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Circulating tumor cells in lung cancer: Detection methods and clinical impact*

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Abstract Circulating tumor cells (CTCs) are tumor cells that enter the blood circulation after detaching from the primary tumor and can migrate to reach distant organs, where they can give rise to aggressive metastasis. Clinical studies have revealed that the presence of CTCs in peripheral blood is correlated with disease progression in lung cancer. However, as CTCs are rare cancer cells released from tumors into the bloodstream, both enrichment and sensitive detection methods are technically challenging. In order to best understand how CTCs are currently being deployed, this review mainly focuses on the different detection methods for CTCs. Furthermore, we will describe the clinical impact of circulating tumor cells in lung cancer and discuss their potential use as biomarker to guide the prognosis.

Key words circulating tumor cells (CTCs); lung cancer; enrichment method; detection method

Lung cancer is the leading cause of cancer death in Western countries [1]. The majority of late stage lung cancer patients die within 18 months of diagnosis [2]. The poor prognosis of lung cancer patients is associated with early dissemination of the disease, as well as late diagnosis due to unspecific and late symptoms from the primary tumor. Circulating tumor cells (CTCs) are tumor epithelial cells shed from the primary solid tumor into the bloodstream. Accordingly, CTCs can be transported to distant sites to form metastases which is responsible for the majority of cancer deaths [3]. The presence of CTCs was first reported by Thomas Ashworth in 1869 [4].

The challenge of CTC enumeration and detection faced are that CTCs are extremely rare in the bloodstream. Therefore, a CTC detection platform will need to comprise highly sensitive and specific enrichment. There have been many technologies developed to detect CTCs, many of which have been explored and evaluated with samples from cancer patients [5–8]. However, many questions still remain unanswered regarding the biology of CTCs, the optimal method to enumerate and characterize them and the path to regulatory and general clinical acceptance of technology platforms still under development. Numerous studies in recent years have shown that CTCs may be used as a marker to predict disease progression and survival in various cancers [9–12]. However, the prognostic significance of CTCs in lung cancer is unclear. This review covers previously reported studies of the clinical relevance of CTC detection in lung cancer. A greater understanding of CTCs will open new methods for early diagnosis and treatment of lung cancer in the future.

Enrichment and detection of CTCs

In general, methods for CTC detection can be divided into cytometric and nucleic-acid based techniques. Both techniques are composed of enrichment. Due to the very low events occurring of CTCs in blood (one cell per 10^6–10^7 leukocytes) [13], enrichment is generally essential to increase sensitivity and specificity to measurable levels. In the past decades, there had been numerous efforts, and many researches focused on the development of CTC enrichment and identification. Moreover, each existing enrichment or detection method had its own disadvantages. Some devices which combined two methods could overcome some shortcomings. In an attempt to clarify the hierarchy in the various techniques, we have depicted their major advantages and disadvantages in enrichment of CTCs.

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Table 1 CTC enrichment methods

Enrichment methods		Advantages	Disadvantages
Density gradient		Easy and cheap technique	Low specificity
		Applicable for all tumor types	Relatively low purity of sample
		Not marker dependent	Blood cross-contamination
		Feasible for negative selection	
Size based	ISET	Easy, rapid and cheap technique	Low specificity
		Applicable for all tumor types	Tumor cells are heterogeneous in size
		Feasible with EpCAM positive and negative tumour cells	Small CTC maybe lost
			Enrichment of large leukocytes
	OncoQuick	Density gradient based	Low specificity
		Feasible for EpCAM positive and negative tumour cells	Isolation of large leukocytes
		Cross contamination reduced because of additional barrier	Limited data for clinical validation
	ScreenCell	Isolated cells remain intact	The lack of specificity
			Ready loss of tumor cells
Immunomagnetic based	MACS/Dynal	Flexible	Nonspecific antibody binding
	Magnetic Beads	Multiple antibodies available	Non-tumour cells may be express same antigen
		Specific enrichment	
		Isolated cell can further analysis	Specific markers of CTCs may be lost
	AdnaTest	Combined MUC1 and EpCAM enrichment	No flexibility
		Specific enrichment	Non-tumour cells may be express same antigen
		Higher capture efficiency and improves the processing speed	Specific markers of CTCs may be lost

Methods for enrichment

As previously mentioned, CTCs occur at a very low frequency in the bloodstream. Enrichment can be based on cell characteristics, such as size or density, or on immunoseparation, using magnetic beads, ferrofluids or rosettes. The following technologies have been developed based on different characteristics. The different methods are summarized in Table 1.

Density gradient centrifugation

Traditionally, enrichment of tumor cells has been performed using density gradient centrifugation. Density gradient centrifugation is a basal technique that is very effective and inexpensive as a first enrichment step. The basis of this cell separation assay is the lower density of CTCs and nucleated blood cells, which results in the separation of different cell types into distinct layers, with the epithelial tumor cells enriching in the mononuclear lymphocyte cell fraction at density gradient medium [14]. Oncoquick (Greiner Bio-One) is another separation method, this method is based on density gradient, which has the advantage to prevent cross contamination of the different layers due to a porous barrier keeping them separate [15]. However, density based approaches face the challenge of insufficient purity and CTC losses, both of which may result in typically require further enrichment [14].

Size-based filtration

Size-based isolation mainly base on the difference of the larger size of CTCs (20–30 μm), compared to that of blood cells (8–12 μm). The most acknowledged microfiltration is the isolation by size of epithelial tumor cells (ISET). It is a kind of filter which uses a randomly track-etched polycarbonate preferentially and captures tumor cells larger than 8 μm from fixed blood samples [16–18]. In recent years, techniques of microfilter with silicon substrates and electroformed nickel and three-dimensional microfilter have been developed. These approaches demonstrate improved CTC purity and mitigate cell damage [19–20]. Although microfiltration enables extremely high throughput processing of full tubes of blood within minutes, the sensitivity of this method has been questioned as no validation studies have been confirmed that CTCs are indeed never smaller than 8 μm or smaller CTCs and CTC fragments may be missed.

Magnetic beads

Immunological capture techniques utilize antibodies that bind target proteins present at the cell surface [21]. The antibodies employed in the positive selection methods target the epithelial tumor cell surface markers, while those used in negative selection assays are directed against the surface markers expressed abundantly in hematopoietic cells of different lineages. The epithelial cell adhesion molecule (EpCAM), which is expressed in most cancers of epithelial origin, serves as the most targeted marker described on cell surface [7]. Several immunomagnetic bead separation systems are commercially available, including the magnetic-activated cell sorting system, EasySep cell separation, cell isolation by Dynabeads (Invitrogen, USA)

Table 2 CTC detection methods

Identification process		Advantages	Disadvantages
PCR-based analysis	RT-PCR	High sensitivity	RNA degradation
			False positive results due to unspecific amplification, contaminations, pseudogenes
			No distinction between viable and non-viable cells
			False negative results due to low expression level
	RT-qPCR	High sensitivity; Quantitative	No visualisation of CTCs; No further analysis possible
Cytometric analysis	FAST	Scan analysis of large volume of sample	Lack of validation studies in clinical settings
		Cell loss minimised	
		Quick analysis (up to 300 000 cells/s)	
	LSC	Fast; High specificity	Technically challenging; Low sensitivity
	Flow cytometry	High specificity; Multiple parameters	Low sensitivity
	CellSearch	Semi-automated; High sensitivity	Only EpCAM+/CK+/CD45 CTCs detected
		CTC quantification; Reproducible	Subjective images interpretation
		Recognition of fixed marker (EpCAM, CKs, CD45)	No further analysis possible
		FDA approved	
	CTC-chip	98% Cell viability	Only EpCam positive CTCs detected
		High detection rate	Not commercially available
		Further analysis possible	Lack of validation studies in clinical settings
	EPISPOT	Analysis only on viable cells	Further analysis not possible
		High sensitivity	Need of active protein secretion
			Technically challenging
	FISH	Genetic analysis	Further analysis not possible

and the CellSearch system. The CellSearch system (Veridex) allows automated immunomagnetic enrichment of CTCs expressing EpCAM followed by immunocytochemical identification of the tumor cells [22]. This method has been approved for the detection of CTCs in patients with metastatic breast, colon, or prostate cancer.

The AdnaTest™ is based on the use of specific antibodies against epithelial and tumour markers, associated to magnetic beads, and then RT-PCR is performed for the analysis of the selected markers, thus genetically identifying the CTCs [23]. Compared with CellSearch, the AdnaTest™ achieves higher capture efficiency and improves the processing speed [24]. Even so, several technical challenges are associated with these methods. Many studies have confirmed that not all CTCs express cell surface markers, the expression of EpCAM and/or CK may be down-regulated due to the Epithelial to Mesenchimal Transition (EMT) process [25–26]. Moreover, some tumor-like antigens may express on the surface of normal blood cells.

Microchip microfluidic technologies

Microfluidic immunoaffinity-based isolation of CTCs can be achieved by the flowing the blood sample through a straight microchannel whose inside surface has been combined with an antibody against CTC [27]. An affinity-based microfluidic chip (CTC-chip) has gained considerable attention in recent years. This new technology uses microfluidic immunoaffinity-based technique to design a microchip, consisting of 78 000 microposts coated with anti-EpCAM. Whole blood is pumped through the chip and CTCs are captured. After immobilization, CTCs are stained, counted and investigated by semi-automated scanning of microposts [28].

In recent years, other variations of the micropost-enabled immunocapture which uses different materials, different micropost size and distributions. A new microchip coated with antibodies targeting prostatespecific membrane antigen (PSMA) achieves a higher capture efficiency and purity. The “herringbone chip” designs specific herringbone-shaped grooves. This pattern increases cell to surface contact and makes the capture efficiency much higher [29–30]. The OnQChip™ (On-Q-ity, MA, USA) and the CEE™ chip (Biocept Laboratories, CA, USA) are two commercialized microfluidic devices, and they use a micropost design for adhesion-based isolation of CTCs. Lately, a more advanced “CTC-Chip” is discovered and it enables either positive anti-EpCAM CTC selection or leukocyte depletion after enrichment step and hydrodynamic focusing [31].

Detection of CTCs

CTC detection has not been complicated until the enrichment associated with the applying of the other analysis technique to confirm species and sources of the cell. Therefore, conforming to the specific detection techniques is the key procedure of the detection. The most popular methods for CTCs are briefly discussed below

and summarized in Table 2.

Immunocytological detection of CTCs

Immunocytochemistry is the current most reliable and specific technique for CTC detection and enumeration. In immunocytochemistry, the cells are stained with antibodies. Antibodies against epithelial-specific proteins indicate how many CTCs exist. By fluorescence or through enzymatic color reactions the cells are analyzed. Then fluorescence or a light microscope is used for CTCs detecting and counting. A CTC is often defined as CK+/DAPI+/CD45– intact cell [32–33]. CellSearch™ uses a combined EpCAM selection with staining of CK-8, CK-18, CK-19, CD45, and DAPI. The defined criteria for identifying CTCs include (EpCAM+), CK+/DAPI+/CD45– [34]. The novel CTC-Chip and HB-Chip also implement this identification of (EpCAM+), CK+/DAPI+/CD45– cells [30].

The EPISPOT assay is a functional cell culture assay based on the enzymelinked immunosorbent assay (ELISPOT). With EPISPOT, it is possible to identify and count only viable and not apoptotic cells. The EPISPOT has been applied to samples of breast, prostate, and colon cancer patients [35]. Fiber-optic Array Scanning Technology (FAST) uses automated digital microscopy and exploits a laser raster over a very large field of view to rapidly scan a large substrate. It is a very fast scanning technology that is capable of analyzing 300 000 cells per second. This optical technique has been validated for identification of CTCs and takes the advantage of minimized the risk of cell loss [36].

Fluorescent in situ hybridization

Fluorescent *in situ* hybridization (FISH) technique is used to analyze the presence or absence of specific DNA sequences. Several groups employed FISH technique to analyze the genetic characterization of isolated CTCs, and this has been proposed as a valid method for CTC genotyping. For example, some genes such as the androgen receptor locus (AR), ERBB2 (HER2) and ERG that are relevant for specific targeted therapies in prostate and breast cancer are probed by FISH [37]. Another studies analyzed CTCs isolated from breast cancer patients by FISH using probes for HER-2 gene and the result showed that some level of non-concordance between HER2 status determined by the analysis of primary tumors and with their novel CTC assay [38]. More recently, a study utilized FISH technique shows the result that ALK-rearrangement can be identified in non-small cell lung cancer patients harboring ALK translocations [39].

PCR-based detection of CTCs

The reverse-transcriptase polymerase chain reaction (RT-PCR) is a potentially more sensitive nucleic acid-based approach for detection of CTCs [40]. This is generally the method of choice to amplify mRNAs which are targeted by oligonucleotide primers in CTCs [41]. A variety of biomarkers such as CEA, PSA, and Survivin genes have been used in many different studies [42–44]. PCR methods can effectively pick out one target cell from a large sample volume which contains 10^6–10^7 normal cells. Several studies show that compared with immunocytochemistry, RT-PCR may provide enhancement in detection, and that target markers are sufficiently specific [45].

Although RT-PCR offers impressive sensitivity, some technical limitations may reduce its specificity and accuracy. One important limitation is the possibility of false positive results due to employ unspecific markers or the expression of target genes in normal cells. Sometimes, inflammation, invasive diagnostic operation, or surgery could make tissue and organ specific markers detected in peripheral blood sample [46]. In addition, as CTCs are destroyed by RT-PCR cell counting and analyzing individually are impossible [47].

In order to improve the sensitivity and the specificity of RT-PCR, Quantitative reverse transcription-PCR (RT-qPCR) has been developed [48]. RT-qPCR technique designs a cut-off value, which can reveal the accuracy and the validity of the level transcripts. However, the high sensitivity of RT-PCR tests carries an unstable factor, which the level of transcripts is variable and cannot be estimated reliably if the subjects or samples change [49].

Clinical implications of CTC detection in lung cancer patients

The clinical relevance of CTCs in lung cancer has not been as extensively investigated as for other epithelial cancers, such as breast, colorectal and prostate cancer [50–52]. However, there are some relevant published studies available for lung cancer.

Clinical implications by RT-PCR

Yamashita *et al* [53] were the first to investigate whether CEA mRNA could be used as a surrogate marker for CTC detection in peripheral blood from lung cancer patients. The comparison of patients without CEA mRNA proves that those with CEA-mRNA in the preoperative blood samples had a poor survival. The multivariate analysis selected the preoperative CEA mRNA expression (P = 0.0004) and the pathologic stage of disease (P = 0.0002) as the independent prognostic factors for survival.

In a single-center prospective study, Du *et al* [54] reported the prognostic and predictive value of survivin mRNA-circulating tumor cells (CTCs) in peripheral blood of patients from 78 patients with stage IIIB and IV NSCLC before (C0) and after 1 cycle (C1) and 3 cycles (C3) of chemotherapy. Cox proportional hazards model revealed that the presence of CTCs after one and three

chemotherapy cycles is a significant independent factor for worse PFS and OS.

Liu *et al* [55] investigate the prognostic value of CTCs in patients with lung cancer, the blood cells from 134 lung cancer patients, 106 benign pulmonary disease, and 80 healthy individuals were evaluated by RT-PCR to detect the presence of TSA-9, KRT-19, and Pre-proGRP. A significant correlation was observed between the number of positive markers and disease stage and progression. In another study, Yoon *et al* [56] evaluated the prognostic significance of TTF-1 and CK19 by nested real-time RT-PCR for detection of CTCs in presurgery and postsurgery peripheral blood samples from 79 surgically resected NSCLC patients. Cases with postsurgery TTF-1 (+) and/or CK19 (+) CTCs was more associated with disease progression (P = 0.004) and shorter disease progression-free survival (P = 0.006) as compared to those without postsurgery CTCs.

More recently, Li *et al* [57] investigate serial blood samples from 68 patients with stages I–IIIA NSCLC were examined by RT-qPCR targeting LUNX mRNA before (T0) and after surgery (T1) and after the completion of adjuvant chemotherapy (T2). Results showed that detection of LUNX mRNA-positive CTC after surgery and the completion of adjuvant chemotherapy in patients with stages I–IIIA NSCLC are highly predictive for DFS and OS.

Clinical implications by CellSearch

Tanaka *et al* [58] prospectively evaluated CTCs in 7.5 mL samples of peripheral blood sampled from patients with a suspicion or a diagnosis of primary lung cancer. CellSerach was used to capture CTCs. Of 150 eligible patients, 25 were finally diagnosed as having nonmalignant disease, and 125 were diagnosed as having primary lung cancer. CTCs were detected in 30.6% of lung cancer patients and in 12.0% of nonmalignant patients. CTC count was significantly higher in lung cancer patients than in nonmalignant patients. CTC is a useful surrogate marker of distant metastasis in primary lung cancer.

In a single-center prospective study, Krebs *et al* [59] investigated peripheral blood samples from 101 patients with previously untreated, stage III or IV NSCLC were examined by CellSerarch before and after administration of one cycle of standard chemotherapy. The resulting data demonstrated the number of CTCs in 7.5 mL of blood was higher in patients with stage IV NSCLC compared with patients with stage IIIA or IIIB disease. Furthermore, in the multivariate analysis the CTC number remained as an independent prognostic factor for PFS and the predictor of OS. CTCs analysis was feasible before and after one cycle of chemotherapy.

In support of this, a recent paper by Hirose *et al* [60] evaluated the relationship between CTC count and chemotherapy activity in 33 metastatic NSCLC patients treated with Gemcitabine and Carboplatin. This study showed that 12 (36.4%) had positive CTCs and 5 (15.2%) had five or more CTCs before chemotherapy. The rate of tumor progression was significantly higher in the CTC-positive patients (66.7%), compared with the CTC-negative (23.8%) patients (P = 0.02). Possibly, CTCs not only serve as a prognostic marker in selected tumor types, but also be useful as pharmacodynamic marker in drug development.

CTCs have been hypothesized to be a prognostic factor in small-cell lung cancer (SCLC). Normanno *et al* [61] assessed the prognostic value of CTCs in patients with extensive SCLC. CTCs were assessed with the CellSearch system in 60 extensive SCLC patients. CTCs were identified in 90% (54/60) of patients at baseline. CTC count was strongly associated with the number of organs involved.

Clinical implications by TelomeScan

Igawa *et al* [62] evaluated the prognostic value of CTCs in patients with SCLC with the TelomeScan system. They investigated peripheral blood samples from 30 patients with SCLC patients who had commenced chemotherapy or chemoradiotherapy. Peripheral blood specimens were collected from the SCLC patients prior to and following the initiation of treatment CTCs were detected in 29 patients (96%). A multivariate analysis demonstrated that the baseline CTC count was an independent prognostic factor for survival time (hazard ratio, 3.91; P = 0.026). Among the patients that achieved a partial response to treatment, patients who had a CTC count of < 2 cells/7.5 mL following two cycles of chemotherapy tended to have a longer median progression-free survival compared with patients who had a CTC count of ≥ 2 cells/7.5 mL. Therefore, CTCs may be detected in SCLC patients and CTCs have a strong prognostic factor.

Given the available evidence, the presence of CTCs also correlates with the poor prognosis in lung cancer patients. However, some studies were rather small; resulting in low statistical power, different detection methods and biomarker choices further complicated the picture. Nevertheless, larger studies exploring the clinical impact of CTC analysis in lung cancer are also highly warranted.

Conclusion

Although there are some different results, our review of the present literature seems to support the prognostic and predictive role of CTCs in lung cancer. However, the challenge exists in this field due to ever-increasing evidence which proves the heterogeneous of CTC population. Therefore, focusing on methods to enrich specific CTC populations and characterize them will most likely improve the clinical value of CTC investigations in the future.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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