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Clinical significance of perioperative EMT-CTCs in rectal cancer patients receiving open/laparoscopic surgery

W. YIN[1,#], Y. M. HAN[2,3,#], Z. L. LI[2], Z. X. HUANG[2], L. HUANG[2], X. G. ZHONG[2,*]

[1]Department of Pathology, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning 530021, Guangxi, China; [2]Department of General Surgery, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning 530021, Guangxi, China; [3]Department of General Surgery, The First People's Hospital of Xuzhou, Xuzhou, Jiangsu, China

**Correspondence: xiaogangzhong@163.com*

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The objective of this study was to explore the clinical significance of perioperative CTCs (circulating tumor cells) counts and EMT-CTCs (epithelial-mesenchymal transition-CTCs) in rectal cancer patients. A total of 30 patients with rectal cancer who underwent radical resection of rectal cancer at the Guangxi Zhuang Autonomous Region People's hospital were enrolled. Five ml peripheral blood was withdrawn from 30 patients with rectal cancer before the operation and seven days after the operation and at the corresponding time also from 20 healthy volunteers. CanPatrol™ CTC detection technique was used to enrich and identify CTCs and IER3 expression simultaneously. We found out that the preoperative total CTCs were correlated with lymph node metastasis (p=0.008) and tumor size, and mixed CTCs were closely correlated with lymph node metastasis (p=0.009). The number of IER3-positive total CTCs and mesenchymal CTCs were statistically associated with tumor size, p=0.034 and 0.043, respectively. The number of CTCs varied significantly before and after the operation in all patients (p=0.049). There were significant differences in CTCs variations between the open operation group and the laparoscopic operation group. In the laparoscopic operation group, the average number of single-cell CTCs was 6.9 before operation and 3.5 after the operation (p=0.013). In the open operation group, the average number of single-cell CTCs was 5.9 before operation and 4.2 after the operation. To conclude, surgery is associated with a decrease of CTCs in rectal cancer patients, especially in patients receiving laparoscopic surgery. The number of CTCs before the operation in rectal cancer patients is related to the size of tumors and regional lymph node metastasis. CTCs detection and characterization may be useful for clinical staging and lymph node dissection during operation.

Key words: rectal cancer, CTCs, EMT, open radical resection, laparoscopic radical resection

Colorectal cancer is the third most common malignant tumor worldwide and over 50% of all patients will ultimately develop relapse or metastatic diseases [1]. Currently, surgery is the preferred choice for the treatment of this disease. With the improvement of technology, laparoscopic surgery is getting more and more applications in many surgery domains [2]. Due to the considerations on costs and oncological safety, the development of laparoscopic surgery for rectal cancer is relatively slow compared to other abdominal solid tumors [3]. A large amount of clinical data has shown that the local relapse rates of both laparoscopic and open surgery for rectum cancer were 5%, no statistically significant difference in 3-year disease-free survival (DFS) and overall survival (OS) between these two groups was observed [4, 5]. However, several studies demonstrated that laparoscopy was inferior to open surgery in the positive margin and survival [2, 6, 7]. Therefore, more reference biomarkers are required to help to choose the appropriate mode of surgery for every patient.

Circulating Tumor Cells (CTCs) are rare tumor cells found in the peripheral blood of cancer patients, and the presence of CTCs is considered the "seeds" of metastatic disease that accounts for 90% of all cancer-related deaths [8]. Several studies were conducted in various types of cancers (breast, lung, colorectal, prostate, melanoma, etc.) and presenting CTCs have proven prognostic value in these cancer types [9–13]. Advanced colorectal patients with 5 CTCs/7.5 ml or higher CTCs had a significantly worse survival than those in which CTCs were less than 5 [11]. In patients with non-metastatic colorectal cancers, the detection of CTCs was predictive for therapeutic effect and progression [14–18].

Epithelial-mesenchymal transition (EMT) is a cellular process in which cells lose their epithelial characteristics and acquire mesenchymal features [19]. EMT has been associated

with various tumor functions, including tumor initiation, tumor cell migration, intravasation to the blood, resistance to therapy, and metastasis [20]. Studies of CTCs have identified significant heterogeneity of epithelial and mesenchymal marker expression, as well as the presence of biphenotypic cells that express markers of both cell lineages [21]. Increased mesenchymal marker expression correlated with triple-negative breast cancer and also was suggestive of therapeutic resistance [22]. However, few studies have focused on the EMT-CTC variations in rectal patients before and after the operation.

The immediate early response gene X-1 (IEX-1), also known as IER3, belongs to the family of the immediate early response genes [23]. Unlike other members of the family, IER3 lacks a DNA-binding domain and may function as a co-activator or co-repressor [23, 24]. Differences in IER3 expression between tumor tissue and adjacent normal cells can be helpful in both the prognostic prediction and clinical management of cancer patients [25]. Current data suggest that the absence of IER3 expression was associated with poor prognosis in ovarian cancer [26], while positive expression of IER3 predicted progression in breast cancer and myeloma [25, 27]. As in colorectal cancer, the prognostic prediction of IER3 expression remained controversial yet [25, 28, 29].

In this study, CanPatrol™ (Surexam, Guangzhou, China) technique [30] was used to identify perioperative CTC counts and CTC subpopulations in rectal patients receiving laparoscopic/open surgery. We evaluated the relationship between CTCs, IER3 expression of CTCs, and characteristics of rectal patients, as well as CTC changes after surgery.

Table 1. Characteristics of patients included in this study (n=30).

Factor	Subgroups	n	%
Age	<55	10	33.3
	≥55	20	66.7
Gender	male	22	73.3
	female	8	26.7
Histology	adenocarcinoma	30	100
Differentiation	moderate	30	100
T stage	2–3	10	33.3
	4	20	66.7
N	N0	12	40
	N+	18	60
TNM stage	I	4	13.3
	II	8	26.7
	III	18	60
Tumor size	≤3 cm	19	63.3
	>3 cm	11	36.7
Vascular/perineural invasion	yes	8	26.7
	no	22	73.3
Type of surgery	open	15	50
	laparoscopy	15	50
Surgical margins	negative	30	100
	positive	0	0
CTCs	negative	2	6.7
	positive	28	93.3

CTCs – circulating tumor cells

Patients and methods

Patients and healthy volunteers. Thirty patients with rectal cancer and 20 patients with benign diseases were consecutively enrolled between September 2016 and December 2017 at Gastrointestinal Surgery, the People's Hospital of Guangxi Zhuang Autonomous Region. All colon cancer patients received laparoscopic or open surgery. Peripheral blood specimens (5 ml) for CTC analysis were withdrawn one day before surgery and 7 days after surgery. Healthy volunteers also collected 5 ml peripheral blood at the corresponding time point. This study protocol was approved by the Institutional Ethics Committee, and written informed consent was obtained from all the patients.

CTC analysis. The enrichment and characterization of CTCs were conducted with CanPatrol™ technique. In brief, CTCs from the blood samples were isolated and enriched by the filtration method through a calibrated membrane with 8 μm pores as previously described [30].

mRNA in situ hybridization (ISH). A tri-color ISH assay was conducted to identify and classify CTCs. Specific capture probes for epithelial markers included EpCAM, CK8, CK18, and CK19, while these for mesenchymal markers included Twist1 and Vimentin. A CD45 probe was used to identify leukocytes. After incubation with all three kinds of probes, cells on the membrane were stained with DAPI for another 5 min before analyzed with an automatic fluorescence microscope. The ISH results were further verified by qualified pathologists. Red fluorescence, green fluorescence, and bright white fluorescence indicated the expression of epithelial markers, mesenchymal markers, and leukocyte biomarker, respectively. Epithelial CTCs were characterized as red fluorescence-positive $CD45^{-}DAPI^{+}$, while biphenotypic CTCs were characterized as red and green fluorescence-positive $CD45^{-}DAPI^{+}$, mesenchymal CTCs were characterized as green fluorescence-positive $CD45^{-}DAPI^{+}$. The expression of IER3 in each CTC was analyzed by mRNA-ISH using purple fluorescence as described above.

Follow-up analysis. Follow-up was performed by outpatient visit, telephone, or hospital stay. The first outpatient visit was performed one month after surgery. Serum tumor marker, CT, or MRI were performed for evaluation. The second and third outpatient visit was performed 3 months after surgery with routine blood tests, and 6 months after surgery with colonoscopy, respectively. The subsequent follow-up was conducted by telephone and outpatient visit, in turn, every 3 months. Postoperative treatment was performed if required. The latest follow-up time was May 1, 2019.

Statistical analysis. All data were analyzed using SPSS 23.0 software package (SPSS Inc., Chicago, IL, USA). Comparisons of CTCs between groups were assessed by non-param-

eters tests. Spearman correlation test was used to evaluate the relations between clinicopathological features and IER3 expression. A chi-squared test was used for comparison between groups where appropriate. A p-value <0.05 on a two-sided level was considered statistically significant.

Results

Patient characteristics. A total of 30 rectal cancer patients (22 males, 8 females; median age 62 years, range 32–82 years) were enrolled in this study. The basic characteristics of 30 rectal cancer patients are summarized in Table 1, including age, gender, histology, differentiation, TNM stage, tumor size, lymphatic metastasis, vascular/perineural invasion, types of surgery, and surgical margins.

Classification of CTCs. The CTCs were classified into three subpopulations according to the epithelial-to-mesenchymal transition (EMT) status by using multiplex RNA-ISH assay, including epithelial CTCs, mesenchymal CTCs, and biphenotypic epithelial/mesenchymal CTCs (Figure 1A). The results also showed that no CTC could be detected in healthy donors. Analysis of CTCs in all 30 patients with rectal cancer revealed that 93.3% were CTC-positive. The distribution of CTCs in each patient is summarized in Table S1.

Association between CTCs number, phenotypes, and patient pathological features. Next, we explored the correlations between CTC counts or EMT status and clinicopathological characteristics, as shown in Table 2. The total CTCs median of advanced stages (III) rectal cancer patients was higher than that of early stages (I+II) patients (6.0 CTCs/5 ml vs. 3.0 CTCs/5 ml; p<0.05), indicating that higher total CTCs number was associated with advanced tumor stages (Figure 2A). Patients without lymph node metastasis presented lower total CTCs numbers, and biphenotypic CTCs compared with patients presenting lymph node metastasis (3.0 CTCs/5 ml vs. 6.0 CTCs/5 ml, p=0.006; 0.5 CTCs/5 ml vs. 3.5 CTCs/5 ml, p=0.004, respectively, Figure 2B). Similarly, patients with smaller tumor size (≤3 cm) showed significantly lower total CTCs, biphenotypic CTCs, and mesenchymal CTCs than patients with tumor diameter over 3 cm. In addition, younger patients (<55 y) had higher total CTC counts than older patients (6.5 CTCs/5 ml vs. 3.5 CTCs/5 ml, p=0.019). The distribution of CTC counts or EMT-CTC status presented no significant difference in gender, T stage, vascular/perineural invasion or type of surgery.

The number of IER3-positive CTCs correlates with tumor size in rectal cancer. Total CTCs number was ≥1 CTC/5 ml in 28 out of 30 rectal patients. We investigated

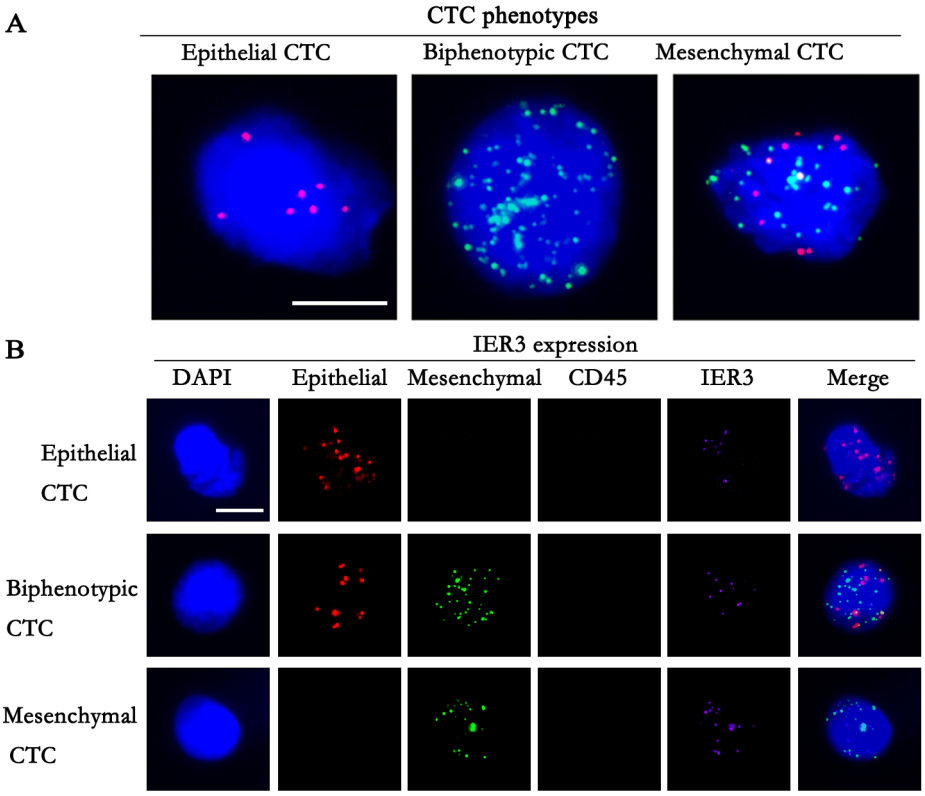

Figure 1. EMT phenotypes and IER3 expression of CTCs detected by the RNA in situ hybridization in rectal cancer patients. A) Fluorescence microscopy images of three types of CTCs with positive expression of epithelial markers (EpCAM and CK8/18/19, red dots), biphenotypic markers (red dots and green dots and mesenchymal markers (Vimentin and Twist, green dots). B) IER3 expression (Alexa Fluor 647) in the epithelial, hybrid, and mesenchymal CTCs. Scale bar = 10 μm.

the expression of IER3 gene in CTCs from these 28 patients. The distribution of IER3 positive CTCs in each patient is summarized in Table S1. As shown in Table 3, the number of IER3-positive total CTCs and mesenchymal CTCs were statistically associated with tumor size, p=0.034 and 0.043, respectively. There was no significant relationship between the number of IER3-positive CTCs and clinicopathological variables of rectal cancer, including age, gender, type of surgery, TNM stage, and vascular/perineural invasion (Table 3).

CTC changes of different operative approaches. One half of 30 rectal patients received open surgery, while another half received laparoscopic surgery. As shown in Table S2, no significant difference in clinical parameters between the two

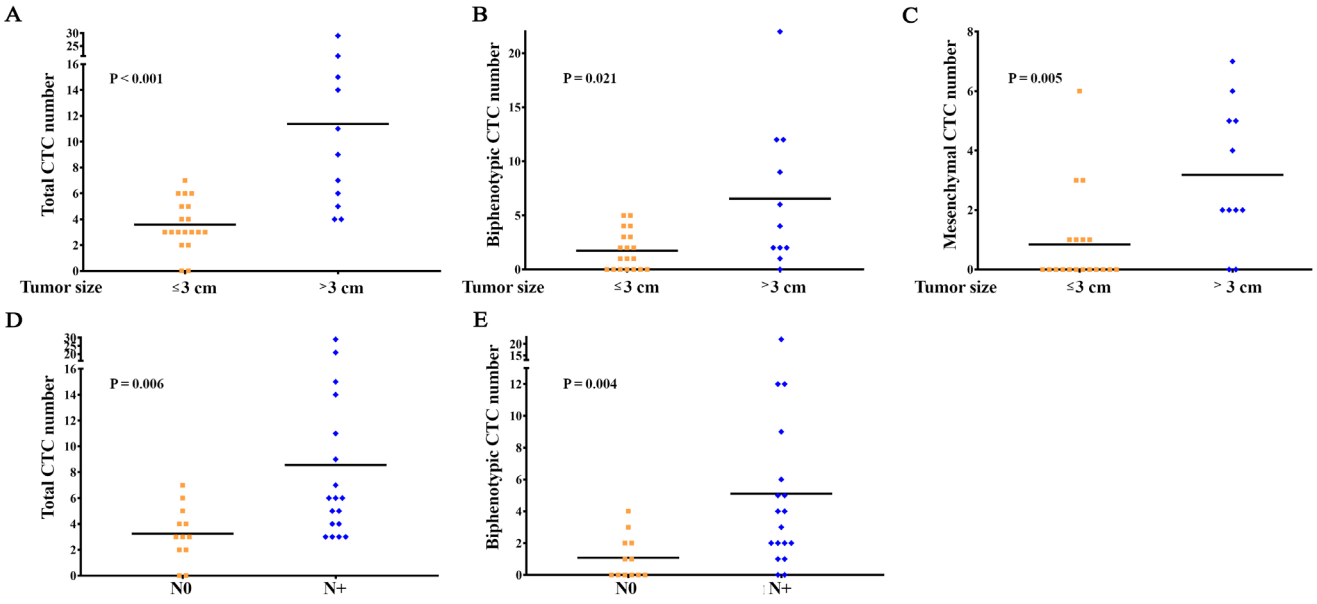

Figure 2. Correlation of CTCs/CTC subpopulations and clinical characteristics. A-C) the distribution of total CTCs, biphenotypic CTCs, and mesenchymal CTCs between patients with tumor size ≤ 3 cm and > 3 cm, respectively. D and E) the distribution of total CTCs, and biphenotypic CTCs between patients with/without lymph node metastasis.

Table 2. Correlations between CTCs counts three types of CTCs and clinical data of rectal cancer patients (n=30).

		n	Total CTCs median	p-value	Epithelial CTCs median	p-value	Biphenotypic CTCs median	p-value	Mesenchymal CTCs median	p-value
Factor	Subgroups									
Age	< 55	10	6.5	0.019	0.0	0.948	4.0	0.1	1.5	0.267
	≥ 55	20	3.5		1.0		1.5		0.0	
Gender	male	22	5.0	0.237	1.0	0.393	2.0	0.534	0.5	0.629
	female	8	4.0		0.0		2.0		1.0	
T stage	2–3	10	4.5	0.812	0.5	0.746	2.0	0.812	1.0	0.65
	4	20	4.5		1.0		2.0		0.5	
Tumor size	≤ 3 cm	19	3.0	<0.001	0.0	0.35	1.0	0.021	0.0	0.005
	> 3 cm	11	9.0		1.0		4.0		2.0	
N	N0	12	3.0	0.006	1	0.692	0.5	0.004	0	0.134
	N+	18	6.0		0.5		3.5		1.5	
TNM stage	I– II	12	3.0	0.006	1.0	0.692	0.5	0.004	0	0.134
	III	18	6.0		0.5		3.5		1.5	
Vascular/perineural invasion	yes	8	4.5	0.696	0.0	0.534	3.5	0.277	1.0	0.765
	no	22	4.5		1.0		2.0		0.5	
Types of surgery	open	15	3.0	0.137	1.0	0.87	1.0	0.015	0.0	0.744
	laparoscopy	15	5.0		1.0		4.0		1.0	

N – lymph node; CTCs – circulating tumor cells; bold font means p<0.050

Table 3. Correlations of IER3-positive total CTCs and three types of CTCs with clinical parameters.

Characteristics	Parameter	IER3-positive CTC			
		Epithelial	Biphenotypic	Mesenchymal	Total
Age	ρ[a]	0.232	0.004	0.137	0.183
(≤55 / >55)	p[b]	0.217	0.982	0.470	0.332
Gender	ρ	0.206	–0.145	–0.083	–0.067
(male / female)	p	0.274	0.444	0.663	0.727
Type of surgery	ρ	–0.073	–0.153	0.177	–0.067
(laparoscopy / open)	p	0.701	0.419	0.35	0.726
Vascular/perineural invasion	ρ	0.066	0.036	0.070	0.023
(yes / no)	p	0.731	0.852	0.713	0.902
Tumor size	ρ	0.021	0.232	**0.388**	**0.372**
(≤3 cm / >3 cm)	p	0.912	0.217	0.034	0.043
TNM stage	ρ	0.027	–0.014	0.209	0.111
(I–II / III)	p	0.889	0.939	0.267	0.561
T stage	ρ	0.213	–0.017	0.251	0.231
(2–3 / 4)	p	0.258	0.931	0.181	0.220
N	ρ	–0.009	–0.026	0.090	0.048
(N0 / N+)	p	0.961	0.893	0.637	0.802

[a]Spearman's Rho, [b]Probability, bold font means p<0.050, N – lymph node; CTCs – circulating tumor cells

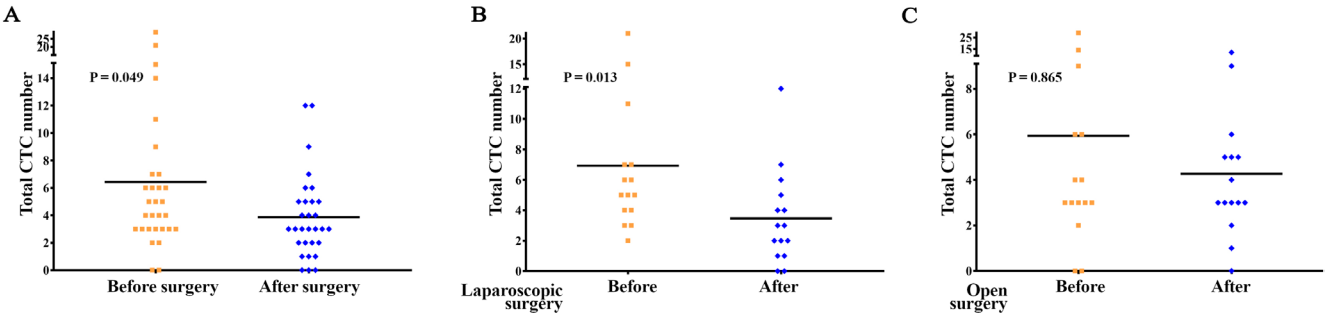

Figure 3. The distribution of total CTCs before and after surgery. A) the distribution of total CTCs in all patients before and after surgery. B and C) the distribution of total CTCs in patients received laparoscopic and open surgery, respectively.

groups was discovered. All 30 patients screened for CTC counts before and after surgery. A comparison of the preoperative and postoperative CTC counts is shown in Figure 3. The preoperative and postoperative CTC positive rates were 93.3% and 90.0%, respectively. Compared with preoperative CTC counts (mean 3.9 cells), the mean CTC counts after operation decreased significantly (mean 6.4 cells), p=0.049, while no significant difference was discovered between preoperative and postoperative CTC subpopulations. In the laparoscopic surgery group, the mean preoperative CTC count was 6.9 CTCs/5 ml, and the mean postoperative CTC count was 3.5 CTCs/5 ml, p=0.013. In the open surgery group, the mean preoperative CTC count was 5.9 CTCs/5 ml, and the mean postoperative CTC count was 4.2 CTCs/5 ml, p>0.05. CTC subpopulations or IER3-positive CTCs at both time points showed no obvious difference in either mode of operation (open or laparoscopic).

Prognostic significance of perioperative CTCs changes. The latest follow-up time was May 1, 2019. The median follow-up time was 19.5 months (13–26 months). Fifty-nine patients were successfully followed up at regular intervals. One patient in the laparoscopic group was lost to follow up. Longer follow-up will be required to determine the prognostic significance of perioperative CTCs changes.

Discussion

Compared to traditional invasive approaches, such as tissue biopsy and molecular imaging, monitoring for CTCs is relatively noninvasive, requiring only a peripheral blood sample. CTCs are rare cells that are detached from primary tumors and metastatic deposits into the blood circulation. The acquisition of this invasive phenotype of tumors cells is hypothesized to correlate with EMT. During this process,

tumor cells downregulate the expression of specific epithelial markers including EpCAM, E-cadherin, and cytokeratin, upregulate the expression of mesenchymal proteins such as vimentin and N-cadherin, Twist, and so on [31]. After entering the bloodstream, CTCs can revert to the epithelial state through mesenchymal to epithelial transition (MET), implying the presence of a transition state between epithelial and mesenchymal [32]. These surface biomarkers are important for CTC identification and classification.

EpCAM-based CTCs enrichment and identification technique (CellSearch™, Janssen Diagnostics, LLC, USA) has been widely used for clinical prognostic assessment of advanced cancer [9]. The dependence of CTC isolation on epithelial cell-specific technique may lead to an underestimation of the actual number of CTCs by missing tumor cells that underwent EMT [33–35]. CanPatrol™ technique is a combination of membrane filtration and epithelial/mesenchymal biomarkers-based identification and enables the isolation of CTCs with low/no epithelial markers possible.

In the present study, CTC counts, as well as biphenotypic CTCs were significantly related to the TNM stage and lymph node metastasis. It is consistent with previous reports showing that CTCs number positively correlate with stage [18, 36, 37]. These results support the idea of CTCs as a reference marker for preoperative staging and lymph node dissection. Interestingly, we found that younger patients had more CTC counts than older patients, indicating the role of CTCs in identifying high-risk patients.

Surgery remains an effective choice for rectal cancer patients. Our results showed that CTC counts in rectal cancer patients with TNM stage I–III decreased significantly after the operation, which is in line with previous findings in colorectal cancer and hepatocellular carcinoma [17, 38]. However, other studies about hepatocellular carcinoma and squamous cell carcinoma of the head and neck reported the elevation of CTCs after surgery [39, 40]. The discrepancy of these research results may possibly be due to the differences in CTC detection and time of blood sampling. In the laparoscopic surgery group, postoperative CTC counts decreased significantly comparing with preoperative CTC counts. Compared with open surgery, lower postoperatively CTC counts of laparoscopic surgery could be justified by the advantage of laparoscopy, including less blood loss, light pain, earlier return of bowel movement, and reduced length of hospital stay [4, 6]. However, due to the relatively short follow-ups, the prognostic prediction of perioperative CTC counts and EMT-CTCs in rectal patients receiving laparoscopic/open surgery is currently impossible.

Furthermore, we analyzed the expression of IER3 in perioperative CTCs from rectal cancer patients for the first time. Previous studies showed that the expression of IER3 is distinct even in the same tumors with different disease stages [41]. The positive IER3 expression predicted good outcome and prognosis in the early stage of colorectal cancer patients [42]. Inconsistent with a previous report [41], we found that IER3 expression in CTCs was only associated with tumor size. No significant correlations were observed between IER3 expression and other clinical parameters such as age, sex, TNM, lymph node metastasis, and mode of surgery. This discrepancy may be attributed to differences in sample size, technological issues or others. Additional larger sample studies with longer follow-ups to ascertain the clinical value of IER3 are warranted.

Taken together, our findings suggested both CTC counts and biphenotypic CTCs were significantly related to the TNM stage and lymph node metastasis, implying the potential value of CTCs and EMT-CTCs as a reference marker for preoperative staging and lymph node dissection. Furthermore, our study presented, for the first time, the perioperative CTC counts and EMT-CTCs between rectal patients receiving laparoscopic or open surgery. However, the main limitation of our study is the small sample size and short follow-ups, which might affect our results. Therefore, larger studies with adequate follow-ups are required for validating our results and drawing a convincing conclusion.

Supplementary information is available in the online version of the paper.

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