Cellular Oncology (2020) 43:31–49
https://doi.org/10.1007/s13402-019-00470-y

REVIEW

Prognostic and therapeutic significance of circulating tumor cells in patients with lung cancer

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Accepted: 6 August 2019 / Published online: 11 December 2019

Abstract

Background Lung cancer is the second most common cancer and the main cause of cancer-related mortality worldwide. In spite of various efforts that have been made to facilitate the early diagnosis of lung cancer, most patients are diagnosed when the disease is already in stage IV, which is generally associated with the occurrence of distant metastases and a poor survival. Moreover, a large proportion of these patients will relapse after treatment, heralding the need for the stratification of lung cancer patients in addition to identifying those who are at a higher risk of relapse and, thus, require alternative and/or additional therapies. Recently, circulating tumor cells (CTCs) have been considered as valuable markers for the early diagnosis, prognosis and risk stratification of cancer patients, and they have been found to be able to predict the survival of patients with various types of cancer, including lung cancer. Additionally, the characterization of CTCs has recently provided fascinating insights into the heterogeneity of tumors, which may be instrumental for the development of novel targeted therapies.

Conclusions Here we review our current understanding of the significance of CTCs in lung cancer metastasis. We also discuss prominent studies reporting the utility of enumeration and characterization of CTCs in lung cancer patients as prognostic and pharmacodynamic biomarkers for those who are at a higher risk of metastasis and drug resistance.

Keywords Circulating tumor cells · Lung cancer · Metastasis · Drug resistance

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1 Introduction

Lung cancer is the second most common cancer worldwide and the main cause of cancer-related mortality, contributing to approximately 25.3% of all cancer deaths [1]. Most of the patients with lung cancer are diagnosed when the disease is already in stage IV, which is generally associated with daunting metastases [2, 3]. According to data from 2008 to 2014, the 5-year survival of lung cancer patients with distant metastases is only 18.6% [1]. This poor prognosis highlights the importance of diagnosing and treating patients before the establishment of overt metastases. Importantly, given that neither surgical resection nor repeated invasive biopsies are part of standard care in metastatic lung cancer, access to clinically relevant tissues is limited. Various efforts have been made to improve the early diagnosis of lung cancer including computerized tomography (CT) scanning [4], bronchoscopy [5] and sputum cytology analysis [6]. However valuable these diagnostics may be, they do not confer further information for risk

stratification of patients with primary lung cancer for a timely application of potentially life-saving treatments in those who are at a higher risk of recurrence and metastasis.

More recently, the detection of circulating tumor cells (CTCs) has developed into an evident diagnostic and prognostic approach for patients with lung cancer [7]. CTCs are tumor cells that are shed from the primary tumor and released into the blood circulation. The migration of CTCs is an early event in the progression of cancer. Hence, patients with tumor-free lymph nodes have tested positive for CTCs in their circulation [8]. Therefore, the presence of CTCs in blood allows for an early diagnosis of asymptomatic tumors. Most of the CTCs are cleared by cells of the immune system, but a few of them may survive and reach distant organs and, consequently, establish metastases [9, 10]. In addition to the fact that the early diagnosis, detection and enumeration of CTCs in blood heralds metastatic spread, it may as well be a strong predictive factor of disease recurrence in patients with a primary solid tumor. In this regard, the detection of CTCs has been incorporated in recent international tumor staging systems [11]. Due to its non-invasiveness and the possibility of longitudinal assessments, CTC detection is useful for the evaluation of cancer progression and assessment of the efficacy of therapies. As a result, it serves as a tool for monitoring minimal residual disease. Recent advances have also facilitated the characterization of the genotypes and phenotypes of CTCs, providing new insights into the make-up of CTCs as well as the identification of genetic alterations that may help to predict sensitivity or resistance to anti-cancer drugs. Recent studies on CTCs obtained from patients with colorectal cancer have, for instance, indicated a high degree of heterogeneity of KRAS mutations, which predict inefficiency of EGFR-targeting therapies in this type of cancer [12, 13]. Other examples include mutations in EGFR and HER2, which block the efficacy of therapies that target these particular molecules in lung [14] and breast [15] cancer, respectively. As “liquid biopsy”, CTCs represent an attractive alternative source to tumor biopsies and bone marrow aspirations with a better patient compliance and being less invasive for monitoring the changing burden of the disease during tumor progression or the course of treatment. Hence, CTCs may provide further complimentary prognostic information and may assist in clinical decision-making.

Numerous technologies have been developed for the isolation of CTCs, such as separation of tumor cells based on their physical properties (size and density), the use of microfluidic devices, or by using immunomagnetic separation methods [16, 17]. However, clinical validations still need to be carried out for most of them [18]. To date, the CellSearch technology is the only US Food and Drug Administration (FDA)-approved system that has been clinically validated using thousands of samples across multiple research laboratories. Epithelial markers, such as epithelial cell adhesion molecule (EpCAM) and cytokeratins (CKs), are usually expressed in carcinoma cells because they originate from epithelial tissues. These markers are, however, not expressed in normal blood cells since these cells have a mesenchymal origin [19, 20]. The CellSearch platform takes advantage of positive immunostaining for EpCAM and negative immunostaining for a common leukocyte antigen, CD45, to exclude leukocytes. Currently, CellSearch is considered as the ‘gold standard’ method for the detection of CTCs. It has been used in large trials and has shown clinical relevance of EpCAM-positive CTC detection. However, given that EpCAM-negative CTCs are not detected by the CellSearch system, the development of novel technologies to detect both EpCAM-positive and EpCAM-negative CTCs, especially marker-independent approaches, is needed [21]. Using the CellSearch system, it has been found that the detection and enumeration of CTCs may serve as an independent prognostic factor for progression-free survival (PFS) and overall survival (OS) of patients with e.g. breast, colorectal and prostate cancer [22–24]. Furthermore, culturing CTCs ex vivo and/or the generation of CTC-derived xenograft (CDX) models through direct implantation of cultured CTCs into recipient mice, have been found to be helpful to overcome the problem of scarcity of CTCs and may hold promise for deep sequencing of CTCs to choose the best therapies over the course of the disease [25, 26]. It has, for instance, been reported that ex vivo culturing and increasing the number of CTCs taken from breast cancer patients may allow for deep sequencing of these cells and for detecting pre-existing mutations in the *PIK3CA* gene and newly-acquired mutations in the estrogen receptor gene (*ESR1*), among others. Testing of drug sensitivities in these CTCs with multiple mutations has revealed new therapeutic targets [25]. Here, we review our current knowledge on the biology and the application of CTCs in lung cancer in general (both small-cell lung cancer, SCLC, and non-small-cell lung cancer, NSCLC). In addition, we elude on the clinical validation of CTCs as predictive and prognostic markers for this particular disease.

2 CTCs and the metastatic cascade

CTCs refer to cancer cells that are shed from the primary tumor into the vasculature and are found at very low levels in the bloodstream. According to the ‘seed and soil’ hypothesis [27], detached tumor cells or “seeds”, which are now called CTCs, require favorable environments for seeding. The dissemination of tumor cells into the circulation may occur at early steps of tumor formation and may eventually lead to blood-borne metastasis [28]. Initially, cancer cells may lose their connections with adjacent cells and the extracellular matrix (ECM), henceforth allowing them to migrate from their original location to other sites either through hematogenous

intravasation or via the lymphatic system [29]. Although blood-borne spread has been the accepted as a route of cancer dissemination and distant organ metastasis, dissemination to the lymphatic system may lead to nodal metastasis [30, 31]. CTCs are able to enter the blood circulation at the end of the lymphatic transport system because lymphatic vessels eventually return to the bloodstream via subclavian veins [32]. It has been found that only 2.5% of CTCs form micro-metastases and 0.01% are able to form macro-metastasis, a process referred to as 'metastatic colonization' [33]. Therefore, metastasis is an inefficient process, in that only very small amounts of CTCs are able to finally develop into macro-metastases. It has been found that CTCs may travel either as single cells or as clusters of cells (2 to more than 50 cells, known as circulating tumor micro-emboli (CTM)) [34]. Although CTC clusters have been reported to be associated with a worse prognosis in locally advanced lung cancer [35] and in other cancers, including head and neck cancer [36], much remains to be learned about the nature and clinical relevance of these clusters. During their parallel progression, metastatic cells acquire features different from their primary tumors [37, 38]. Therefore, the characterization of CTCs may bear relevance for the identification of patients who may benefit from complementary therapies [39]. When CTCs arrive at the bone marrow, they are referred to as 'disseminated tumor cells' (DTCs). These tumor cells may reside in the bone marrow for many years, hence creating a 'tumor reservoir' [40]. DTCs usually lack the expression of Ki-67, contributing to their stationary phase and resistance to chemotherapy [41] and, thereby, facilitating their impending progression to metastasis.

2.1 Metastasis initiation and CTC formation

In theory, tumor cells may invade the basement membrane, enter the circulation and disseminate throughout the body via EMT-mediated or non-EMT-mediated pathways. Via the EMT-mediated pathway, cancer cells actively take on cellular programs to interrupt the basement membrane, migrate through the extracellular matrix (ECM) and enter the blood circulation. Via the non-EMT pathway, the invasion of cancer cells into the blood circulation occurs based on centrosome amplification [42] or on a passive shedding process [43]. Through both pathways, CTCs can leave tumors as single cells or as clusters. In the following two paragraphs, we describe the EMT- and non-EMT-mediated mechanisms involved in the formation and dissemination of CTCs (Fig. 1).

2.1.1 EMT-mediated formation and dissemination of CTCs

EMT is considered as a crucial process for the transformation of epithelial cells into mesenchymal cells, which exhibit a greater motility and resistance to apoptosis, thereby enabling efficient invasion into the blood stream and transport to distant sites to form metastases. EMT is associated with debilitated cell-cell interactions, apoptosis and anoikis, along with increased drug resistance [44]. EMT-related transcription factors (EMT-TFs) (Snail-1, Snail-2 (Slug), Twist, ZEB-1) and extracellular molecules (TGFβ, HGF, FGF, Wnt and Notch) secreted from tumor cells and/or inflammatory cells, as well as specific signaling pathways (Wnt/β-catenin, MAPJ, NF-k and PI3K) regulate these changes. These changes, which turn tumor cells into stem-like cells, increase their mobility and survival, and eventually allow them to invade other tissues [45, 46]. In addition, these stem-like cells are resistant to chemotherapy and, as such, they may give rise to cancer recurrence.

Tumor cells that have undergone EMT have been found to express different markers. Low expression of epithelial markers such as EpCAM, cadherin, claudin, plackoglobin and CK, and high expression of mesenchymal markers such as vimentin, laminin and N-cadherin are characteristic of EMT. EpCAM and CK are generally being used to detect CTCs, although these markers may be insufficient due to the fact that they may be poorly expressed on CTCs after having undergone EMT [47]. Recently, studies have been carried out to detect EMT markers in CTCs of various cancers, including lung cancer. The identification of these markers may play an important role in both primary tumor detection and in tumor metastasis detection. Larsen et al. reported that the expression of ZEB1 and the resultant induction of EMT is increased in early stages of tumor formation in patients with non-small cell lung cancer (NSCLC), and that this marker can be considered as an important molecular target in reducing the metastasis of lung cancer and human bronchial epithelial cells (HBECs) [45]. In another study, Tsoukalas et al. assessed the relationship between immunohistochemical expression of E-cadherin, vimentin and Ki-67 in tissue microarray (TMA) samples of 121 patients with NSCLC. They observed a direct relationship between the expression of E-cadherin and the overall survival rate, and an inverse relationship between the expression of vimentin and Ki-67 and the overall survival rate, indicating a role of high vimentin and Ki-67 expression and low E-cadherin expression in NSCLC progression and metastasis [48]. To unravel the role of EMT in the dissemination of CTCs, Yu et al. established a quantifiable RNA-in situ hybridization (ISH) assay [49]. Using this assay, they assessed circulating breast tumor cells for the expression of seven epithelial markers (CKs -5, -7, -8, -18 and -19, EpCAM and CDH1) and three mesenchymal markers (FN1, CDH2 and SERPINE1/PAI1). The found that CTCs exhibit dynamic changes in epithelial and mesenchymal composition during tumor progression. Their results indicated that rare primary tumor cells may simultaneously express epithelial and mesenchymal markers. Subsequent serial monitoring of CTCs during tumor progression revealed that the mesenchymal markers

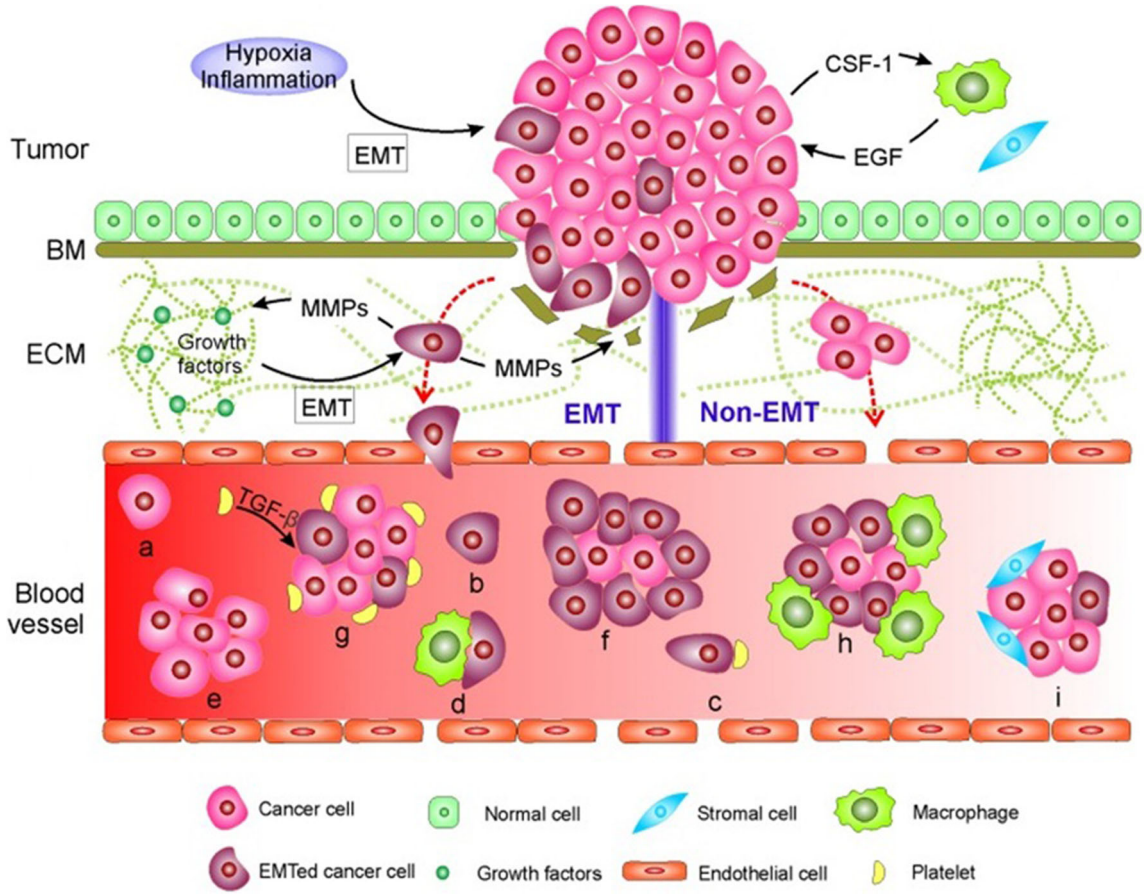

Fig. 1 Mechanisms of CTC formation and various CTC subpopulations existing in the circulation. CTCs can be generated through EMT- or non-EMT-mediated mechanisms. In the EMT-mediated mechanism, tumor cells actively undergo different changes at the cellular and molecular levels, leading to break-down of the basement membrane, migration across the ECM and entrance into the circulation. In contrast, in the non-EMT-mediated mechanism, tumor cells travel to the blood via passive infiltration by external forces or centrosome amplification-mediated invasion. Through both mechanisms, CTCs can leave the primary tumor as single cells or as cell clusters. Once entering the circulation, CTCs may be present as single cells with different EMT phenotypes (a-d) that may subsequently bind to immune cells, or as clusters of cells with different EMT phenotypes (e-i) that may be covered by immune or stromal cells. Reprinted with permission from Creative Commons Attribution (CC BY-NC 4.0) license [181]

were highly enriched in CTCs occurring both as single cells or as multicellular clusters (CTM) [49]. These data underscore a role of EMT in blood-borne metastasis.

The Notch signaling pathway is known to be involved in processes of normal growth, embryonic development, neuroendocrine (NE) hormone production and EMT. Its receptors include four transmembrane proteins, NOTCH 1–4. Hassan et al. found that a high expression of NOTCH3, followed by an increased expression of NOTCH1, Hes1 and Jagged1, plays a role in NSCLC progression via EMT induction. Conversely, it was found that this pathway prevents EMT in small cell lung cancer (SCLC) patients [50].

Fucosyltransferase IV (FUT4) and its synthetic cancer sugar antigen Lewis Y (LeY) have been found to be abnormally expressed in many cancers, including lung cancer [51–54]. FUT4 catalyzes the transfer of Fucose to N-acetylglucosamine on sugar chains, turning it into an important enzyme in the synthesis of LeY. LeY induces the glycosylation and activation of EGFR and promotes the migration of tumor cells. Tian et al. [53] revealed a role of Ginsenoside Rg3 in the inhibition of FUT4, EMT and metastasis, in addition to an decreased activation of EGFR in lung cancer. Therefore, the direct relationship observed between high FUT4 expression and EMT in patients with lung cancer and its important role in metastasis and tumor invasion indicates that FUT4 may be considered as a therapeutic target for cancer [53].

2.1.2 Non-EMT-mediated formation and dissemination of CTCs

Although EMT-related tumor metastasis is generally accepted as an established route for cell invasion and metastasis, non-EMT routes have also been found to be involved in tumor cell dissemination [46, 55]. Non-EMT cancer metastasis may occur through a number of mechanisms and, consequently, lead

to the formation of pro-metastatic CTCs [56]. In this regard, it has been shown that increased Arp2/3-dependent actin polymerization and reduced cell-cell adhesion upon centrosome amplification may convey invasive properties to cancer cells [55]. Collective migration of cancer cells is known as another form of non-EMT-associated dissemination [57]. In this process, E-cadherin-positive epithelial cell clusters act as invasive leader cells intravasating into the surrounding vasculature [9]. This collective migration is thought to be a potential contributor to the successful establishment of metastases and to contribute to the resistance of tumor cells to anoikis-mediated cell death. These cancer cells retain their epithelial-like characteristics allowing cell-cell adhesion at distant sites [9, 56, 58]. Interestingly, after intravenous injection of EMT-derived cells and non-EMT cells, Tsuji and colleagues found that unlike EMT cells, non-EMT cells were able to initiate lung metastasis [59]. In a study conducted by Fischer et al., an EMT lineage tracing strategy, which was capable of monitoring EMT in vivo, revealed that breast-to-lung cancer metastasis was mainly derived from non-EMT tumor cells, contradicting the original EMT/MET hypothesis [60]. In another study by Zhao et al. it was found that non-EMT tumor cells maintain their epithelial phenotype during lung metastasis and that EMT is dispensable for the establishment of lung metastases [61]. In addition, migration in vascularized areas inside the tumor mass, early dissemination at the stage of carcinoma in situ and passive shedding, which represent other prevalent routes of cell migration, have been shown to trigger cancer metastasis [56, 62].

2.2 Dispersion of CTCs into the vasculature

It has been estimated that when CTCs enter the circulation, they survive for approximately 1–2.5 h [63]. After this time period, most of the CTCs (specially as single cells) undergo apoptosis in circulation [64]. It has also been reported that about 1 million tumor cells may enter the circulatory system every day and that the immune system clears more than 85% of these cells within minutes after leaving the original tumor mass [33]. Compared with single cells, CTC clusters are relatively rare in the circulation, but they are more resistant to apoptosis [65]. So, although pro-metastatic CTCs are relatively rare in the blood circulation, they can extravasate through several mechanisms and ultimately form distant metastases [66].

Within the circulatory system, CTCs are faced with various factors, including hemodynamic forces of the blood flow, immune responses and endothelial cells lining the vessel walls. All these factors create a challenge for the survival of CTCs. Once CTCs enter the blood stream, single tumor cells may be bound to platelets and/or macrophages, after which the clusters may be further covered by platelets, macrophages and/or reactivated stromal cells [55]. Therefore, an increased platelet count (thrombocytosis) is often observed in many cancer types. The attachment of CTCs to platelets yields aggregated platelets, a process that is termed tumor cell-induced platelet aggregation (TCIPA) [67]. TCIPA plays a key role in the survival of the tumor cells in circulation. CTCs trigger TCIPA by direct contact or through the release of agonistic mediators, such as ADP, thrombin, TXA2 or tumor-associated proteinases [68]. Platelets are activated in TCIPAs and, as a consequence, bind to the surface of CTCs through GPIIb-IIIa-fibrinogen bridges, and are up-regulated by P-selectin [68]. It has been shown that by treating mice with a highly specific antagonist of thrombin, coagulation events are blocked and lung metastasis is inhibited [69]. The tight coating of CTCs by activated platelets may help to avoid physical stress and/or their recognition and destruction through the immune system [70]. TCIPA also helps CTCs to undergo EMT. Thereby, it enhances the ability of the tumor cells to resist apoptosis. Platelets also protect CTCs against natural killer (NK)/T cells and mediate the enrollment of neutrophils, which further enable the adhesion of CTCs to endothelial cells and, hence, extravasation. In Fig. 2 interactions of CTCs with immune cells and platelets, resulting in immune escape, are depicted. In addition to platelets, in the majority of cases CTCs are associated to neutrophils. In a recent study, Szczebra et al. isolated and characterized individual CTC-associated neutrophils from breast cancer patients and used single-cell RNA sequencing to compare transcriptome profiles of CTCs associated with neutrophils with those of CTCs alone [26]. Their study revealed a number of differentially expressed genes that underlie cell cycle progression and trigger metastasis. They also identified cell-cell junction and cytokine-receptor pairs that are involved in the clustering of CTCs with neutrophils, representing key vulnerabilities for therapeutically targeting metastasis [26].

Tumor cells may also escape from the immune system by other mechanisms, including down-regulation of the expression of certain HLA-I types, loss of antigens, alterations in the expression of MHC molecules, involvement of NK-cell ligands, FAS/FAS ligand (FASL)-induced apoptosis, and immune-checkpoint molecules, such as CD47 and programmed cell death-ligand 1 (PD-L1) (Fig. 2) [71]. In lung cancer, immune escape can occur by reducing the expression of HLA through allele-specific HLA loss events. Loss of heterozygosity (LOH) at the HLA locus has been observed in 40% of NSCLC patients. This loss facilitates evasion from the immune system and allows a cancer to transit to the escape stage, allowing CTCs to attach to the vascular endothelium, leave the circulatory system, and survive at distant locations to form metastases [72].

2.3 Extravasation of CTCs to distant organs

When CTCs are dispersed through blood vessels, they may become trapped within capillaries due to their small diameters

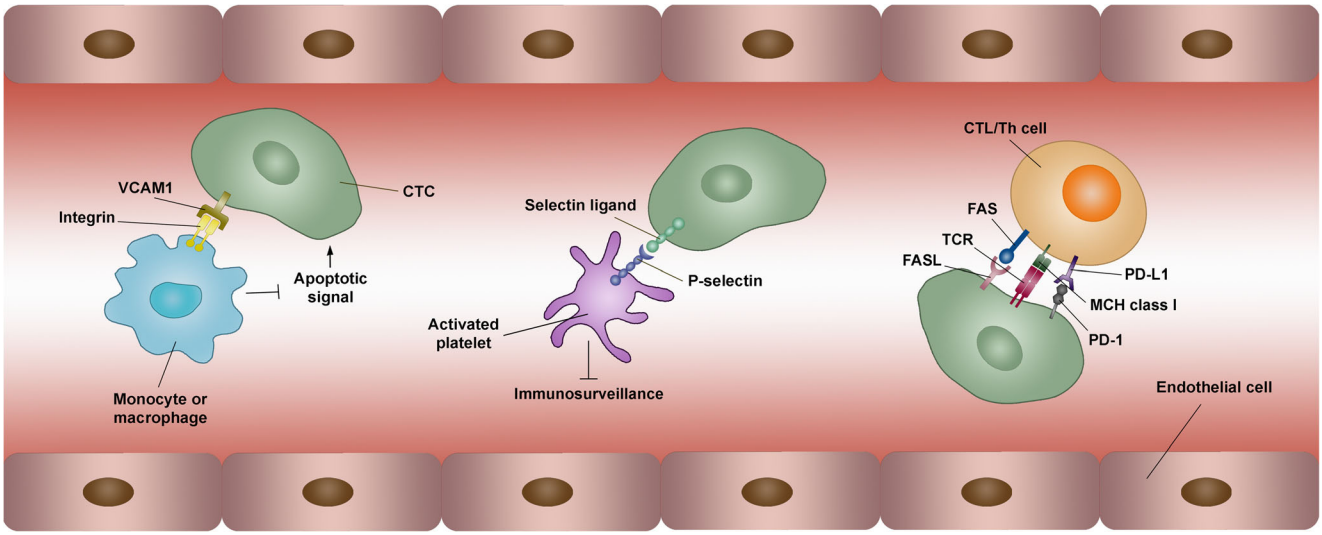

Fig. 2 Mechanisms of escape of CTCs from immunity in blood

(3–8 μm) which are, in general, less than the size of a tumor cell (9–30 μm) [73]. As such, CTCs can anchor to the luminal surface of endothelial cells and extravasate through the subepithelial extracellular matrix (ECM). Given the notion that CTC clusters may seem too large to pass through narrow capillaries, it was previously thought that only individual tumor cells can reach distant organs. However, recent studies have challenged this assumption. Using microscale devices designed to mimic human capillary constrictions and in vivo studies, it has been shown that CTC clusters can reversibly be reorganized into single-file chains and subsequently transverse through capillaries [74, 75].

Interactions between CTCs and endothelial cells during extravasation occur through ligand-receptor binding processes. For example, integrins, which are present on CTC surfaces, can bind to intercellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion molecule 1 (VCAM-1) on endothelial cell surfaces. Platelets and leukocytes mediate this process. Interactions between CTCs and platelets induce the expression of C-C chemokine ligand 5 (CCL5), which in turn leads to an increase in the recruitment of leukocytes to the CTCs [76]. In addition, platelet-derived TGF-β and PDGF have been found to induce EMT in CTCs and to provide CTCs with the migratory and invasive properties needed to break through the ECM of blood vessel walls [76]. Leukocytes may act as linker cells between CTCs and endothelial cells [77]. Cancer cells are capable of producing chemokine ligands (e.g. CXCL12), which may promote organ-specificity of metastatic tumor growth depending on expression of the respective receptors (e.g. CXCR4). This process relates to how lymphocytes become destined for homing to distant organs according to chemokine ligand-receptor trafficking processes.

3 CTC detection and enrichment

Compared to more invasive tests, the detection and sampling of CTCs in the peripheral blood is easier and more reproducible. In addition, it has the advantage of a better patient compliance. However, the low number and short half-life of CTCs are challenging factors that are faced during their separation, i.e., ~1 CTC may be found among 10^5–10^6 peripheral blood mononuclear cells [18, 78]. Therefore, enrichment of CTCs is an important step in their detection as well as in their actual use for the diagnosis and prognosis of cancer. Several methods have been developed and applied for enriching specific cell types within blood samples, and some current CTC detection methods combine a CTC enrichment step with tumor-specific molecular analyses. Both enrichment and isolation techniques take advantage of unique characteristics of CTCs. Below, we will discuss the basic approaches that can be used for these purposes.

Enrichment/isolation techniques can be classified according to their physical and biological characteristics. Both the enrichment and isolation of CTCs based on their physical characteristics are encountered with several difficulties due to the associated cell stress. Therefore, new approaches have been designed based on the expression of specific proteins, which can be used with less variation. The two main techniques used for CTC enrichment include density gradient centrifugation, either by Ficoll-Hypaque or by OncoQuick®, and immunomagnetic enrichment of tumor cells using cell surface antigens. One disadvantage of immunomagnetic enrichment is that CTCs are highly heterogeneous in their expression of antigens [73, 79, 80] and, as a result, methods using EpCAM as an enrichment marker may miss the population of EpCAM -negative CTCs [45]. Therefore, it would be more reasonable to use tumor-specific antigens, for example CD17, for the

enrichment of CTCs [81, 82]. Furthermore, CTCs can be isolated by size. A great advantage of isolation by the size of the tumor cell (ISET) is that by this technique, CTCs are not modified during the enrichment process. Accordingly, they can subsequently be used for fluorescence in situ hybridization (FISH)- or polymerase chain reaction (PCR)-based characterization methods. The RosetteSep™ system offers a negative selection of tumor cells by cross-linking unwanted blood cells to erythrocytes, thereby simplifying their removal during density gradient centrifugation [82].

The CellSearch system is the 'gold standard' immunocytological diagnostic technique for the detection of CTCs. This system is based on antibodies that have been generated against specific epithelial markers, such as EpCAM, CK19, CK18 and CD45, to exclude leukocytes [83]. The measurements are attained according to fluorescence microscopy. This system allows a sensitive and positive capture of CTCs through utilizing EpCAM coated with ferrofluids [83]. The CellSearch system is a widely used system with many advantages, providing prognostic information with respect to PFS and OS [15]. However, it suffers from a number of disadvantages, including a low sensitivity and a failure to detect EpCAM-negative cells [84, 85]. That is why recent strategies are focused on the development of marker-independent methods. These methods are not only able to detect both EpCAM-positive and EpCAM-negative CTCs, but can also better detect CTC clusters, which are considered imperative in the process of metastasis. The OHSV1-hTERT-GFP technique has been used to detect CTCs in lung cancer patients and its sensitivity was compared with that of the CellSearch system. In doing so, more CTCs were detected using the oHSV1-hTERT-GFP technique compared to the CellSearch system, which suggests a relatively high sensitivity of the former technique. [86]. The Nanoelectromechanical Chip (NELMEC) technique represents a new nano-based method to differentiate whole tumor cells (WTCs) and chymase-positive mast cells (MCTCs) from white blood cells (WBCs) without the need for labeling and marking. In addition, it is devoid of the problems confronted in the EMT process. This technique is based on the use of SiNG/Au nanoelectrodes and is able to separate CTCs from WBCs with a capture yield of 92% to 97% [87].

The Fiber-Optic Array Scanning Technology (FAST) is based on a fast scanning technique, which is widely used for the rapid detection of CTCs by analyzing 300,000 cells per second. This technique has the ability to examine large volumes of samples as well as the ability to detect CTCs using antigen-agnostics and size-agnostics. An automated digital microscopy (ADM) platform and a laser raster are used in this technique. It has also been shown to be able to detect PD-L1 and vimentin surface markers in patients with NSCLC [88, 89].

Two other diagnostic methods for detecting CTCs may be used, including flow cytometry and immunofluorescence assays. A high specificity along with multiple parameters (DNA-content, cellular markers and cell size) are considered important advantages of the flow cytometry method. The detection of heterogeneous CTCs by immunomagnetic nanospheres modified with antibodies against EpCAM and folate receptor α (FRα) markers in NSCLC cells by applying fluctuation cytometry and immunofluorescence techniques have been shown to yield a high sensitivity and a high performance in diagnosis. Hence, this evidently indicates the implication of flow cytometry for the detection of CTCs [90]. Epithelial Immuno-SPOT (EPISPOT) and enzyme linked immunosorbents, which are used to detect specific secretion proteins (CK19, PSA, HER2 and VEGF), can detect non-apoptotic and viable cells with a high sensitivity relative to the CellSearch system [81, 91, 92]. It has been found that an active subset of breast cancer cells with metastatic properties can be detected by the presence of CK19 proteins via the EPISPOT assay (63).

Nucleic acid-based diagnostic techniques (RNA and DNA) and aptamers represent yet other strategies widely used for the detection of CTCs in conjunction with surface markers [93, 94]. Several studies have been carried out to detect FR^{+}-CTCs using LT-PCR techniques in lung cancer patients. FR is a glycoprotein receptor that can be found on the surface of many tumor cells, including ovarian and lung cancer cells [95, 96]. The basis of this technique is labeling CTCs with conjugated ligands or tumor-specific oligonucleotides and, subsequently, performing PCR. This technique has shown a high sensitivity (81.8%) and specificity (93.2%) for the detection of CTCs [97]. Quantitative reverse transcription PCR (RT-qPCR) is another diagnostic technique for the detection of CTCs. One of the advantages of this technique is a quantitative detection with a high sensitivity as well as a simultaneous analysis of more than one marker [98]. In order to detect CTCs with RT-qPCR, several enrichment steps need to be carried out. The AdnaTest is a commonly used technique that is based on immunomagnetic enrichment and, subsequently, CTC detection using RT-qPCR. As such, the RT-qPCR technique has been applied to detect NSCLC CTCs using CK7, CK19, fibronectin 1 (FN1) and human epithelial glycoprotein (EGP) for enrichment [99]. FISH is another technique used for the detection of CTCs. This method has shown a high sensitivity (75.0%) and specificity (100%) for the detection of CTCs in the cerebrospinal fluid (CSF) of patients with lung cancer meningeal metastasis [100].

The isolation of platelet-covered CTCs may be another interesting approach to capture CTCs. Compared to conventional CTC isolation/enrichment methods, which are dependent on the presence of surface markers on tumor cells, platelet-targeted isolation can be applied to capturing CTCs of both epithelial and mesenchymal phenotypes. In a recent

study by Jiang et al., free platelets were first depleted by means of hydrodynamic size-based sorting, followed by immuno-affinity-based isolation of platelet-covered CTCs with antibodies directed against human platelets (CD41 HB-Chip) or CTCs (EpCAM HB-Chip) via a herringbone micro-mixing device. This method enabled the isolation of CTCs from 66% of lung cancer, 60% of breast cancer and 83% of melanoma (mesenchymal) samples tested [101]. The various systems for the detection of CTCs are listed in Table 1.

4 CTCs as diagnostic and prognostic factors in lung cancer

During the last decade, there has been a pressing need for the development of non-invasive, blood-based biomarkers for the early diagnosis and prognosis of human cancers, as well as for the monitoring of therapeutic responses. The fact that CTCs can be found in blood samples of patients with a primary tumor before the establishment of an overt metastasis, has been an impetus for continued studies to grasp hypothesis-generating information on the predictive and prognostic significance of CTCs in patients with lung cancer. In this regard, various approaches have been developed to detect CTCs in blood of cancer patients and to evaluate their correlation with the course of the disease. In this section, we will mainly focus on state-of-the-art translational studies, which have been devoted to unraveling the prognostic significance of CTCs in patients with lung cancer, with the ultimate aim to carry them to clinical application.

4.1 CTCs and early diagnosis of lung cancer

As mentioned above, thus far most studies performed on CTCs have been focused on the risk stratification and prognosis of patients with an established diagnosis of cancer. However, tumor cells may disseminate through the circulatory system years before a diagnosis is even made. Based on this notion, several studies have considered CTCs as the 'Achilles heel' of this cancer entity and asked whether sensitive detection methods may permit the early diagnosis of lung cancer.

In 2009, Tanaka et al. reported a first detailed study on the diagnostic significance of CTCs in patients with a suspicion or a diagnosis of primary lung cancer using the CellSearch system [102]. They detected CTCs in 17 of 88 (19.3%) stage I patients. According to their study, although the CTC counts were significantly higher in patients with lung cancer than in nonmalignant patients, its discriminatory capacity was insufficient in performance, with an area under each receiver operating characteristic curve (AUC-ROC) of 0.598 compared to the moderate performance of serum CEA (AUC-ROC, 0.747). This insufficient capacity was mainly attributable to a low sensitivity and a negative predictive value of CTC detection among patients. It is important to note that the CTC status was significantly competent in predicting tumor progression as well as in predicting the occurrence of impeding metastasis (AUC-ROC, 0.783), whereas the detection of serum CEA was insufficient. Therefore, the most important advantage of CTC evaluation in early stage lung cancer may be the prediction of micro-metastases, which are undetectable using conventional diagnostic methods.

In another study, Ilie et al. attempted to examine the presence of CTCs in chronic obstructive pulmonary disease (COPD) patients devoid of any clinically detectable malignancy. The purpose was to identify patients with COPD who were at a high risk for lung cancer [103]. COPD and lung cancer share a common pathophysiology, and it has been shown that the presence of COPD, even in its early stage, is a risk factor for NSCLC [104]. According to their study, CTCs were detected in 5 of 168 (3%) COPD patients. Furthermore, one to four years after the detection of CTCs, CT scan screening revealed lung nodules in these patients, which ultimately led to the histopathological diagnosis of early-stage lung cancer and subsequent surgical resection. Interestingly, follow-up of these patients by CT scan and ISET one year after surgical resection revealed no tumor recurrence. This result highlights the importance of early diagnosis in the efficacy of tumor management.

4.2 Enumeration of CTCs as a prognostic factor in SCLC and NSCLC

Allard et al. reported the first proof-of-principle on the possibility to enumerate CTCs in lung cancer patients using the CellSearch platform [21]. They used this platform to analyze 964 samples from different cancer patients, including 90 with lung cancer. Their results revealed that 36% of all samples and nearly 20% of samples from patients with metastatic lung cancer showed positive CTC counts at baseline. The baseline was defined as ≥2 CTCs in 7.5 ml blood. This study was a prelude to consider the incorporation of CTCs as biomarkers in clinical trials for lung cancer patients. However, they did not report on the prognostic significance of CTC positivity in the patients, nor specified whether these samples were obtained from patients with SCLC or NSCLC. Subsequent studies indicated that the median number of CTCs in blood of patients with SCLC is relatively high (28; range, 0–44,9) [105], while this number is smaller in NSCLC patients (1; range, 0–146) [106]. The abundance of CTCs in blood of patients with SCLC concurs with the rapid doubling time, the metastatic proficiency and the aggressive behavior of SCLC [107], and supports a rationale for studying CTCs independently in SCLC and NSCLC.

Amongst human solid tumors, SCLC is an exemplary cancer entity that initially is relatively sensitive to chemotherapy, but invariably relapses with a fatal chemo-resistant phenotype

Table 1 Methods for the detection/enrichment of CTCs

	Detection method	Enrichment method	CTC markers	Cancers	Ref
Immunocytological technologies	CellSearch system	Immunomagnetic beads	+EpCAM, CK8, CK18, K19, -CD45, +DAPI	colorectal cancer, lung cancer, breast cancer	[86, 141–143]
	CTC-chip	EpCAM-Abcoupled microposts	+EpCAM, -EpCAM CK8, CK18, CK19, -CD45, PSA, +DAPI	lung cancer and malignant pleural mesothelioma, prostate cancer, pancreatic cancer, breast cancer, colon cancer, NSCLC cancer	[144–147]
	Flow cytometry	Immunomagnetic enrichment	EpCAM, FRα, +CK, CD45	NSCLC cancer, epithelial cancers such as squamous cell carcinoma of the head and neck (SCCHN)	[90, 148]
	AdnaTest	Immunomagnetic beads	EpCAM, MUC-1 and HER2, DAPI, CD45	breast cancer, NSCLC cancer, metastatic colorectal cancer	[141, 143, 149]
	MagSweeper	Immunomagnetic beads	EpCAM, DAPI, CK, CD45	lung cancer	[150]
	FAST	No pre-enrichment	CK, CD45-, DAPI, HER2, ER, ERCC1	lung cancer (NSCLC), breast cancer	[88, 89, 151]
	Ariol® system	Immunomagnetic beads	+EpCAM, CK8, CK18, K19, -CD45, +DAPI, PD-L1/PD-1	lung cancer (NSCLC)	
	HB chip	Immuno-staining	EpCAM, CSPG4, MCAM, TYRP1, and α-SMA (platelet markers CD41, CD45, CD61)	lung cancer, breast cancer, melanoma	[101]
Molecular (RNA-DNA based) technologies	RT-qPCR	AdnaTest, negative enrichment	CK8, CK18, CK19, CK7, CK19, EGF, FN1, EpCAM, MUC1, mRNA,	lung cancer, breast cancer	[39, 99, 152]
	RT PCR	Immunomagnetic enrichment, negative enrichment	CK19, EGFR mRNA, CEA, CK7, EGFR, ELF3, EphB4, EpCAM, MUC1, ERBB2 -	breast cancer, gastric cancer, lung cancer (NSCLC)	[9, 39, 153–155]
	Droplet digital PCR	Immunomagnetic enrichment	CK19, CEA, CA19.9, KRAS, EGFR	colorectal cancer, lung cancer (NSCLC)	[156, 157]
	LT-PCR	Lysis of erythrocytes and Immunomagnetic depletion of leukocytes	FR, CEA, NSE, Cyfra21–1	lung cancer (NSCLC)	[97, 158]
	FISH and Immuno-FISH technology	Filtration enrichment, Size-based enrichment, Negative depletion (EasySep™), Density-based, Immunomagnetic beads	ALK, CK, vimentin E-cadherin, CD45, CK, DAPI, HER2, BRCA1	lung cancer, NSCLC, breast cancer, prostate cancer	[9, 100, 129, 159, 160]
Functional assays	EPISPOT-assay	Negative depletion, Density-based	HER2, EGFR, VEGF PSA, MUC1, CK, TG, CK19	non-metastatic prostate cancer (PCa), breast Cancer	[161, 162]
	Versatile label free biochip	Cell size deformability	CK, -CD45, DAPI, VEGF	lung cancer, prostate cancer	[163, 164]
Others	OHSV1-hTERT-GFP technique		Evaluation of hTERT and GFP expressions in EpCAM-positive or -negative CTCs	lung cancer, malignant tumor cells	[86, 165]
	Nanoelectromechanical (NELMEC) chip		–	breast cancer	[87]
	Cluster-Chip	A specialized bifurcating trap under low shear-stress conditions for isolation of CTC clusters	–	metastatic cancers of the breast, prostate and melanoma	[166]
	CTC-iChip	Deterministic lateral displacement, inertial focusing and magnetophoresis	-CD45, -CD66b	breast and pancreatic cancers	[167]
	Parsortix™	Size and deformability	–	prostate and breast cancer	[168]
	^{Hb}CTC-Chip	Turbulent flow	EpCAM	NSCLC	[123]
	A multi-flow microfluidic system	Phenomenal effect of size-dependent inertial migration	–	spiked cancer cells, NSCLC	[169]

[107]. Therefore, it is of utmost importance to develop new tools to monitor minimal residual disease in this malignancy before the establishment of macroscopic metastases. Although often considered as a neuroendocrine tumor, SCLC is an epithelial tumor expressing epithelial markers along with neural and endocrine markers [108]. The first studies using unvalidated manual technologies for the detection of epithelial markers in blood revealed CTCs in a small number of SCLC patients. The results, however, were not robust enough to prove the significance of CTCs in the studied patients [109, 110]. In 2009, Hou et al. published a first report on the use of the CellSearch system for the detection and enumeration of CTCs in 88 SCLC patients undergoing standard chemotherapy [105]. They also noted CD56 positivity in the CTCs, thereby confirming their neoplastic origin. They found that CTCs were detectable in 86% of the patients and their enumeration was found to serve as a strong prognostic marker for patient survival and for monitoring therapy response as determined by univariate analysis. In addition, they found that patients without detectable CTCs exhibited early stages, raising the question whether CTC enumeration is capable of stratifying patients according to chemoradiotherapy outcome. Importantly, the number of CTCs was found to decrease in all patients after one cycle of chemotherapy, suggesting that CTCs may serve as pharmacodynamic biomarkers for SCLC. Therefore, CTC detection may help to identify high risk SCLC patients prone to metastatic relapse.

In 2011, Krebs et al. published a first study reporting the prognostic significance of CTCs in patients with NSCLC using the CellSearch system [106]. Using blood samples obtained from 101 patients with previously untreated stage III and IV NSCLC, they found that CTCs were detectable in 21% of the patients with advanced NSCLC at baseline (≥ 2 CTCs in 7.5 ml blood). In addition, they found that the numbers of CTCs were higher in patients with stage IV NSCLC compared to stage IIIA and IIIB patients. They also assessed the prognostic capacity of CTC enumeration in NSCLC (cutoff ≥ 5 CTCs) and found that both PFS and OS were higher in patients with fewer than five CTCs compared to patients with five or more CTCs. Using multivariate analysis, they showed that CTC enumeration was the strongest predictor of OS. Interestingly, they found that the hazard ratio prominently increased with the incorporation of a second CTC enumeration following one cycle of chemotherapy [106]. More recently, Lindsay et al. explored the use of CTCs as prognostic markers in 125 patients with treatment-naive stage IIIb-IV NSCLC who were prospectively recruited for CellSearch analysis. In line with previous reports, this study validated a prognostic cutoff of ≥ 5 CTCs through OS analysis and confirmed the clinical utility of CTC enumeration as an independent and strong prognostic factor for patients with advanced NSCLC [111]. Although both of these studies are considered as early indications for a 5 CTC-cutoff in NSCLC, they are consistent with previous validated reports for breast, colorectal and prostate cancer, which led to FDA approval of the CellSearch system for the enumeration of CTCs in these cancers [22, 23, 112]. Although additional clinical studies have reported the prognostic significance of CTC enumeration in NSCLC using the CellSearch system, the numbers of patients in these studies were small and validation was lacking (Table 2).

5 Characterization of CTCs as potential biomarkers for therapy response and targeting of lung cancer

Molecular characterization of CTCs in peripheral blood can be employed for real-time cancer monitoring and patient evaluation [113]. As such, CTCs may have a great potential as biomarkers for prognosis assessment. CTCs may also serve as biomarkers for novel targeted therapies aimed at inhibiting metastatic recurrence and improving patient management [114]. Since CTCs have been found to be derived from clones of the primary tumor [16], they may reflect tumor burden at all stages of its development. Thus, in addition to playing a potential role in early diagnosis and prognosis, CTCs may also be used for the detection of genetic and immunophenotypic changes during tumor progression. As such, CTC characterization may be instrumental for the development of new targeted therapies [17, 18]. A particularly important attribute of blood sampling is that it is safe and can be performed repeatedly, whereas repeated invasive procedures, including bone marrow aspiration, may result in limited patient compliance. A limitation of using CTCs to monitor disease progression and therapy response may be their scarcity among blood cells. Recent findings have, however, shown that in vitro culturing of CTCs and/or the generation of cell-derived xenografts (CDXs) may help to overcome this limitation. Such strategies may result in sufficient starting material for CTC characterization via e.g. deep sequencing. Recent work has, for example, shown that CTCs from patients with either chemo-sensitive or chemo-refractory SCLC can form tumors in immune-compromised mice, and that deep sequencing revealed that somatic mutations are stably maintained between primary patient tumors and CDXs. Consequently, the resultant CDXs were found to reflect the donor patient's responses to etoposide and platinum and recapitulated the evolving drug sensitivities of the patients [115, 116].

Various studies have shown that molecular characterization of tumor cells may facilitate the specific targeting of oncogenic variations in patients with advanced NSCLC [117, 118]. But, since the sizes of tumors related to NSCLC are usually small, molecular screening may be a challenge, especially when carried out during early stages of the disease as well as during the course of treatment with e.g. tyrosine kinase inhibitors (TKIs). Maheswaran et al. noted an increase in the

Table 2 Reports on the CTC detection/enumeration in patients with lung cancer using the CellSearch and ISET method

Group (Ref.)	Primary tumor	Method	Size of cohort	Detection rate (%)	Sensitivity (%)	Specificity (%)	Results
Tanaka et al. [102]	SCLC/NSCLC	CellSearch	150	30.6%	71.0	83.0	Insufficient capability of the CTC test in the discrimination between lung cancer and nonmalignant diseases/ CTC count significantly increased along with tumor progression
Allard et al. [21]	Not specified	CellSearch	99	20%	–	–	No prognostic significance with the detection of CTCs.
Wang et al. [170]	SCLC	CellSearch	96	50.0	–	–	Serum neuron-specific enolase (NSE) was found to be associated with CTC thresholds/ no significant differences were observed for an association of any threshold CTC count with the treatment response
Normann et al. [171]	SCLC	CellSearch	60	90	–	–	CTCs have a useful prognostic role in extensive SCLC, but only the change of the CTC count after the first cycle of chemotherapy provides clinically relevant information
Hou et al. [172]	SCLC	CellSearch	88	86	–	–	
Krebs et al. [106]	NSCLC	CellSearch	101	21	26	100	CTCs are detectable in patients with stage IV NSCLC
Lindsay et al. [111]	NSCLC	CellSearch	125	40.8	–	–	The baseline presence of ≥5 total CTCs in advanced NSCLC confers a poor prognosis/CTCs from the EGFR-mutant NSCLC expresses EMT characteristics, not seen in the CTCs from patients with the KRAS-mutant adenocarcinoma
Hirose et al. [173]	NSCLC	CellSearch	33	36.4	–	–	The enumeration of CTCs could be a useful predictive factor for the effectiveness of cytotoxic chemotherapy in patients with metastatic NSCLC
Punnoose et al. [174]	NSCLC	CellSearch	41	78	–	–	The greater sensitivity for the mutation detection was observed in ctDNA rather than in the CTCs and the detected mutations were strongly concordant with the mutation status in the matched tumor/ the correlation between decreases in the CTC counts and radiographic response by either the FDG-PET or RECIST in patients with advanced NSCLC
Muinelo-Romay et al. [175]	NSCLC	CellSearch	43	41.9	–	–	The high levels of total CK positive events were associated with a poor prognosis in the group of patients with <5 CTCs. Regarding the therapy and monitoring, the patients presenting with increased levels of CTCs during the treatment demonstrated lower OS and PFS rates
Juan et al. [176]	NSCLC	CellSearch	37	24	–	–	CTCs ≥2 at the baseline were detected in only 24% in this group of patients with advanced NSCLC as well as a poor performance status/No significant differences were observed in the PFS and OS between the patients with or without CTCs at the baseline
Chudasam et al. [177]	NSCLC	CellSearch	23	80.6	–	–	The presence of CTCs analyzed by ScreenCell did not necessarily lead to a poorer prognosis in the patients with lung cancer after curative surgery
Hofman et al. [178]	NSCLC	CellSearch / ISET	210	69	–	–	The presence of CTCs detected by both the CellSearch and ISET correlated even better with a shorter DFS at a univariate and multivariate level/ the CellSearch and ISET are complementary methods for the detection of CTCs in preoperative radical surgery for NSCLC
Hofman et al. [179]	NSCLC	ISET	208	49	–	–	The presence and level of 50 or more circulating nonhematologic cells (CNHC) are associated with a worse survival in patients with resectable NSCLC
Isobe et al. [180]	NSCLC	CellSearch	24	33.3	–	–	The presence of CTCs was correlated with the positivity of the EGFR mutation in cfDNA

sensitivity of detecting EGFR mutations in CTCs using a CTC-chip [14]. Subsequently, they used a Scorpion amplification refractory mutation system (SARMS) to detect EGFR mutations in both circular tumor DNA (ctDNA) and CTCs, as well as for the detection of primary tumor sample mutations. From 27 NSCLC patients tested, 20 were found to harbor EGFR mutations in the tumor samples and in 19 of them (95%) CTCs with EGFR mutations were detected. Other studies were carried out to test various methods such as real time-PCR and melting curve analysis (sensitivity 100%) [119], next-generation sequencing (sensitivity 84%) [120] and SARMS (sensitivity 50%) [121]. Detecting EGFR mutations in CTCs may improve the prognosis of NSCLC patients, whereas longitudinal molecular screening of CTCs gives may lead to the detection of new mutations underlying e.g. therapy resistance. Maheswaran et al. were, for example, able to recognize an exon 20 T790M EGFR hot spot mutation in 64% of the NSCLC patients who suffered from relapse after EGFR TKI treatment [14]. Yafang Liu et al. conducted a comprehensive meta-analysis and demonstrated that EGFR mutations can be detected in CTCs with a high specificity (99%) and sensitivity (91%), and an additional AUC-ROC of 0.99 illustrated a high diagnostic performance of CTCs for the detection of EGFR mutations [122]. Sundaresan et al. found that, while CTC- and ctDNA-based genotyping failed to detect the T790M EGFR hot spot mutation in 30% and 20% of the patients harboring this mutation in their tumor, the combined use of these two assays enabled its detection in all patients from whom a blood sample was available [123]. Moreover, using these two assays, they detected the T790M mutation in 35% of the patients in whom the concurrent biopsy was negative or indeterminate [123]. Therefore, complementary CTC and ctDNA analyses [124] may result in a complete assessment of each patient's cancer and, thus, may reliably predict their responses to T790M-targeted inhibitors.

Several studies have demonstrated that also ALK and ROS1 rearrangements may serve as actionable genetic alterations that can be detected in CTCs from NSCLC patients. Ilie et al. showed, for instance, that FISH and immunohistochemistry (IHC) could reliably be used for screening of CTCs (isolated by ISET test) in 87 NSCLC patients [125]. By doing so, they showed that 6% of the patients exhibited ALK rearrangements in paired samples of CTCs and tumor biopsies, while both the CTCs and tumor samples from the remaining 82 patients were lacking ALK rearrangements. Thereby, they illustrated an excellent concordance between CTCs and tumor samples for the presence of ALK mutations [125]. Pailler et al. developed a semi-automated microscopy approach that was able to merge phenotypic and cytomorphological information with filter-adapted FISH for the detection of filtration-enriched CTCs (isolation by size of epithelial tumor cells, ISET). Through this approach they were able to detect ALK-rearranged CTCs in 82% of the patients with ALK-rearranged NSCLC [126]. Similarly, filter-adapted FISH can be used for the detection of ROS1 rearrangements in CTCs [127]. Additional molecular abnormalities related to NSCLC that can be screened in CTCs are KRAS and BRAF mutations [113, 128]. Recently, the significance of CTCs with an aberrant ALK-FISH pattern (ALK-rearrangement or ALK-copy number gain (ALK-CNG)) has been evaluated using filter-adapted FISH (FA-FISH) in NSCLC patients treated with the ALK inhibitor crizotinib [31]. They found that decreases in CTC numbers with ALK-CNG upon crizotinib treatment were significantly associated with a longer PFS [31]. Therefore, ALK-CNG may serve as a predictive biomarker for crizotinib and may be used for real-time patient monitoring and clinical outcome prediction. It has also been found that ALK-rearranged CTCs may express the mesenchymal markers vimentin and N-cadherin, suggesting that ALK rearrangements and mesenchymal phenotypes may arise from clonal selection of tumor cells with a metastatic CTC proficiency [129].

Various studies have indicated that PD-L1 expression may increase during radiotherapy and/or chemotherapy, providing a rationale for combining PD-1/PD-L1 inhibitors with chemotherapy and/or radiotherapy [130]. Given the fact that obtaining serial biopsies from lung cancer patients is difficult, evaluation of PD-L1 expression in CTCs may allow real-time monitoring of immune activation in lung tumors. Several studies have indicated that a high PD-L1 expression in patients with early stage NSCLC [131], or before/during chemotherapy [132] and radiotherapy [133], is associated with a poor prognosis. In addition, it has been shown that abundant CTCs with a high PD-L1expression facilitate selection of patients for PD-1/PD-L1 blockade therapy [134]. It has also been reported that CTCs with a high PD-L1 expression may be characterized by a partial EMT phenotype, which suggests that co-expression of PD-L1 and EMT markers may help CTCs to escape from immunity [135].

Large-scale screening of multiple abnormalities may allow their simultaneous detection in CTCs and, thus, enable the development of new targeted therapies also directed against NSCLC. Park et al., for example, conducted a high-throughput multiplexed approach for single-cell mutation profiling of CTCs from 35 stage IV lung adenocarcinomas. They also evaluated the expression and mutation status of four genes in single CTCs and reported an increased sensitivity [136]. Yoo et al. performed a large scale molecular screening of 381 cancer-associated genes in single CTCs from 13 NSCLC patients, and subsequently confirmed the results using droplet digital PCR. Point mutations, primarily found in the EGFR and TP53 genes, were detected in 62% of the blood samples and 85% of the tumor samples. Furthermore, they found several mutations in the CTCs that were not detected in the related tumor samples, suggesting that the genetic profiles of CTCs may differ from those of tumor samples. This

observation indicates a parallel progression of tumor cells in the blood [137]. In another study, Jiang et al. used cerebrospinal fluid CTCs (CSF CTCs) as a potential "liquid biopsy" approach for the detection of leptomeningeal metastases (LM) in advanced NSCLC [138]. They utilized a next-generation sequencing (NGS) panel to screen 416 cancer-associated genes on CSF CTCs from LMs. By doing so, they were able to show that the molecular alterations detected in CSF CTCs were 89.5% (17/19) similar to the genetic profiles identified in the primary tumors [138]. Carter et al. assessed copy number alterations (CNAs) in CTCs from two pretreatment SCLC cohorts (13 patients as a training set and 18 patients as a validation set) with whole-genome amplification (WGA) to identify genetic alterations that occur differently in chemo-sensitive and chemo-refractory diseases. They were able to distinguish 83% of the tumor samples as chemo-refractory versus chemo-sensitive according to the CNA profiles [139]. Ultimately, whole transcriptome analyses (WTA) of CTCs in lung cancers has been found to allow the assessment of global gene expression profiles and to enable the identification of main regulators of lung cancer cells. In a study of 42 NSCLC patients with EpCAM-positive CTCs, for example, WTA indicated that the most extremely dysregulated genes in CTCs were linked to cell adhesion, cell-cell communication and cell movement. According to their observations, NOTCH1 was identified as a potential prognostic biomarker for CTCs. Additionally, the authors suggested that NOTCH1 may have the potential to be used as a molecular target for the prevention of lung cancer metastasis [140].

6 Conclusions and perspectives

During the last decade, CTC detection and enumeration approaches have been explored for the early identification of micro-metastases and their development into overt metastases. These efforts have resulted in FDA-approval of the CellSearch system for the prognosis of breast, colorectal and prostate cancer. In addition, it has been found that CTCs may serve as pharmacodynamic biomarkers that may fulfill the criteria for appropriate surrogate response biomarkers. Additional research is needed to define the significance of CTCs in other human cancers, including lung cancers, which are not easily accessible for sampling. The CellSearch system, although valuable, suffers from a relatively low sensitivity and an inability to detect EpCAM-negative CTCs, which may provide valuable prognostic information. Therefore, other reliable methods are needed to enhance the accuracy and sensitivity of CTC detection/enumeration. Taking advantage of the high number of CTC markers available, a multi-gene panel may allow the development of more efficient CTC-chips for the detection of CTCs. Alterations in the expression of CTC markers during the process of EMT may represent another challenge in the detection of CTCs, especially since most metastasis-forming CTCs undergo EMT and, thus, acquire more invasive phenotypic profiles. This challenge should be addressed when developing new CTC-detection methods. As such, the development of marker-independent strategies may hold promise. Taken together, we conclude that additional knowledge on the biology of CTCs and the development of new technologies for the detection/isolation of CTCs with clinical applicability will be instrumental for improving the prognosis of human cancer patients, especially lung cancer patients. In addition, we conclude that novel anomalous/dysregulated markers in CTCs may be useful to better reflect the tumor burden, to allow an improved clinical management of the disease and to develop personalized therapeutic strategies.

Acknowledgements This study was supported by the Hematology, Oncology and Stem Cell Transplantation Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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