Topic: Reviews of Recent Advances in Research and Treatment for Gastroenterological Malignancies

The clinical significance of circulating tumor cells in gastrointestinal cancer

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ABSTRACT

Circulating tumor cells (CTCs) are originated from the primary tumor lesion into the blood stream. CTCs could lead to recurrence of gastrointestinal (GI) cancers, even after a curative resection and colonizing in the distant organs to facilitate tumor distant metastasis; however, it has been challenging in clinic to detect CTCs for a long time, such as detection methodology or molecular markers for identification of CTCs. This review discussed the recent technical advances and biomarkers in the detection of CTCs and the molecular mechanism of CTC in cancer progression and metastasis. Moreover, novel concepts, such as cancer stem cells and epithelial-mesenchymal transition, could lead to CTCs and tumor progression and metastasis. Nevertheless, the involvement of CTCs varies greatly among cancer types in the GI and much remains to be learned. Thus, further study will provide more insightful information from a clinical and translational viewpoint to use CTCs for cancer early diagnosis or prediction of tumor recurrence and investigation of tumor progression and metastasis as well.

Key words: Cancer stem cells, circulating tumor cells, epithelial-mesenchymal transition, gastrointestinal cancer, tumor progression and metastasis

Introduction

Tumor recurrence often occurs in patients with gastrointestinal (GI) cancers, even after a curative resection, which may be because undetectable tumor cells depositor enter into the blood stream at the time of operation. In some cases, tumor recurs despite adjuvant chemotherapy after curative surgery suggesting that chemotherapy failed to eradicate all cancer cells that persist after curative surgery. Thus, tumor cells could be disseminated before surgery. The concept of the circulating tumor cells (CTCs) has been, therefore, established and indicates that tumor cells are in blood stream, which will facilitate tumor progression and metastasis although detection of CTCs in peripheral blood was described more than a century ago.[1] Recent advance on research of CTCs largely contributed to diagnosis and treatment of GI cancers. However, the clinical relevance of CTC detection in GI cancers is still the subject of controversies, and their biology is poorly understood.

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Indeed, detection of CTCs becomes a promising means to early diagnosis and prediction of prognosis and tumor recurrence for several types of human cancer.[2-5] Furthermore, the study of CTCs could also elucidate the molecular biological profile of CTC and lead to better understanding of cancer metastasis. To date, standard procedures of CTC detection have to be established, and the clinical relevance should be confirmed by a large-scale clinical study. In this review, we updated and discussed recent progress regarding CTCs in GI cancer. These new data could improve our understanding of the mechanisms of cancer progression and metastasis as well as therapy resistance. This information may also lead to the development of novel clinical targets and improve the clinical management of GI cancer.

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Methodology in Detection of Circulating Tumor Cells

In general, methodology in the detection of CTCs consists of two steps, that is, enrichment and detection process. The enrichment process is required because of the rarity of CTCs in peripheral circulation (one CTC per 1×10^6 to 1×10^7 mononuclear cells). To enrich CTCs from blood mononuclear cells, density gradient centrifugation (Ficoll-Hypaque or OncoQuick separation), immunomagnetic or size filtration procedures are used.[6,7] After enrichment, the identification of CTCs is then performed. For identification techniques, nucleic acid methods and cytometric methods are usually used. Recently, the development of molecular techniques can make molecular and genetic analysis of CTCs after enrichment and identification of CTCs, leading to developing CTC characterization.

Enrichment Techniques

Cell morphologic-based enrichment

Isolation of CTCs using the size of epithelial tumors is based on the size of tumor cells without functional modification and complex enrichment procedures. It is usual to utilize 5-8 μm probe filters to enable and to separate small leukocytes from the large epithelial cell and the isolation sensitivity threshold is approximately one tumor cell per milliliter.[8,9] These techniques have a valuable advantage in isolation of CTCs without damaging cells and enable further immunocytochemical or immunofluorescence evaluation of CTCs. Although it is easily handled and cheap, it is considered to be not highly sensitive and poorly specific.

Furthermore, density gradient separation using Ficoll-Hypaque is an alternative technique to separate CTCs and mononuclear cells from other blood cells and granulocytes. However, Ficoll-Hypaque can be toxic to CTCs. CTCs can also be easily to lose due to the migration of cells to the plasma layer. OnkoQuick was developed to avoid the cross-contamination of different layers, resulting in higher recovery rate of CTCs.[10,11] Recently, RosetteSep™ (Stem Cell Technologies, Vancouver, British Columbia, Canada) developed a novel method based negative selection to improve the specificity of standard gradient separation.[12,13]

Immunomagnetic circulating tumor cell enrichment

The immunomagnetic CTC enrichment technique is a magnetic bead-based separation method. To date, there have been two methods to identify CTCs expressing targeting-specific biomarkers. One is using the epithelial cell-specific marker, e.g. epithelial cell adhesion molecule (EpCAM) or cytokeratin (CK) expressed on the surface of tumor cells from epithelial origin. Another is using the tumor-specific markers, such as α-fetoprotein, Her2-neu, or carcinoembryonic antigen (CEA) expressed on a particular type of cancer cells. Immunomagnetic isolation technique utilizes monoclonal antibodies that are labeled magnetic microbeads and separates CTCs from the leukocytes background by magnetic force. To separate leukocytes, the negative selection is performed using an anti-CD45 antibody recognizing surface marker of leukocytes. Based on this technique, the Magnetic Activated Cell Sorting System (MACS™ Miltenyi Biotec, Bergisch Gladbach, Nordrhein-Westfalen, Germany) is a useful technology for detecting and analyzing CTCs because it avoids cell lysis and enables cell count by immunocytochemistry and immunofluorescence assay.[14] CellSearch System™ (Veridex, Warren, NJ) approved by the US Food and Drug Administration (FDA) is a semi-automated analyzer enriching the CTCs with ferrofluid nanoparticles coated with anti-EpCAM antibodies. This system is proved to be useful for detecting and analyzing CTCs with patients with breast, colorectal and prostate cancer in the clinic.[15] However, Alunni-Fabbroni and Sandri argued that this technology has two possible limitations, that is, there is no "universal marker" available for each type of tumor, while epithelial marker (EpCAM) could be down-regulated in epithelial tumor cells after tumor cells undergo epithelial-mesenchymal transition (EMT).[16] Thus, this method could only detect selected CTCs.[17,18]

Enrichment Techniques

Nucleic acid-based analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) based techniques can increase the specificity of the molecular methods to discriminate between the higher levels of molecular changes in cancer patients and the low background level in normal cells. Expressions of epithelial or tumor-specific markers are detected using RT-PCR to evaluate and identify CTCs. Nowadays, multiplex RT-PCR approach has been established to screen at the same time more than one single biomarker. Furthermore, quantitative RT-PCR improves the specificity of detection for CTCs by defining a cut-off value for biomarker expression. However, there are some limitations of this method: (1) contamination of non-malignant epithelial cells such as skin cells; (2) false positive due to unspecific markers; and (3) amplification of cell-free nucleic acids. Therefore, it is essential to select the appropriate marker that is expressed specifically by tumor cells to boost the specificity and reliability of CTC detection.

Cytometric-based analysis

Cytometric-based technique can isolate and count CTCs using monoclonal antibodies against various antigens. To detect CTCs, CK and EpCAM are most commonly used. It enables to keep CTCs intact during analysis because cell lysis is not necessary. Furthermore, this technique provides information of high statistical

precision and subpopulation quantification with high specificity due to simultaneous analysis using multiple parameters. However, in contrast to RT-PCR technique, the disadvantage of this technique has a lower sensitivity.

Fiber-optic Array Scanning Technology, a rapid and accurate CTC location cytometric system, is a scanning technology characterized by a large field of view.[19] It allows analyzing large volumes of samples without any purification step and minimizing the risk of cell loss. Additional scanning systems such as ACIS (Automated Cellular Imaging System, DAKO, Spatial Technology, USA) and ARIOL (Applied Imaging Corp, Wetzlar, Germany) are available on the market.

Recent Advances in Detection of Circulating Tumor Cells

As discussed above, the detection of CTCs is involved in two steps of enrichment and identification; thus, the development of automated techniques could offer at the same time enrichment, staining and scanning of the samples. The Cell Search System® enriches the CTCs with ferrofluid nanoparticles coated with anti-EpCAM antibody. The enriched EpCAM+ population is stained with phycoerythrin-conjugated antibodies against CK-8, -18 and -19 with allophycocyanin-conjugated antibodies specific for leukocytes (anti-CD45 antibody) and with the nuclear dye 4', 6-diamino-2-phenylidole (DAPI) for the nucleic acids staining. The $CK^+/DAPI^+/CD45^-$ cells are then counted as CTCs using CellSpotter analyzer (Veridex, Warren, NJ), a four-color semi-automated fluorescent microscope.[20] More recently, CTC-chip based on a microfluidic platform has also developed to isolate a high rate of CTCs.[21] CTC-chip consists of an array of 78,000 microspots coated with anti-EpCAM antibodies. Whole blood is pumped through this chip, and EpCAM+ cells are captured and detected by cameras identifying their morphology, viability and the expressions of tumor markers.[14] However, the relevance of this technology in clinical setting remains unclear and clinical validation study is required. Finally, based on enzyme-linked immunospot assay technology, epithelial immunospot (EPISPOT) assay can identify CTCs by detecting specific CTC-secreted proteins (CK, mucin or prostate specific antigen).[22,23] EPISPOT makes it possible to detect the only viable CTCs because dying CTCs are unable to secrete an adequate amount of proteins to be detected. Sensitivity of EPISPOT is superior to ELISA assay in a two-order magnitude while detecting the release of CK-19 from tumor cells.[24]

Recent Development of Molecular and Genetic Characterization of Circulating Tumor Cells

The next desirable step is to elucidate the molecular and genetic characterization of CTCs after enrichment and isolation of CTCs. This step may help us to comprehend the mechanism of cancer metastasis, leading to the development of treatments of tumor metastasis. However, the molecular and genetic characteristics of CTCs are not fully clarified when compared with corresponding tumors in GI cancer. The molecular and genetic characteristics of CTCs are usually analyzed by PCR-based methods, fluorescent *in situ* hybridization (FISH) or comparative genomic hybridization (CGH). There have been no reports about CTCs characterization analyzed by FISH and CGH in gastric cancer, whereas abnormal copy number alteration was detected in CTCs from patients with metastatic prostate cancer.[25-27] Using PCR-based methods, conventional detection system with epithelial markers such as CEA and CK has been previously performed to show the clinical significance of CTCs in gastric cancer.[28-32] However, Mimori *et al.*[33] showed in a large-scale study that CTCs circulate even in early stages of the disease indicating that the simultaneous presence of CTC and VEGFR-1 expression is clinically significant for disease progression. It is also well known that there is discordance of expression profile between CTCs and primary tumor, and several markers for regulating metastasis and prognosis have been determined by PCR-based methods.[34-37] Furthermore, a comprehensive molecular profiling using the cDNA microarray was performed to identify novel genes to predict gastric cancer metastasis, recurrence and prognosis, suggesting that expression of MT1-MMP in peripheral blood identified by the cDNA microarray technique in gastric cancer was a powerful indicator of distant metastasis, especially for peritoneal dissemination.[38] van de Stolpe *et al.*[39] reported that CTCs were heterogeneous and differed among different cancer types. In addition to differences across cancer types, CTCs have heterogeneity within the same patient. Although the heterogeneity of primary tumors has been known, Klein *et al.* showed that early disseminated cancer cells are genomically very unstable, as well as the primary tumor.[40] In this case, gastric cancer is well known to have histological heterogeneity in primary lesion. Various histological types and differentiation of gastric cancer cells are frequently observed in the same specimens.[41] Therefore, histological heterogeneity may make it difficult to the molecular and genetic characterization of CTCs in GI cancer.

Clinical Relevance of Circulating Tumor Cells in Gastrointestinal Cancer

To date, there are a number of methodologies in the detection of CTCs and the clinical relevance of GI cancer have been reported. In clinical setting, the detection of CTC is expected to be useful in early diagnosis of cancer, monitoring of treatment responses and disease progression. In the following, we summarized a comprehensive update of the studies with more than 50 patients or with an outcome analysis and discussed their clinical implications in selected GI cancers.

Esophageal cancer

There are only a few studies of esophageal cancer available as compared to gastric and colorectal cancers

[Table 1]. In esophageal cancer, RT-PCR was the main technique to detect CTCs in previous studies.[42-45] As for available molecular markers, CEA and SCC are useful predictive markers for tumor recurrence and survival. Most recently, a large-scale of study using CellSearch System®, morphological technique are also reported.[46,47] Matsushita *et al.*[46] revealed that CTC detection by CellSearch System® was useful to evaluate the clinical efficacy of chemotherapy and chemoradiation therapy on esophageal cancer patients. Reeh *et al.*[47] reported that patients with positive CTCs had significantly poorer overall survival and progression-free survival rate; therefore, preoperative CTC detection by CellSearch System® was an independent prognostic indicator for patients with esophageal cancer. However, most of previously reported patients had esophageal squamous cell carcinoma. There are some differences of biological behaviors between esophageal squamous cell carcinoma and adenocarcinoma; therefore, further study of esophageal adenocarcinoma is needed.

Gastric cancer

A number of studies of CTC detection in patients with gastric cancer have been reported previously as summarized in Table 2. Although the several methodology of CTC detection including RT-PCR and CellSearch System® [Table 2], it remains unclear which is the best method and molecular marker for detection of CTCs in gastric cancer patients. Recently, various meta-analyses demonstrated that presence of CTCs was associated with poor prognosis and advanced clinicopathological factors.[48-50] It has been reported that detection of CTCs in gastric cancer may be useful in early diagnosis and monitoring of treatment responses and prognosis. However, as for diagnosis, a recent meta-analysis showed that CTC detection alone cannot be recommended as a screening test for gastric cancer because of lower and inconsistent sensitivity estimates for CTC.[51] Furthermore, Mimori *et al.*[33] showed that CTCs occurred in early stages of the disease, and CTC

Table 1: Clinical relevance of CTC in esophageal cancer

Author	Year	Case	Method	Molecuar marker	Clinical relevance
Kaganoi	2004	70	RT-PCR	SCC	Prediction of recurrence
Setoyama	2006	106	RT-PCR	CEA	Prediction of recurrence
Liu	2007	53	RT-PCR	CEA	Prediction of recurrence
Hashimoto	2008	147	RT-PCR	CEA	Prediction of recurrence and prognosis
Cao	2009	108	RT-PCR	Survivin	Prediction of haematogenous recurrence and prognosis
Tanaka	2010	244	RT-PCR	CEA, SCC	Predictor for hematogenous and local recurrences
Yin	2012	72	RT-PCR	CEA, CK-19, Survivin	Clinical efficacy of RT
Matsushita	2014	90	CellSearch	EpCAM, CK-8, 18, 19	Clinical efficacy of CT or CRT
Reeh	2015	100*	CellSearch	EpCAM, CK-8, 18, 19	Prediction of recurrence and prognosis

*Esophageal adenocarcinoma is included. RT: Radiotherapy; CRT: Chemoradiation therapy

Table 2: Clinical relevance of CTC in gastric cancer

Author	Year	Case	Method	Molecuar marker	Clinical relevance
Wu	2006	64	MAH	hTERT, CK-19, CEA, MUC1	Associated with recurrence
Pituch-Noworolska	2007	57	ICC	CK-8, 18, 19	No prognostic impact
Ito	2010	65	ICC	GFP, EpCAM,	Shorter OS
Majima	2000	52	RT-PCR	CK-19, 20	Shorter OS
Miyazono	2001	57	RT-PCR	CEA	Associated with liver metastaisis, recurrence
Sumikura	2003	106	RT-PCR	CEA	Associated with recurrence
Illert	2005	70	RT-PCR	CK-20	Shorter OS
Ikeguchi	2005	59	RT-PCR	CEA	No association with recurrence
Uen	2006	52	RT-PCR	MUC1, c-Met	Shorter OS
Koga	2008	101	RT-PCR	CK-18, 19, 20	Shorter OS (CK-19 is better)
Yie	2008	55	RT-PCR, ELISA	Survivin	Predictive marker for DFS
Mimori	2008	810	RT-PCR	CK-7,19, 20, VEGFR1	Associated with hematogenous metatasis
Bertazza	2009	70	RT-PCR	Survivin	Predictive marker for OS
Qiu	2010	123	RT-PCR	CEA	Predictive marker for DFS
Arigami	2010	94	RT-PCR	B7-H3	Shorter OS
Arigami	2011	95	RT-PCR	B7-H4	Shorter OS
Cao	2011	98	RT-PCR, ELISA	Survivin	Predictive marker for DFS
Arigami	2013	93	RT-PCR	STC2	Shorter OS
Matsusaka	2010	52	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS (CTC level after Cx)
Uenosono	2013	148	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS

MAH: Membrane-array hybrudization; ICC: Immunocytochemistry; hTERT: Human Telomerase reverse transcriptase; ELISA: Enzyme-Linked ImmunoSorbentAssay; DFS: Disease free survival; OS: Overall survival; PFS: Progression free survival

alone can not be a predictor of cancer metastasis in a large-scale study. This study also revealed that elevated expression of VEGFR-1 facilitated the establishment of hematogenous metastases of gastric cancer and that the simultaneous presence of CTC and VEGFR-1 expression at premetastatic sites was clinically significant in disease progression.

Colorectal cancer

To date, there are a large number of studies of CTC detection in colorectal cancer as compared to other GI cancers as summarized in Table 3. RT-PCR and the CellSearch System® have been mainly reported methods to detect CTC in colorectal cancer and data showed that CTCs were associated recurrence and overall survival of patients. For example, Cohen *et al.*[2] revealed that the number of CTCs detected by the CellSearch System® before and during treatment was an independent predictor of PFS and OS in 430 patients with metastatic colorectal cancer in a prospective multicenter clinical trial. The CellSearch System® using in colorectal cancer was the first CTC detection system that was approved by US FDA.[2] Furthermore, a previous meta-analysis reported the aprognostic significance of CTC detected by the CellSearch System® has been reported.[48] Eleven studies including 1,847 colorectal cancer patients were analyzed in this study and the presence of CTCs was significantly associated with overall and progression-free survival as reported by the previous meta-analysis.[52] In a previous prospective study of non-metastatic colorectal cancer, preoperative CTC detection was a strong and independent prognostic marker.[53] The treatment response rate was significantly lower in CTC-positive patients than that of CTC negative patients at base line and during treatment.[23,54-57] Another previous study demonstrated potentially clinical application in detection of KRAS mutational in CTCs for selecting metastatic colorectal cancer patients for cetuximab therapy.[58] In addition, recent development of molecular and genetic characterization of single-CTC demonstrated that there was intra- and inter- heterogeneity of EGFR expression and genetic alterations of EGFR, KRAS and PIK3CA,

Table 3: Clinical relevance of CTC in colorectal cancer

Author	Year	Case	Method	Molecuar marker	Clinical relevance
Wong	2009	132	ICC	CK-20	Predictive marker for OS
Tanigich	2000	53	RT-PCR	CEA	Predictive marker for DFS
Yamagichi	2000	52	RT-PCR	CK-20, CEA	Shorter OS
Hardingham	2000	94	RT-PCR	CK-19, 20, MUC2	Shorter DFS
Bessa	2001	68	RT-PCR	CEA	No prognostic impact
Ito	2002	99	RT-PCR	CEA	Shorter DFS
Bessa	2003	66*	RT-PCR	CEA	No prognostic impact
Sadahiro	2005	93	RT-PCR	CEA	No prognostic impact
Koch	2006	90	RT-PCR	CK-20	Shorter DFS
Douard	2006	121	RT-PCR	CK-20, CGM2	No prognostic impact
Iinuma	2006	167	RT-PCR	CK-20, CEA	Shorter DFS and OS
Katsumata	2006	57	RT-PCR	CK-20	Strong relation to LN metastais and OS
Allen-Mersh	2007	113*	RT-PCR	CK-20, CEA	Shorter DFS
Wang	2007	157*	RT-PCR**	CK-19, 20, CEA, hTERT	Shorter DFS
Uen	2007	194	RT-PCR**	CK-19, 20, CEA, hTERT	Shorter DFS
Sadahiro	2007	200*	RT-PCR	CEA	Shorter DFS
Uen	2007	438*	RT-PCR	CK-19, 20, CEA, hTERT	Shorter DFS
Yie	2008	86	RT-PCR, ELISA	Survivin	Predictive marker for DFS
Lu	2011	141*	RT-PCR**	CK-19, 20, CEA, hTERT	Shorter DFS and OS
Iinuma	2011	735	RT-PCR	CK-19, 20, CEA, hTERT	Shorter DFS and OS
Pilati	2012	50	RT-PCR	CK-19, 20, CEA, CD133, VEGF, EGFR, Survivin	Predictive marker for OS (CD133 CTC)
Cohen	2008	430	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for OS (CTC ≥ 3)
Matsusaka	2011	64	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS (CTC level after Cx)
Tol	2010	467	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS (CTC level before Cx)
Sastre	2012	180	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for PFS and OS (CTC level before Cx)
Aggarwal	2013	209	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for OS (CTC level before Cx)
Gazzaniga	2013	119	CellSearch	EpCAM, CK-8, 18, 19	Predictive marker for OS (CTC ≥ 1)
Kuboki	2013	63	CellSearch	EpCAM, CK-8, 18, 19	Shorter DFS and OS
Sotelo	2015	472	CellSearch	EpCAM, CK-8, 18, 19	No prognostic impact (stage III)
Seeberg	2015	194	CellSearch	EpCAM, CK-8, 18, 19	Predict nonresectability and impaired survival

*Post-operative mesuament. **Membrane array. ICC: Immunocytochemistry; ELISA: Enzyme-Linked ImmunoSorbent Assay; hTERT: Human Telomerase reverse transcriptase; LN: Lymphnode metastasis; DFS: Disease free survival; OS: Overall survival; PFS: Progression free survival; Cx: Chemotherapy

which possibly explained the variable response rates to EGFR inhibition in patients with colorectal cancer.[59] Therefore, the information on the molecular status of CTCs might be useful for stratification of molecular-directed therapy.

Future Perspectives

Epithelial-mesenchymal transition

There are two main approaches in the detection of CTCs, that is, immunological assays using monoclonal antibodies and PCR-based molecular assays, exploiting tissue-specific transcripts.[60] Immunocytochemical detection of epithelial or tumor-associated antigens is widely accepted.[61] Recent studies have shown that EMT plays a critical role in cancer progression and metastasis in epithelial malignancies including gastric cancer.[62] Our previous study implied that vimentin-positive tumor cells were able to survive in the peripheral circulation and in the bone marrow and that vimentin-positive cancer cells that invade intratumoral vessels must have undergone mesenchymal transition. We assume that not all detected CTCs but rather only a few, which have undergone EMT could give rise to tumor metastasis or recurrence.[17] Most recently, Wu *et al.*[63] reported that mesenchymal CTCs classified using EMT markers were more commonly found in patients in metastatic stages of the disease in different types of human cancers. Therefore, it is possible that conventional detection system using epithelial markers fail to detect that population of CTCs.

Cancer stem cell

Furthermore, the concept of rare subpopulations of cancer stem cells (CSCs) has created a novel focus in cancer research but arises a question whether CTCs have CSC property. It is expected that CTC with CSC property may be disseminated from the primary tumor lesion to a distant metastatic site. This hypothesis is supported by the similarities between the properties of CTCs and CSC and suggests that the founder cells of metastases arise from the CTC population. It has been reported that stem cell markers are frequently overexpressed in the CTCs of patients with metastatic breast cancer, and the most CTCs have stem cell phenotypes that are non-proliferating and resistant to chemotherapy. For example, Iinuma *et al.*[64] revealed that multi genetic markers of CSC, CEA/CK/CD133 in peripheral blood samples could be a useful predictor for recurrence and prognosis. Pilati *et al.*[65] reported that CD133-positive CTCs might represent a suitable prognostic marker to stratify the risk of patients who undergo liver resection for CRC metastasis.

Conclusion

An increasing number of studies have shown that CTC is associated with GI cancer progression, metastasis and resistance to pharmacotherapy. However, the clinical evidence supporting the role of CTC in cancer progression still remains inconclusive. Therefore, further analysis and clinical trials are required to achieve clinical utility of CTC detection in GI cancers.

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Conflicts of interest

There are no conflicts of interest.

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