Liquid biopsies in lung cancer—a narrative review

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Background and Objective: The use of liquid biopsies to detect not only genetic and epigenetic alterations in circulating DNA but also to identify circulating non-coding RNAs (ncRNAs) is a promising tool for diagnosis, prognosis and prediction of therapy outcome of cancer patients. The objective of the narrative review is to describe the genetically and epigenetically altered circulating DNA as well as the identification of microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) in both peripheral blood and other bodily fluids, but also in exosomes as potential biomarkers for lung cancer identification.

Methods: In the following article we summarized important aspects from the studies listed in database PubMed using keywords "circulating, cell-free DNA, RNA, ncRNAs, microRNAs, lncRNAs, circRNAs, exosomes" and "lung cancer" from 1985–2022. A total of 450 articles were considered and 149 relevant articles were included in the review.

Key Content and Findings: In lung cancer patients, new molecular technologies of circulating tumor DNA and miRNAs have been shown to increase the odds of finding the occurrence of targetable mutations in this deadliest malignancy worldwide. The clinical application of relevant non-invasive DNA biomarkers, such as EGFR, ALK, ROS-1, MET and PD-L1, has been investigated for introduction into daily clinical practice. Cell-free EGFR mutations have been approved in selecting patients for treatment with tyrosine kinase inhibitors and assays for their detection have already entered clinical practice.

Conclusions: Although the application of (I) circulating miRNAs for the detection of lung cancer has not entered clinical practice, and (II) circulating DNA has only been exploited in some cases, their utility for diagnosis, prognosis, and prediction of different treatment strategies is promising. In particular, the consideration of exosomes containing these molecules is essential since they are important factors for the propagation of cancer.

Keywords: Lung cancer; exosomes; circulating mutant DNA; circulating miRNA; circular RNAs (circRNAs); long non-coding RNAs (lncRNAs)

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Introduction

Primary lung cancers (PLCs) are comprised of two main types, namely, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Some 80–85% of these will be NSCLCs with the remainder tending to be SCLCs (1). The former is termed neuroendocrine tumors due to their commencement in the lung neuroendocrine cells. In contrast, the NSCLC types are primarily adenocarcinomas, squamous cell carcinomas and large cell carcinomas representing some 90% of primary lung tumors (2). Less common are lung sarcomas and lymphomas plus mesotheliomas. A very high percentage of PLCs are due to tobacco smoking being the case of some 90% of male and

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75% of female lung cancers (3).

The 5-year survival rate of lung cancer is about 19% and lower than many other cancer types, because of its late detection, when the tumor has already progressed. The 5-year survival rate for lung cancer is 56% but only for cases with still localized disease. New targeted therapies have improved clinical outcomes of a significant proportion of advanced NSCLC patients. Tyrosine kinase inhibitors (TKIs) that target alterations of epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), *ROS1*, rearranged during transfection (*RET*), B-type Raf proto-oncogene (*BRAF*) *V600E* and mesenchymal-epithelial transition (*MET*) exon 14 are important tools for the treatment of NSCLC patients. For example, TKIs may inhibit the permanent emission of growth signals contributing to the uncontrolled growth of the tumor caused by activating EGFR mutations of exon 19 and 21 (4).

Liquid biopsies have been used for many years since Ashworth [1869] first identified circulating cancer cells (CTCs) in human blood and Mandel & Metais (5) demonstrated both cell-free DNA (cfDNA) and cell-free RNA (cfRNA) in human blood sampled from a variety of patients.

They are now well-established in both research and clinical use with an expansion from their original concept of "A test made on a sample of blood to determine the presence of either CTCs or circulating DNA fragments (cirDNA) from a tumor circulating in the blood" (6,7) to one in which a variety of sources in addition to blood, e.g., sputum, saliva, urine, cerebro-spinal fluid, peritoneal fluid, breast milk, tears, bronchial lavage fluid (BALF) and seminal fluid can be exploited. In addition to being used as a marker indicating a tumor presence, this cfDNA may also be used to monitor both treatment management and prognosis (8-11).

It is important to consider that a range of components circulate in human blood including cfDNA and cfRNA. The cfDNA will include circulating tumor DNA (ctDNA) that has been derived by either apoptosis or cell and tissue necrosis or pyroptosis or ferroptosis or netosis or sepsis or mitochondrial DNA or haemopoietic release or transposons or retrotransposons as well as vesicles such as exosomes and virtosomes and the presence of bacterial, viral and parasite DNA in healthy individuals (12-14).

Since much of the cfDNA and cfRNA is contained in exosomes (Exs), these vesicles have become the favourite structures to isolate in order to detect mutant DNA/RNA relevant to cancer. Exs are spherical bodies, 30–150 nm in diameter containing DNA, RNA, protein and lipids. They have been shown to carry intraluminal and transmembrane proteins including heat shock proteins (HSP70, HSP90), integrins, and tetraspanin proteins (CD9, CD63, CD81, CD82). Furthermore, proteins involved in both membrane transport and fusion (e.g., Rab GTPases, annexins, flotillins) and multiple vesicular body biogenesis [e.g., Alix and tumor susceptibility gene 101 (Tsg101)] may also be present. Additionally, they can contain ceramides and cholesterol (15).

ExDNA, both genomic (100 b–17 kb) and mitochondrial (present in whole or damaged mitochondria), has been found in both normal and pathological environments (16-20). Under experimental conditions, DNA-containing Exs have been linked to the initiation of both glioma and colorectal cancer (16,18). Importantly, Exs have been found to contain a wide range of RNAs including mRNA, miRNA, rRNA, tRNA, lncRNA, piRNA, circRNA and snRNA. Using deep-sequencing, the dominant fraction appears to be mRNA (42.32%) in human plasma-derived exosomal RNAs. Remaining fractions included rRNA (9.16% of all mappable counts), lncRNA (3.36%), piRNA (1.31%), tRNA (1.24%) and snRNA (0.18%). The five most common of the 593 miRNAs detected were miR-99a-5p, miR-128, miR-124-3p, miR-22-3p and miR-99b-5p so providing 48.99% of all mappable miRNA sequences (21).

Exs are released by both healthy and tumor cells with a view to sending, e.g., information and healthy mitochondria, to recipient cells. The latter may be either healthy cells or tumor cells. Thus, Exs may move from either healthy to healthy, healthy to tumor or tumor to healthy cells. Hence, the use of Exs for the identification of tumor cell markers, i.e., DNA and RNAs, will depend primarily on the presence of tumor cell Exs. Studies of Ex DNAs have shown them to contain some 90% of cfDNA that may be contained either within the Exs or bound to their outer surface (22). The remainder of the DNA will be either free or bound to protein in the blood. There appears to be no such data available for RNAs that are presumed to be only in the Exs. In using only Exs for the detection of RNA/DNA tumor markers, it is worth noting that a portion of the Exs present in blood will be removed by the liver. The impact of this on tumor detection has been considered by Khier & Gahan (23). It should be noted further that in working with just the Ex fraction of cfDNA from blood, some 10% of the cfDNA will not be employed in any ctDNA analysis.

Some of the potential biopsy fluids will contain CTCs in various states of competence as well as ctDNA and ctRNAs. However, it should be noted that CTCs have already been

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Table 1 Methods and search terms used for this review

Items	Specification
Date of search	1st August 2022
Database	PubMed
Search terms used including free text search terms and filters	Circulating, cell-free DNA, RNA, ncRNAs, microRNAs, lncRNAs, circRNAs, exosomes, lung cancer
Time frame	1985–2022
Inclusion and exclusion criteria	Case reports, research studies, systematic reviews and meta- analysis were included; abstract, conference proceedings, letters to the editor and articles in language other than English were excluded
Selection process	Two authors (PBG and HS) conducted the search independently

well-studied and in consequence, this narrative review will concentrate on circulating nucleic acids.

In particular, this narrative review will focus on ctDNA and ctRNAs, both cell-free and exosomal, contained in the biopsy fluid, i.e., blood and other bodily fluids namely, bronchial aspirates, sputum, saliva, urine, cerebrospinal fluid and ascites fluid. We present the following article in accordance with the Narrative Review reporting checklist (available at https://exrna.amegroups.com/article/view/10.21037/exrna-22-16/rc).

Methods

In the following article, we used the database PubMed applying keywords "circulating, cell-free DNA, RNA, ncRNAs, microRNAs, lncRNAs, circRNAs, exosomes" and "lung cancer" from 1985–2022. Two authors (PBG and HS) conducted the search independently. Research studies, systematic reviews and meta-analysis were included. Abstract, conference proceedings, letters to editors and articles in language other than English were excluded. A total of 450 articles were considered and 149 relevant articles were included in the review. The methods and search terms are detailed in *Table 1*.

Technologies of cfDNA

Preclinical variables

Due to blood cell lysis that occurs during clotting contaminating serum with genomic DNA, plasma is preferred to serum for the extraction of cfDNA. Plasma should be processed in less than 6 h at room temperature in order to avoid the release of genomic DNA. There are specialized tubes on the market, such as Qiagen PAXgene tubes, Streck Inc. BCT tubes and Roche Diagnostics cfDNA collection tubes, that collect blood without cell lysis. They allow the storage of blood plasma for up to 48 h at room temperature prior to processing. The processed plasma samples can be stored at −80 ℃ for a year without degradation of cfDNA. Longer storage time at −80 ℃ is not recommendable because of degradation of cfDNA to avoid manipulation of the data analyses. Several freeze-thawing steps of blood plasma are also not recommended [detailed reviewed in (24)].

cfDNA extraction

Blood sample collection, handling, and storage are essential for quality of cfDNA analysis. For plasma extraction, 10–20 mL blood is usually collected in anticoagulant-treated tubes, e.g., EDTA-treated or citrate-treated tubes. In contrast, serum is collected after the blood is allowed to clot which is removed by centrifugation. Plasma should be preferred to serum since the clotting process may lead to contamination of genomic DNA. Processing of the blood sample should be performed within a few hours, to avoid cfDNA degradation and release of genomic DNA by lysed white blood cells. The percentage of ctDNA is often low, and accounts for 1–5% of the total cfDNA (24).

CfDNA and cfRNA have been extracted from the various bodily fluids with standard commercial extraction kits.

Genetic technologies of cfDNA

To date, several analytical methods have been developed for the identification of molecular alterations in cfDNA. They differ between targeted (narrow) approaches and untargeted (broad) approaches. Almost all PCR-based methods belong

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to the targeted approaches, whereas next-generation sequencing (NGS)-based methods belong to untargeted (broad) approaches.

Real-time or quantitative PCR

Real-time (RT) PCR differs from classic PCR using specific probes which emit fluorescent light the intensity of which is measured in every cycle so allowing the estimation of the quantity of the ctDNA sample based on the number of cycles and a threshold fluorescent signal. In this regard, the TaqMan quantitative polymerase chain reaction (qPCR) is widely used method (25,26).

For example, cobas *EGFR* mutation test is a PCR-based test for molecularly analyzing cell-free EGFR mutations in NSCLC patients. This commercially available kit from Roche Molecular Diagnostics identifies exon 19 deletions plus L858R and T790M mutations from the plasma DNA. Data of the ENSURE study showing the relevance of detecting the EGFR mutations in lung cancer patients who benefit from erlotinib treatment (27) led to the only technology approved by the U.S. Food and Drug Administration (FDA) and as the first liquid biopsy test. However, plasma EGFR mutations were only found in the plasma of 76.7% patients with EGFR mutations, confirmed in tumor tissues. Therefore, plasma-negative patients have been recommended to be re-tested using tissue specimens.

Digital droplet PCR (ddPCR)

ddPCR is a microfluidic technology that partitions a sample so that each droplet contains either 1 or 0 DNA molecules to be amplified. The initial ratio of mutant-to-*wt* DNA can be estimated by detecting the signal from each droplet combined with the use of the Poisson distribution (26). The sensitivity for detecting *EGFR* T790M mutation in plasma can vary according to the technology, but may reach 93% using ddPCR (28).

BEAMing

BEAMing (beads, emulsions, amplification and magnetics) uses DNA templates bound to magnetic microbeads before suspension into droplets. After amplification, thousands of copies of DNA are bound to each bead which can be isolated through centrifugation and/or use of a magnet. Optical scanning or flow cytometry are used to quantify the DNA on the beads (29). In the phase I AURA study, the sensitivity of detecting *EGFR* mutations in patients' plasma treated with osimertinib was 82% for exon 19 deletion and 86% for L858R mutation using BEAMing (30).

NGS

NGS allows the sequencing of nucleic acids through a large number of parallel reads and their subsequent alignment to a genomic reference standard. For example, deep sequencing implements tens of thousands of reads of the same strand. Whole genomes or panels of hundreds of targeted regions of exons or introns can be sequenced by NGS. Numerous DNA alterations can also be detected by NGS, such as mutations, insertions, deletions, amplifications, rearrangements (inversions and translocations). Nowadays, NGS platforms can simultaneously detect mutations, copy number variations and genomic rearrangements.

The most used NGS-based platforms are Illumina and Ion-Torrent (ThermoFisher Scientific) Illumina that simultaneously identify DNA bases, while incorporating them into a DNA strand, by using four-color optical imaging of fluorescently labeled nucleotides. Instead of using optical signals, Ion-Torrent applies a semiconductor to measure a change in pH referring to the release of an H^+ ion following the addition of a nucleotide (31).

Epigenetic technologies of cfDNA

Usually, epigenetic alterations are more abundant than genetic alterations in cancer and thus, global methylation analysis may result in a higher sensitivity. A detailed description of the following techniques of cfDNA methylation analyses is given in the review article by Galardi *et al.* (32).

Restriction enzyme-based methods

A classical approach is the application of methylation-sensitive and -insensitive restriction enzymes with subsequent PCR for assessing methylation patterns in cfDNA (32).

Bisulfite-based conversion methods

Since 1992, the application of bisulfite treatment has been an important breakthrough in analyzing DNA methylation. In this approach, bisulfite converts all unmethylated cytosines to uracil, while methylated cytosines remain unchanged. Many techniques are based on bisulfite-based conversion, such as methylation-sensitive PCR (MSP), whole genome bisulfite sequencing (WGBS), reduced-representation bisulfite sequencing (RRBS), MCTA-seq, targeted bisulfite sequencing, and methylation array (32).

Immunoprecipitation-based methods

Methylated DNA immunoprecipitation sequencing

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(MeDIP-seq) and methyl-CpG binding domain protein (MBD) capture sequencing (MBD-seq) are based on affinity enrichment array analysis. MeDIP uses antibodies specific for methylcytosines. High-quality methylomes can be sequenced by combining MeDIP along with NGS, which provides 1 to 300 bp resolution. MBD-seq uses magnetic beads to pull down MBD-coated methylated DNA fragments (32).

Technologies of microRNAs

Different technologies, such as NGS, real-time PCR, northern blotting, and microarrays are used for assessment of miRNA expression. The extraction of RNA is usually performed by using commercially available kits containing phenol/guanidinium products, such as TRIzol (Life Technologies), and column-based extraction kits, such as MirVana (Life Technologies) and miRNeasy (Qiagen) (33).

To obtain large-scale profiles of circulating miRNAs and to determine candidate miRNAs for further analyses, the extracted miRNAs are subjected to NGS or miRNA microarray (34,35). In most studies, miRNAs are amplified by real-time PCR, such as TaqManPCR. TaqManPCR kits are available with a probe specific for one miRNA or as microarrays for several miRNAs. The data obtained from the different assays should be normalized with a reference miRNA. However, establishment of endogenous controls for data normalization remains challenging for a reliable miRNA quantification. To date, no definitive reference miRNA has been established. RNU-6B, RNU-48 and miR-16 are still commonly used as endogenous controls (36).

Challenges of the application of liquid biopsy using these technologies

As described above and in detailed reviews (6,37-39), there is a variety of techniques, some of which allow the analysis of the entire exome or genome, while others target specific genes. Each technique has specific advantages and disadvantages. Notably, real-time PCR allows the analyses of a few genetic alterations in cfDNA in one assay. Droplet digital detection can detect a low level of ctDNA and ncRNAs in plasma with a high specificity and sensitivity, within few hours. Because of the growing number of guideline-recommended oncogene targets to be assessed in advanced NSCLC, testing of plasma ctDNA should be performed by NGS. NGS provides a large profiling of hundreds of mutations. The limit of detection rate (1–0.03%) depends on the platform used. TAm-Seq is the most sensitive, detecting allelic frequencies as low as 0.03%. However, only 0.004% of the lung cancer genome is commonly mutated so making the relevant fraction of cfDNA to be analyzed still smaller. MSP is the main detection method employed for detecting methylated ctDNA in cancer patients. Its limit of detection has been reported to be as low as 0.01%, with a high sensitivity and specificity for the detection of differentially methylated ctDNA.

Nevertheless, there are obstacles when using liquid biopsies. So far, a discordance between tumor tissue and plasma DNA patterns have been described that may arise from the limitations of ctDNA detection: Firstly, the concentration of ctDNA is low and varies from 1 to 10 ng/mL in blood. Therefore, higher volumes of blood are needed. In addition, the half-life of cfDNA in the bloodstream varies largely between 16 min and 2.5 h. Secondly, cfDNA is fragmented due to its release from apoptotic cells and to the presence of DNA nucleases in body fluids. Thirdly, liquid biopsies form a pool of cfDNA of different origins. Apart from tumor cell DNA, normal healthy cells and leukocytes also contribute to the cfDNA content, resulting in increased false positives data and masking the ctDNA (40). To avoid such genomic contamination and so successfully detect ctDNA, a recent article presented a new method that uses a small amount of extracted DNA to accurately quantify high-molecular weight DNA and hence, to adjust the ctDNA input amount for optimal NGS assay performance (41). In contrast, the screening of ncRNAs is easier since they are far more abundant than ctDNAs. Their concentration, and not their genetic alterations, are usually determined. On the other hand, ctDNA and miRNAs can be considered as a real-time snapshot that reflects genetic and epigenetic alterations as well as alterations in their levels. These features are important aspects for guiding targeted therapy for precision medicine (42).

Great efforts have been made to improve the ctDNA assays, e.g., for the use for stages I or II lung cancer to reach a better sensitivity and specificity (43). Mutations in *KRAS* and *TP53* could be detected in stored sputum samples from individuals up to 1 year before lung cancer diagnosis (44). To date, the European Medicines Agency and the FDA have approved EGFR mutation testing using ctDNA for therapy guidance in NSCLC patients. For NSCLC patients treated with immune checkpoint inhibitors, ctDNA was shown to be an early marker of therapeutic efficacy and could

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bpredict survival outcomes (45).

Drugs used in lung cancer therapies

Erlotinib

Erlotinib is a TKI that targets the *EGFR* and was approved in the EU (European union) in 2005 for the treatment of locally advanced or metastatic NSCLC patients who have failed at least one prior chemotherapy regimen and harbor an EGFR L858R mutation. Erlotinib binds reversibly to the adenosine triphosphate (ATP) binding site of the intracellular EGFR TK domain (4).

In the randomized phase III EURTAC study, the effect of erlotinib was compared for the first time with the platinum-based chemotherapy in EGFR-mutated advanced NSCLC patients in western countries. The demographic parameters of the 86 patients in the erlotinib and 87 patients in the chemotherapy group were comparable. The response rate was 58% with erlotinib and 15% with chemotherapy, with two patients who completely respond to erlotinib. A partial response was achieved in 56% of patients on erlotinib and 15% on chemotherapy (46).

Gefitinib

In EU, the TKI gefitinib has been approved for the treatment of locally advanced or metastatic NSCLC with proven deletion of exon 19 or L858R mutation in exon 21 of the EGFR since June 2009 (4).

Afatinib

Afatinib is a TKI of the ErbB family, binds to all EGFR members of the ErbB family (ErbB1), HER2 (ErbB2), ErbB3 and ErbB4 homo- and heterodimers and irreversibly blocks signal transduction via these receptors. Since September 2013, afatinib has been commercially obtainable. Afatinib is used for the treatment of locally advanced NSCLC with activating EGFR mutations, even if it has already metastasized (4).

Osimertinib

Since June 2018, the TKI osimertinib has been approved for the first-line treatment of advanced lung cancer with EGFR mutations. In Germany, Osimertinib has already been approved for the treatment of advanced lung cancer with an EGFR T790M mutation since March 2016 (4).

Dacomitinib

Since April 2019, the TKI dacomitinib has been approved for the treatment of locally advanced or metastatic NSCLC with proven deletion of exon 19 or L858R mutation in exon 21 of the EGFR (4).

Checkpoint inhibitors

Antibodies, such as pembrolizumab, nivolumab and ipilimumab specifically change the communication between tumor cells and T-lymphocytes resulting in improved recognition of the tumor cells by the immune system. In particular, blockade of programmed cell death (PD-1), a negative regulator of T cell activity, has been shown to be effective in a variety of malignancies. In April 2019, FDA approved pembrolizumab for the first-line treatment of stage III NSCLC patients who were not candidates for surgical resection or definitive chemoradiation or metastatic NSCLC. Then, in May 2020, FDA approved nivolumab with ipilimumab and two cycles of platinum-doublet chemotherapy as first-line treatment for metastatic or recurrent NSCLC patients with no EGFR or anaplastic lymphoma kinase (ALK) genomic tumor aberrations (4).

Genetic alterations of cfDNA in blood

Table 1 indicates our methods of literature research while *Table 2* summarizes the most important genetic alterations in blood. Half of NSCLC patients with EGFR T790M mutation acquires resistance to EGFR-TKI. Zheng *et al.* detected T790M ctDNA in the plasma of 55 of 117 (47%) NSCLC patients using ddPCR. Patients received TKI treatment at second line or later. The T790M ctDNA positive group had significantly shorter overall survival (OS) than the negative group (61). In the phase III multicenter study FASTACT-2, the occurrence of EGFR mutations was compared in combined serum/plasma and tumor tissue of 238 NSCLC patients who received six cycles of gemcitabine/platinum plus sequential erlotinib or placebo, using the Cobas tissue and blood test. Concordance between tissue and blood tests was 88%, with a blood test sensitivity of 75% and a specificity of 96%. A dynamic change in *EGFR* status in blood samples was linked with clinical outcomes. Those with *EGFR* mutation-negative assessment had better outcomes of progression-free survival

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Table 2 Examples of essential DNA markers in lung cancer

Markers	Function	Ref.
Genetic markers		
EGFR mutations	Transmembrane glycoprotein, acquired resistance to first and second-generation EGFR-TKIs	(47)
ALK fusions	Tyrosine kinase receptor, active form of the kinase	(48)
MET amplification	Tyrosine kinase receptor	(49)
MET exon 14 skipping	Angiogenesis	(49)
KRAS mutations	G protein, hyperactivation of downstream signaling cascades leading to cell proliferation, survival	(50)
ROS1 fusions	Receptor tyrosine kinase, differentiation of pithelial tissues	(51)
BRAF mutations	Serine/threonine-Kinase B-Raf, cell proliferation, survival	(52)
RET fusions	Receptor tyrosine kinase, brain metastasis a low immune infiltrate	(53)
HER2 mutations	Receptor tyrosine kinase, proliferation, differentiation migration	(54)
Epigenetic markers		
APC	Negative regulator in Wnt/beta-catenin signaling pathway	(55)
p16	Cell cycle pathway	(56)
SHOX2	Homeobox protein, transcriptional regulator	(57)
RASSF1A	Molecular scaffold protein	(57)
HOXA9	Homeobox protein, transcriptional regulator	(58)
RARβ2	Nuclear receptor activated by retinoic acid	(59)
CHFR	E3 ubiquitin-protein ligase, cell cycle	(60)

(PFS) and shorter OS than those whose samples were still *EGFR* mutation-positive, suggesting that this dynamic change may predict benefit of treatment with erlotinib (62).

In 8,388 advanced NSCLC patients, driver and resistance mutations were examined using plasma-based comprehensive genomic profiling results. Somatic alterations were detected in 86% of samples. Activating alterations of oncogenes were identified in 48% of patients, including EGFR (26.4%), MET (6.1%) and BRAF (2.8%) alterations as well as ALK, RET, and ROS1 fusions (2.3%) (63). Digital Sequencing of ctDNA was performed by Guardant360 in 88 Patients with advanced lung adenocarcinoma. Seventy-two patients (82%) had more than one ctDNA alteration. The most frequent alterations were in *TP53* (44.3%), *EGFR* (27.3%), *MET* (14.8%), *KRAS* (13.6%) and *ALK* (6.8%) genes. The concordance rate for *EGFR* alterations was 80.8% between ctDNA and tissue test (64). In a multicenter study, plasma samples of 101 advanced NSCLC patients positive for *ALK* and 27 patients positive for *ROS1* alterations were analyzed using InVisionFirst-Lung assay. Sensitivity was 67% (18 of 27) for *ALK/ROS1* fusion detection. Higher detection was observed for *ALK* fusions at TKI failure (33 of 74, 46%) versus in patients with therapeutic response (12 of 109, 11%). The absence of ctDNA mutations at TKI failure was associated with prolonged median OS (65).

In the FLAURA trial, osimertinib showed higher efficiency compared with standard of care, i.e., EGFR-TKIs in advanced NSCLC patients who were evaluated by their plasma using the NGS Guardant360 assay. In the osimertinib arm, there was no evidence of acquired resistance to EGFR T790M. The most acquired resistance mechanism was MET amplification (14 of 91, 15%) and EGFR C797S mutation (6 of 91, 7%), followed by HER2 amplification, PIK3CA and RAS mutations (2–7%). In the standard of care arm, the most common resistance mechanisms were T790M mutation (60 of 129, 47%), MET amplification (5 of 129, 4%) and HER2 amplification (3 of 129, 2%) (66).

Methylation of cfDNA in blood

Table 2 summarizes important epigenetic alterations in

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blood. The first methylation analyses of ctDNA from lung cancer patients were performed on adenomatous polyposis coli (*APC*) and *p16* by Usadel *et al.* and Bearzatto *et al.* in 2002, respectively (67,68). It has been shown that in lung cancer, Ras association domain family protein 1 isoform A (*RASSF1A*) and *p16* are the two most frequently methylated genes detected in ctDNA (69).

DNA methylation of short stature homeobox 2 (*SHOX2*) determined in plasma samples from 411 lung cancer patients and controls by RT-PCR may be used as a biomarker to differ between malignant lung disease and controls at a sensitivity of 60% and a specificity of 90%. Lung cancer patients with stages II (72%), III (55%), and IV (83%) were detected at a higher sensitivity than stage I patients. SCLC (80%) and squamous cell carcinoma (63%) were identified at the highest sensitivity when compared with adenocarcinomas (70). At 7–10 days after therapy initiation, 36 advanced NSCLC and SCLC patients who clinically responded to chemo-/radiotherapy demonstrated a decrease in methylation of *SHOX2* in their plasma. Furthermore, higher methylation levels of *SHOX2* both before and 7–10 days after starting therapy were indicative of shorter OS (71).

Methylation levels of *APC*, homeobox A9 (*HOXA9)*, retinoic acid receptor β2 (*RARβ2*) and *RASSF1A* were assessed in 129 plasma samples using quantitative methylation-specific PCR. Interestingly, higher methylation ctDNA levels of *HOXA9* and *RASSF1A* were found in SCLC than in NSCLC. For SCLC detection, *HOXA9* displayed a high sensitivity of 63.8%, whereas *RASSF1A* disclosed a high specificity of 96.2% in ctDNA (72). To assess chemotherapy efficiency and toxicity, 316 patients with advanced lung cancer who were treated with cisplatin-based therapy were enrolled. Higher methylation levels of *APC* and/or *RASSF1A* within 24 h after cisplatin-based chemotherapy were detected and also associated with increased OS (73). Methylation of *APC* and *RASSF1A* at diagnosis was also an independent predictor of increased disease-specific mortality in lung cancer patients with a 3.9-fold risk of dying from lung cancer compared to those lacking DNA methylation (74).

The clinical outcome of second-line chemotherapy or EGFR TKIs was assessed in 179 stage IV NSCLC patients. OS due to EGFR TKIs of these patients with unmethylated checkpoint protein FR (*CHFR*) was 21.4 months, and 11.2 months for those treated with chemotherapy. Second-line EGFR TKIs improved survival in patients receiving first-line cisplatin-based treatment. Thus, NSCLC patients with unmethylated *CHFR* depicted longer OS when treated with EGFR-TKI compared to those treated with chemotherapy as second-line therapy (75).

RNAs

Although a range of RNAs have been detected in various liquid biopsy samples, e.g., mRNAs, small nucleolar RNAs and piwi interacting RNAs, there are three particular RNAs, the presence of which in a liquid biopsy are important for diagnostic purposes—miRNAs, lncRNAs and circRNAs.

miRNAs

These are derived from an initial RNA strand of a host gene by means of splicing, capping and polyadenylating. This results in the development of mature, active 21–23 nucleotide miRNAs (76,77). The resultant miRNA is then integrated with an RNA-induced silencing complex (RISC) that will subsequently target such mRNAs that need to be either degraded or inhibited (78). Although RNAse is present in the blood, its levels rising during cancer (79,80), miRNAs do not appear to be affected. This is probably due to their presence in extra-cellular vesicles. Thus, they are useful markers for the monitoring of the presence, resulting treatment as well as the possible reappearance of a particular cancer type.

LncRNAs

Although lncRNAs are comprised of more than 200 nucleotides they lack the possibility of coding for proteins (81,82). Given their multifactorial functions in gene regulation and expression, they are implicated in a range of biological processes. These include apoptosis, imprinting, cell growth, differentiation (81,82). The expression levels of lncRNAs become dysregulated in patients having an association with tumorigenesis, cancer progression and metastases (38). More than 210,000 lncRNAs have been identified (83), with 106,063 of them being associated with man (lncRNAWiki, 2015). In contrast, Gencode—lncRNA microarray [2014] has recorded only 14,470 of them (http://www.gencodegenes.org/lncrna_microarray.html). Additional analyses indicated only 1,867 human lncRNAs to be biologically active (40). In the case of lung cancer, the differential expression of lncRNAs has been considered to be associated with diverse tumor prognosis (84). Thus, SRY-Box transcription factor 2 (SOX2)-overlapping transcript (SOX2-OT) that is frequently detected in lung squamous

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cell carcinomas as opposed to lung adenocarcinomas (85,86).

Circular RNAs (circRNA)

CircRNA are a class of non-coding RNAs formed on 3'-5' ligation of an RNA molecule. Three forms of circRNA occur depending upon their nuclear origin. Thus, they can be formed from either introns (ciRNA) or exons (ElciRNA) or exon-introns (ecRNA). CircRNAs are important in the regulation of miRNAs through acting as miRNA sponges in, e.g., the presence of cancer (87,88). Primarily found in the cytosol, circRNAs—mainly circular RNA sponge for miR-7 (CiRS-7) and Sry circRNA—act as sponges with the former having over 60 binding sites for miR-7 (CiRS-7) (89) and the latter 16 binding sites for miR-138 (76).

Consequently, a mechanism is present leading to a reduction in the number of miRNAs, e.g., miR-7 in the cell. Nevertheless, when the cell needs such RNA molecules, they can be released from the sponge. This possibility is based upon CiRS-7 being spliced by miR-671 so providing a system capable of releasing miRNAs as necessary (90). circTRIM33-12 has been shown to act as a sponge for miR-191 in hepatocellular carcinoma (HCC) (91). The downregulation of circTRIM33-12 was able to upregulate ten-eleven translocation methylcytosine dioxygenase 1 (TET1) expression on sponging miR-191. In addition, downregulation of circTRIM33-12 in HCC significantly correlated with malignant characteristics that affected OS as well as recurrence-free survival after surgery. It appears that circTRIM33-12 sponging of miR-191 and upregulating TET1 expression led to significantly reduced 5-hydroxymethylcytosine levels in HCC cells.

Yao *et al.* (92) found that circRNA_100876 was abnormally expressed in NSCLC and that the higher expression level of circRNA_100876 lowered the survival rate. Hence, it may be possible to use circRNA_100876 as a biomarker for early detection and screening of lung cancer. In addition, hsa_circ_0013958 in lung cancer cells appears to promote lung cancer cell proliferation while inhibiting apoptosis (93). In contrast, circRNA of hsa_circRNA_001141 in lung cancer tissues has been demonstrated to suppress the development of lung cancer through enhancement of its parental gene, ITCH, expression (94). In addition, circRNA hsa_circ_0102231 has been demonstrated to sponge miR-145 in the promotion of NSCLC cell proliferation through the up-regulation of the expression of RBBP4 (95).

Liquid biopsy

Bronchoalveolar lavage fluid

To date, little attention has been placed on the use of either circRNAs (96,97) or lncRNAs (98) present in bronchioalveolar lavage fluid (BALF) concerning the early identification of lung cancer. However, miRNAs have been shown to be of interest in this liquid biopsy form.

ctDNA

Hur *et al.* (99) have compared tissue determined NSCLC with both BALF and pleural effusion extracellular vesicle (EV) EGFR-TK1 sensitive mutations. Overall average sensitivity and specificity of BALF EV-based EGFR genotyping (n=137) was 75.9% and 86.7% respectively. The sensitivity showed a significant increase as the disease progressed. Hence, T1 stage sensitivity was 40% increasing to 75% at T2 and to 100% at T3 and T4 stages. N staging also affected sensitivity with T1 at 63.3%, 75% at T2 and 100% at the N3/N4 stages. The presence of metastasis was a major feature to reach 100% sensitivity. Such results demonstrate that an increased release of EVs containing oncogenic EGFR mutant DNA likely depends upon an increment of tumor size and metastatic behavior.

Diaz-Lagares *et al.* (100) identified novel epigenetic biomarkers in stage I lung cancer by using an integrative genome-wide restrictive analysis of two large public databases.

They identified nine cancer-specific hypermethylated genes in primary tumors. Four of these genes were consistent with CpG island hypermethylation when compared with non-malignant lung and, as might be expected, were linked to transcriptional silencing. A diagnostic signature was determined based on a combination of BCAT1, CDO1, TRIM58, and ZNF177. Clinical diagnostic value was also validated in multiple independent cohorts to yield a high diagnostic accuracy in all cohorts tested. When the four genes were combined in a logistic regression model, a significant area under the curve (AUC) of 0.85 [95% confidence interval (CI): 0.78–0.93, $P<0.001$] with an optimism-corrected value of 0.83 was obtained. Furthermore, a higher diagnostic precision was obtained with BALF samples than with conventional cytology.

miRNAs

As early as 2015, an investigation by Rehbein *et al.* (101) using qPCR, demonstrated the significant upregulation

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of five miRNAs (U6 snRNA, hsa-miR 1285, 1303, 29a-5p, 650) from BALF of lung cancer patients. It was considered that these miRNAs could be used for early lung cancer identification. Kim *et al.* (102) examined exosomal miR-7, miR-21, miR-126, Let-7a, miR-17 and miR-19, all of which were considered to be of interest for the diagnosis of lung adenocarcinoma (LA). Of these miR26 and Let-7a were shown to be significantly higher in the BALF of LA patients than in controls.

Later, Moretti *et al.* (103) attempted to resolve the inconsistencies in the various proposals for miRNA biomarkers from BALF for screening of stage I–II NSCLC patients. Four circulating miRNAs, miR-223, miR-20a, miR-448 and miR-145, each with high sensitivity (>0.80) and area under the curve (AUC) (>0.80), could be used as biomarkers for identifying stage I–II NSCLC. In addition, miR-628-3p, miR-29c, miR-210 and miR-1244 also showed a high specificity (>0.90). Finally, a two-step screening for stage I–II NSCLC was proposed in which if the first four-miRNA panel was positive, then the other four miRNAs could be used for confirmation of the result. This combined model offered more sensitivity (0.916) and specificity (0.934). The following year, Lu *et al.* (104) using a plasma source, developed a panel employing six miRNAs (miR-17, miR-190b, miR-19a, miR-19b, miR-26b, and miR-375), to distinguish between lung cancer patients and healthy donors. A second panel involved three miRNAs (miR-17, miR-190b, and miR-375) from the first panel in order to distinguish with a high diagnostic accuracy between SCLC and NSCLC. It is proposed that this approach could well be adopted for BALF miRNAS and lung cancer.

Saliva liquid biopsy

Saliva, like urine, is a minimally invasive method for liquid biopsy, though less useful than sputum at present. Whilst lncRNAs have been reported to be present in saliva, only those acting as possible biomarkers for oral squamous cell carcinoma have been identified (105,106).

ctDNA

As noted by Wei *et al.* (107) EGFR offered promise as a biomarker for lung cancer. More recently, this approach has been further developed by Li *et al.* (108). As described earlier (109), much ctDNA occurs as short pieces of DNA and they found the majority of EGFR L858R ctDNA fragments detected generally to be <80 bp and 40–60 bp in patient saliva. Using both cell lines and patient biofluids, they further indicated the majority of such short DNA fragments to be present mainly in exosomal fractions. However, the sensitivity of such markers tended to be low at 46.2%. EGFR 19-del EGFR 21-L858R have been reported as possible saliva biomarkers (110,111). These workers did not discuss the sensitivity of such biomarkers.

miRNAs

While there are no reports of saliva containing miRNAs, a number have been found to be present in exosomes, namely let-7b-5p, let-7e-5p, miR-24-5p, and miR-21-5p miR-181-5p + miR-361-5p miR-320b + miR-10b-5p and miR-126 (112,113). However, it is not clear that exosomal miRNAs have been investigated in saliva (113).

CircRNAs

Some 422 salivary circRNAs (114) were shown to be involved in signal transduction and inflammatory response in human cell-free saliva. Given that the occurrence and development of tumors are primarily influenced by inflammation, saliva circRNAs may play an important role in tumorigenesis. Whilst hsa_circ_0001874 and hsa_circ_0001971 (115) have been shown to be involved in oral squamous cell carcinoma, there has been no indication of circRNAs in saliva being linked to lung cancer.

Sputum liquid biopsy

In addition to ctDNA, sputum has been found to contain a variety of miRNAs, lncRNAs and circRNA. They have been demonstrated to be stable, even resisting freezing and thawing. Whilst there has been no definitive biomarker for lung cancer found amongst them, there are suggestions for possible ways forward in exploiting their presence.

ctDNA

Initial studies by Hubers *et al.* (116) indicated that hypermethylated gene DNA in sputum could be useful in the identification of lung cancer. Initially they started with a learning set of DNA hypermethylated RASSF1A, APC, cytoglobin, 30ST2, PRDM14, FAM19A4 and PHACTR3 to analyze sputum samples from both symptomatic lung cancer patients and controls. However, they finally chose to assay sputum with a panel of the ctDNA of the hypermethylated genes, RASSF1A, 30ST2 and PHACTR3 (sensitivity 67.1%, specificity 89.5%).

A subsequent study was made by Hubers *et al.* (117), as a part of the NELSON lung cancer low-dose spiral

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CT screening trial to detect lung cancer at the preclinical stage. They investigated DNA hypermethylation of *RASSF1A*, *APC*, cytoglobin, *3OST2*, *FAM19A4*, *PHACTR3* and *PRDM14* as possible biomarkers in sputum. It was determined that that RASSF1A could be used for the identification of invasive lung cancer given its high specificity of 93%, though its sensitivity was only 17%. However, using a panel of *RASSF1A*, *3OST2* and *PRDM14*, 28% of lung cancers were detected within 2 years. Parallel assays with sputum cytology did not detect any lung cancers. Thus, this offers an initial basis for detection of lung cancers by ctDNA.

As an improvement on the reliability of this approach for the prediction of lung cancer, Hulbert *et al.* (118) suggested the combining of positive computed tomography (CT) screening together with the use of ctDNA hypermethylated genes of interest. This would also aid in the elimination of false positive results obtained by CT.

miRNAs

Early studies showed the possibility to use miRNAs in sputum as possible markers of lung cancer. Thus, Xie *et al.* (119) using RT-PCR examined sputum miRNAs miR-21 and miR-155 and found that sputum miRNA-21expression was significantly higher in NSCLC patents than controls and could clearly distinguish between cancer patients and healthy controls by a discriminative receiver-operator characteristic (ROC) curve profile. The detection of miR-21 expression yielded 69.66% sensitivity and 100.00% specificity. Moreover, a better performance was obtained with sputum miR-21 expression (69.66% sensitivity; 100.05% specificity) when compared to sputum cytology (47.82% sensitivity; 100.00% specificity).

This was followed by study of lung adenoma carcinoma (120) when of the seven miRNAs found in sputum, four were selected to differentiate between heathy and lung adenocarcinoma (LA) patients, namely miR-21, miR-486, miR-375, and miR-200b. mi486 was down- regulated whilst the others were upregulated. Used in combination, they produced the best approach for differentiating between LA patients and normal subjects (80.6% sensitivity; 91.7% specificity).

More recently, Xing *et al.* (121) proposed, a panel of three miRNA biomarkers (miRNA-21, miRNA-31 and miRNA-10) for the identification of malignant solitary pulmonary nodules (82.93% sensitivity; 87.84% specificity). In earlier studies, Xing *et al.* (122) used microarray- based platforms to determine expression of 818 human mature miRNAs for NSCLC and paired normal lung tissues. A set of 12 miRNAs (miRs-21, 31, 126, 139, 182, 200b, 205, 210, 375, 429, 486 and 708) were identified in sputum displaying dysregulation in NSCLC (120,122,123). These workers further demonstrated that 10 of the 12 miRNAs (miRs-21, 31, 126, 182, 200b, 205, 210, 375, 486 and 708) with abnormal expressions could be linked with lung cancer (120,122). Roa *et al.* (124) were able to identify, via sputum miRNA profiling of lung cancer, the relative expressions of 11 miRNAs in sputum (miRs-21, 145, 155, 205, 210, 92, 17-5p, 143, 82, 372, and let-7a) of which, five sputum miRNAs (miRs-21, 143, 155, 210, and 372) related to lung cancer.

Thus, a number of miRNAs appear to be considered as possible biomarkers in sputum for lung cancer detection and treatment monitoring. Of these, miR-21 and miR-31 could be important since they appear in more than one test panel studied. However, it is clear that miRNAs will be best used as panels if a satisfactory identification of lung cancer is to be achieved.

LncRNAs

In an analysis of the data published in 28 articles relating to 3,044 patients with lung cancer and 2,598 controls, Dai *et al.* (125) reported that lncRNA yielded a high diagnostic efficiency with a combined 95% sensitivity and a 95% specificity. The combined AUC was also 95%. However, the diagnostic efficiency was improved if metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and growth arrest specific 5 (GAS5) were employed together.

Whilst it has been reported that Peng *et al.* (126) found no lung cancer lncRNAs to be present in sputum, that does not mean that they are absent from sputum. Thus, Fan *et al.* (98) have listed 14 lncRNAs in exosomes though to date, there appears to have been no study on this aspect of analysis.

circRNAs

A study by Yao *et al.* (92) on circRNAs for diagnostic purposes identified cirT + RNA_100876 for this purpose with respect to NSCLC. This RNA was found to be significantly upregulated in NSCLC tissue as opposed to the adjacent healthy tissues. A close correlation was also observed between cirT + RNA_100876 upregulation and lymph node metastasis and tumor staging. Furthermore, the up-regulation of this cirRNA correlated with shorter survival time in NSCLC patients when compared to those with low expression. The fact that cirT+RNA_100876

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can be found in the sputum of LSCLC patients offers the possibility of its use as a biomarker.

Urine biopsy

ctDNA

ctDNA has been more studied than the ncRNAs in urine concerning lung cancer. NSCLC biomarkers already shown to be circulating in plasma and serum have been identified in urine and exploited for the demonstration of the presence of such tumors both before and after treatment. Such biomarkers include EGFR mutations L858R, D Exon 19, T790M (115) and KRAS mutations that are mainly present in codons 12 and 13 (127).

EGFR mutations have been successfully identified in urine using ddPCR for NSCLC (128), and for both early stage and advanced stage NSCLC (129). Interestingly, Li *et al.* (129) indicated poor specificity (42%) and concordance (75%) in early-stage NSCLC, but high specificity (93%) and concordance (100%) in the advanced stage NSCLC. Using ddPCR, KRAS was identified for 200 patient urine samples with 92% specificity and for 100 patient samples with 92% specificity and 77% early stage and 93% late-stage concordance (130). Also using ddPCR, Hu *et al.* (131) demonstrated that the mutant DNA concentrations varied between patients, but that the mean value was 220± 237 copies/mL. Post-treatment patients had urine either completely devoid of ctDNA or with a reduced amount. However, six months post treatment EGFR mutations were found to be significantly increased. In addition to the use of EGFR and KRAS mutated DNA in urine to determine the presence of NSCLC, the use of methylated DNA could provide another approach. Thus, Bach *et al.* (132) made a prospective study of metastatic NSCLC patients who, over 2 days, collected six urine samples each morning, afternoon, and evening. Urinary cfDNA concentrations and methylation levels of *CDO1*, *SOX17*, and *TAC1* were determined for each set of samples. A large variation was observed in the concentrations of each methylated ctDNA throughout the day and between days. Nevertheless, this approach, employing multiple urine samples per patient per analysis, could provide a basis for determining patients with NSCLC based on observations with CDO1 and SOX17.

MiRNAs

The presence of miRNAs in exosomes has been known for some time. As yet, they have not been considered as possible markers for lung cancer.

CircRNAs

Although there are a number of circRNAs present in exosomes relating to cancer in general, to date, none appear to have been studied specifically in the case of lung cancer.

LncRNAs

It might be expected that urine will be a good reservoir of ctDNA relating to the urinary system, but exosomes are also present and contain lncRNAs related to NSCLC. An initial study of lncRNAs by Wang *et al.* (133) identified three potential markers for the initiation of lung cancer namely, upregulated lnc-CCAT1, lncDQ786227 and lnc-H19. However, a study of three selected lncRNAs for each of the up- and down-regulated exosomal lncRNAs present in the urine of 20 NSCLC patients versus healthy controls was made by Lin *et al.* (134). They intimated that potential urinary biomarkers for NSCLC are lnc-FRAT1-5, lnc-SPR-11 and lnc-RNAse13-1 up-regulated together with down-regulated lnc-RP11-80A15.1.1-2, lnc-ARL6IP6-4 and lnc-DGKQ-1.

Peritoneal fluid (PF) biopsy

Whilst a number of cancers have possible PF biomarkers, few observations have been made for lung cancer. Thus, to date no ctDNA appears to have been identified in PF from lung cancer patients. Nevertheless, a number of possible biomarkers are present in the exosomal fractions from plasma and serum that may form the basis for the identification of lung cancer biomarkers in exosomes present in PF.

MiRNAs

So far, the few studies that have been made on miRNAs in body fluids for the early identification of lung cancer tend to be limited to those present in exosomes that have been extracted from either plasma or serum (135). Plasma exosomal miR-23b-3p, miR-10b-5p and miR-21-5p levels were shown to be elevated in NSCLC patients (136) whilst Dejima *et al.* (137) found the levels of plasma exosomal miR-21 and miR-4257 to be significantly higher in NSCLC patients than in healthy controls.

There appears to be little information concerning the serum exosomal miRNAs though preliminary data showed that they may also be useful biomarkers for treatment monitoring (135).

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Cerebrospinal fluid biopsy

Cerebrospinal fluid can be employed in liquid biopsy to determine the presence of ctDNA and ctRNAs related to lung cancer (136,138-141). However, the link is only with metastatic developments and so is not useful for the detection of PLC.

Liquid biopsy in the management of lung cancer

The cobas EGFR mutation test was the first FDA approved ctDNA-based companion diagnostic test (27). Since the publication of the first International Association for the Study of Lung Cancer (IASLC) liquid biopsy statement in 2018, large advances have been made in the field of liquid biopsy, leading to changes in the therapeutic decision-making algorithm for advanced NSCLC (142). With the development of high-sensitive ctDNA assays, the application of ctDNA as a diagnostic and predictive biomarker may be realized in the clinic. The following paragraph addresses some exemplar studies on lung cancer screening in liquid biopsy that may promise useful clinical applications in lung cancer (37).

The FDA-approved Guardant360 CDx is a companion diagnostic test to identify *EGFR* mutations in patients with advanced NSCLC who might benefit from the treatment with osimertinib. In addition, this test has clinical validity for identifying patients with *KRAS*p.G12C–mutant NSCLC (143,144). The FoundationOne Liquid CDx is a further FDA-approved comprehensive pan-tumor liquid biopsy test with a high specificity of 80–95% and a sensitivity varies of 60–85% for *EGFR* driver mutations (145). The AURA3 study showed that early clearance of mutations in ctDNA was associated with the outcome of patients treated with osimertinib. Furthermore, in the AURA2 study, plasma was tested for *EGFR* T790M-resistant mutations using real-time PCR (146). In the FLAURA3 study, *MET* amplification, *HER2* amplifications plus *PIK3CA*, *RAS*, and *EGFR C797S* mutations were identified as playing a role in the resistance to the first-line osimertinib therapy (147). CtDNA has also been successfully used for the detection of *ALK/ROS1*fusions, *BRAF V600E*, *RET* fusion and *MET* exon 14 skipping mutations. The phase II/III BFAST study screened plasma samples from 2,200 patients. Patients who received alectinib had a response rate of 92% with the 1-year duration of response being 78% (148). In the phase 3 trial ADAURA, patients with completely resected EGFR mutation-positive NSCLC were treated with either osimertinib or a placebo. In patients with stage IB to IIIA EGFR mutation-positive NSCLC, disease-free survival was significantly longer among those who received osimertinib than among those who received placebo (149).

In summary, liquid biopsy may be the preferred method of molecular testing in some clinical settings. The application of liquid biopsy is exclusive in serial monitoring and detection of minimal residue disease (MRD) and therapy management.

Conclusions

As has been demonstrated on a number of studies, serum and plasma ctDNA and ctRNAs can be successfully employed in the diagnosis of lung cancer. However, in peripheral blood, the majority of cfDNA and cfRNAs originate from leukocytes, and only a small fraction is released from primary tumor, CTCs and metastatic sites. In addition, most studies have been performed for stages II, III and IV. In contrast, there have been few markers identified for early stage I and disease recurrence. Preliminary examinations of other bodily fluids have shown the concentrations of ctDNA and ctRNAs to be higher than those found in either plasma or serum. However, beside a higher quantity of circulating nucleic acids which is easier to analyze, their quality should also be considered. Although these liquid biopsies are well eligible for ctDNA and ctRNA analyses, the fragmentation of these circulating molecules may be higher in some body fluids, e.g., urine and saliva, than in blood, impairing their analyses. Possibly, the development of liquid biopsies other than those of plasma/serum should permit a more successful approach for the early detection of stage I lung cancer since the earlier lung cancer is detected, the better treatable is lung cancer and lower the patient death rate. To date, this approach has been little explored as is clear from the data available for BALF, urine, saliva, sputum and CBF studies. It would also be interesting to compare the genetic and methylated patterns in the different liquid biopsies and to analyze their relevance to correlate with diagnosis, prognosis and therapy regimens. It is to be expected that further development of the alternative liquid biopsies will permit a more confident analytical process for the early diagnosis of lung cancer, treatment monitoring and an early detection of tumor recurrence. Thus, urgency should be given to the aspects of stage I lung cancer identification and prediction of recurrence. The use of serial liquid biopsies as well as multiplex platforms capturing various circulating biomarkers

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in different fluids for real-time monitoring of early local or distant disease recurrence although challenging, may help to improve patient live quality as well as lowering their death rate.

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