

Determination of the optimal detection time of circulating tumor cells for the postoperative monitoring of colorectal cancer

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Abstract. Circulating tumor cells (CTCs) are widely used in cancer screening and monitoring. The present study focused on investigating the optimal time for the postoperative CTC detection in patients with colorectal cancer (CRC) to obtain more accurate results and facilitate subsequent treatment. By subtraction enrichment immunofluorescence *in situ* hybridization detection of CTCs, the present study demonstrated that different postoperative detection times in CRC substantially influenced the CTC numbers. In total, 134 subjects were enrolled. Among 10 healthy individuals and 20 preoperative patients with CRC, no CTCs were identified in the healthy subjects, and CTCs were detected in 85% (17/20) of the preoperative patients. In total, 104 postoperative patients with CRC (53 males and 51 females) with a mean average age of 57.63 years were studied. The total CTC detection rate was 81.73% (85/104) and the mean average CTC numbers in patients with tumor stage (T) T1, T2, T3 and T4 were 4.00, 3.33, 5.90 and 5.64 per 7.50 ml of peripheral blood, respectively. The CTC number trends in these four tumor stages within 5, 6, 7 and 10 postoperative days were variable, and were the most stable at 7 days. Gradual upward trends in CTC numbers were

observed after 5, 6 and 7 postoperative days, and this upward trend was more obvious after 7 days. Overall, the findings of the present study suggest that CTC detection in CRC should be performed after at least 7 postoperative days rather than within 7 postoperative days.

Introduction

Colorectal cancer (CRC) is a common gastrointestinal malignancy that has no obvious symptoms in the early stage, but causes symptoms including hematochezia, diarrhea, constipation and local abdominal pain following disease progression (1). The incidence and mortality rates of CRC are high, accounting for 9% novel cancer cases in males and 8% in females, in 2019 in the United States (2). Although colonoscopy is the gold standard for CRC diagnosis and can be used to assess the location of a tumor, the invasiveness of this method may affect patient participation; furthermore, other auxiliary methods, such as fecal occult blood tests and rectal finger examinations have limited sensitivity for disease detection (1).

In recent years, liquid biopsy technologies, such as circulating tumor DNA and circulating tumor cell (CTC) detection have substantially advanced the screening and monitoring of CRC (3-5). CTCs, which shed from carcinoma tissue, have been widely used in the screening and monitoring of cancers, including CRC, breast cancer, small cell lung cancer and other solid tumors. CTC monitoring and screening has been incorporated into the Guidelines for Tumor Staging formulated by the American Joint Committee on Cancer (5-8). The CTC detection results obtained using a binary-blend fiber-based capture assay are identical to those from the pathological analysis of colonoscopy biopsies (9). A study demonstrated that when 55 patients with CRC underwent CTC detection, the median CTC number was 30.8 cells/ml (range, 5.8-431.3/ml), and the number of CTCs was associated with the prognosis of patients with CRC (10). Yang *et al* (11) demonstrated that postoperative CTC positivity was independently associated with a shorter 3-year recurrence-free survival rate compared with preoperative CTC positivity. In a study by Wu *et al* (12), the detection rates of epithelial CTCs, mesenchymal CTCs (M-CTCs), epithelial/mesenchymal CTCs and circulating tumor microembolus (CTMs) in 126 patients with CRC were

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Abbreviations: ACD, acid-citrate-dextrose; CTCs, circulating tumor cells; CRC, colorectal cancer; COX-2, cyclooxygenase-2; M-CTC, mesenchymal CTC; CTM, circulating tumor microemboli; SE-iFISH, subtraction enrichment-immunofluorescence *in situ* hybridization

Key words: circulating tumor cells, colorectal cancer, postoperative monitoring

76.98, 42.06, 56.35 and 36.51%, respectively. Additionally, the metastases of tumors in patients with CRC who had CTMs and M-CTCs had a higher risk of tumor metastasis compared with patients in the other CTC subgroups (12). A study also revealed that the expression of leucine-rich repeat-containing G protein-coupled receptor 5 in CTCs was significantly associated with the incidence of CRC metastasis (13). Patients with CRC who had metastasis had a higher expression of cyclooxygenase-2 (COX-2) compared with patients without metastasis, and the expression of COX-2 was positively correlated with the expression of mesenchymal cell markers (14). Thus, numerous studies that have investigated the screening and monitoring of CRC with CTCs have demonstrated a good monitoring effect.

Studies have investigated CRC screening, recurrence and metastasis; however, very few studies have focused on selecting the best CTC detection time for postoperative monitoring of CRC. The results of the present study demonstrated different detection times had a significant impact on CTC results, which influenced the formulation of treatment plans and monitoring of tumor progression. In addition, different detection methods also have a substantial impact on CTC results. CTC detection by the CellSearch system revealed a low detection rate based on a positive antibody capture method (15), while subtraction enrichment-immunofluorescence *in situ* hybridization (SE-iFISH) performed better (16). SE-iFISH is based on the subtraction enrichment of hematogenous cells and subsequent removal of red and white blood cells, ultimately leaving only the CTCs (17). SE-iFISH is currently used widely in the detection of CTCs (17).

In order to establish an appropriate time for CTC detection, to determine the diagnostic effect, the present study detected CTCs in postoperative patients with CRC at different times by SE-iFISH (18) and defined the influence of different detection times on CTC results, and determined the optimal time for CTC detection.

Materials and methods

Clinical samples. Subjects were enrolled at Anyang Tumor Hospital (Anyang, China) between January 2017 and April 2019. There were a total of 134 subjects, including 10 healthy individuals (6 males and 4 females, age range 46-66 years) and 124 patients with CRC (64 males and 60 females, age range 23-84 years) (Table I). Permission to use peripheral blood samples was obtained from the Ethics Committee of Anyang Tumor Hospital. All subjects signed an informed consent form for the study. The SE-iFISH testing group included 10 healthy people and 20 patients with CRC before treatment, and the experimental group included 104 postoperative patients with CRC. The tumor node metastasis staging standard for CRC was from the Union for International Cancer Control 8th Edition (19). Peripheral blood specimens (7.5 ml each) were obtained from patients, collected in Vacutainer acid-citrate-dextrose (ACD) tubes (BD Biosciences) and stored at room temperature, and subsequent assays were performed in a timely manner.

SE-iFISH. SE-iFISH CTC detection was performed using the CTC Enrichment kit (Cytelligen) according to the manufacturer's instructions. Briefly, 7.5 ml of peripheral blood were

collected in an ACD anticoagulant tube and centrifuged at 800 x g for 8 min at room temperature to remove plasma, for CTC enrichment. Blood cells were transferred into centrifuge tubes containing 3 ml hCTC separation matrix and subsequently centrifuged to discard red blood cells at 450 x g for 8 min at room temperature. The Buffy coat cells were collected in tubes and incubated with an immunomagnetic particle conjugated anti-CD45 antibody (part of the kit) on horizontal rotators. Subsequently, the tubes were centrifuged at 200 x g for 20 min at room temperature and placed on a magnetic stand (Corning Inc.; cat. no. IMAG-150-I-G) to remove leukocytes and obtain CTCs. Cytelligen Fixative reagent (part of the kit) was added to the CTCs, and the mixture was tiled onto a glass slide and dried overnight at 30°C.

For CTC identification, the dried cells were treated with 20 μ l of FR1 and 180 μ l of an FR2 mixture for 10 min, washed with FR3 buffer and dehydrated with ethanol. After air drying for 5 min, 10 μ l of probe solution (fluorescence-labeled CEP8 probes), part of the kit, was added to the glass slide, which was subsequently subjected to fluorescence *in situ* hybridization with denaturation at 76°C for 10 min and hybridization at 37°C for 4 h. Subsequently, the cells were immunostained with anti-CD45 (1:200) and anti-CK18 (1:200) antibodies (part of the kit) in the dark at room temperature for 20 min. After washing with Washing Solution (part of the kit) three times, the slide was dyed with DAPI reagent for 1 min at room temperature and observed under a fluorescence microscope (Nikon Corporation; magnification, x20). The CTCs were confirmed for CK18⁺CD45⁺DAPI⁺CEP8=2, CK18⁺CD45⁺DAPI⁺CEP8>2 and CK18⁺CD45⁺DAPI⁺CEP8>2. The white blood cells were confirmed for CK18⁺CD45⁺DAPI⁺CEP8=2 (18).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 statistical software (GraphPad Software, Inc). The data are presented as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CTC detection by SE-iFISH is suitable for CRC screening. To assess the efficacy of SE-iFISH for the detection of CTCs in CRC and to evaluate the performance of this method, 10 healthy individuals and 20 patients with CRC who had received prior treatment were enrolled in the present study. Peripheral blood specimens (7.5 ml) were obtained from the subjects, and serum, red blood and white blood cells were removed. CTCs were retained for SE-iFISH detection. The results showed that no CTCs were detected in samples from the 10 healthy individuals, and 85% (17/20) of the patients with CRC had CTCs (Fig. 1A and B). The average numbers of CTCs present in the samples of these 20 patients with T1, T2, T3 and T4 stages were 2.25, 2.40, 5.00 and 11.00, respectively, and the trend was consistent with disease progression (Fig. 1B). However, due to individual differences and the small number of samples, some differences existed among the patients, even among those who had disease in the same pathological stage (Fig. 1B). This indicated that SE-iFISH may be used for the detection of CTCs in CRC and had high specificity.

Table I. CTC counts in pre and postoperative patients with CRC.

Subjects	Male, n	Female, n	Total, n	Age, mean \pm SD	CTC count, mean \pm SD
Healthy individuals	6	4	10	59.30 \pm 6.10	0.00 \pm 0.00
Preoperative patients with CRC	11	9	20	61.00 \pm 6.07	5.00 \pm 3.76
T1	2	2	4	66.00 \pm 3.56	2.25 \pm 1.71
T2	3	2	5	58.40 \pm 4.04	2.40 \pm 2.30
T3	3	4	7	65.57 \pm 1.90	5.00 \pm 1.83
T4	3	1	4	53.50 \pm 6.35	11.00 \pm 1.83
Postoperative patients with CRC	61	53	104	57.63 \pm 13.23	4.92 \pm 6.04
T1	7	3	10	55.20 \pm 8.01	4.00 \pm 4.22
T2	13	17	30	54.60 \pm 9.50	3.33 \pm 4.65
T3	22	20	42	61.24 \pm 13.86	5.90 \pm 6.26
T4	11	11	22	56.00 \pm 16.94	5.64 \pm 7.63

CTC, circulating tumor cells; CRC, colorectal cancer; T, tumor; SD, standard deviation.

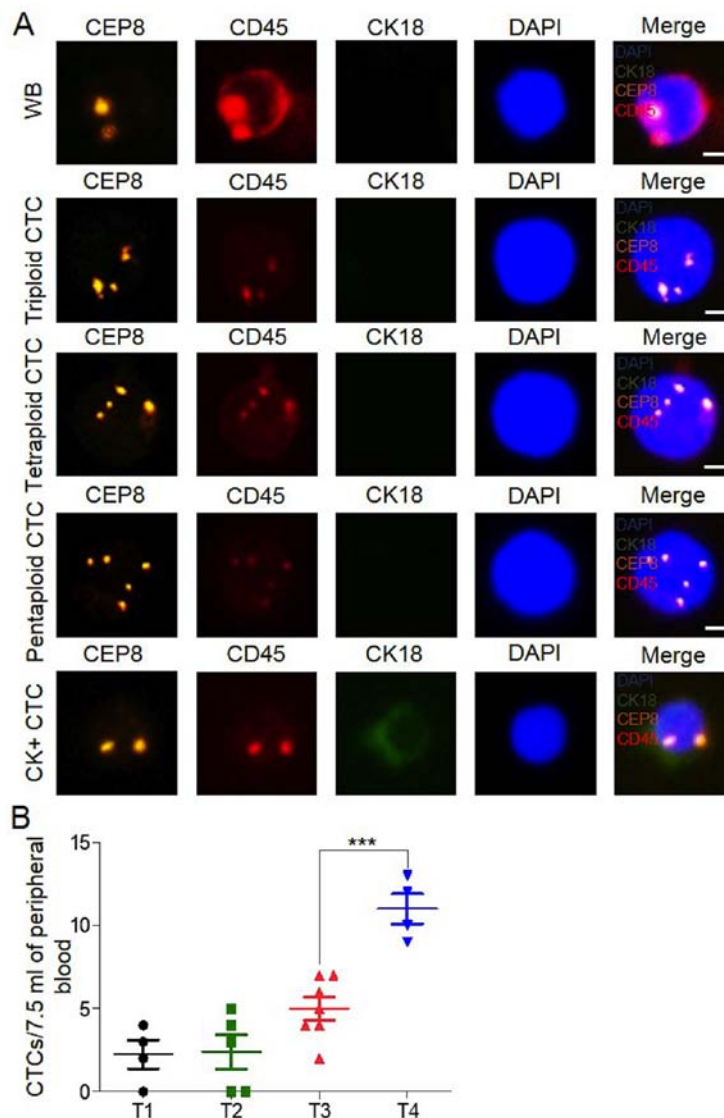


Figure 1. Detection of CTCs in blood of patients with CRC, by using subtraction enrichment-immunofluorescence *in situ* hybridization. (A) CTCs and white blood cell image, CEP8 (orange), CD45 (red), CK18 (green), and DNA (blue). Scale bar, 5 μ m. (B) CTC numbers in 20 patients with CRC from 7.5 ml of peripheral blood. T1, n=4; T2, n=5; T3, n=7 and T4, n=4. ***P<0.001. CTC, circulating tumor cell; CRC, colorectal cancer.

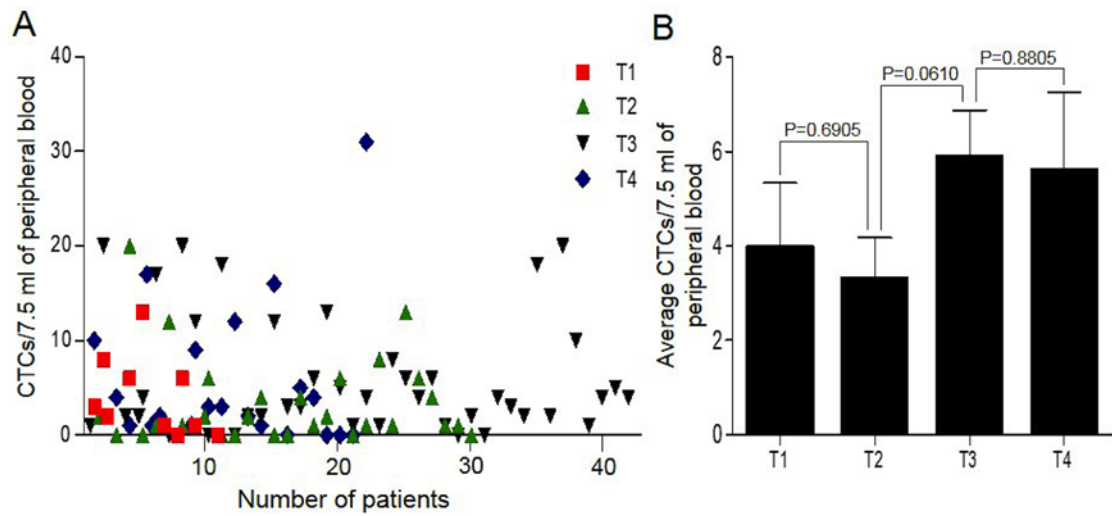


Figure 2. Detection of CTCs in 104 postoperative patients with CRC. (A) CTC numbers of 104 postoperative patients (B) CTC numbers in different tumor stages. T1, n=10; T2, n=30; T3, n=42 and T4, n=22. CTC, circulating tumor cells; CRC, colorectal cancer.

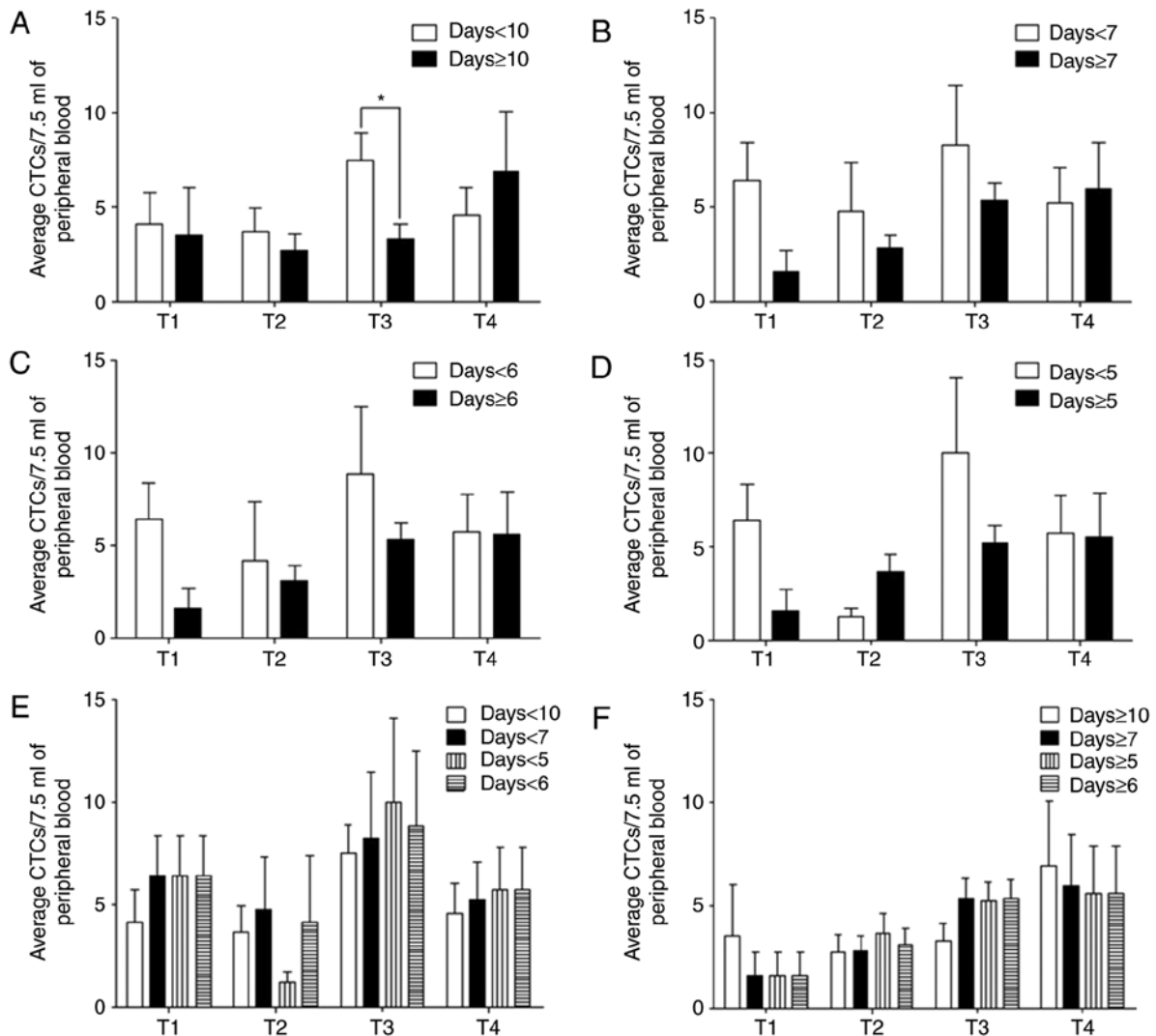


Figure 3. CTC detection at different times. (A) The CTC numbers within 10 postoperative days (T1, n=8; T2, n=19; T3, n=26; T4, n=12) and after 10 postoperative days (T1, n=2; T2, n=11; T3, n=16; T4, n=10). (B) The average CTC numbers within 7 postoperative days (T1, n=5; T2, n=8; T3, n=8; T4, n=9) and after 7 postoperative days (T1, n=5; T2, n=22; T3, n=34; T4, n=13). (C) The CTC numbers within 6 postoperative days (T1, n=5; T2, n=6; T3, n=7; T4, n=8) and after 6 postoperative days (T1, n=5; T2, n=24; T3, n=35; T4, n=14). (D) The average CTC numbers within 5 postoperative days (T1, n=5; T2, n=4; T3, n=6; T4, n=8) and after 5 postoperative days (T1, n=5; T2, n=2; T3, n=36; T4, n=14). (E) Comparison of the CTC numbers within 5, 6, 7 and 10 postoperative days. (F) The CTC numbers after 5, 6, 7 and 10 postoperative days. *P<0.05. CTC, circulating tumor cells; CRC, colorectal cancer.

Table II. Circulating tumor cell counts in postoperative patients with colorectal cancer.

Days	T1, mean \pm SD	T2, mean \pm SD	T3, mean \pm SD	T4, mean \pm SD
≥ 10	3.50 \pm 3.54	2.73 \pm 2.80	3.31 \pm 3.20	6.90 \pm 10.08
<10	4.13 \pm 4.58	3.68 \pm 5.49	7.50 \pm 7.16	4.58 \pm 5.04
≥ 7	1.600 \pm 2.51	2.82 \pm 3.32	5.35 \pm 5.46	5.92 \pm 8.99
<7	6.40 \pm 4.39	4.75 \pm 7.30	8.25 \pm 9.02	5.22 \pm 5.61
≥ 6	1.60 \pm 2.51	3.13 \pm 3.71	5.31 \pm 5.38	5.57 \pm 8.73
<6	6.40 \pm 4.39	4.17 \pm 7.81	8.86 \pm 9.56	5.75 \pm 5.75
≥ 5	1.60 \pm 2.51	3.65 \pm 4.91	5.22 \pm 5.34	5.57 \pm 8.73
<5	6.40 \pm 4.39	1.25 \pm 0.96	10.00 \pm 9.94	5.75 \pm 5.75

T, tumor; SD, standard deviation.

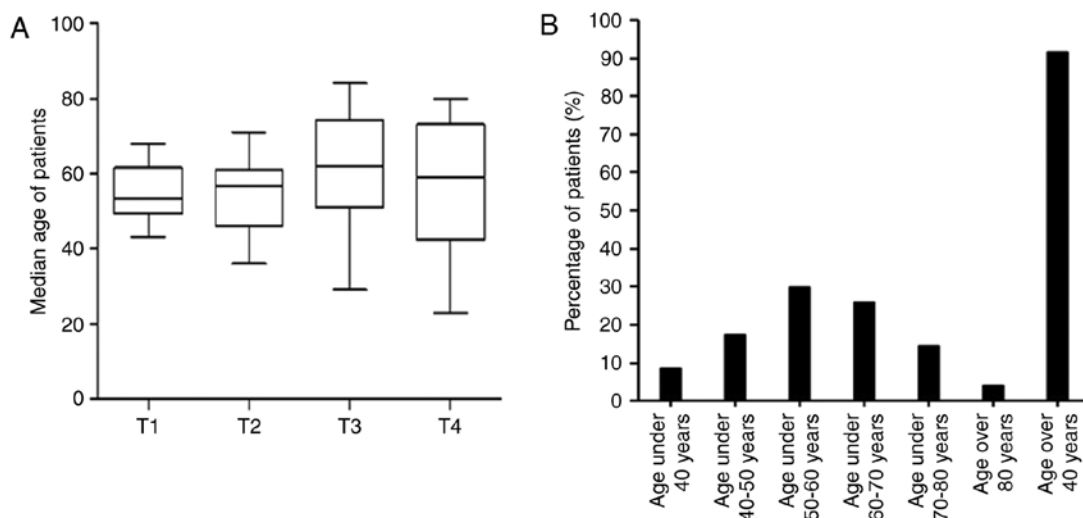


Figure 4. Effect of age on colorectal cancer incidence. (A) Ages of patients in different tumor stages; T1, n=10; T2, n=30; T3, n=42 and T4, n=22. (B) Percentages of patients with cancer in different age groups; <40 years, n=9; 40-50 years, n=18; 50-60 years, n=3; 60-70 years, n=27; >80 years old, n=4 and >40, n=95. CTC, circulating tumor cells.

Detection of CTCs after 7 days postoperation may have clinical value. After verifying the validity of the CTC detection method, the optimal CTC detection time for postoperative patients with CRC were investigated and the impact of different test times on the CTC results were investigated. In total, 104 postoperative patients with CRC (4-20 days postoperation) were enrolled in the present study. The patients had no cancer metastasis, and 53 were male and 51 were female with a mean average age of 57.63 years. CTCs were detected in 81.73% (85/104) of the patients, and the mean average CTC number was 4.92/7.50 ml of peripheral blood (Fig. 2A). Despite the difference between T1 and T2 ($P=0.6905$), those between T2 and T3 ($P=0.0610$) and T3 and T4 ($P=0.8805$) were not significant. The average CTC numbers of the T1, T2, T3 and T4 tumor stages were 4.00, 3.33, 5.90 and 5.64, respectively (Fig. 2B; Table I). The results indicated that CTCs could be detected in the peripheral blood of postoperative patients with CRC and were not completely eradicated in a short time despite the tumor tissue having been removed.

Postoperative patients were divided into four groups based on the 4 tumor stages (T1, T2, T3 and T4), with a possibility of

one patient being assigned into multiple groups. For instance, if a patient presented with CTCs 10 days after surgery, this patient would be grouped under both the '7 days postoperative' and '6 days postoperative' subgroups. The CTC numbers in each group were analyzed within 5, 6, 7 and 10 postoperative days or after 5, 6, 7 and 10 postoperative days (days ≥ 5 , days ≥ 6 , days ≥ 7 and days >10). For the CTCs detected within 10 postoperative days, the average CTC numbers of the T1, T2, T3 and T4 tumor stages were 4.13, 3.68, 7.50 and 4.58, while those after 10 postoperative days were 3.50, 2.73, 3.31 and 6.90, respectively (Fig. 3A; Table II).

The average CTC numbers within 5, 6, 7 and 10 postoperative days demonstrated a fluctuating trend, while the average CTC numbers after 5, 6 and 7 postoperative days demonstrated a linear trend and was more consistent with the cancer stage (Fig. 3A-D). By comparison, the trend within 7 and 10 postoperative days had smaller fluctuations compared with within 5 and 6 postoperative days (Fig. 3E; Table II). The trend of CTC numbers after 7-10 postoperative days had a more consistent trend with cancer staging than that after 5-6 postoperative days (Fig. 3F; Table II). Overall, the average number of CTCs

detected within 5, 6, 7 and 10 postoperative days were higher compared with those after 5, 6, 7 and 10 postoperative days. These results suggest that CTC detection after 7 postoperative days provide accurate data for patients and may be used for improved follow-up diagnoses and monitoring for patients.

Age factor effect on CRC incidence. The mean ages of the 104 patients with CRC within the T1, T2, T3 and T4 tumor stages were 53.50, 56.50, 62.00 and 59.00 years, respectively. The trend of age increase was partly consistent with that of cancer progression (Fig. 4A). The cancer incidence rates in the different age groups of the 104 patients were investigated, which revealed that patients <40 years of age had the lowest incidence rate of CRC (8.65%; 9/104), patients between 50-60 years of age had the highest incidence (29.81%; 31/104). Patients >40 years accounted for 91.35% (95/104) of the cohort (Fig. 4B). Therefore, the incidence of CRC in patients >40 years is the highest.

Discussion

A number of studies have investigated the screening and monitoring of CRC by CTCs, demonstrating that different detection methods have a substantial influence on CTC detection results (16,20,21). By using the CTC detection method SE-iFISH in the present study, and the positive rate of CTCs in healthy individuals was revealed to be 0%, and the positive rate in patients with CRC was 85%, demonstrating higher sensitivity and specificity in CRC screening compared with the CellSearch system (16). Additionally, 81.73% of postoperative patients also had CTCs in peripheral blood samples. This phenomenon may explain why patients with cancer are prone to recurrence or metastasis after surgery (20,21).

Among the patients in the present study, the numbers of CTCs in the T1 and T4 phases were not significantly different from each other. There may be a number of reasons for this lack of significance. First, the pretreatment sample should be used to evaluate the association between the CTC number and cancer stage to avoid the interference of surgery, chemotherapy or neo-adjuvant chemotherapy on the CTC number. However, as the present study focused on patients after surgery, the CTC numbers may have been affected by surgery. Secondly, this phenomenon also showed the importance of the time choice for CTC detection. Although the trend of CTC numbers in stages T1-T4 were not exactly coordinated with the cancer stage, the trend of CTC numbers after 7 days of detection corresponded to the cancer stage and showed an upward trend. However, the CTC number exhibited a fluctuating trend within 7 days and this result was more inconsistent with the tumor stage. Therefore, it is important to choose a detection time that accurately reflects the CTC numbers and the actual condition of patients.

The results of the present study demonstrated that different CTC detection times in postoperative patients had a substantial effect on CTC numbers and further affected the accuracy of the efficacy evaluation. Detection of CTCs in 104 postoperative patients with CRC elucidated a phenomenon in which the CTC number trend was consistent with the corresponding patient stages and showed an upward trend. The reason for this result may be that although the cancer tissues were resected, certain CTCs remained in the blood

circulation system and could not be completely cleared in the short term. On the other hand, the result may have been due to the surgical technique leading to the spread of some cancerous tissue cells into the blood circulation. The average numbers of postoperative CTCs in patients with CRC within 5, 6, 7, and 10 days were compared with those after 5, 6, 7 and 10 days post-surgery in the present study. This revealed that the numbers of postoperative CTCs within 5, 6, 7 and 10 days fluctuated remarkably, whilst those after 7 days showed a more stable upward trend than those after 5, 6 and 10 days. To summarize, CTCs should be detected at least 7 days post-operatively to obtain accurate results.

The present study identified that the incidence of CRC may be associated with age of patients with CRC. Patients >40 years accounted for 91.35% of the total population, and patients >50 years accounted for 74.04%. These results are in agreement with a previous study that revealed that people >50 years of age had a high risk of CRC (22). Therefore, people >40 years of age have a higher incidence of CRC and require further investigation. It was noteworthy that there were 4 patients (two T3 patients and two T4 patients) >80 years of age in the present study. The >80 years age group had a lower incidence of CRC compared with the other age groups, which may be due to the low clinical visit rate, or patient death due to disease progression. Larger-scale follow-up studies are needed to firmly establish the effect of age on CRC incidence.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

TH, XZ and FZ designed and conceived the present study, drafted the initial manuscript and reviewed the manuscript. JX and QW acquired the samples and clinical information. CX and DB adjusted the test method and detected samples by SE-iFISH. YW and YZ analyzed and annotated the data. XZ and FZ supervised the study. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Anyang Tumor Hospital Ethics Committee and written informed consent was obtained from all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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