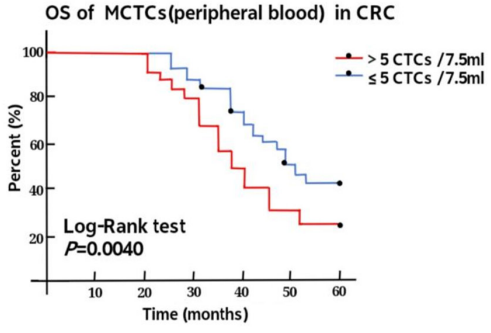
≤ 5 CTCs /7.5ml
Percent (%)
Log-Rank test
P=0.0025
Time (months)

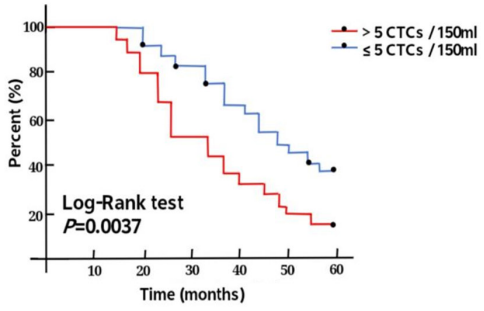
C


FPS of MCTCs(peripheral blood) in CRC
> 5 CTCs /7.5ml
≤ 5 CTCs /7.5ml
Percent (%)
Log-Rank test
P=0.0031
Time (months)

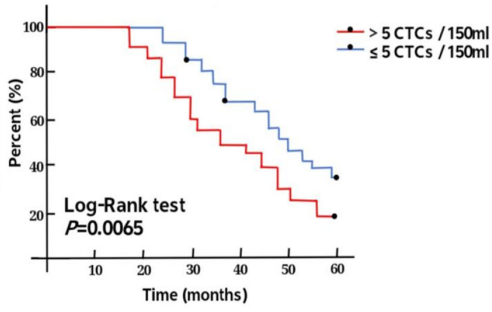
D


OS of MCTCs(peripheral blood) in CRC
> 5 CTCs /7.5ml
≤ 5 CTCs /7.5ml
Percent (%)
Log-Rank test
P=0.0040
Time (months)

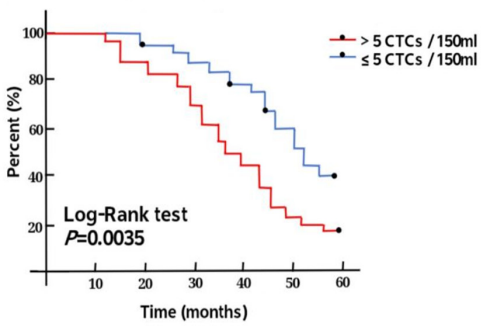
E


FPS of CTCs(Flushing solution before excision) in CRC
> 5 CTCs /150ml
≤ 5 CTCs /150ml
Percent (%)
Log-Rank test
P=0.0037
Time (months)

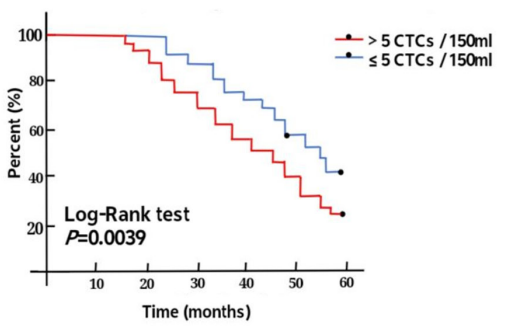
F


OS of CTCs(Flushing solution before excision) in CRC
> 5 CTCs /150ml
≤ 5 CTCs /150ml
Percent (%)
Log-Rank test
P=0.0065
Time (months)

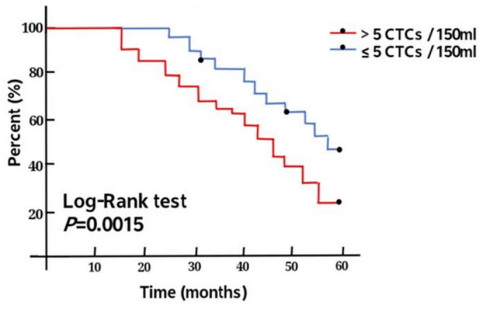
G


FPS of CTCs(Flushing solution after excision) in CRC
> 5 CTCs /150ml
≤ 5 CTCs /150ml
Percent (%)
Log-Rank test
P=0.0035
Time (months)

H


OS of CTCs(Flushing solution after excision) in CRC
> 5 CTCs /150ml
≤ 5 CTCs /150ml
Percent (%)
Log-Rank test
P=0.0039
Time (months)

I


FPS of CTCs(Flushing fluid after tumor mesangium opening)
> 5 CTCs /150ml
≤ 5 CTCs /150ml
Percent (%)
Log-Rank test
P=0.0015
Time (months)

J

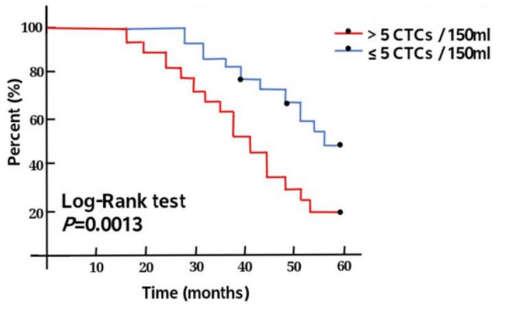

OS of CTCs(Flushing fluid after tumor mesangium opening)
> 5 CTCs /150ml
≤ 5 CTCs /150ml
Percent (%)
Log-Rank test
P=0.0013
Time (months)

Figure 5. Kaplan-Meier survival analysis of CTCs and MCTCs in peripheral blood and surgical lavage fluid of CRC patients. A. PFS of patients with >5 CTCs vs. ≤5 CTCs in peripheral blood; B. OS of patients with >5 CTCs vs. ≤5 CTCs in peripheral blood; C. PFS of patients with >5 MCTCs vs. ≤5 in peripheral blood; D. OS of patients with >5 MCTCs vs. ≤5 in peripheral blood; E. PFS of patients with >5 CTCs vs. ≤5 in lavage fluid collected before surgical resection; F. OS of patients with >5 CTCs vs. ≤5 in the lavage fluid collected before surgical resection; G. PFS of patients with >5 CTCs vs. ≤5 in lavage fluid collected after surgical resection; H. OS of patients with >5 CTCs vs. ≤5 in lavage fluid collected after surgical resection; I. PFS of patients with >5 CTCs vs. ≤5 in lavage fluid after tumor tissue mesangium opening; J. OS of patients with >5 CTCs vs. ≤5 in lavage fluid after tumor tissue mesangium opening. CTCs, Circulating Tumor Cells; MCTCs, Micrometastatic Circulating Tumor Cells; CRC, colorectal cancer; PFS, Progression Free Survival; OS, overall survival.

analysis of longitudinal changes in PFS and OS. Future studies should optimize experimental conditions, expand the sample size, and include longer follow-up periods to validate and extend these findings.

In summary, this study comprehensively investigated both the relationship between CTC levels and clinicopathological features of CRC, as well as the association between CTC detection and patient survival outcomes. CTCs and MCTCs in peripheral blood represent valuable biomarkers for predicting CRC prognosis. In particular, the quantification of CTCs in surgical lavage fluid provides additional prognostic insight. Therefore, the detection of CTCs in both peripheral blood and intraoperative lavage fluid has significant clinical implications and holds promise as a novel biomarker for evaluating CRC prognosis. This approach may also guide the formulation of personalized treatment strategies, facilitate evaluation of tumor biological behavior, and support the implementation of tailored, comprehensive therapeutic interventions.

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Disclosure of conflict of interest

None.

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