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Detection of circulating tumour cells in colorectal cancer: Emerging techniques and clinical implications

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Abstract

Despite several advances in oncological management of colorectal cancer, morbidity and mortality are still high and devastating. The diagnostic evaluation by endoscopy is cumbersome, which is uncomfortable to many. Because of the intra- and inter-tumour heterogeneity and changing tumour dynamics, which is continuous in nature, the diagnostic biopsy and assessment of the pathological sample are difficult and also not adequate. Late manifestation of the disease and delayed diagnosis may lead to relapse or metastases. One of the keys to improving the outcome is early detection of cancer, ease of technology to detect with uniformity, and its therapeutic implications, which are yet to come. "Liquid biopsy" is currently the most recent area of interest in oncology, which may provide important tools regarding the characterization of the primary tumour and its metastasis as cancer cells shed into the bloodstream even at the early stages of the disease. By using this approach, clinicians may be able to find out information about the tumour at a given time. Any of the following three types of sampling of biological material can be used in the "liquid biopsy". These are circulating tumour cells (CTCs), circulating tumour DNA, and exosomes. The most commonly studied amongst the three is CTCs. CTCs with their different applications and prognostic value has been found useful in colorectal cancer detection and therapeutics. In this review, we will discuss various markers for CTCs, the core tools/techniques for detection, and also important findings of clinical studies in colorectal cancer and its clinical implications.

Key Words: Circulating tumour cells; Colorectal cancer; Tools and techniques; Clinical implications

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Core Tip: Circulating tumour cells (CTCs) in the blood have been found to be mainly associated with the stage of the disease and serve as a prognostic marker for survival in colorectal cancer. Some studies have also reported its role in the diagnosis and treatment monitoring. By focusing molecular research on rare CTCs, targeting cellular markers of CTCs, and discovering new cellular markers may improve the management of colorectal cancer and play a role in prevention of metastatic disease. Patients at high risk might benefit from additional individualized treatment which can be investigated in future clinical trials.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, which stands second and third in women and men, respectively, across the globe with more than 1.2×10^6 new cases and 608700 mortalities annually[1]. It develops due to genetic and epigenetic alterations in human genome and environmental factors. Mode of presentation of CRC can be inherited, familial, and sporadic. Inherited CRC accounts for 5%-10% of all cases, for example, Lynch syndrome, familial adenomatous polyposis, and Peutz-Jeghers syndrome. Among all the CRCs, familial CRC accounts for 20%-30% and sporadic cases approximately 70% of all CRCs which are associated with somatic mutations[2]. There are many invasive and non-invasive diagnostic and prognostic tools with varying sensitivity and specificity, and each has its limitation. There is a need for new tools which may be simpler, non-invasive, cheaper, reproducible, and easily available with high sensitivity and specificity. "Liquid biopsy" is currently the most recent area of interest in oncology, which may provide important tools regarding the characterization of the primary tumour as well as metastasis because tumour cells shed into the bloodstream at the early stages of the disease. In "Liquid biopsy", one of the three types of sampling of biological material can be used, which are-circulating tumour cells (CTCs), circulating tumour DNA, and exosomes. CTCs are one of the main components of liquid biopsy, where subsets of tumour cells can disseminate from the primary tumour and intravasate to the circulatory system. CTCs are non-invasive and safe in comparison to traditional tissue biopsy, and can be used for monitoring of tumour progression and tumour response to therapy in real time. CTCs in peripheral blood serve as a source of valuable tumour markers. The present review will describe the main areas of the ongoing investigation on CTCs with particular emphasis on different tools and techniques used for CTC capturing and analysis, and also currently available data of clinical relevance of CTCs.

CTCs

A tumour cell contains millions of cells maintaining genetic mutations driving them to grow, divide, and invade the local tissues. Some cells separate from the edges of a tumour and are released into the bloodstream or lymphatic system. These cells are CTCs. CTCs can also be defined as cells spreading into vasculature by a primary tumour and they keep circulating in the bloodstream of cancer patients[3]. It was Ashworth (1869) who reported the CTCs for the first time and described the presence of tumour cells with resemblance to the cells from the primary tumour, in the blood of a patient with metastatic breast carcinoma. Later, in 1955, evidence of the presence of CTCs in the blood of a patient with primary and metastatic carcinoma was found by immunohistochemistry. In 1990, Moss and Sanders in their study found evidence for CTCs in seven out of ten disseminated neuroblastoma patients by immunostaining. In CRC, CTCs were first reported in 1993 with the help of conventional cytology and cytokeratin staining. Tumour cells were isolated from 42 patients who underwent

resection with the help of density gradient centrifugation, immune histological evidence for CTCs was reported in 4 out of 42 patients. Above mentioned studies have showed that tumour cells could be detected by traditional immunochemistry techniques; however, their results were based on small sample size and single-center studies.

Some studies have also reported CTC circulation in the body fluids before metastasizing to other parts of the body even in the early stages of the disease[4,5]. Wang *et al*[6] analysed the prognostic role of CTCs, highlighting the importance of CTC count before and after chemotherapy. They found that the presence of CTCs during chemotherapy is an unfavorable but independent factor and may play a role in deciding overall survival (OS) and survival without disease progression [progression free survival (PFS)] in advanced CRC cases. From this study, it was clear that CTCs in peripheral blood can be used as useful tumour markers. Characterization and early detection of CTCs have been reported to play an important role as a prognostic and predictive factor in different types of solid tumours[7,8]. Many epithelial cancers, including breast, prostate and lung cancers, have also been found to be associated with CTCs[9,10].

Early diagnosis, prediction of prognosis, assessment of recurrent risk, individualized treatment, and treatment with curative intent have focused research in the field of CTCs[11]. CTCs have faced difficulties for years because of their very low number (1–10 cells *per* 10 mL of blood) in many studies, and they have a short half-life which ranges from 1 to 2.4 h in blood[12,13], hence posing difficulty in further study. Their detection, quantification, and characterization of molecular features are also difficult. At present, there are several limitations to available CTC isolation techniques. Moreover, only a very small number of CTCs possess metastatic property[14]. Hence, it is very important to characterize them exactly so as to differentiate the non-metastatic CTCs from metastatic ones. There are several techniques which are described here for isolation and detection of CTCs effectively.

CIRCULATING TUMOUR CELL ISOLATION AND DETECTION METHODS

Basic principles

Investigation of CTCs can provide helpful clinical information. However, as described earlier, blood stream harbors very few CTCs and every single CTC is surrounded by 10^6 – 10^7 mononuclear white blood cells (WBCs). To isolate CTCs and detect their characteristics, it is crucial to isolate them from whole blood cells.

Although there are several methods described for isolation of CTCs (Table 1), there are only two basic approaches. The first one is isolation methods based on the detection of specific surface markers for CTCs, which is also termed as “label-dependent methods” (or cell surface markers), and the second method is based on physical or biological properties of CTCs, termed as “label-independent methods”. These approaches are not based on antibodies or other markers for labeling the cells of interest, but they enrich them by use of the difference of physical properties.

Label-dependent methods

In these methods, CTC isolation is based on specific markers. The majority of label-dependent methods use specific epithelial tissue marker-epithelial cell adhesion molecule (EpCAM). EpCAM is the most commonly used method of capturing CTCs because its expression is virtually universal in the cells of epithelial origin and is absent in blood cells. Cell capture with conjugated antibodies followed by purification of captured cells *via* the magnetic field was initially used to enrich CTCs from the blood of patients with prostate or breast cancer. The CellSearch system (Veridex) is a commercial platform which is based on this feature; CTCs are characterized as a population of EpCAM-captured cells that are confirmed to be negative for CD45 and positive for cytokeratins[15]. Other markers are also used, like human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), and cytokeratins[16,17].

The CellSearch CTCs system (Veridex) is commonly used, and in today's scenario it is the gold standard and the only FDA-approved method for CTC detection. It was approved in 2004 for extracting CTCs in metastatic breast cancer[18] and later in colorectal[19] and prostate cancers[20]. Equipment cost ranges from 600000–800000 USD.

The CellSearch CTCs Kit is generally used for the enumeration of CTCs of epithelial origin (CD45–, EpCAM+, and cytokeratins 8+, 18+, and/or 19+) from whole blood sample and works on the principle of anti-EpCAM immunomagnetic

Table 1 Techniques for circulating tumour cell isolation, markers, and their limitations

No.	Name	Property	Markers	Limitations	Ref.
1	CellSearch	Isolation by anti-EpCAM antibody coated immunomagnetic beads	EpCAM, CKs, CD45, DAPI	Only suitable for cancer of epithelial origin but not for that undergoing the EMT Cells are not viable after detection	[48]
2	AdnaTest	Separation by way of anti-EpCAM and anti-MUC1 antibody coated immunomagnetic beads	EpCAM, MUC1, mucin-1, HER2	Possible false-positive finding due to expression of a selection marker being present in other cells other than CTCs Cells are not viable after detection	[49]
3	MACS	Immunomagnetic CTC enrichment by antibodies against cell surface markers	CK19, EpCAM, Her-2, MUC-1 CK7, CK8, CK18, CK19	Lengthy processing time and low sensitivity	[50]
4	MagSweeper (Illumina Inc)	Immunomagnetic isolation of CTC by antibodies against EpCAM and cell surface markers Captured cells are viable with intact RNA	EpCAM, CD45, DAPI	Less sensitive during the early stages of tumour development	[51]
5	CTC Chip	Utilizes bifurcating traps to capture CTCs, release <i>via</i> flow reversal	EpCAM, CKs, CD45, DAPI	Identification of CTCs is lower than other methods	[52]
6	GEM chip	Geometrically enhanced mixing chip structure that allows enhanced capture of CTC on antibody coated surfaces	EpCAM, DAPI, CD45, cytokeratin	Low sensitivity	[53]
7	Onco Quick (Greiner BioOne, Frickenhausen, Germany)	Separation of erythrocytes and some leukocytes from CTC. High sensitivity, Quantification	CCNE2, DKFZp762E1312, EMP2	No morphology confirmation; not really capture CTCs	[54]
8	ISSET (Rarecells Diagnostics)	Rapid processing; non-antigen dependent; Filter based approach	CKs, EGFR, VE-cadherin, Ki67	Size-dependent, manual processing	[55]
9	EPISPOT	Removes leukocytes <i>via</i> CD45 depletion Can detect viable CTCs	CD45, CK19, mucin-1, cathepsin-D	Problem arises when antigen levels are lower or binding efficiency is reduced	[56]
10	Ficoll + RT-PCR	Separation of CTC based on size dependent enrichment. High Sensitivity	CK-19, HER2, h-MAM, CEA, maspin, GABA A, B726P	No morphology confirmation	[57]
11	Cyttel Method	Negative immune-magnetic selection of WBC (CD45 antibody)-High detection rate	CD45	-	[58]
12	MetaCell	Size-based enrichment and separation for viable CTCs	CK-18, -19, -20, CK-7, EPCAM, MUC1, HER2, EGFR	Lengthy processing time	[59]

MACS: Magnetic-activated Cell Sorting; CTC: Circulating tumour cell; RT-PCR: Real-time polymerase chain reaction.

enrichment. For the CTC enumeration step, the CTC kit has reagents to stain and fix the cells. The protocol has been described in detail by Coumans and Terstappen[21]; EpCAM is unique for epithelial cells and is expressed in most carcinomas in a very strong manner, while its expression is limited to embryonic stem cells in non-epithelial cells[22]. However, EpCAM is not a universal cancer marker. EpCAM expression is quite absent in squamous carcinoma or down-regulated if cancer cells undergo epithelial-to-mesenchymal transition (EMT); such cancer cells can escape the capturing process. A gene expression study on breast cancer showed that EpCAM was down-regulated in mesenchymal lines relative to the epithelial cell lines[23] and EMT-induced breast cancer cells[24].

Despite its high specificity and efficiency, some of the disadvantages of the CellSearch system are: (1) It is only suitable for cancer of epithelial origin but not for cancer cells undergoing EMT; (2) CTCs cannot be further analysed in real-time and live-cell conditions, because CTCs cannot be kept alive for a long time; and (3) The use of expensive antibodies leads to high detection cost.

Other label dependent methods

AdnaTest is the second most common method used for CTC detection after the CellSearch. It is a commercially available positive selection method in which immuno-

magnetic beads are coated with a combination of antibodies for the increased capture and enrichment of CTCs. Through gene expression testing of specific tumour markers in the captured cells and comparison of this with their primary and metastatic tumour equivalents, clinicians may analyze the clinical implications of CTCs. Therefore, it has both diagnostic and prognostic value. CTCs captured by magnetic beads coated with antibodies (EpCAM, MUC-1, *etc.*) are then analyzed by multiplex real-time polymerase chain reaction (RT-PCR) gene panels.

The other techniques which are used for CTC enrichment are as follow: (1) Magnetic-activated cell sorting system: This system works on immunomagnetic CTC enrichment by antibodies against cell surface markers. Magnetic-activated cell sorting offers both positive and negative enrichment for the high-efficient and accurate isolation of CTCs (Clinical value: Prognosis and diagnosis); (2) MagSweeper: This system works on immunomagnetic isolation of CTCs by antibodies against EpCAM and other cell surface markers. It can process large amount of blood (approximately 9 mL/h) and can detect 1–3 CTCs *per* 1 mL of whole blood (Clinical value: Prognosis); (3) GEM chip: This is geometrically enhanced mixing chip that permits increased identification of CTCs on antibody-coated surfaces (Clinical value: Treatment monitoring and prognosis); (4) Onco cell enrichment and extraction: This platform uses microfluidic chip with internal surfaces functionalized with an antibodies group against bio-tumour-associated and mesenchymal markers (Clinical value: Treatment monitoring, prognosis, and diagnosis); (5) Graphene oxide chip: In this platform, graphene oxides (GO) nanosheets are used to capture antibodies against cell surface markers of CTCs with a high sensitivity (Clinical value: Prognosis); (6) Ephesia (CTC-chip): Micromagnetic particles are functionalized with EpCAM antibodies which can be self-assembled in a micro-fluidic platform (Clinical value: Prognosis and diagnosis); (7) Quadrupole magnetic separator: This separator works as negative CTC enrichment after it combines with viscous flow stress and magnetic force for the recovery of unlabelled CTCs (Clinical value: Treatment monitoring, prognosis, and diagnosis); and (8) CTC-iChip: This chip works on lateral displacement, inertial focusing, and magnetophoresis for fast isolation of leukocytes by using anti-CD45 and anti-CD66B antibodies in negative enrichment or EpCAM activated beads for CTC enrichment in positive enrichment of CTCs (Clinical value: Prognosis and diagnosis).

Label independent methods

Many newly studied methods for CTC recognition have been reported[25]. Separation of circulating tumor cells by physical properties, *i.e.*, density gradients and gravity, using microfluidic technology[26,27] have been found to be able to capture CTCs efficiently.

The different tools and techniques described for CTC isolation in this category are as follows: (1) ISET: Filter based isolation and enrichment (Clinical value: Treatment regimen and prognosis); (2) MetaCell system: Size-based enrichment and separation (Clinical value: Diagnosis and prognosis); (3) Parylene filter: Filter based isolation and enrichment (Clinical value: Diagnosis and prognosis); (4) ScreenCellCyto: Filter based size-exclusion separation and enrichment (Clinical value: Diagnosis); (5) Cell sieve: Microfilter based isolation and enrichment (Clinical value: Diagnosis and prognosis); (6) Parsorti technology: Micro fluidic separation of CTC based on their size and deformability (Clinical value: Diagnosis and prognosis); (7) RosetteSep CTC enrichment/CD45 depletion: This is an immuno-density negative selection method for CTCs using tetrameric antibody complexes that identify CD45, CD66, and glycophorin on WBCs and red blood cells (RBCs) (Clinical value: Prognosis); (8) Onco Quick: Isolation of RBCs and some leukocytes from CTCs by using filtration through porous membrane followed by density-gradient centrifugation for better CTC enrichment (Clinical value: Prognosis); (9) Cytel method: Based on the negative immuno-magnetic selection of WBCs (antibody CD45) followed by gradient centrifugation and smearing through slides of isolated CTCs (Clinical value: Prognosis and treatment regimen); (10) AccuCyte-CyteFinder: Automated rapid imaging of single rare cells in CTCs, followed by density-based cell separation method (Clinical value: Prognosis); (11) EPISPOT: Negative enrichment using CD45 depletion (Clinical value: Prognosis); (12) Cyto Track: Use of fluorescently labeled cells against EpCAM and scanned with the help of beam (Clinical value: Prognosis); (13) Fiber optic array scanning technology (FAST) (Clinical value: Prognosis); (14) Image Stream: Immunogenetic sorting of blood followed by flow cytometry and enumeration of CTCs by fluorescent microscopy (Clinical value: Diagnosis); (15) DEPArray: Moving dielectrophoretic cages for cell capture coupled with Sanger sequencing (Clinical value: Tumour monitoring and prognosis); (16) Vortex: CTC extraction using microscale vortices and inertial focusing (Clinical value: Diagnosis, prognosis, and treatment planning); (17) ClearCell FX: CTC

separation based on size using Dean Flow Fractionation (Clinical value: Diagnosis); and (18) qRT-PCR: Separation of CTCs based on size-dependent enrichment using CD45, CK19, and CK20 (Clinical value: Prognosis).

COMPARISON OF CELLSEARCH SYSTEM WITH OTHER TECHNIQUES

The high sensitivity and specificity of CTC detection methods have a great effect in improving patient outcomes. Politaki *et al*[28] have compared CTC detection rates and prognostic significance in breast cancer patients by comparing three commonly used methods including CellSearch, qRT-PCR, and double immunofluorescence (IF) microscopy. They analyzed early diagnosed ($n = 200$) and metastatic ($n = 164$) breast cancer patients before the start of adjuvant or first-line chemotherapy. They compared CellSearch system, qRT-PCR for *CK19* mRNA detection, and double IF microscopy by using A45-B/B3 and CD45 antibodies and concluded that patients were more likely to be CTC-positive using the CellSearch (37%) than qRT-PCR (37% *vs* 18.0%, $P < 0.001$) or IF (37% *vs* 16.9%, $P < 0.001$). In another study[29], CellSearch was compared with Adna Test and RT-PCR in breast cancer, and it was found that multimarker qRT-PCR showed a superior sensitivity for the detection of CTCs in metastatic breast cancer patients compared with the CellSearch system and the AdnaTest. There is limitation of the assessment by PCR as it provides the number of target transcripts based on the actual number of CTCs present in a sample[30] and does not allow the morphological assessment of cells. Two cell-based detection assays, the CellSearch and Onco-Quick (for density gradient centrifugation), on comparison revealed that the CellSearch was a far more accurate and sensitive method to detect and enumerate CTCs[31].

There is one study by Gervasoni *et al*[32], in which they compared the capacity of three methods, multimarker RT-PCR assay, standardized CellSearch method, and dHPLC-based gene mutation analysis, to detect CTCs in the blood of 20 CRC patients (stage I = 5, stage II = 8, stage III = 6, and stage IV = 1). They found CTC positivity in 75% of samples by RT-PCR, 20% by CellSearch method, and only 14.3% of samples were found to be gene mutated with the presence of CTCs by HPLC method. These results show that out of these three methods tested, multimarker RT-PCR assay provides the maximum probability of CTC detection. Future studies, by using the above three distinct methods for follow-up, may provide more information about the prognostic significance of CTCs detected through single method assay *vs* combination of different assays[32].

CIRCULATING TUMOUR CELLS AND THEIR CLINICAL APPLICATIONS IN COLORECTAL CANCER

CTC characterization and number may be useful in several ways where they can be used both as a prognostic marker for survival as well as prediction of response to cancer treatment[33]. A multivariate analysis[34] demonstrated that CTC count is the strongest prognostic biomarker for patient survival. If the CTC number increases or remains static, the treatment can be deemed to be ineffective, whereas, if CTC number decreases, the treatment may be effective. Several studies have shown that the presence of as few as 3 to 5 CTCs in 7.5 mL of blood is associated with poor PFS and OS rates[35]. Studies with the CellSearch system and others have shown that high numbers of CTCs are associated with lower DS and OS rates[36]. In a study of 413 metastatic CRC patients being treated with first, second, or third-line therapy, patients with a baseline CTC number of more than 3/7.5 mL had significantly poor median PFS (4.4 mo *vs* 7.8 mo, $P = 0.004$) and OS (9.4 mo *vs* 20.6 mo, $P < 0.0001$) compared with patients with less than 3 CTCs/7.5 mL[37,38]. CTC evaluation, during treatment, may be used as a prognostic predictive marker to determine progression-free survival (PFS) and OS. The CellSearch system has its own limitation; the method of isolation utilizes EpCAM expression on the cell surface of the tumour, which is expressed in 75% of cancer types. A study by Fang *et al*[39] (2016) analyzed the expression of cell surface markers CD133, CD54, and CD44 with the help of flow cytometry to analyze the correlation between cellular subpopulations and colorectal liver metastasis. They observed that the expression of cellular subpopulations (CD133+, CD54+, and CD44+) was higher in the peripheral blood of CRC liver metastasis in comparison with those with no metastasis ($P < 0.001$). In a study by Lalmahomed *et al*[40] (2015) on peripheral blood of 151 CRC patients who underwent liver metastasectomy, CTCs were detected

by the CellSearch system after a density-gradient-based enrichment step. They found that CTCs were detected in 75 samples (43%), out of which 16% had 3 CTCs/7.5 mL of blood. Patients with or without detectable CTCs have an almost similar 1-year recurrence rate (47% *vs* 48%, respectively). A similar recurrence rate was also reported with low *vs* high CTC count (< 3 or 3 CTCs/7.5 mL of blood: 50% *vs* 47%, respectively). In their report, no difference was found in disease-free survival and OS among patients with or without CTCs. A report by Shimada *et al*[41] (2012) found that detecting CEA/CK/CD133 mRNA in tumour drainage blood (RT-PCR method) could act as a prognostic marker in patients with Duke's stages B and C CRC. The findings of the CTC isolation techniques and their clinical significance have been given in detail in Table 2. Hendricks *et al*[42] (2020) used qRT-PCR for indirect CTC detection, which was already applied in previous studies on CRC patients and found to have prognostic value. An earlier study by Sastre *et al*[43] (2008) reported that the CellSearch system could identify CTCs in CRC patients and that CTC positive cases were correlated with the stage of the disease ($P = 0.005$) but there was no significant correlation between CEA levels, tumour locations, grade of differentiation, and lactate dehydrogenase (LDH) levels. A meta-analysis by Katsuno *et al*[44] (2008) of a total of nine studies found that CTC-positive patients (in blood samples by RT-PCR), correlated with lymph node (LN)-positive patients (50%) *vs* LN-negative patients (21%).

Guadagni *et al*[45] (2020) have published a couple of studies about the role of CTC based therapeutic decision making in CRC. In the first study[45], they included 62 patients with advanced unresectable rectal cancer and reported that where the patients were selected for the treatment based on CTCs (HPP/target-therapy group, $n = 43$); the disease control rate was significantly higher (PFS = 8 mo, OS = 20 mo) as compared to those given systemic chemotherapy ($n = 19$) based on age, co-morbidity, and performance status (PFS = 4 mo, OS = 8 mo). The second study[46] was performed on 106 advanced unresectable CRC patients. The therapy was decided based on CTCs (HAI/targeted, $n = 44$), age, and co-morbidity performance status (systemic chemotherapy, $n = 62$). The authors found that the group where treatment was given based on CTCs had longer PFS and median survival (MS) (PFS = 5 mo, MS = 14 mo) as compared to those given therapy based on age and co-morbidity performance status (PFS = 3 mo, MS = 8.5). Finally, they concluded that CTCs can be used to choose therapeutic options in unresectable CRC.

Inherited or acquired resistance in response to specific treatment can be assessed with CTCs which may also work as pharmacodynamic markers. CTCs have enhanced our knowledge and understanding about the primary mechanisms of cancer metastasis. This understanding may be useful in therapeutic manipulation with the help of new targets. CTCs were evaluated in phase I trial based on their count and the expression of insulin-like growth factor-1 receptor (IGF-1R) to find out their therapeutic applications. The CellSearch system was used, either alone or in combination with docetaxel, to count CTCs in patients treated with monoclonal antibodies against IGF-1R. Positive IGF-1R and CTC response was seen in 23 out of 26 patients. These patients responded better in case of combined treatment than in case of the remaining three patients who were negative for IGF-1R. From these findings, it was concluded that CTCs can be used as a potential marker for the selection of chemotherapy[47].

CHALLENGES IN CIRCULATING TUMOUR CELL IDENTIFICATION

CTC interpretation is quite promising but has limitations such as factors like requirement of large volume of blood, small size of the cancer patient population, and the standard value for comparison (*i.e.*, CellSearch, blood sample, other micro-devices, *etc.*). Till now, many reports have enlightened the prospects for cancer patient monitoring, and for few years researchers have focused on CTCs to explore their biological metastatic property and role in cancer treatment monitoring. Among the several important clinical applications for CTC technology is the correlation of CTC count with OS and PFS as a measure of clinical outcome.

The presence of CTCs in the blood sample is also a major challenge. If they are present, their heterogeneity of unknown extent is also present. Because of this nature, it demands an ongoing diversity in the detection and characterization of CTCs using the present available and upcoming methods in the future.

Table 2 Studies showing postoperative isolation of circulating tumour cells in colorectal cancer—markers, techniques, and clinical implications

No.	Technology	Markers	Number of patients	TNM stage	Correlation	Clinical significance	Ref.
1	CellSearch system	EpCAM	164	I-III	With stage	N/A	[60]
		EpCAM	24	IV	With therapy response	May be used in monitoring response to therapy	[61]
		EpCAM	97	II	With stage	Correlates with stage	[62]
		CD133+, CD54+, CD44+	15 (nmCRC); 95 (mCRC)	I-IV	≥ 5 CTCs were 8 times more likely to develop distant metastasis. CTC counts show good correlation with colorectal neoplasm	Independent prognostic marker for nmCRC	[63]
		hTERT, CK19, CK20, CEA	438	I-III	-	Poor relapse free survival	[64]
		hTERT, CK19, CK20, CEA	157	I-III	With stage	Poor relapse free survival and overall survival	[65]
		Survivin, CK20 and CEA	156	I-III	With stages (Duke's) and lymph node metastasis.	Useful as an adjunct in detection of CRC patients	[66]
		CD133, CEA, CK20, CK19,	197	II-III	CEA/CK/CD133 expression and stage (Duke's)	Prognostic significance (Duke's stages B and C)	[44]
2	Flow-cytometry with immunofluorescence	CTCs	18	I-III	With stage and also detected in an early cancer stage.	Predictive	[68]
		CTCs	18	I-III	With stage and also detected in an early cancer stage.	Predictive	[68]
3	Pyrosequencing	KRAS (Codon 12/13)	26	IV	No association	Prognostic	[69]
4	MetaCell separation method	CTCs	98	I-IV	CTC-positive in 83% CTC-negative in 17%	Prognosis and predictive	[70]
5	Mag Sweeper	PIK3CA	242	-	Mutational discordance found between CTCs, DTCs, and metastases, and among CTCs; DTCs from this patient propagated <i>in vitro</i> contained a PIK3CA mutation	Investigating new drug therapies	[71]
6	CTC-Chip	EpCAM, HER2, and EGFR	-	-	Efficiency of 87.5%	<i>In situ</i> protein expression, and culture CTCs from the same set of cells	[72]

CRC: Colorectal cancer; DTC: Disseminated tumour cells; GAPDH: Glyceraldehydes 3-phosphate dehydrogenase; nmCRC: Non-metastatic CRC; mCRC: Metastatic CRC.

CONCLUSION

CTCs have become a hot pursuit and in recent years many new CTC detection technologies have emerged. Discoveries of these technologies from laboratory to clinical practice are non-trivial. Only a few systems are available for routine use in the clinical setting, but not freely available. CTC detection is challenging because of the small number of circulating cells but has been found both in metastatic and non-metastatic cancer (Table 3). It has been well correlated with the stage of the disease, prognosis, and survival but has a limited role in therapeutic decision-making. There is a need for the development of newer, cheaper techniques of CTC detection which can be used as an alternative to invasive diagnosis and treatment monitoring. Future research is required as the current literature has limited information on its use in routine clinical practice but the future is promising.

Table 3 Circulating tumour cells in metastatic vs non-metastatic colorectal cancer

No.	Type of CRC	Markers used	Detection method used	Relevance	Clinical implications	Limitations of the study	Ref.
1	nmCRC	CEA, CA19-9, CA72-4	Cyttl	Diagnostic/prognostic/predictive	Combination of CTCs and CEA: Diagnostic and prognostic indicators	Small sample size, weak power of the study	[73]
2	mCRC	CK, CD45	Immunomagnetic separation	Prognostic/predictive	The number of CTCs before and during treatment is an independent predictor of PFS and OS in patients with mCRC	The baseline unfavourable CTC was low (26%) and overall CTC yield was less than in other epithelial cells	[74]
3	mCRC	ALDH1, CD44, CD133, MRP5, Survivin	qRT-PCR	Prognostic	Poor prognosis and chemo therapy non-responsiveness Survivin and MRP5 selection of mCRC patients resistant to 5-FU and L-OHP	Require further molecular analyses of CTCs for selection of targeted agents	[75]
4	mCRC	CEA	Cyttl method, immunofluorescence <i>in situ</i> hybridization technologies (imFISH)	Prognostic	PFS, OS	Small sample size Lack of dynamic enumeration of CTCs	[6]
5	mCRC	VEGF, CD133+, CD34+/KDR + EPC, CD-34 ⁻ VEGFR2	Flow cytometry/IHC	Prognostic	Treatment response; PFS, OS	-	[76]
6	nmCRC	CD133, CD166, CD44, EpCAM, ALDH1	Tissue microarray, IHC	Prognostic	No association with poor clinical response; OS	Treatment information was missing (local recurrence, distant metastasis, and postoperative therapy)	[77]
7	nmCRC	CK19, MUC1, CD44, CD133, ALDH1	Flow-cytometry, CellSearch, Cytomorphology, qPCR	Prognostic	May be useful as a therapeutic target; PFS, OS	-	[78]

CRC: Colorectal cancer; nmCRC: Non-metastatic CRC; mCRC: Metastatic CRC; OS: Overall survival; PFS: Progression-free survival; IHC: Immunohistochemistry.

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