

# Circulating tumor cells versus circulating tumor DNA in lung cancer – which one will win?

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**Abstract:** Liquid biopsies appear to be a reliable alternative to conventional biopsies that can provide both precise molecular data useful for improving the clinical management of lung cancer patients as well as a less invasive way of monitoring tumor behavior. These advances are supported by important biotechnological developments in the fields of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). Analysis of CTCs and ctDNA may be useful in treatment selection, for response monitoring, and in studying resistance mechanisms. This review focuses on the most recent technological achievements and the most relevant clinical applications for lung cancer patients in the CTC and ctDNA fields, highlighting those that are already (or are close to) being implemented in daily clinical practice.

**Keywords:** Lung cancer, circulating tumor cell (CTC); circulating tumor DNA (ctDNA); biomarker; liquid biopsy

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## Introduction

Lung cancer is the leading cause of cancer-related mortality among men and women worldwide, with more than 1.8 million estimated new cases each year (1). Despite advances in biomedical research and improvements in both the diagnostic tools and therapeutic options that have become available over the past few decades, lung cancer still has a 5-year overall survival (OS) rate of 18% for all stages (2). The main reason for this poor outcome for this cancer type is late diagnosis: a high percentage of the patients are diagnosed at advanced stages when curative surgery is no longer possible. The clinical management of lung cancer in advanced stages is also changing; better understanding and descriptions of the molecular abnormalities present in lung

cancer have opened up new therapeutic options in specific disease subsets.

### *Lung cancer: driver alterations, predictive biomarkers, and intratumor heterogeneity*

The development of a new generation of molecular techniques has led to substantial advances in the knowledge of cancer genomes, and specifically in lung cancer, facilitating the discovery of oncogenic-driver mutations/alterations that cause aberrant signaling and proliferation in certain tumor subsets. These findings have allowed the development of new treatment strategies based on molecular targets and thus have reshaped tumor classification from classical histological

methods towards molecular pathology approaches. Indeed, non-small cell lung cancer (NSCLC) is one of the most genomically diverse tumor types and there is a wide variety of molecularly-defined patient subsets characterized by specific driver-mutations, involving different genes such as epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), or V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) (3-7) among others.

The identification of driver mutations located in the tyrosine kinase domain of EGFR as the primary oncogenic event in a subset of lung adenocarcinomas (8-13) led to the development of specific tyrosine-kinase inhibitors (TKIs) for this receptor, resulting in a dramatic change in the treatment of advanced NSCLC patients harboring EGFR mutations. The use of EGFR-TKIs has produced a particularly large increase in progression-free survival (PFS) with a negligible toxicity profile as well as an increase in OS to more than 24 months (11,14-18). Unfortunately, the effect of TKIs is limited over time because of the emergence of drug resistance. A number of molecular mechanisms underlying acquired resistance to EGFR-TKIs have been reported, including the secondary EGFR Thr790Met (T790M) mutation found in around 50% of patients, loss of the EGFR mutant allele, MET amplification, hepatocyte growth factor (HGF) overexpression, phosphatase and tensin homolog (PTEN) downregulation, epithelial-mesenchymal transition (EMT), BRAF mutations, and other mechanisms (19-22). Resistance is frequently related to the emergence of a clone thought to be initially present at a low percentage in the tumor, thus emphasizing the role of intratumour heterogeneity as one way of explaining resistance mechanisms to targeted agents (23,24).

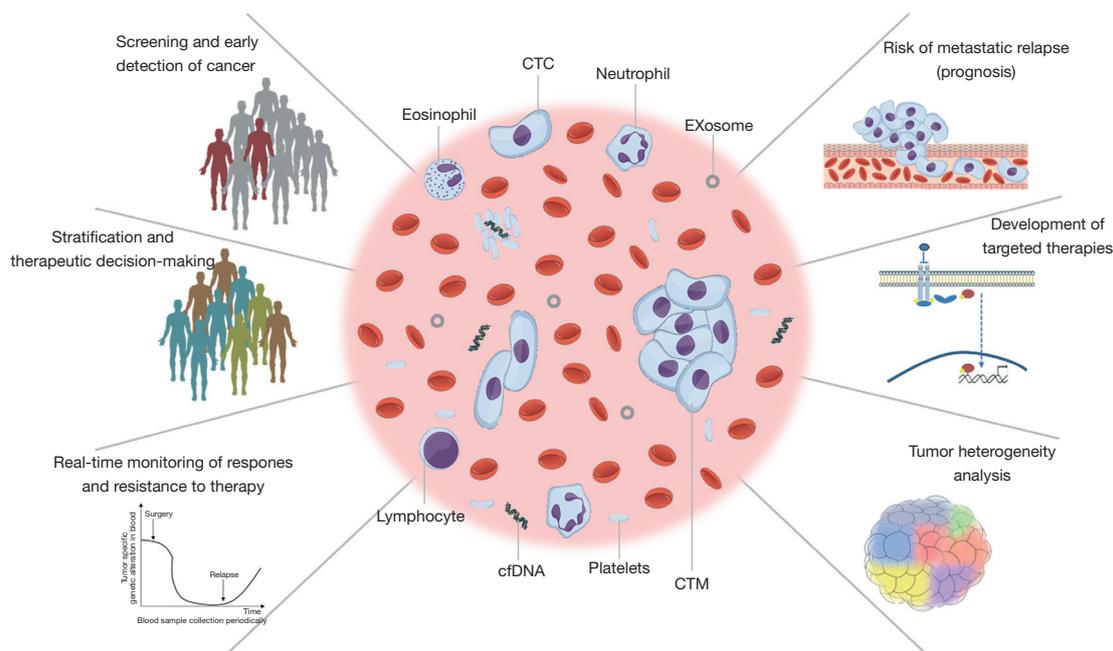
Other driver mutations in lung cancer have also been targeted by these new drugs but, again, the emergence of resistance is a common event in patients treated with targeted-therapies (25,26). In addition, metastases are responsible for roughly 90% of cancer-associated patient mortality, through largely unknown mechanisms. Therefore, future studies must aim to directly analyze metastatic cells in order to better understand the mechanisms of cancer spread (27). However, metastasis biopsy is an invasive procedure limited to certain locations, and additionally, recent work has shown that different metastatic sites harbor different genomic aberrations and so biopsy of only one or two accessible metastases may not be enough to represent the whole tumor genome (24,28). Finally, solid tumors also exhibit temporal heterogeneity, evolving over time under selection-pressure with treatment (29-32). Even though,

and considering the heterogeneity of the tumors, it becomes difficult to have a complete scenario of the whole tumor based on the information obtained from small biopsies, and in several cases from a restringed number of tumor cells which, in turn, could lead to erroneous therapeutic decisions.

Since the beginning of the era of targeted therapies, there has been a clear need to understand the mechanisms of resistance and therefore rebiopsies at the time of clinical progression or the emergence of treatment resistance were gradually incorporated into clinical practice. Considering all the above-mentioned facts about lung cancer: presence of driver mutations, tumor heterogeneity, tumor dynamics, drug sensitivity, drug resistance, it is of remarkable importance the development of a non-invasive way to obtain this valuable biologic information, which includes the ability to diagnose, prognose and monitor lung cancer evolution. At this point, liquid biopsies seem to be the approach that covers all these requirements. In lung cancer, blood samples are the most explored type of liquid biopsy, and have been used to improve the diagnosis as well as for the searching prognostic and predictive biomarkers. Liquid biopsies have several advantages: (I) they allow rapid biomarker assessment in lung cancer patients for whom solid biopsies are impossible because of restricted or extremely risky access possibilities; (II) they can easily be repeated during cancer patient follow-up in order to control treatment efficiency; and/or (III) they can be used to detect genomic alterations occurring as result of resistance to therapy.

### *Liquid biopsies in lung cancer*

In lung cancer liquid biopsies, blood samples are mainly used as a sample source for analyzing circulating tumor cells (CTCs) or circulating tumor DNA (ctDNA), in addition to other biomarkers of interest, such as circulating microRNAs, circulating RNA, platelets, plasma/serum metabolites, or exosomes (*Figure 1*). To explore the data available regarding the above in this review we used MEDLINE to perform a comprehensive literature search for original and review articles related to the terms “liquid biopsy” and “CTCs”, “ctDNA” and “NSCLC”, or “lung cancer”. Furthermore, to complete our search we also reviewed the bibliographies of these articles; only those published in English and in peer-reviewed journals were included. Our purpose was to describe the current contribution of CTC and ctDNA detection and analysis



**Figure 1** The potential clinical benefits of CTC and ctDNA analyses in cancer care. CTC, circulating tumor cell; cfDNA, circulating free DNA; CTM, circulating tumor microemboli.

in lung cancer patients and to compare the advantages and disadvantages of these two approaches.

### CTCs versus ctDNA: which one will win?

There are an increasing number of scientific reports pointing out the advantages of, and difficulties in, both detecting mutations and isolating CTCs and ctDNA in both metastatic and non-metastatic lung cancer (*Table 1*). The discrepancies in sensitivity, reproducibility, and concordance with tissue biopsies are likely due to the different approaches and methodologies used as well as their clinical settings.

### CTCs

CTCs are tumor cells from solid tumors that spread via blood and/or lymphatic vessels. CTCs are shed into the vasculature from primary tumors and are postulated to contain subpopulations of cells with the potential to spread and initiate distant metastases (33). They were observed for the first time by Thomas Ashworth in 1869 in the blood of a man with metastatic cancer (34), but they only became relevant in modern cancer research a couple of decades ago with the demonstration of their presence early in the course of malignant disease (35). Several models have been

suggested to describe the dissemination process whereby tumor cells leave the primary tumor to colonize distant organs, either when they become competent to metastasize or because of physical tumor extension (27,36).

CTCs can circulate in the bloodstream of lung cancer patients as single cells or as aggregates known as circulating tumor microemboli (CTM) (37-39). In this regard, the phenotype of single or aggregated CTCs can be different and so may present different levels of potential aggressiveness (37,38,40,41). Similar to single migratory mesenchymal-like CTCs, CTMs appear to be enriched in mesenchymal markers, an indicator of increased potential plasticity, which in turn seems to be related to more aggressive behavior, thus supporting their role in both tumor cell dissemination and the initiation of metastatic outgrowth (38,42-44). The presence of CTMs has been reported as a negative prognostic factor in lung cancer patients (38,40,45).

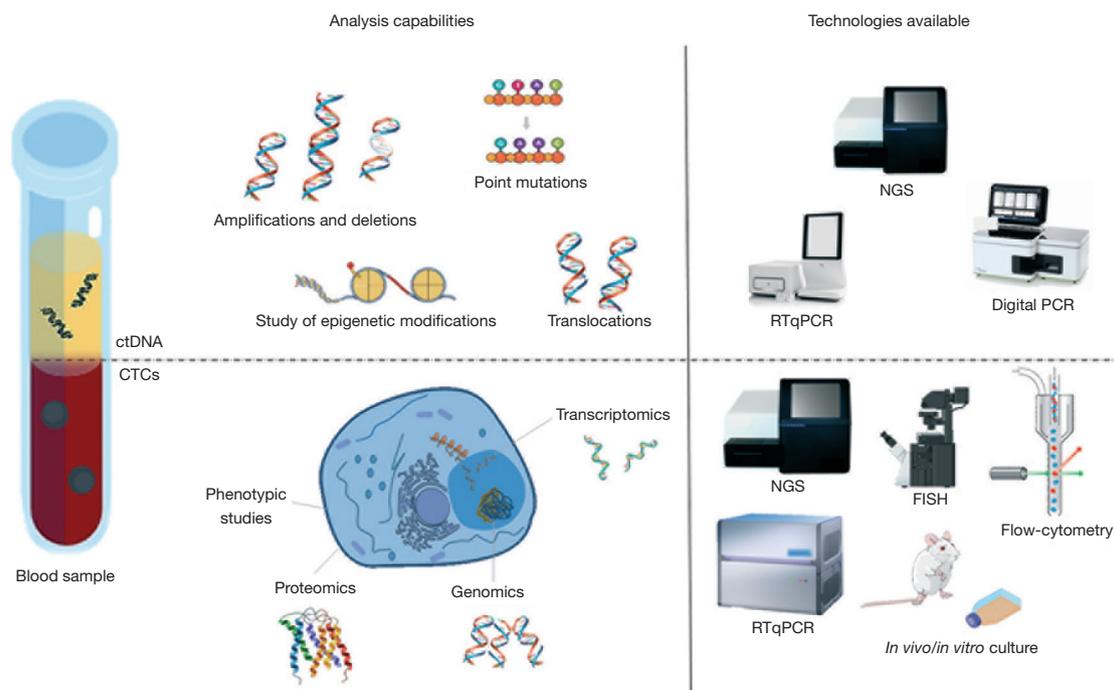
### Isolation and detection of CTCs

Although many technologies have been developed over the past few years to detect and isolate CTCs in the peripheral blood of NSCLC patients (44,46-48), this task remains challenging (*Figure 2*). In advanced lung cancer patients,

**Table 1** Advantages and limitations of CTCs and ctDNA liquid biopsy assays

	Advantages	Limitations
CTCs	<p>Constitute a minimally-invasive procedure with high specificity</p> <p>Address intra-tumor heterogeneity and supplies an adequate reflection of the tumor</p> <p>Allow structural evaluation of cancer phenotype</p> <p>Give information about tumor progression and metastasis</p> <p>Permit different <i>in vitro</i> and <i>in vivo</i> assays</p> <p>Make molecular characterization of the disease possible</p> <p>Offer the use of immunolabeling techniques</p> <p>Provide complementary information to ctDNA</p> <p>Could facilitate therapeutic decision-making</p>	<p>Prospective collection is required for targeting pre-analytical differences</p> <p>Constitutes a small and fragile population</p> <p>Analytical methods with very high sensitivity and specificity are needed</p> <p>False-negative and false-positive errors</p> <p>Low signal-to-noise ratio, mostly in early-stage disease</p> <p>Heterogeneity of CTCs complicates identification</p> <p>Disparity of techniques used for CTC isolation</p> <p>—</p> <p>—</p>
ctDNA	<p>Constitutes a minimally-invasive procedure with high specificity</p> <p>Addresses intra-tumor heterogeneity and supplies an adequate reflection of the tumor</p> <p>Extremely high sensitivity for detection of cancer burden even after curative care</p> <p>Might predict acquired treatment resistance</p> <p>Could facilitate therapeutic decision-making</p>	<p>Prospective collection is required for targeting pre-analytical differences</p> <p>False-negative and false-positive errors</p> <p>Low signal-to-noise ratio, mostly in early-stage disease</p> <p>Lack of standardization of pre-analytical conditions</p> <p>No protein or functional studies available</p>

CTCs, circulating tumor cells; ctDNA, circulating tumor DNA.



**Figure 2** Comparison of the analysis capability and the technologies available for CTCs and ctDNA. CTC, circulating tumor cell; ctDNA, circulating free DNA; NGS, next generation sequencing; RTqPCR, real-time quantitative PCR; FISH, fluorescence in situ hybridization.

CTCs are rare cells present in very low concentrations in the bloodstream; approximately 1 mL of whole peripheral blood contains 1–10 CTCs against a background of  $10^6$ – $10^7$  nucleated blood cells and around  $10^9$  red blood cells (49). Therefore, to reach the extreme sensitivity required to detect CTCs, an enrichment step is often required to increase their concentration before trying to detect or capture them. In this context, different methods for cellular enrichment, characterization, and identification of lung CTCs have been developed, including CTC microchips, filtration devices, quantitative reverse-transcription PCR assays, automated microscopy systems, etc. (46,50,51).

CTC detection or capture methods can be broadly categorized as: (I) label-dependent, based on positive enrichment involving cell surface markers such as epithelial cell adhesion molecule (EpCAM); or (II) label-independent, based on negative selection, such as size, or other differential biophysical properties of CTCs. Besides these two main approaches, other strategies include direct CTC imaging and functional assays (46,47,52–54). The first label-dependent studies tried to detect lung CTCs based on the assumption that circulating tumoral cells maintain the same characteristics as their tissue of origin, therefore most lung CTC categorization was based on the expression of epithelial-specific markers such as cytokeratin (CK) or intermediate filaments (IF) (41,55–60). Therefore, positive enrichment methods define lung CTCs as nucleated cells present in the bloodstream that express epithelial CKs and EpCAM and do not express the white blood cell surface antigen CD45 (59,61,62).

One of these methodologies is the CellSearch<sup>®</sup> system (Veridex, Raritan, NJ, USA) which has been approved by the Food and Drug Administration (FDA) for monitoring metastatic breast cancer (63), castration-resistant prostate cancer (64), and colon cancer patients (65). It has also been shown to be of prognostic significance for small cell lung cancer (SCLC) but the test has not yet been FDA-approved for this cancer type (40,66,67). The method is based on an initial enrichment of EpCAM positive cells followed by immunofluorescent staining using epithelial markers (CK 8, 18, 19), a leucocyte marker (CD45), and 4', 6-diamidino-2-phenylindole (DAPI) for nuclear staining. The CellSearch<sup>®</sup> definition of CTCs is any intact EpCAM+/CK+/CD45–cell at least 4  $\mu$ m in size and with a nucleus occupying at least 50% of the cytoplasm (41,55–61). Using EpCAM-dependent assays, CTCs can be detected in approximately 20–40% of NSCLC patients (41,55–58,60). Unfortunately, technologies relying on EpCAM positive selection cannot

detect CTCs that have undergone EMT or any cancer stem cells that have not yet started epithelial differentiation. In lung cancer, CK-negative CTCs, which potentially represent tumor cells undergoing EMT, have also been identified. Consequently, the use of EpCAM as a positive selection marker should be carefully considered when trying to detect CTCs in NSCLC patients. Unfortunately, so far no reliable surface markers, which distinguish lung CTCs from normal epithelial cells and can be used for such label-dependent approaches, have yet been identified.

Label-independent approaches to CTC detection in lung cancer are an attractive alternative. One such method, Isolation by Size of Epithelial Tumor cell (ISET<sup>®</sup>, developed by Rarecells Company, France), used for cells isolated from lung cancer patients, involves a CTC enrichment step based on size by using a filtration device followed by cytological characterization. This system has been used to detect lung CTCs in both metastatic and non-metastatic NSCLC patients and has shown increased sensitivity in a wider range of patients compared to label-dependent methods such as CellSearch<sup>®</sup> (41,55,68–75).

Another direct technology, known as ScreenCell<sup>®</sup>, is also based on the size of CTCs but, in addition, allows their isolation so that they can be subjected to further morphological and molecular studies (76,77). Interestingly, it has been shown that lung CTCs isolated with different systems, can be cultured *in vitro* which is of particular interest for generating *in vitro* and *in vivo* models. As a first step towards this goal, data has already been generated for successful short-term cultures (up to 28 days) of CTCs isolated from patients with lung cancers (78–80). Such model systems could be used to study drug susceptibility or to generate sufficient numbers of cells for systematic deep analysis of their molecular profiles or biological behavior (52,81). Several recent studies have reported the development of mouse xenografts generated directly from CTCs or from breast, colorectal, prostate, hepatocellular, small cell lung, or gastric cancer CTC cultures (82–87). In particular, CTCs enriched from blood samples from SCLC patients were subcutaneously implanted into immunocompromised mice as CTC-derived explants (CDX); the CTCs were tumorigenic at densities of more than 53 CTCs/1 mL of blood, however, such large numbers of CTCs are not always obtained from advanced patients, thus highlighting one of the biggest challenges associated with these approaches (86).

Current models generated either *in vitro* or *in vivo* are also potentially limited if the clones they are grown from

do not accurately reflect the true heterogeneity of the tumor (e.g., there may be a selective advantage for highly aggressive clones). Furthermore, *in vivo* xenograft models do not recapitulate tumor-host interactions that may play a role in drug resistance. Direct comparison between label-dependent and label-independent CTC isolation methods shows that both approaches have pros and cons. Label-dependent methods are more specific but they are rendered ineffective when antigen expression is lost in certain CTC subpopulations, and the cells become less viable after isolation. On the other hand, label-independent approaches are less specific but do not depend on CTC phenotype, and seem to better preserve CTC viability for downstream applications. There are currently many other technological developments focused on exhaustive lung CTC characterization in the pipeline at several diagnostic companies.

### **CTCs: clinical applications**

CTC analysis is considered an interesting approach for early diagnosis, prognosis assessment, prediction of treatment efficacy, and early detection of lung cancer relapse. The most relevant lung cancer CTC studies are summarized in *Table 2*.

Larger numbers of CTCs are recovered from SCLC patients than from NSCLC patients (40,56). In addition, some researches have reported a positive association between the number of CTCs and clinical stage or the presence of distant metastases in primary lung cancer (56,57,73,88,93), whereas other studies failed to find any significant differences (50,92). Regarding other clinical applications, the majority of articles on CTCs in lung cancer focus on their prognostic role. In a large population of NSCLC patients, one group showed that isolation of more than 50 CTCs per 10 mL sample (using ISET<sup>®</sup>) is of prognostic value and is associated with shorter OS and disease-free survival (DFS) (89). Similar results were reported by Krebs *et al.* showing that PFS and OS was significantly better in advanced NSCLC patients with fewer CTCs (41). However, in another NSCLC patient cohort it was reported that although the median survival time tends to be shorter in CTC-positive than in CTC-negative patients, the difference was not significant (92).

In SCLC a significant association between higher numbers of CTCs and shorter survival has been described, and at least one study has reported that CTCs are a better predictor of survival than disease stage and tumor response

(40,66,90). Moreover, a reduction in the number of CTCs after chemotherapy was also significantly associated with better outcomes in SCLC (66,94). Regarding the role of CTCs as biomarkers for therapeutic monitoring in NSCLC, comparisons between studies performed on samples collected before and after chemotherapy have consistently found that survival rates were significantly worse for patients with CTC counts that remained positive during treatment (95,96). In a group of patients treated with erlotinib and pertuzumab, the authors found that the decrease in CTC count upon treatment were significantly associated with disease response (91).

One of the main difficulties of working with CTCs in the field of lung cancer is their use as a theranostic tool for detecting somatic mutations (97). However, in 2008 Maheswaran and Sequist identified the presence of EGFR-activating mutations in 11 out of 12 (92%) of CTCs isolated from EGFR-mutated patients. During follow-up the authors detected the T790M mutation (which confers drug resistance) in CTCs collected from patients who progressed to TKI treatment (98). Moreover, *EGFR* mutations in CTCs from NSCLC patients were recently successfully assessed using sensitive next generation sequencing (NGS) (59). Similarly, in 2012 Paul Hofman's group published results from ALK-specific fluorescence in situ hybridization (FISH) analyses performed on archived lung cancer patient CTC samples. Their blind analysis of CTCs and corresponding tumor tissue showed a perfect match: 5 patients positive for ALK rearrangement in both CTC and tumor tissue were found while 82 were negative for this mutation in both CTC and tumor tissue (99).

In summary, there are currently 343 studies registered on "ClinicalTrials.gov" concerning CTCs and lung cancer, but despite the numerous scientific publications on this topic, these cells are still not used in routine clinical practice. This can be explained by the large number of methods available for CTC detection, and by the difficulty of selecting a reliable lung CTC marker. Despite the efforts made by the scientific community in the CTC field to try to improve lung cancer management, the analytical specificity and clinical utility of these methods must still be demonstrated in large prospective multicenter studies in order to obtain the evidence required for their introduction into the daily management of lung cancer patients.

### **ctDNA**

The field of ctDNA analysis originally started almost 70 years

Table 2 Diagnostic and prognostic impact of CTC analysis in lung cancer

Study	Patients included	Histology	Stage	Technology	Sensitivity (%)	Diagnostic and/or prognostic significance	Refs.
Stenel et al. [2003]	62	NSCLC	I-IIIa	Ficoll-Hypaque	18	Disseminated cancer cells in the PV blood are detectable in patients with operable NSCLC and are associated with a poor clinical outcome	(88)
Tanaka et al. [2009]	125	NSCLC	I-IV	CellSearch	30-71	CTC count is a useful surrogate prognostic marker, but insufficient for discriminating malignant and non-malignant patients	(56)
Hou et al. [2009]	97	SCLC	IIB-IV	ISET/CellSearch	85	M30, M65, lactate dehydrogenase, and CTC number are prognostic for patient survival. Detection of CTM in 32% of patients	(39)
Hofman et al. [2011]	208	NSCLC	I-III	ISET	49	The presence and level of 50 or more CNHCs are associated with worse survival	(89)
Hofman et al. [2011]	210	NSCLC	I-IV	ISET/CellSearch	39-69	The presence of CTCs detected by CellSearch and ISET shows the best correlation with shorter DFS at a univariate and multivariate level	(55)
Krebs et al. [2011]	101	NSCLC	III-IV	CellSearch	21	PFS and OS are significantly higher for patients with $\leq 5$ CTCs before chemotherapy	(41)
Hiltermann et al. [2012]	59	SCLC	I-IV	CellSearch	73	Absolute CTCs after one cycle of chemotherapy in patients with SCLC is the strongest predictor for response on chemotherapy and survival	(66)
Hou et al. [2012]	97	SCLC	I-IV	ISET/CellSearch	85	Pretreatment CTCs, change in CTC number after one cycle of chemotherapy, CTM, and apoptotic CTCs are independent prognostic factors	(40)
Naito et al. [2012]	51	SCLC	I-IV	CellSearch	68	Patients with $\geq 8$ CTCs had worse survival than those with $< 8$ CTCs at baseline, posttreatment, or relapse	(90)
Hofman et al. [2012]	250	NSCLC	I-IV	ISET/Cytology	49	49% of patients showed CNHCs, corresponding to malignant (41%), uncertain malignant (6%), and benign cells (2%)	(71)
Punnoose et al. [2012]	41	NSCLC	III-IV	CellSearch/RT-PCR	78	There is a correlation between decreases in CTC counts and response to radiography by either FDG-PET or RECIST in advanced NSCLC patients	(91)
Paillet et al. [2013]	18	NSCLC	IIB-IV	CellSearch/ISET/RT-PCR	100	ALK rearrangement can be detected in the CTCs of patients with ALK-positive NSCLC, enabling both diagnostic testing and monitoring of crizotinib treatment	(72)
Funaki et al. [2013]	130	NSCLC	I-IV	Rosette Sep	74	DFS rate was significantly worse in patients where CTMs were detected	(45)
Juan et al. [2014]	37	NSCLC	IIB-IV	CellSearch	24	No significant differences in PFS and OS were observed between patients with or without CTCs at baseline	(92)
Muñelo-Romay et al. [2014]	43	NSCLC	IIB-IV	CellSearch	42	Patients with $\geq 5$ CTCs at baseline presented worse PFS and OS. In addition, patients with increased levels of CTCs during the treatment showed lower OS and PFS rates	(61)
Carlsson et al. [2014]	129	NSCLC	I-IV	HD-CTC assay	50	The presence of CTMs with clinical and imaging data significantly discriminates diagnostic accuracy in NSCLC patients	(37)
Chudasama et al. [2015]	20	NSCLC	III-IV	Screen Cell	25	An increase in CTCs following EC is observed in 75% of patients, which may have implications for tumor dissemination	(77)

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; CTCs, circulating tumor cells; CTM, circulating tumor microemboli; DFS, disease-free survival; PFS, progression-free survival; OS, overall survival; CNHCs, circulating non-hematological cells; PV, pulmonary vein; CK, cytokeratin; EC, endobronchial cryotherapy; NS, non-specific.

ago (100); higher levels of circulating free DNA (cfDNA) were identified in cancer patients compared to healthy controls, suggesting that this correlated with malignancy and tumor stage (101-103). To date, two main mechanisms for releasing ctDNA have been postulated: “passive” and “active”. The passive mechanism involves the release of nucleic acids into the bloodstream, either directly from apoptotic and necrotic tumor cells or indirectly by necrotic tumor cells engulfed by macrophages (104). Data about the size distribution of cfDNA has revealed an enrichment in 150–180 bp fragments which reflects the nucleosomal pattern of DNA fragmentation characteristic of apoptotic processes (105-107). In contrast, fragments of tumoral nucleic acid can also be actively released into the circulation by living cells. One potential explanation hypothesizes that cancer cells release nucleic acids to transform the targeted recipient cells at distant locations, although the mechanisms are not completely understood (108). It is important to consider that ctDNA may represent a small proportion of the total cfDNA, at levels corresponding to one genome equivalent in 5 mL of plasma (0.01% allele fraction), and thus it may be undetectable with routine sampling (103,109). Apart from this, ctDNA levels can vary according to tumor burden and stage, anatomical proximity to vasculature, and biological features like apoptotic rate and metastatic potential (110,111).

### *Detection and quantification of ctDNA*

The most common sample source used for ctDNA isolation is plasma collected in standard EDTA tubes. However, considering the low percentage of ctDNA present within total isolated cfDNA, it is important to control both the analytical and pre-analytical steps that can significantly affect ctDNA detection in blood samples (112). Plasma samples should be processed and stored immediately after blood collection to avoid increases in genomic DNA released from white blood cell lysis that could modify the relative levels of ctDNA. Therefore, the uses of standardized protocols in conjunction with specialized preservative-containing tubes (e.g., Streck Cell-Free DNA BCT) are strongly recommended (113).

The amounts of ctDNA present in lung cancer patient samples give important diagnostic and prognostic information about the disease (114,115). However, the most important advantage of this technology is that it enables such analyses via easily obtained, minimally-invasive samples which are likely to reflect any genomic abnormalities present in the original neoplasm, giving

insights into the types of mutations, indels, chromosomal rearrangements, chromosomal region gains or losses, and epigenetic modifications present (54,116-118). Given the small proportion of ctDNA present in the total cfDNA samples obtained, it is important to select the correct methods for its isolation and analysis; several highly sensitive techniques have been developed for the latter, ranging from PCR-based to more complex methodologies using NGS (summarized in *Figure 2*).

In lung cancer a variety of methods have been used for ctDNA analysis, many of them based on real-time PCR, although these approaches are more applicable when a limited number of loci are evaluated. Such systems include peptide nucleic acid (PNA) or locked nucleic acid (LNA) mutant-enriched PCR (ME-PCR) (119-121), amplification-refractory mutation system (ARMS) (122), digital PCR (including droplet-based systems) (123), and the beads, emulsification, amplification, and magnetics (BEAMing) system (124,125). Moreover, recently developed NGS technologies have also shown that it is possible to detect many cancer-associated mutations in single lung cancer patient blood samples (126,127). There are also protocols specifically intended to improve the sensitivity of NGS ctDNA sample analysis; these include tagged-amplicon deep sequencing (TAm-Seq) (128), Safe-Sequencing System (Safe-SeqS) (129), and cancer personalized profiling by deep sequencing (CAPP-seq) (109,130,131), among others.

### *ctDNA: clinical applications in lung cancer*

The clinical applications of ctDNA can be divided into two main categories: (I) quantification of circulating DNA for early diagnosis, prognosis, and response prediction; and (II) analysis of ctDNA in order to profile and characterize molecular tumor alterations (*Table 3*).

Lung cancer patients have increased plasma cfDNA levels compared with control individuals (142,143) and the amount of cfDNA has been associated with tumor stage and burden in lung cancer (109). However, there are data demonstrating that this absolute cfDNA amount is limited as diagnostic tool in the absence of contextual knowledge of any associated tumor mutations (114). High levels of cfDNA have been reported as an indicator of poor outcome in lung cancer patients (115,138,142), but in other studies pretreatment levels of total cfDNA were not prognostic of survival (144,145). One possible restriction of these approaches is that cfDNA is also present at high levels in the blood of patients with benign diseases such as hepatic

Table 3 Studies of the mutation status in cfDNA samples from NSCLC patients

Study	Patients included	Stage	Technology	Sample	Sensitivity (%)	Specificity (%)	Conclusions	Refs.
Rosell <i>et al.</i> [2009]	164	I-IV	ME-PCR	Serum	59	NA	Ninety-seven of 164 patients carried mutations (del19 and L858R) in 64 and 33, respectively	(119)
Taniguchi <i>et al.</i> [2011]	44	III-IV	BEAMing	Plasma	73	NA	From 44 patients that were confirmed to have activating mutations in their primary tumor, 32 mutations were detected in the plasma DNA	(125)
Goto <i>et al.</i> [2012]	194	III-IV	ARMS	Serum	43	100	Fewer patients were EGFR mutation positive when assessed using pretreatment cfDNA (23.7%) vs. tumor tissue-derived DNA (61.5%)	(132)
Huang <i>et al.</i> [2012]	822	III-IV	DHPLC	Plasma	70	80	Concordance of EGFR mutations between tissue and plasma samples was 77%. In patients with two or more lines of EGFR-TKI therapy, EGFR mutation status in plasma samples was a predictor for PFS	(133)
Zhao <i>et al.</i> [2013]	111	I-IV	ME-PCR	Plasma	36	95.5	The sensitivity was 10% in early-stage patients and 56% in advanced-stage patients. Patients with poorly differentiated tumors showed the highest sensitivity (77.8%)	(134)
Kim <i>et al.</i> [2013]	60	III-IV	PNA/LNA PCR	Plasma	17	100	There was no statistically significant difference in clinical parameters between patients with EGFR mutations in plasma and those without EGFR mutations	(121)
Kim <i>et al.</i> [2013]	57	III-IV	PNA/LNA PCR	Serum	73	91	The status of EGFR and KRAS mutation in serum was not prognostic in patients with advanced NSCLC	(135)
Wang <i>et al.</i> [2014]	134	III-IV	ARMS	Plasma	22	97	Patients with high TGF- $\beta$ 1 levels showed shorter OS and worse response to EGFR-TKIs than patients with low TGF- $\beta$ 1 levels	(136)
Couraud <i>et al.</i> [2014]	107	I-IV	Ion Torrent PGM	Plasma	58	87	In tumor DNA, 50 mutations (36 EGFR, 5 ERBB2, 4 KRAS, 3 BRAF, and 2 PIK-3CA) were identified, of which 26 were detected in cfDNA	(127)
Weber <i>et al.</i> [2014]	199	II-IV	Cobas® EGFR blood test	Plasma	61	96	Patients with activating EGFR mutations in plasma DNA had a longer PFS than patients with wild-type status	(137)
Karachaliou <i>et al.</i> [2015]	97	III-IV	PNA/LNA PCR	Plasma	78	NA	Median OS was shorter in patients with the L858R mutation in cfDNA than in those with the exon 19 deletion	(138)
Thress <i>et al.</i> [2015]	38	IV	Cobas®/Therascreen®/DDPCR/BEAMing	Plasma	78-100	93-100	For the T790M mutation, the sensitivity and specificity were 73% and 67%, and 81% and 58% with the Cobas®Mutation Test and the BEAMing dPCR, respectively	(139)
Wei <i>et al.</i> [2016]	50	IV	DDPCR	Plasma	76	88	Concurrent mutant T790M DNA detection of lung cancer patients at baseline achieved 82% concordance with matched tissue analysis	(140)
Newman <i>et al.</i> [2016]	142	III-IV	CAPP-Seq	Plasma	92	100	In silico elimination of highly stereotypical background artifacts with a molecular barcoding strategy improves the sensitivity of cancer profiling by CAPP-Seq	(131)
Zheng <i>et al.</i> [2016]	25	III-IV	DDPCR	Plasma	81	100	In patients receiving 2 <sup>nd</sup> line or later TKI treatment, the T790M cfDNA positive group had a significantly shorter OS than the negative group	(141)

cfDNA, circulating free DNA; ctDNA, circulating tumor DNA; TKI, tyrosine-kinase inhibitor; PFS, progression-free survival; OS, overall survival; ME-PCR, mutant-enriched PCR; BEAMing, beads, emulsification, amplification, and magnetics; DHPLC, denaturing high performance liquid chromatography; ARMS, amplification-refractory mutation system; PNA/LNA PCR, peptide nucleic acid/locked nucleic acid PCR; DDPCR, droplet digital PCR; CAPP-Seq, cancer personalized profiling by deep sequencing.

disorders, diabetes, cardiovascular diseases, non-neoplastic lung diseases, or infections (75).

Regarding the prognostic information provided by ctDNA, monitoring tumor-specific alterations present in ctDNA isolated from plasma from early stage NSCLC patients following surgical resection identified patients with residual disease and was able to detect disease recurrence (109,145). However, despite the reporting of some controversial results, when *KRAS* mutations in ctDNA were assessed as a prognostic marker in NSCLC patients (135,146,147) *KRAS* status in plasma ctDNA was associated with poor tumor response to EGFR-TKIs in NSCLC patients and so it could be used as a predictive marker for selecting appropriate NSCLC patients for such treatments (148-150). Is in this latest aspect, the presence of circulating DNA containing tumor-specific sequences, where we find the most widespread and important applications of ctDNA.

Several reports have analyzed the concordance between genomic alterations (such as *EGFR* mutations) present in lung cancer tissues and corresponding ctDNA samples (119,125,132-134,137): depending on the technology used, the agreement ranges between 60% to more than 90% (122,123,125,138,139,149,151,152). The EURTAC trial was one of the first to explore the feasibility of using ctDNA as a surrogate for tumor biopsy and to correlate mutations in plasma with PFS and OS (120). Since then, several other clinical trials in lung cancer have incorporated the analysis of plasma as a sample source for studying genomic tumor alterations (138).

In the context of metastatic disease the use of ctDNA is particularly useful for patients with tumors that are difficult to biopsy, those with contraindications for biopsy procedures, or where tumor samples have been exhausted; in these cases the possibility of determining the presence of genomic tumor alterations in ctDNA have brought forward the prospect of implementing precision oncology. In addition, ctDNA can be used for real-time monitoring of therapeutic responses to targeted-agents (132,136,152-154) as a valid surrogate for the current use of invasive rebiopsies. In this respect, a number of research groups have recently shown that ctDNA analysis can sensitively and specifically detect T790M clones early, i.e., before therapy or their emergence during EGFR-TKI treatments, demonstrating that this approach represents also an elegant way to overcome the problems arising from tumor heterogeneity(19,29,140,141,155).

For broader applicability, ctDNA analysis platforms focus on not only maximizing analytical sensitivity, but also on providing sufficient genomic coverage to be

able to analyze multiple molecular markers in the same sample, thus providing the possibility of anticipating the molecular alterations expected as tumors evolve. Therefore, ongoing and future prospective studies should aim to test if treatment strategies informed by the unique data provided by ctDNA could yield superior clinical outcomes compared to tissue-based approaches.

### The war: strengths, and limitations

There are many studies aiming to detect and/or characterize CTCs or ctDNA in lung cancer patients; the question is which of these two approaches will become the eventual gold standard for managing these patients in the era of precision oncology. In this “war” the usefulness of CTCs for *ex vivo* models, including in functional studies such as cultures, mouse xenografts, or real-time *in vitro* assays for drug sensitivity evaluation, is undisputed. CTC enumeration as a prognostic biomarker in lung cancer research has not been as successful as it was in breast, prostate, and colon cancers for which there is a FDA-approved CTC method; even so, the adoption of this approach in these cancers in daily oncological practice remains low.

Reports on comparative mutation analyses of CTCs and cfDNA have shown an interesting relationship between them in cancer patient blood samples (57,91). Maheswaran *et al.* analyzed *EGFR* mutations in CTCs and ctDNA obtained from the same NSCLC patients and determined that genotyping seemed to be more sensitive in CTCs than in cfDNA and that the associated CTC quantification provided an important context in which to interpret these genotyping results (98). Thus, with the emergence of extremely sensitive technologies, complete genomic and transcriptomic profiles, drug sensitivity testing in CTC-derived cell cultures or in single cells might soon become a reality. Until now, the use of ctDNA has remained limited to research scenarios. However, an *EGFR* plasma test (TheraScreen<sup>®</sup> *EGFR* plasma PCR kit) has recently been approved in Europe and China for screening advanced NSCLC patients where it is impossible to obtain a tumor biopsy, allowing subsequent treatment with gefitinib in appropriate cases. Hence, new perspectives for implementing ctDNA in clinical settings are starting to open up (122).

There is mounting scientific evidence supporting the use of ctDNA for profiling and characterizing lung tumor molecular alterations as well as for monitoring therapies and identifying mutations associated with acquired drug

resistance (91,118,119,122,126,144,149). In this context ctDNA, rather than CTC analysis, is more appealing because plasma samples can be collected and analyzed without requiring prior enrichment and there is no need to isolate a rare cell population. Although pre-analytical conditions for ctDNA analysis must be further standardized, it seems that ctDNA, therefore, is likely to be the preferred option for genotyping and treatment-response monitoring. However, one important limitation of working with these samples is that *in situ* and morphological analyses using FISH and ICC (of particular interest in lung cancer for assessing *ALK* or *ROS1* status) cannot be performed with these samples (49,156). Another drawback of ctDNA is that, because of the high sensitivity of the different methodologies used, it also detects clinically irrelevant molecular changes.

In order to fully incorporate liquid biopsies into clinical practice some critical points must still be addressed: (I) a consensus is required regarding the best matrix of detection (CTC or ctDNA) for each required application; (II) a consensus regarding the ideal technical approach for each application is mandatory; (III) the pre-analytical phase should be standardized to obtain robust and reproducible results; (IV) investment and uptake of the currently available techniques is required in order to bring down prices which presently limit accessibility to patients.

## Conclusions

Liquid biopsy has great potential for the management of lung cancer patients. Despite the numerous techniques and experimental approaches that have been established in this field, the common objective of all of them is to develop a useful, sensitive, specific, and real-time prognostic, predictive, and monitoring system using minimally-invasive samples, which can be easily transferred into the clinical practice. From our point of view, ctDNA analysis should be chosen for analysis of mutations, copy number aberrations, and DNA methylation changes, whereas CTC analysis provides the unique opportunity to study whole cells, thus allowing DNA, RNA, and protein-based molecular profiling, as well as use *in vivo* studies. It is likely that both CTCs and ctDNA will have complementary roles as cancer biomarkers and might be used in parallel for earlier lung cancer diagnosis, prediction of treatment responses, or detection of disease progression. Taking all of these arguments into account, we consider the real victory in this “war” is the genuine possibility these technologies create for

translating the concept of precision oncology into clinical practice. Liquid biopsies represent an important advance in the management of lung cancer in which CTCs and ctDNA are both expected to play complementary roles based on their relative strengths and limitations.

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## Footnote

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