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Clinical significance of circulating tumor cells testing in auxiliary diagnosis of gastrointestinal cancer

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Routine diagnostic tools and serum biomarkers of gastrointestinal (GI) cancer have limitations in detecting early and micro-metastasis, and circulating tumor cells (CTCs) have emerged as a promising metrics to complement this gap. The present study is designed to explore technical feasibility of using CTCs as an auxiliary diagnostic tool in GI cancer. Over all, 70 inpatients with GI cancer and 30 healthy volunteers were recruited, and 10 mL of peripheral venous blood was collected from all subjects. CTCs were detected by microfluidic blood rare cell analysis technique, and the sensitivity and specificity of CTCs in GI cancer diagnosis were derived from comparison with the pathological diagnosis results and serum tumor marker results. Compared with the healthy volunteers, the CTCs levels of the patients in gastrointestinal cancer group were significantly increased. Advanced stage subjects demonstrated higher level of CTCs, yet without statistical significance. The sensitivity of CTCs to diagnose stage I to IV disease were 84.62, 94.12, 94.44, and 100.00%, respectively, yielding a comprehensive sensitivity of 92.56% and specificity to be 89.66%. Combined detection of CTCs and four tumor serum markers was helpful in detecting positivity rate, but without statistical signifiance compared with detecting CTCs alone. Our study demonstrates the value of CTCs as an auxiliary diagnostic method for gastrointestinal cancer, and could meet the deficiency of routine tissue biopsy, which can be used alone or in combination with conventional serum tumor markers and thereby facilitate the clinical diagnosis of gastrointestinal cancer.

Keywords: Serum tumor marker

Gastrointestinal cancer is the most common malignant tumor of the digestive system in clinical practice in our country. Its morbidity and mortality are much higher than the global average, and the incidence is higher and more younger patients are being diagnosed with this disease[1]. One of the important indicators affecting the prognosis of gastrointestinal cancer is tumor staging. After treatment for early disease, the 5-year survival rate of patients can reach more than 90-95%, but because more than 70% of patients with gastrointestinal cancer have no obvious symptoms in the early-stage of the disease onset, the early detection rate is only less than 10% in China[2]. The vast majority of patients are already in the intermediate- and advanced-stages of the disease at the time of diagnosis, at this time, gastrointestinal cancer is prone to vascular and lymphatic metastasis, and the efficacy and prognosis are relatively poor. The 5-year survival rate hovering around 10% for a long time[3,4]. At present, the routine clinical detection methods for gastrointestinal cancer include ultrasound, X-ray, serum tumor markers, endoscopy, computed tomography (CT), and tissue biopsy, etc.[3,4], but traditional detection methods and techniques are rather difficult to detect early and micro-metastasis. For example, imaging cannot detect early, and particularly the micro-metastasis. When the metastasis is found by bone scan, the disease has already progressed to advanced-stage. The comprehensive evaluations of tumor score and clinical stage can only judge the prognosis and cannot prompt the tumor metastasis in real time. Serum tumor markers such as cancer antigen (CA)125, CA199, CA724, carcinoembryonic antigen (CEA), etc., have been widely used in curative effect and recurrence monitoring, but their tumor markers have low sensitivity, specificity, and effectiveness; so they cannot provide physicians with accurate detection basis. Therefore, there is an urgent need to find a newer type of marker for real-time monitoring.

Circulating Tumor Cells (CTCs) mainly refer to tumor cells that enter the peripheral blood. The

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content of CTCs in the healthy subject's blood is extremely rare, accounting for only $1/10^6$ ~ $1/10^7$ of peripheral blood leukocytes, which can migrate with the blood circulation to related tissues or organs, it develops into tumor lesions under appropriate conditions[5], which is closely related to the clinical stage, progression-free survival, overall survival, drug efficacy, and early recurrence and metastasis of cancer patients[6]. CTCs detection is to use special methods to separate and enrich CTCs in peripheral blood, and to detect the obtained CTCs by means of cell counting or gene level analysis. A large number of studies have shown that CTCs have real-time monitoring functions, and have important clinical application value for early cancer diagnosis, prognostic assessments, curative effect evaluation, individualized medication guidance, and tumor metastasis and recurrence monitoring[7-9]. It is a non-invasive new diagnosis, and which is also called "liquid biopsy"[6,10]. Compared with routine clinical detection methods for gastrointestinal cancer, CTCs have the advantages of high sensitivity, high specificity, high accuracy, convenient sampling, multiple and repeatable detection, and relatively shorter turn-around-time for test results.

In this study, the CTCs in blood samples of subjects with gastrointestinal cancer of different stages (n=70) and control healthy volunteers (n=30) were detected and compared with the results of pathological diagnosis and tumor serum markers, aiming to provide a reference basis for the search of a highly sensitive, specific, safe, and minimally invasive method for the auxiliary diagnosis of gastrointestinal cancer.

Materials and Methods

Research subjects

Seventy inpatients with gastrointestinal cancer from Gaomi People's Hospital were enrolled into this study from January to December 2019. After signing the informed consent form, collecting basic information, medical history data, and a whole blood sample 10 mL on admission were collected at postoperative pathological diagnosis information for TNM (tumor, node and metastasis) staging (gastrointestinal cancer group). Also, as control, 30 healthy volunteers were recruited, basic information and 10 mL of peripheral venous blood was collected (Healthy Volunteer Group). Due to inclusion/exclusion criteria, final analyses included only 54 patients and 29 healthy volunteers, which formed the basis of our study (Fig. 1).

Enrollment criteria for healthy volunteers: regular comprehensive physical examinations can exclude gastrointestinal cancer and other tumors. Inclusion criteria for the gastrointestinal cancer group: all

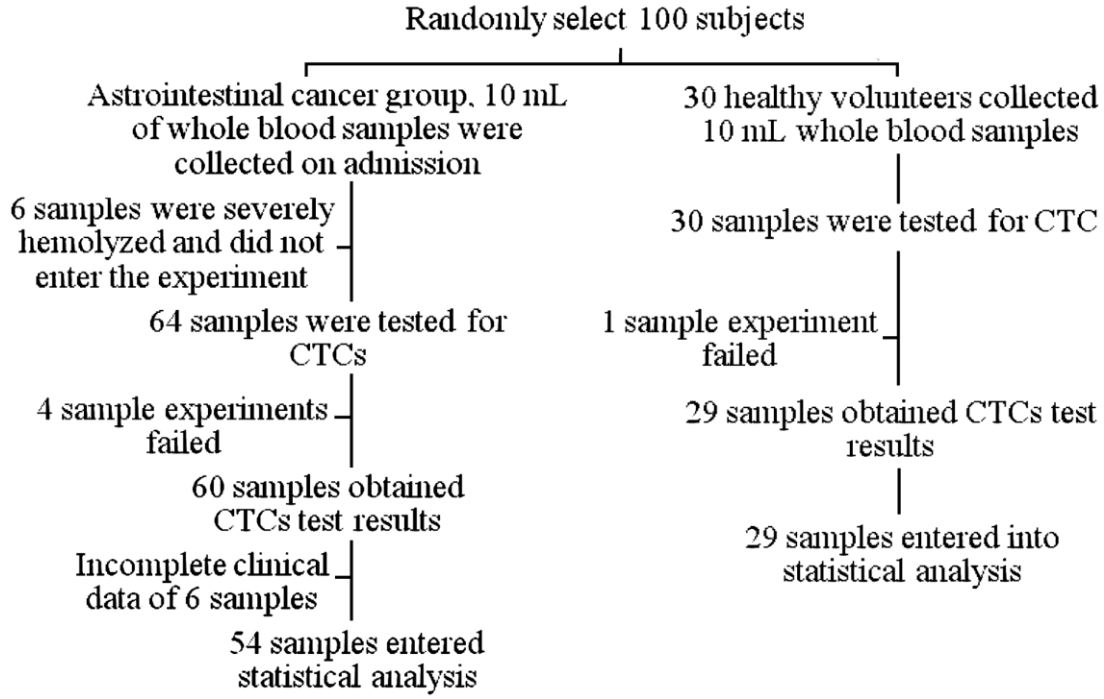

Fig. 1 — Schema of the experimental research plan for the subjects enrolled in the study analyses

patients who were clinically diagnosed as gastrointestinal cancer and were scheduled to undergo surgical treatment. Exclusion criteria: 1) there were obvious absolute contraindications to surgery; 20 patients whose tumor clinical stage was stage IV and gave up subsequent treatment; and 3) patients who refused to accept follow-up observation and/or treatment. This study was approved by the hospital ethics committee. There was no significant difference in age and gender between the two groups of subjects (all P >0.05) (Table 1). The postoperative TNM pathological staging indexes of patients in the GI cancer group refer to the 7th edition of the American Cancer Federation standards, as shown in Table 2.

Laboratory Assessments

CTCs detection

We took 10 mL of the subject's whole blood into a dedicated anticoagulant cell preservation tube, gently inverted and mixed to avoid coagulation, stored and transported at room temperature, and delivered to Shenzhen Tsure Biotechnology Co.,Ltd.. for CTCs detection within 72 h. CTCs detection adopted the microfluidic blood rare cell analysis technology of American Fluxion company for CTCs enrichment, the enriched cells were subjected to immunofluorescence staining and the Japanese Nikon company's ECLIPSE Ti-E inverted electric fluorescence microscope was used for image scanning and analysis. Immunofluorescence staining reagents included FITC-labeled recombinant human cytokeratin (CK) monoclonal antibody (CK-FITC), Cy3-labeled rabbit antihuman C45 monoclonal antibody (CD45-Cy3), and nuclear dye 4',6-diamidino-2-phenyl indole (DAPI). The identification criteria for CTCs were: CK positive, leukocyte common antigen (CD45) negative, DAPI positive. All images were obtained by two independent technicians according to the CTCs identification standard, the pictures were independently analyzed, and the samples with inconsistent interpretation were confirmed by a third person.

Table 1 — Comparison of baseline data between gastrointestinal cancer group and healthy volunteer group [cases (%)]

Index	Gastrointestinal cancer gr. (54 cases)	Healthy volunteer gr. (29 cases)	Stats.	P value
Gender			χ^2=0.904	>0.05
Male	39 (72.22)	18 (62.07)		
Female	15 (27.78)	11 (37.93)		
Age (years old, $\bar{x} \pm s$)	60.70±13.48	57.21±9.29	t=–1.246	>0.05

Table 2 — Tumor TNM (tumor, nodes, and metastases) staging of patients in gastrointestinal cancer group [cases (%)]

Postoperative pathological staging	Whole group (54 Cases)
Tumor T staging	
T1	5 (9.3)
T2	9 (16.7)
T3	25 (46.3)
T4	15 (27.7)
Tumor N staging	
N0	27 (50.0)
N1	14 (25.9)
N2	13 (24.1)
Tumor M staging	
M0	48 (88.9)
M1	6 (11.1)
Tumor TNM staging	
I	13 (24.1)
II	17 (31.5)
III	18 (33.3)
IV	6 (11.1)

Detection of serum tumor markers (*CA125, CA72-4, CA19-9, and CEA*)

Serum tumor biomarkers were detected using the Elecsys2010 immunoassay analyzer produced by Roche Diagnostics, using electrochemiluminescence and Roche's supporting CA125, CA72-4, CA19-9, and CEA detection kits.

Statistical methods

SPSS 19.0 software was used to analyze the data, and the measurement data conforming to the normal distribution were expressed as $\bar{x} \pm s$, the two independent sample t-tests for comparison between groups, and One-way ANOVA analysis for comparison between multiple groups; The measurement data that did not conform to the normal distribution were represented by the median (25th percentile to 75th percentile). The Mann-Whitney U test was used for comparison between the two groups, and the Kruskal-Wallis H test was used for comparison between multiple groups. The count data was expressed as a percentage (%), and the comparison adopted χ^2 tests; The receiver operating characteristic (ROC) curve was drawn, the cut-point was screened, the area-under-the-curve (AUC) was calculated, and the predictive effect of CTCs in the diagnosis of gastrointestinal cancer was evaluated. When the value was P <0.05, the difference between the comparison groups was considered statistically significant.

Results

CTCs enrichment, immunofluorescence staining and counting

Different numbers of individual CTCs were seen in the gastrointestinal cancer group samples, and circulating tumor microemboli (CTM) were seen in

samples. Parallel stained positive quality control products (gastrointestinal cancer cell lines) highly express CK but not CD45. All healthy control samples highly express CD45, and little-to-no cells show CK expression, as shown in Fig. 2.

Comparison of CTCs results between the two groups of subjects

The distribution of CTCs detection results of the two groups of subjects is shown in Fig. 3. Compared with the the healthy volunteer group, the blood CTCs levels of gastrointestinal cancer group subjects were significantly increased (P <0.01). In addition, the blood CTCs levels of subjects in each clinical stage of the patients group were compared with the healthy volunteers' group, and the differences were statistically significant (P <0.01). The blood CTCs levels of subjects in each clinical stage of the gastrointestinal cancer group increased with the increase of the clinical stage, but the difference between the groups was not significant (P >0.05) Table 3.

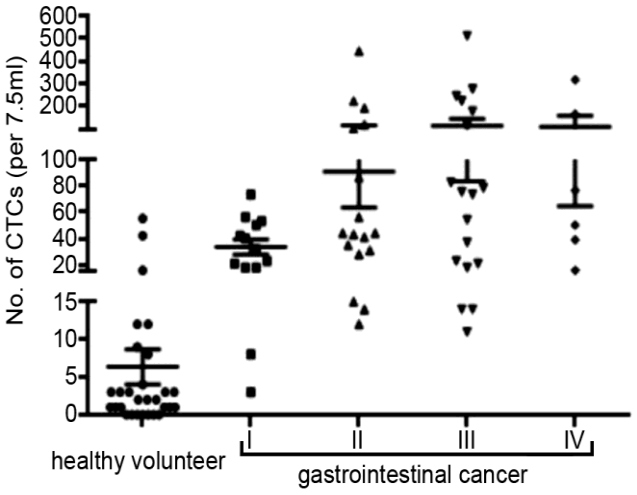

Fig. 2 — Comparison of circulating tumor cells (CTCs) results between the two groups of subjects.

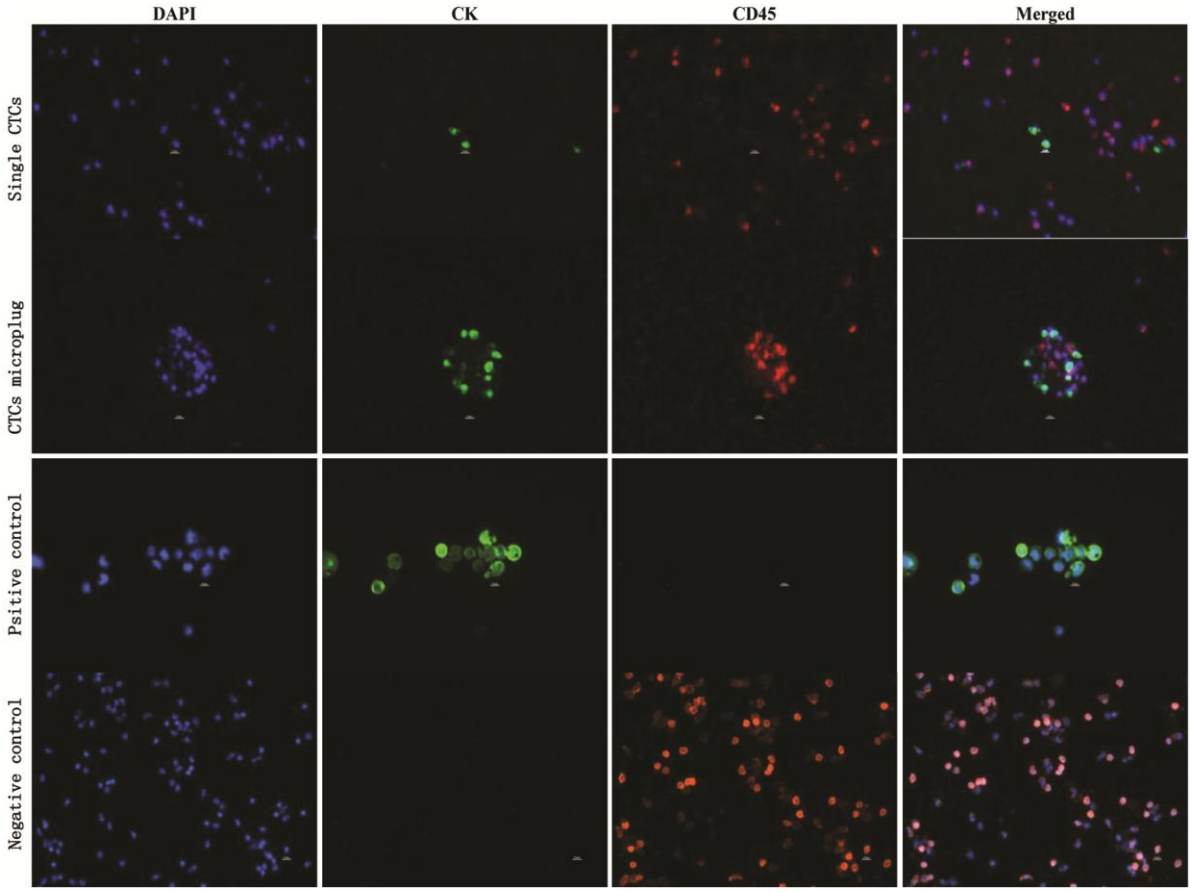

Fig. 3 — Circulating tumor cells (CTCs) immunofluorescence staining image. [The four groups of pictures shown in the figure are the pictures after immunofluorescence staining of single CTCs, CTCs microplugs, positive control products and negative control products. Each group of pictures contains the results of three fluorescence channels under the same field of view, where DAPI stands for 4',6-diamidino-2-phenylindole, CK stands for cytokeratin, CD45 stands for white blood cell common antigen, Merged: The merged image of the three fluorescence channels]

ROC analysis

The ROC curve was used to analyze the detection results of CTCs (Fig. 4). The AUC was 0.950 (95% confidence interval value was 0.897 to 1.000), indicating that the diagnostic efficiency of CTCs detection was very good. The ROC curve determined that the critical value of CTCs was ≥13 CTCs/7.5 mL of peripheral blood. The sensitivity of CTCs to stage I to IV gastrointestinal cancer was 84.6, 94.1, 94.4 and 100%, respectively, and the comprehensive sensitivity was 92.6%, the specificity was 89.7%.

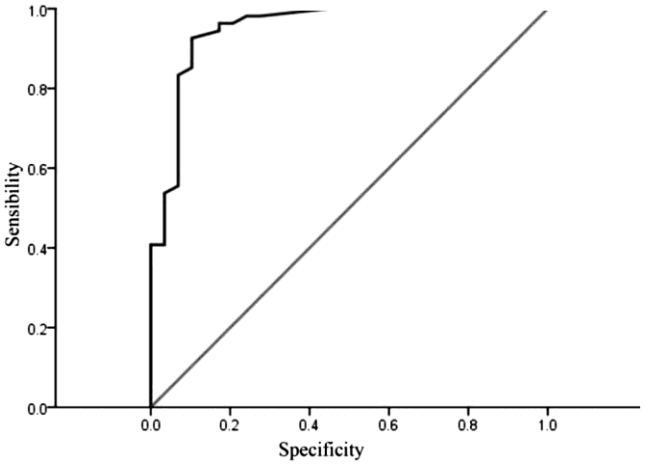

Fig. 4 — Receiver operating characteristic (ROC) curve of circulating tumor cells (CTCs) for gastrointestinal cancer

Table 3 — Comparison of circulating tumor cells (CTCs) results between gastrointestinal cancer group and healthy volunteer group

Group	No. of samples	CTCs Results [M (P25 ~ P75)]
Healthy Volunteer Group	29	2 (0.50~6.00)
Gastrointestinal cancer group	54	44 (21.00 ~ 90.25)*
Stage I	13	32 (18.00 ~ 51.50)*
Stage II	17	44 (29.50 ~ 111.00)*
Stage III	18	74 (20.25 ~ 189.25)*
Stage IV	6	63 (33.25 ~ 204.00)*

[*P <0.01 *vs*. the healthy volunteer group; Comparison between cases of each stage in the gastrointestinal cancer group P >0.05]

Comparison of the positive rates of CA125, CA19-9, CA72-4, CEA, and CTCs in the gastrointestinal cancer group

The positive rates of CA125, CA19-9, CA72-4, CEA, and CTCs in the gastrointestinal cancer group were 11.1, 27.8, 1.9, 33.3 and 92.56%, respectively. The positive rate of CTCs in the gastrointestinal cancer group was significantly higher than CA125, CA19-9, CA72-4, CEA, and other serum tumor markers, and the combination of four tumor markers (a positive for any marker was regarded as a combination positive), and the difference was significant (P <0.05). The combined detection of CTCs and four tumor markers can help increase the positive rate of detection, but compared with the detection of CTCs alone, the difference was not statistically significant (P >0.05) (Table 4).

Discussion

Gastrointestinal cancer is the most common type of malignant tumor in the world. In 2015, there were 1.7 million new cases of colorectal cancer and 832,000 deaths, ranking second in the number of deaths caused by cancer; 1.3 million new cases of gastric cancer and 819,000 deaths, ranking third in the number of deaths caused by cancer[11]. Since most of the early symptoms of gastrointestinal tumors were not obvious, the incidence was mostly insidious. Most patients were in the intermediate or late stage when they had obvious symptoms, and were often accompanied by lymph node metastasis, so the survival rate of patients was relatively low.

Tissue biopsy was still the gold standard for cancer diagnosis, which could assist clinical diagnosis of lesions or provide clues for disease diagnosis, help understand the nature and development trend of lesions, determine the prognosis of the disease, verify and observe the efficacy of drugs, and provide reference for clinical medication. However, tissue biopsy was complicated, expensive, longer turn-around-time for test results, and difficult to sample

Table 4 — Comparison of the positive rates of serum carcinoembryonic antigen (CEA), cancer antigen (CA)19-9, CA125, and CA72-4 and blood circulating tumor cells (CTCs) in the gastrointestinal cancer group [case (%)]

		Markers							
		CA125	CA19-9	CA72-4	CEA	CEA, CA19-9, CA125, CA72-4 combination	CTCs	CEA, CA19-9, CA125, CA72-4, CTCs combination	Sum
Positive	Cases	$6^{a,b,c}$	$15^{c,d}$	1^{b}	$18^{a,c,d}$	28^{d}	50^{e}	51^{e}	171
	%	11.1%	27.8%	1.9%	33.3%	51.9%	92.6%	94.4%	45.2%
Negative	Cases	$48^{a,b,c}$	$39^{c,d}$	53^{b}	$36^{a,c,d}$	26^{d}	4^{e}	3^{e}	207
	%	88.9%	72.2%	98.1%	66.7%	48.1%	7.4%	5.6%	54.8%
Sum	Cases	54	54	54	54	54	54	54	378
	%	100%	100%	100%	100%	100%	100%	100%	100%

[Note: The columns with the same subscript letters were not significantly different from each other on the P value of 0.05 level]

continuously/processing, it was traumatic and may cause tumor implantation and dissemination. Due to the heterogeneity of tumor tissue, conventional tissue biopsy can only represent a specific area and specific time of tumor tissue, it was difficult to summarize the whole picture. Therefore, other more accurate, safe and relatively easy-to-operate auxiliary diagnosis methods were needed as supplements in clinical practice.

At present, CTCs, circulating free DNA (cfDNA) and serum tumor markers are emerging as clinical auxiliary diagnostic methods for malignant tumors, and the former two are collectively referred to as "liquid biopsy"[12]. CTCs was the earliest "liquid biopsy" method used in clinical practice. Notably, Johnson & Johnson's CellSearch CTCs detection system based on cell adhesion molecule (EpCAM) and immunomagnetic bead method had been approved by the U.S. FDA for clinical use. CTCs can be used as indicators for prognostic judgment and curative effect monitoring of gastrointestinal cancer, and molecular biological detection based on CTCs was expected to provide a theoretical basis for further revealing tumor heterogeneity in the clinic[13,14]. However, due to the low CTCs enrichment efficiency of the CellSearch system, most clinical samples could only detect a few CTCs, which lead to low sensitivity of detection. On the other hand, with such a small number of cells, it was difficult to proceed to the next in-depth molecular analysis. Therefore, the development of CTCs detection technology with higher enrichment efficiency and further improvement of the sensitivity of CTCs detection were essential to further the application of CTCs detection technology in clinical practice. In this study, microfluidics combined with specific markers for gastrointestinal cancer cells were used to detect CTCs, and the sensitivity and specificity were both above 94%, which greatly improved the sensitivity of CTCs detection while maintaining good specificity. In 54 patients with gastrointestinal cancer, the level of CTCs increased with the clinical stage. Most patients with gastrointestinal cancer could capture dozens to hundreds of CTCs. Compared with the CellSearch system, the capture efficiency of CTCs was greatly improved, laying the material foundation for downstream molecular analysis of CTCs.

Commonly used clinical serum tumor markers for gastrointestinal cancer included CA125, CA19-9, CA72-4, and CEA. Systematic review data showed that the overall positive rates of CA724, CA19-9, and CEA in gastric cancer patients were only 29.9% (829/2774), 27.0% (1431/5,300), and 24.0% (1,945/8,104) respectively, and the positive rates of these three serum tumor markers in patients with early gastric cancer were all lower than 20% (stage I patients: CA724 12.0%, CA199 9%, and CEA 13.7%; (stage II patients: CA724 15.6%, CA19-9 19.9%, and CEA 23%)[15]. The positive rates of CEA in colorectal cancer patients were 5% for stage 0, 10% for stage I, 33% for stage II, 33% for stage IIIA, 45% for stage IIIB, and 78% for stage IV[15]. The positive rates of CA19-9 in colorectal cancer patients were 5% for stage 0, 4% for stage I, 11% for stage II, 10% for stage IIIA, 13% for stage IIIB, and 52% for stage IV[16]. It could be seen that a single serum tumor marker has low sensitivity in the diagnosis of early gastrointestinal cancer and cannot meet clinical needs[15,16]. The combined use of serum tumor markers could help improve the sensitivity and accuracy of the diagnosis of gastrointestinal cancer. The combined use of four serum tumor markers of CEA, CA19-9, CA72-4, and CA125 could increase the positive detection rate of early gastric cancer to 57.1%, and increase the positive detection rate of advanced gastric cancer to 89.4%[17]. Although it was higher than the use of a single serum tumor marker, they were still needed to improve continuously. In this study, the positive detection rate of serum tumor markers used alone or in combination in patients with gastrointestinal cancer was close to that reported in the peer-reviewed literature[14-16]. However, the positive detection rate of CTCs in gastrointestinal cancer patients was as high as 94.4%, which was significantly higher than the positive detection rate of serum tumor markers.

The combined detection of CTCs and four tumor markers in patients with gastrointestinal cancer can further increase the positive detection rate to 96.3%, but the difference was not statistically significant compared with the detection of CTCs alone, indicating that CTCs can be used as an independent auxiliary diagnostic index for gastrointestinal cancer.

This study showed that the development of a more sensitive and specific CTCs detection method was expected to further promote clinical application of CTCs detection. Recently, Yang et al. developed a

method that utilizing conditional cell culture of CTCs to enrich tumor biomarker and thereby genotyping the genomic mutation, which showed promise for a half invasive sampling for genetic analysis of tumors18. Beyond gastric cancer, anti-epithelial cell adhesion molecule (EpCAM) – based CTCs identification successfully diagnosed pancreatic ductal adeno-carcinoma at early stage[19]. As an auxiliary diagnosis method for gastrointestinal cancer, CTCs are expected to make up for the shortcomings of conventional tissue biopsy. The use of CTCs alone or in combination with conventional gastrointestinal cancer serum tumor markers may have a greater role in improving the clinical diagnosis/prognosis of gastrointestinal cancer. However, due to the limitation of sample size, the difference in CTCs levels of patients with different clinical stages had not been found to be statistically significant, and further research is desirable in this regard.

Conclusion

All stages of GI cancer subjects had higher level of CTCs in the peripheral venous blood as detected by microfluidic blood rare cell analysis technique for which the specificity and sensitivity were at par. This study has demonstrated the value of CTCs to be an auxiliary diagnostic tool in GI cancer. Further studies are warranted to improve the accuracy and robustness.

Conflict of interest

Authors declare no competing interests.

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