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The clinical application of Circulating tumor cells and DNAs as prognostic and predictive biomarkers in gastrointestinal cancer

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Abstract

Gastrointestinal (GI) cancer is one of the most common cancers globally. Genetic and epigenetic mechanisms are involved in its pathogenesis. The conventional methods for diagnosis and screening for GI cancers are often invasive and have other limitations. In the era of personalized medicine, a novel non-invasive approach called liquid biopsy has been introduced for the detection and management of GI cancers, which focuses on the analysis of circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA). Several studies have shown that this new approach allows for an improved understanding of GI tumor biology and will lead to an improvement in clinical management. The aim of the current review is to explore the clinical applications of CTCs and ctDNA in patients with GI cancer.

Key words: Gastrointestinal cancer; Liquid biopsy; CTCs; ctDNA; biomarkers**;** tumor biology

Introduction

Gastrointestinal (GI) cancer is one of the most common causes of cancer-associated death worldwide [1]; it is the fourth most common malignancy in men and the fifth in women. It is predicted that, GI cancer accounts for 20% of new cancer patients and 15% of deaths globally [2]. GI cancer affects the gastrointestinal tract, or accessory organs, including: the colon, esophagus, and intestine [3]. There are various established predisposing factors, such as smoking, obesity, genetic mutations, Helicobacter pylori, or viral infection (Hepatitis B and C) [4].

Over the past decades, the traditional diagnostic test for tumors has been biopsy-based techniques. The tissue biopsies are burden on patients, because they are painful and invasive, and are associated with some complications, including: bleeding, infection, and injury to the surrounding tissues. Furthermore, these conventional methods do not always provide suitable prognostic and predictive information for treatment [5]. For these reasons, the use of non-invasive biomarkers that could diagnose tumor at an early stage is clearly desirable. Precision medicine techniques may provide beneficial information at the most appropriate time and that is dependent on the biological and molecular features of the tumor [6-7].

Liquid biopsy refers to a new approach for providing an individualised biomarker with diagnostic, prognostic and predictive importance. Liquid biopsy offers several benefits, such as low pain, less invasiveness, and easier access [8-9]. In addition, liquid biopsies afford potential information regarding the tumor molecular profiling. Liquid biopsies include the testing of all circulating cancer cell traits, such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating cell-free DNA (cfDNA), circulating miRNAs, as well as exosomes, proteins, mRNAs and others [10-11].

In the present review, we have focused on the source, and detection technologies for CTCs and ctDNA as well as their clinical applications as prognostic and predictive biomarkers.

2. Circulating tumor cells (CTCs)

Circulating tumor cells (CTCs) refer to epithelial cancer cell that enter the peripheral blood [12]. CTCs are of great interest because they play a critical role in tumor metastasis and provide a profile of cancer cells during all stages of development[13]. Furthermore, this novel approach is an effective method to detect, assess and possibly direct the treatment of some cancers. The main challenges in detection of CTCs are their low concentration in blood and the possibility of missing cancer-specific markers. Addressing these problems requires platforms and diagnostic tools of high sensitivity and specificity to increase the detection rate of CTCs [14]. Therefore, the methods for CTC- detection are based on two steps, and consist of an enrichment process and a detection method.

2.2. Methods for CTC enrichment and detection

Techniques for CTCs enrichment are based on numerous approaches that are based on physical and biological properties of CTCs to distinguish them from normal cells. Methods based on physical characters consist of; density-based filtration and gradient centrifugation (Ficoll, OncoQuick) and size-based filtration (ISET, ScreenCell) [14]. Biological methods using immunomagnetic assays to separate CTCs in positive selection or negative form. However in positive selection antibody target the tumor-associated antigens, the negative selection antibody is against antigens that CTCs lack, CD45 common antigen on the surface of leukocyte [15]. The CellSearch system (CS) is the gold standard and FDA approved method for CTCs- detection, a

positive selection based, antibody targets the epithelial cell adhesion molecule (EpCAM) which is specific cancer marker and highly expressed in carcinomas [15]. The other kind of biological approach for CTCs-detection is microfluidic devices providing a microfabricated structure and a biocompatible environment and also handling very low blood concentration[16]. There are different microfluidic methods such as HTMSU (high-throughput microsampling unit), CTC-Chip, HB-Chip or herringbone-chip, and Nano-Velcro technology[17] (Table1.). Microfluidic technology is a new methodology for the detection of rare CTCs. The microfluidic devices provide several advantages, enabling low sample quantities and high surface area to volume ratio which make it highly sensitive and efficient method. Microfluidic devises can capture CTCs using different strategies, such as size-based sorting, immunoaffinity-based capture, and fluorescence-based separation. The recent advances in microfluidics approach via miniaturization of analytical instruments of bench-top, incorporation with nanotechnologies, and in situ analysis of CTCs offer a promising way for enrichment and detection of CTCs[18].

The sensitivity and plasticity of CTC enrichment can be improved by using a combination of different approaches including immunomagnetic and chip technology, resulting in increased sensitivity up to 99% [19]. Another strategy in development for CTC enrichment that improves efficiency more than 90% is by combining aptamer technology, single-stranded RNA or DNA molecules or peptides, with antibodies which incorporated into a microfluidic device[20-21]. The huge advancement in detection of CTCs in blood is nanomaterials, their small size enables microfluidic devices enrich and detect CTCs in higher sensitivities [22].

After enrichment, CTCs need to be isolated to distinguish tumor cells from normal cells. An important method for detection of CTCs is based on their mRNA profile. One of the most common commercial kit based mRNA methods is the AdnaTest that uses PCR (RT-PCR) assays

to detect expression of tumor-specific genes [23]. A second CTC-detection approach is protein-based using antibody with immunofluorescence staining to identify positive marker (CKs), negative marker (CD45), and DAPI (4-, 6-diamidino-2-phenylindole) nuclear staining. The example of protein-based platforms are Epic Sciences, RareCyte and Epispot assay are wildly used in clinical diagnostics[24-25].

However, most of the CTC detection methods have been utilized after enrichment, two current approaches can detect CTCs without an enrichment step which is called direct detection of CTCs. The first one is line-confocal microscope, a fast and automated screening technique based on the microfluidics and uses both avalanche photodiode (APD) signals and fluorescent images to quantitate rare CTCs in the early stages of cancer. The second one, Surface enhanced Raman spectroscopy (SERS) is a suitable, reliable, and rapid analysis method which is based on SERS-active nanoparticles targeting CTCs on the blood by specific ligands [26].

3. Circulating tumor DNA (ctDNA)

Cell-free DNA (cfDNA) can be released into the bloodstream from different sources such as primary tumors and healthy cells (inflamed cells) because of cellular turnover, apoptosis or necrosis[10]. In patients with cancer, the tumor capacity increase, have much greater levels of cfDNA than in healthy subjects. The term ctDNA is referred to as a fraction of cfDNA which originated from primary tumors, metastatic tumor cells, or circulating tumor cells in cancer patients[10, 27]. They play a pivotal role in providing useful real-time information regarding the stage, molecular profile and mutations of tumor, and also metastasis formation. Therefore, ctDNAs are of interest because they can use as biomarkers for early diagnosis and prognosis as well as offering effective treatment for patients with cancer.

The detection of ctDNA in blood because of their usually low concentrations remains the major challenge. In recent years, various techniques which usually consist of a combination of enrichment and detection procedures have been established to solve this problem. Consequently, these techniques are based on the detection of molecular alterations in tumors including point mutations, rearrangement, and also gene copy number variations[28].

3.1. Methods for ctDNA detection and analysis

The approaches for ctDNA detection can be divided into targeted and untargeted sequence determination. The targeted approaches have been focused on known genetic alterations for example hot spot mutations in KRAS and BRAF genes. These known mutations have been used as predictive biomarkers to guide curative therapy. The second one investigates the known and unknown genetic alterations in ctDNA of tumor tissue.

The known mutations can be detected by PCR-based and Digital PCR-based approaches. Real-time allele-specific PCR (qPCR) method is a diagnostic technique which is used for detection of single-nucleotide polymorphism (SNP). Because of its low limit of detection (LOD) (0.01%) this method may be performed in patients with advanced stages of disease[29]. Further PCR-based technique which developed for ctDNA evaluates include; amplification refractory mutations systems (ARMS)[30], and quantitative nested real-time (QNRT) PCR[31]. More recently, Digital PCR-based methods such as droplet digital PCR, BEAMing, and microfluidic digital PCR have overcome the limitations of previous approaches to detect the genetic alteration in ctDNA.

Droplet digital polymerase chain reaction (ddPCR) is based on water-oil droplet technology[32]. Each DNA molecule is separately analyzed by individual end-point PCR reaction. This method can detect rare and multiple mutations with high sensitivity (0.05-0.001%). Moreover, ddPCR is

a useful tool for the detection of copy number variations and microRNA[33]. BEAMing (Beads, emulsion, amplification and magnetics) is a highly sensitive digital PCR based on water- oil emulsion. This method is a combination of emulsion digital PCR and flow cytometry. BEAMing is used to investigate hotspot mutations in cancer, however, the clinical use of this method is limited due to the difficulties of workflow and high cost [34]. Microfluidic digital PCR is an analytic strategy generate millions of droplets, each droplet contains no more than one target gene which amplification with specific prime or probe. Microfluidic methods allow the detection of genetic mutations and the number of copies present in the cell of a small volume of sample[35].

Deep sequencing of ctDNA, a new strategy based on NGS, has an ability to detect unknown mutations, copy number variations and chromosomal rearrangements. During the last few years, NGS methods significantly improved due to some valuable advantages in monitoring DNA profile. Here, we present a portion of these new advanced techniques[29].

Safe-SeqS (Safe-Sequencing System), a high sensitivity approach to detect rare variants between hundreds of millions of template molecules. One of the main advantages of this method is increase the reliability of massively parallel sequencing by implementing a unique identifier (UID) to each amplified template molecule, then amplify each unique molecule[36].

TAm-Seq (Tagged-amplicon deep sequencing) uses a library and statistically-based analysis algorithms to examine a patient's cfDNA, and detect multiple known mutations, or de novo mutations. The major benefits of this method include a reduction of sequencing time and cost [37].

CAPP-Seq (CAncer Personalized Profiling by deep Sequencing) plays a pivotal role in analysis of multiple mutations types, including single nucleotide variants, rearrangements, and copy number changes. The low concentrations of ctDNA from early stage tumors can be detected by

CAPP-Seq. This ultrasensitive technique based on hybridization of the selector, biotinylated DNA oligonucleotides, on the mutated areas[38].

Whole genome sequencing (WGS) and whole exome sequencing (WES) are untargeted approaches that permits sequencing the whole genome and the coding region, respectively, to detect common or novel mutations. Disadvantages of these methods include cost, they are time-consuming, and also the high concentrations of ctDNA are needed for sequencing.

4. Prognostic and predictive value of CTCs in GI cancers

4.1. CTCs in esophageal cancer

EC is the eighth most common cancer and is the sixth leading cause of cancer-related mortality worldwide [39-40]. It is estimated that 17,650 new cases of EC will be diagnosed in the US in 2019, which contributes to about 1% of all new cancer cases [41]. Furthermore, EC is responsible for over 16,000 cancer deaths annually, which contributes to 2.6% of all cancer-related deaths in the US in 2019 [41]. Despite the multidisciplinary treatment of EC, nearly 50% of treated EC patients develop recurrence due to distant metastasis. With a 5-year survival rate of about 20%, EC remains one of the most lethal human cancers and causes serious problems for healthcare systems annually [41]. Therefore, understanding the mechanisms of recurrence and metastasis of EC is an important issue and finding prognostic markers for this cancer would help the patients benefit more from therapies. The most significant mechanism underlying tumor recurrence and metastasis is the dissemination of CTCs from the primary tumor via blood circulation. Therefore, CTCs have been a focus of attention and introduced as an important prognostic and therapeutic marker for EC.

The investigations performed in the last two decades were mostly carried out on Asian EC patients and used nucleic acid-based deletion of CTCs using quantitative reverse-transcription PCR. These studies have revealed a relatively broad range of CTC detection in EC patients (varying from 2-57% in

esophageal squamous-cell carcinomas (ESCC)) which is due to the use of various protocols and different molecular markers [42]. A recent meta-analysis included 13 eligible literature studies, 11 of which were carried out using RT-PCR. In this study, 979 ESCC patients were evaluated, including 424 CTC-positive and 684 CTC-negative cases. This study revealed that the detection of CTCs in the blood of patients with ESCC is associated with shorter survival progression-free and overall survival with hazard ration (HR) of 2.32 and 2.64 respectively [43].

As stated earlier, recent progresses have resulted in the improvement of CTC enrichment and detection using cytometric-based detection assays. These advancements have resulted in the approval of CellSearch system by FDA for some cancers. However, few studies on the significance of CTCs in EC have been published using the CellSearch method. In 2014, a pilot study on 18 EC patients revealed ≥ 2 CTC per 7.5 ml blood in 8 of the patients. After chemotherapy, the response rates were 60% and 38% in patients with < 2 CTC and ≥ 2 CTC per 7.5 ml blood, respectively [44]. Another study reported that CTCs were detected in 25/90 (28%) of the patients with advanced non-treated ESCC [45]. Both of these studies reported that the detection of CTCs is associated with shorter progression-free and overall survival and that patients with no CTC at baseline and patients with changing CTC status from positive to negative after chemotherapy had favorable prognosis. Li et al compared the result of immunomagnetic CellSearch method with the marker-independent method, ISET, which takes advantage of the difference between the size of tumor cells with white blood cells [46]. According to their study, CTC were detected in 33% (20/61) and 2% (1/61) of the ESCC patients by using ISET and immunomagnetic method, respectively. In addition, circulating tumor microemboli (CTM) were detected in 5% (3/61) of the ESCC patients whereas they were not detected in any of the patients [46]. Other similar studies comparing the immunomagnetic CellSearch method and ISET are currently being undertaken in clinical trials of more than 400 patients [47]. These studies holistically show that compared to isolation of CTCs by their size, CellSearch has a less sensitivity. Table 3 summarizes the different studies about the significance of CTCs in EC.

4.2. CTCs in gastric cancer

GC is the fourth most common cancer with over 950,000 new cases per year and the third leading cause of cancer-related mortality worldwide [48]. Despite the important progresses in the diagnosis and therapy of GC, the 5-year survival rate of this disease is still dismal, and approximately, 50% of the patients display tumor recurrence and metastasis after receiving the treatments [49]. Therefore, identification and development of new prognostic markers is of paramount importance to facilitate the diagnosis and improve the prediction of patients' outcome and also to determine the therapeutic responsiveness.

In the last decade, the detection and analysis of CTCs have frequently been reported in GC. In a systematic review and meta-analysis, Tang et al. have reported that the detection of CTCs may have a role in screening of GC patients and could be used as a non-invasive method for the confirmation of GC diagnosis [50]. According to this study, the sensitivity and specificity of CTC detection in the blood of GC patients were 42% and 99% respectively, highlighting the promising role of CTCs as a prognostic marker in these patients. The early studies have mostly focused on the use of reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) for the detection of CTCs in human cancers, 17 studies which included in this meta-analysis, were also performed by using RT-qPCR and detected cytokeratins (CKs) or carcino-embryonic antigen (CEA) as CTC markers [50]. The molecular approaches indirectly detect the surrogate markers, whereas the later studies have focused on detecting tumor cells [51].

Li et al, have reported on the clinical significance of CTCs in 136 patients with newly diagnosed advanced GC (AGC), and particularly, the potential role of CTCs for real-time monitoring of therapy responsiveness was evaluated using CellSearch system [52]. Following 6 weeks of chemotherapy, the baseline of 3 CTCs per 7.5 ml was correlated with the objective response rate (P=0.016) and the disease control rate (P=0.013). In addition, CTCs were independent prognostic markers for a shorter progression-free survival and overall survival. Specifically, a reduction of CTC numbers to < baseline following therapy improved the prognosis, but those who changed to an unfavorable CTC level, displayed a significantly worse outcome [52]. Therefore, according to this study, elevation of CTCs during therapy may

be associated with a poor prognosis. Kang et al, studied CTCs in 116 GC patients and 31 healthy individuals using a microfluidic system [53]. According to this study, considering the baseline of ≥ 2 per 7.5 mL of blood, the sensitivity and specificity was 85.3% and 90.3%, respectively. In this study, although CTCs were not associated with any clinicopathologic features, the results suggested that they could be a biomarker for early diagnosis of GC [53]. Importantly, in a recent study on 40 GC patients, using a novel wedge-shaped microfluidic chip, CTCs were detected in 75% of the patients, whereas CTCs were not detected in 25 healthy donors [54]. In this study, the detection of CTCs was associated with differentiation grade, lymphovascular invasion, and tumor staging. Taken together, these, in association with other studies, indicate that CTCs could be a valuable and independent prognostic marker for the prediction of patients' outcome and response to therapies. Table 3 summarizes the important studies of CTCs in GC using cytometric methods including the CellSearch system.

4.3. CTCs in colorectal cancer

CRC is the third most common cancer in men and the second in women marked by about 694,000 deaths worldwide [55]. It is estimated that 145,600 new cases will be diagnosed in the US 2019, contributing to approximately 8.3% of all new cancer cases. In addition, this cancer was estimated to contribute to over 51,000 cancer-related deaths, involving 8.4% of all cancer deaths in the US in 2019 [56]. With a 5-year survival rate of less than 65%, CRC remains a serious health problem worldwide, and especially in western countries [57]. Early detection of CRC may help improve the prognosis of the disease and allow an efficient treatment [58]. Sigmoidoscopy and colonoscopy, along with double contrast barium enemas are invasive methods. Imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) are expensive and also, their application may be limited by radiation exposure. Therefore, the investigation of non-invasive and reasonably economical methods for early detection of CRC is required. Various non-invasive methods have been developed to screen for CRC. For instance, fecal

occult blood test is being considered as a highly cost-effective method. However, it has high false positives results [58-59].

Recently, the detection of CTCs has been reported to be highly applicable for early diagnosis, prognosis and clinical decision-making in patients with CRC. In fact, enumeration of CTCs in the blood of patients with CRC has been shown to be an independent prognostic factor for progression free survival (PFS) and overall survival (OS) in patients with CRC. These results have led to the approval of CellSearch system by the FDA for the detection of CTCs in CRC, along with patients with breast cancer and prostate cancer [60-62]. In metastatic CRC (mCRC), data from relevant studies have revealed that CTCs could be predictive markers for response to chemotherapy because they herald potential micro-metastases. In addition, in a large proportion of patients with mCRC, where the level of tumor markers such as CEA are not measurable, CTCs have been shown to fulfill a better disease monitoring and, therefore, higher level of CTCs was correlated with tumor relapse because of their cancer stem cell properties which help them start recurrence [63]. CTCs have also been revealed to diagnose liver metastases in CRC patients. Using flow cytometry to detect various subpopulations of CRCs in mCRC with liver metastasis and non-metastatic CRC, it has been observed that the protein expression of CD133, CD54 and CD44 were higher mCRC with liver metastases, compared to non-metastatic CRC [64]. This observation provides evidence for a significant association between these CTC subpopulations and liver metastasis. Given that early diagnosis of liver metastasis allows liver-targeted therapy to improve survival, this subpopulation of CTCs has a valuable prognostic and predictive value and will help improve the patients' survival [64-65].

Regarding the comparison of CTCs with circular tumor DNA (ctDNA), which both are liquid biopsies, CTCs have been shown to represent the phenotype, genotype, cell cultures and PDXs models of tumor, and therefore, may be a more suitable biomarker. However, overall, the mutations detected in CTCs and ctDNA, e.g. mutations in KRAS, BRAF and PIK3CA, have been reported to be in concordance [66]. KRAS has been reported to have major implication in the prognosis of CRC and response to drugs. As stated earlier, studies on CTCs in CRC patients could reflect the high degree of heterogeneity in KRAS

mutations in the primary tumor, which results in inefficiency of for treatment with targeted anti-EGFR monoclonal antibodies like Cetuximab or Panitimumab [67-68]. Another prognostic significance of CTC characterization in CRC patients undergoing treatment is the analysis of programmed death-ligand (PD-L1). The expression of PD-L1 in was quantified at cells membrane, cytoplasm and nucleus and over-expression of this ligand in the nucleus has been reported to be correlated with shorter survival [69]. Recently, Plastin-3 (PLS3) has been introduced as another marker for CTCs in CRC patients. As a molecule involved in the induction of epithelial-mesenchymal transition (EMT and invasiveness, over-expression of PLS3 in CTCs was correlated with poor prognosis and high rate of metastasis in CRC patients [70]. Most of the studies on CTCs in colorectal cancer have been performed using real-time PCR and CellSearch system (Table 3).

5. Conclusion

Given the high incidence and mortality of GI cancers and the lack of sensitive and specific biomarkers for monitoring patients with these conditions, identification of novel biomarkers for diagnosis, prognosis and the prediction of therapy response is of great importance. Enumeration of CTCs and evaluation of ctDNA help predict the prognosis of the patients. In addition, evaluation of CTCs and ctDNAs, as a non-invasive approach, help monitor the patients repeatedly. Given the dynamic processes involved in carcinogenesis and the evolution of tumor cells during the course of disease, longitudinal assessment of these tumors would help understand the characteristics of cancer cells in a real-time manner. Importantly, evaluation of actionable genetic changes will help design targeted therapy for each patient and will help us to reach a precision and personalized treatment. However, CTCs and ctDNA are found in low quantities in the blood of the patients and the current methods for their detection somehow lack the reasonable sensitivity. Therefore, the development of new methods will help improve the field. For instance, the application of digital droplet-PCR has greatly improved the field of

ctDNA technology. In case of CTCs, the development of marker-free methods for the detection of CTCs will help overcome the limitations observed in CellSearch method. For instance, evaluation of the significance of EpCAM-negative CTC populations may give valuable information with regard to the disease. Taken together, the field of liquid biopsy is in its early ages and, upon the development of new technologies, this filed will drastically change the field of tumor markers.

Table1.Comparison of CTC enrichment techniques

Enrichment method	Advantages	Disadvantages	Examples
Density-based filtration	Easy procedure, low cost, separating cells in different layers	Low CTC recovery, low specificity, CTC size not uniform	Ficoll-Hypaque, OncoQuick
Size-based filtration	Simple process	Low specificity, filter clogging by large leukocytes	ISET, ScreenCell
Immunomagnetic assays	FDA approved and a gold standard method, High purity, High specificity	Only EpCAM-positive CTCs collected, expensive,	CellSearch
Microfluidic	High sensitivity, reduced sample size, fast, low cost	Only EpCAM-positive CTCs collected, cell morphology may be changed during microfiltration	HTMSU, CTC-Chip, HB-Chip, Nano-Velcro technology

Table 2. Technologies for detecting circulating tumor DNA (ctDNA).

Technology of detection	Platform	LOD (%)	Target Mutation	Advantages	Disadvantages	Ref
PCR-based						
	Allele-specific PCR	<0.01	Known	-Easy to use -Lowest cost Rapid	-Lower sensitivity -Only detect limited genomic position	[71]
	ARMS	1.00	Known			[72]
	QNRT		Known			
Digital PCR-based						
	dd PCR	0.001	Known	-Lowest cost -High sensitivity -Rapid	- Only detect limited genomic position	[73]
	BEAMing	0.01	Known			[74]
	Microfluidic digital PCR		Known			
Targeted deep sequencing						
	Safe-SeqS	0.10	Known and new	-Relatively inexpensive - High sensitivity -clinical validated	-Long time for analysis -Less comprehensive	[36]
	TAm-Seq	2.00	Known and new			[37]
	CAPP-Seq	0.01	Known and new			[38]
	WGS	5-10	Unknown	-Broad application	-Expensive -Low sensitivity	[75]
	WES	>1-3	Unknown			[76]

LOD, limit of detection; ARMS, Amplification Refractory Mutations Systems; QNRT, quantitative nested real-time; ddPCR, Droplet digital; Safe-SeqS, Safe-Sequencing System; TAm-Seq, tagged-amplicon deep sequencing; CAPP-Seq, cancer personalized profiling by deep sequencing; WGS, whole genome sequencing; WES, whole exome sequencing;

Table 3. Summary of Studies on CTCs in patients with esophageal cancer, gastric cancer and colorectal cancer

Clinical relevance of CTCs in Esophageal cancer					
Author	**Year**	**Size of cohort**	**Detection method**	**Detection rate**	**Result**
Setoyama et al [77]	2006	106	RT-PCR	36.8%	CEA mRNA in peripheral blood during follow-up is a useful marker for recurrence in esophageal squamous cell carcinoma
Liu et al [77]	2007	53	RT-PCR	before surgery: 28.3% immediately after surgery: 60.4% on the 3rd day postoperatively: 42.9%	Esophageal cancer operation results in the dissemination of CTC in peripheral blood, which elevates the change of developing metastasis.
Tanaka et al [78]	2010	244	RT-PCR	before surgery: 13.9% after the thoracic procedure:16.8%	CTC detection after the thoracic procedure is a useful prognostic factor for tumor recurrences.
Matsushita et al [45]	2015	90	CellSearch	27.8%	CTC may be a promising indicator for prognosis and therapy response in patients with ESCC.
Reeh et al [79]	2015	100	CellSearch	18%	Immunomagnetic detection of CTCs is an independent prognostic factor for EC patients' outcome and may improve accuracy of preoperative staging in EC
Su et al [80]	2016	57	Flowcytometry	44.6%	The Enumeration of CTCs before concurrent chemoradiotherapy was an independent prognostic factor in patients with unresectable ESCC.
Han et al [81]	2018	21	CanPatrolTM CTC enrichment technique and characterization according to EMT markers	8.5% e-CTCs 58.9% mix-CTCs 32.6% m-CTCs	Mix-CTCs and mesenchymal -CTCs may play an important role in progression of ESCC; the number of CTCs in ESCC might be a prognostic factor.
Zhang et al [82]	2019	63 ESCC patients 50 healthy donors	EpCAM⁻independent enrichment and immunostaining fluorescence in situ hybridization	34%	Evaluation of CTCs may be a predictive marker for tumor prognosis and the clinical efficacy of treatment in patients with ESCC
Kuvendjiska et al [83]	2019	20	ScreenCell® filtration	60%	The rate of CTC positive findings and the quantity of CTCs changes in the course of multimodal neoadjuvant

					chemoradiation/chemotherapy and surgery.
Clinical relevance of CTCs in gastric cancer					
Author	**Year**	**Size of cohort**	**Detection method**	**Detection rate**	**Result**
Pituch-Noworolska et al [84]	2007	57	FACS	54%	The detection of CTCs has no prognostic value in patients with resectable GC.
Hiraiwa et al [85]	2008	14, Nonmetastatic 27, Metastatic	CellSearch	14.3% 55.6%	The detection of CTCs at baseline correlated with tumor stage, peritoneal dissemination and shorter overall survival. The increase in CTC numbers was correlated with disease progression and chemotherapeutic effect.
Koga et al [86]	2008	101	RT-PCR	11.6%	Among different markers for RT-PCR, CK19 is a better marker than CK18, CK20 and CEA, and could be clinically useful for prognostic and therapeutic purposes.
Mimori et al [87]	2008	810	RT-PCR	37%	simultaneous presence of CTCs and VEGFR-1 expression at pre-metastatic sites is clinically significant for disease progression
Matsusaka et al [88]	2010	52, Baseline 51, 2-week 48, 4-week	CellSearch	33%	CTCs detection may be a surrogate marker for determining response to S-1 based or paclitaxel regimens in advanced GC.
Kutun et al [89]	2010	50	RT-PCR	20% for CEA; 48% for CK19	Elevated levels of CTCs were observed in patients with MVI invasionExpression of both CEA and CK19 in the peripheral blood of gastric cancer patients are strong predictors of MVI and significantly worse survival. rates
Uenosono et al [90]	2013	Resection 148 Non-resectable 103	CellSearch	60.2% 11.3%	CTC detection at baseline predicted tumor progression, and the effect of chemotherapy.
Okabe et al [91]	2015	136	CellSearch	18.4%	CTCs were independent predictor of progression-free survival in AGC and were helpful for selecting treatment.
Kolostova et al [92]	2016	22	MetaCell	59%	CTCs were found to be present in both resectable and non-resectable GC patients. The sensitivity of CTC-detection could be improved by the combination of cytological and molecular analyses.
Li et al [52]	2016	136 advanced GC	CellSearch	42%	The levels of Post-therapy CTCs can help evaluate therapeutic response. Changes in CTCs following therapy are useful in rapidly identifying treatment efficacy and prognosis.
Kang et al [53]	2017	116	FAST	85%	CTCs have a potential role as early diagnostic biomarker of GC.
Pernot et al	2017	106	CellSearch	46%	Quantification of CTCs at baseline may be a

[93]					useful prognostic tool in advanced GOA, as it is associated with shorter survival
Yang et al [54]	2018	40	Microfluidic chip	75%	CTC-ΔChip holds great potential of clinical application for cancer therapeutic guidance and prognostic monitoring in the future.
Significance of CTCs in colorectal cancer					
Author	**Year**	**Size of cohort**	**Detection method**	**Detection rate**	**Result**
Bessa et al [94]	2001	68	RT-PCR	34%	Preoperative detection of CTCs using CEA marker does not have prognostic significance in patients with colorectal cancer.
Ito et al [95]	2002	99	RT-PCR	37%	RT-PCR is a useful technique for assessment of CTCs in the blood of patients with colorectal cancer.
Cohen [96]	2008	430	CellSearch	26%	The number of CTCs before and during treatment is predicts PFS and OS in patients with mCRC. CTCs provide prognostic information in addition to imaging.
Tol [97]	2010	467	CellSearch	29%	
Iinuma [98]	2011	735	RT-PCR	-	Detection of CEA, CK and CD133 mRNAs is useful for determining which patients are at high risk for recurrence
Sastre [99]	2012	180	CellSearch	47.2%	The enumeration of CTC is a strong prognostic factor for PFS and OS in mCRC patients
Aggarwal [100]	2013	209	CellSearch	-	both CEA and CTCs are prognostic factor for survival in patients with mCRC
Gazzaniga [101]	2013	119	CellSearch	17%	The presence of CTCs at baseline predicts poor prognosis in mCRC patients. Patients with 1-2 CTC should be switched from the favorable prognostic group to the unfavorable, deserving a more careful monitoring
Sotelo [102]	2015	472	CellSearch	35%	CTC detection was not associated with shorter DFS and OS in stage III CRC patients
Seeberg [103]	2015	194	CellSearch	19.6%	CTCs predict impaired survival and also should be considered as a tool for decision-making before liver resection in mCRC patients.
Gorges et al [104]	2016	47	CellSearch AdnaTest	33% 30%	Combined analysis of CellSearch and AdnaTest leads to an improved detection of CTCs in mCRC patients
Le et al [105]	2018	24	CellSearch	25%	Tumor cells release from pulmonary metastases in CRC. A correlation of CTC isolated from the tumor outflow with established negative prognostic markers in metastasized CRC was observed

Fig 1. Comparison of the technologies for CTC and ctDNA enrichment and detection. Circulating tumour cells (CTCs) and circulating cell-free tumour DNA (ctDNA) are isolated from blood sample. Techniques for CTCs enrichment are based on their physical and biological properties, for example density-based filtration and size-based filtration are considered as physical methods as well as immunomagnetic assays and microfluidic devices are biological-based methods. After enrichment, CTCs need to be isolated from normal cells. The most common commercial kit are based on mRNA and protein profile. The approaches for ctDNA detection can be divided into targeted and untargeted sequence determination. The known mutations can be detected by PCR-based and Digital PCR-based approaches. Targeted deep sequencing methodes, including; Safe-SeqS, TAm-Seq, CAPP-Seq, WGS, and WES can detect known and known mutation in ctDNAs.

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