The Oncologist®

Lung Cancer

Clinical Utility of Liquid Diagnostic Platforms in Non-Small Cell Lung Cancer

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Disclosures of potential conflicts of interest may be found at the end of this article.

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Key Words. Non-small cell lung cancer • Liquid diagnostics • Cell-free DNA • Circulating tumor cells • MicroRNA

Abstract

A firmer understanding of the genomic landscape of lung cancer has recently led to targeted, therapeutic advances in non-small cell lung cancer. Historically, the reference standard for the diagnosis and genetic interrogation for advanced-stage patients has been tissue acquisition via computed tomography-guided core or fine needle aspiration biopsy. However, this process can frequently put the patient at risk and remains complicated by sample availability and tumor heterogeneity. In addition, the time required to complete the diagnostic assays can negatively affect clinical care. Technological advances in recent years have led to the development of blood-based diagnostics or "liquid biopsies" with great potential to quickly diagnose and genotype lung cancer using a minimally invasive technique. Recent studies have suggested that molecular alterations identified in cell-free DNA (cfDNA) or circulating tumor DNA can serve as an accurate molecular proxy of tumor biology and reliably predict the response to tyrosine kinase therapy. In addition, several trials have demonstrated the high accuracy of microRNA (miRNA) platforms in discerning cancerous versus benign nodules in high-risk, screened patients. Despite the promise of these platforms, issues remain, including varying sensitivities and specificities between competing platforms and a lack of standardization of techniques and downstream processing. In the present report, the clinical applications of liquid biopsy technologies, including circulating tumor cells, proteomics, miRNA, and cfDNA for NSCLC, are reviewed and insight is provided into the diagnostic and therapeutic implications and challenges of these platforms. ***The Oncologist*** 2016;21:1121–1130

Implications for Practice: Although tumor biopsies remain the reference standard for the diagnosis and genotyping of non-small cell lung cancer, they remain fraught with logistical complexities that can delay treatment decisions and affect clinical care. Liquid diagnostic platforms, including cell-free DNA, proteomic signatures, RNA (mRNA and microRNA), and circulating tumor cells, have the potential to overcome many of these barriers, including rapid and accurate identification of de novo and resistant genetic alterations, real-time monitoring of treatment responses, prognosis of outcomes, and identification of minimal residual disease. The present report provides insights into new liquid diagnostic platforms in non-small cell lung cancer and discusses the promise and challenges of their current and future clinical use.

Introduction

Lung cancer remains the leading cause of cancer-related mortality, with a 5-year survival rate of only 17% for all stages [1]. Despite advances in the development of targeted therapies and the emergence of novel immunotherapeutic approaches, significant challenges remain in the diagnosis and treatment of non-small cell lung cancer (NSCLC). First, although the adoption of low-dose helical computed tomography (LDCT) screening for high-risk patients might allow for earlier detection, roughly 65% of patients still present with advanced-stage disease, with more than half dying within 1 year [1]. Additionally, for patients receiving curative intent therapy, currently, no strategies outside of routine imaging are available to detect recurrent or minimal residual disease. Finally, standard tumor biopsies can be cumbersome, put the patient at risk, and might not accurately identify relevant molecular alterations owing to both suboptimal tissue acquisition and tumor heterogeneity. Given these challenges, additional strategies are urgently needed to help diagnose NSCLC

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1083-7159/2016/$20.00/0 http://dx.doi.org/10.1634/theoncologist.2016-0082

Table 1. Diagnostic approaches for the identification of genetic alterations

Method	Analysis	Allele-specific?	Sensitivity	Specificity
Quantitative PCR	Quantitative	No	High	Low (nontumor and tumor-derived DNA)
Sanger sequencing	Qualitative	Yes	Very low	High
Pyrosequencing	Qualitative	Yes	Low	Low
ARMS	Qualitative	Yes	Low	High
TAM-Seq	Qualitative	Yes	High	High
PNA clamp analysis	Qualitative	Yes	Low	High
Emulsion PCR (droplet digital PCR)	Qualitative	Yes	High	High
BEAMing	Qualitative	Yes	High	High
NGS	Quantitative and qualitative	No	High (varies depending on method)	High

Abbreviations: ARMS, amplification refractory mutation system; BEAMing, beads, emulsion, amplification, and magnetics; NGS, next-generation sequencing; PCR, polymerase chain reaction; PNA, peptide nucleic acid; TAM-Seq, tagged-amplicon deep sequencing.

and genotype tumor tissue from patients eligible for selection of molecularly driven therapies.

Recent technological advances have led to the development of blood-based diagnostics or "liquid biopsies" in NSCLC. This noninvasive approach allows for early detection of de novo or recurrent disease, the prognosis of outcomes, identification of genetic alterations to guide targeted therapy, and real-time monitoring of treatment response. Although liquid biopsies have historically referred to circulating tumor cells (CTCs), the definition has expanded to include proteomics, microRNA (miRNA), messenger RNA (mRNA), and, more recently, cell-free DNA (cfDNA). We review the technology of liquid biopsies in NSCLC and discuss the diagnostic and therapeutic implications of these platforms.

cfDNA

First discovered in 1948 by Mandel and Metais, fragmented DNA or cfDNA has since been associated with a number of conditions, including end-stage renal disease, myocardial infarction, stroke, and trauma [2–7]. Multiple studies have shown a correlation between the levels of cfDNA and cellular injury and necrosis, processes relevant in cancer cell survival and propagation. As tumors increase in volume, the capacity of phagocytes to eliminate and clear apoptotic and necrotic fragments can be exceeded, leading to passive release of cfDNA into the bloodstream [8]. Alternatively, in vitro studies have shown that DNA can be released by an active mechanism in which cancer cells spontaneously release DNA fragments into the circulation [9]. Depending on the tumor size and vascularity, the amount of cfDNA released in the circulation can vary from 0.01% to 90% of all DNA present in plasma [10].

Multiple studies have reported elevated cfDNA in lung cancer patients compared with healthy controls and increasing cfDNA concentrations in advanced disease compared with earlier stages of disease [11, 12]. Recent genomic technologies (including digital polymerase chain reaction [D-PCR], amplification refractory mutation system [ARMS], beads, emulsion, amplification, and magnetics [BEAMing], tagged-amplicon deep sequencing, and next-generation sequencing [NGS]) can detect low levels of cfDNA in plasma or serum and identify relevant genetic alterations (Table 1). Investigators have recently used cfDNA not only to identify patients with actionable mutations, including both sensitizing (exon 19 and 21) and resistant (T790M) *EGFR* mutations, but also as a real-time monitor of pharmacodynamic responses to tyrosine kinase inhibitor (TKI) therapy.

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Identifying Mutations in Treatment-Naïve Patients: Concordance With Tissue

One critical issue in blood-based genotyping is whether cfDNA can serve as an accurate molecular proxy for the corresponding tissue in the identification of *EGFR* mutations. Kimura et al. matched tumor and serum samples obtained from 42 advanced-stage NSCLC patients treated with gefitinib [13]. The concordance rate between *EGFR* mutations identified in serum and tissue was 92.9% (matching in 39 of 42 paired samples), with a sensitivity of 85.7%. High concordance rates were also demonstrated in a subset of patients from a phase IV, single-arm study evaluating first-line gefitinib in advanced-stage NSCLC patients [14]. The mutation concordance rate between 652 matched tumor and plasma samples before treatment was 94.3% (95% confidence interval [CI], 92.3–96.0), with a sensitivity of 65.7% (95% CI, 55.8–74.7) and specificity of 99.8% (95% CI, 99.0–100.0). Additional studies evaluating the concordance of plasma/serum and tissue *EGFR* mutations (Table 2) have reported a wide range of concordance rates (27.5%–100%) and sensitivities (17.1%–100%), with consistently high specificities (71.4%–100%). The disparities in sensitivities are heavily technology-dependent. Two recent meta-analyses assessing the diagnostic accuracy of cfDNA *EGFR* mutations demonstrated a pooled sensitivity of 61% and 67.4% and specificity of 90% and 93.5%, respectively [15, 16].

Table 2. Studies evaluating cfDNA *EGFR* mutations in patients with NSCLC

Study/samples	Detection method	Alleles	% Positive in serum/plasma (*n*/total study samples)	Matched tumor and serum or plasma samples (*n*)	Concordance, % (*n*/total)	Sensitivity, % (*n*/total)	Specificity, % (*n*/total)
Kimura et al. [21], 2006	ARMS	Exon 19 del, L858R	48.1 (13/27)	11 serum	72.7 (8/11)	50.0 (2/4)	86.0 (6/7)
Kimura et al. [68], 2006	Direct sequencing	Exon 19 del, L858R	77.8 (21/27)	12 serum	58.3 (7/12)	75.0 (3/4)	50.0 (4/8)
Kimura et al. [13], 2007	Direct sequencing and ARMS	Exon 19 del, L858R	16.7 (7/42)	42 serum	92.9 (39/42)	85.7 (6/7)	94.3 (33/35)
Bai et al. [22], 2009	DHPLC	Exon 19 del, L858R	34.3 (79/230)	230 plasma	74.0 (200/230)	81.8 (63/77)	89.5 (137/153)
Yung et al. [69], 2009	Digital microfluidics	Exon 19 del, L858R	42.9 (15/35)	35 plasma	96.4 (27/28)	92.0 (11/12)	100.0 (16/16)
Mack et al. [70], 2009	ARMS	Exon 19 del, L858R	57.1 (8/14)	14 plasma	57.1 (8/14)	66.7 (4/6)	50.0 (4/8)
Kuang et al. [30], 2009	ARMS; WAVE/ Surveyor	Exon 19 del, L858R	48.8 (21/43)	43 plasma	74.4 (32/43)	91.3 (21/23)	55.0 (11/20)
He et al. [71], 2009	Mutant-enriched PCR	Exon 19 del, L858R	49.3 (66/134)	18 plasma	94.4 (17/18)	88.9 (8/9)	100.0 (9/9)
Brevet et al. [23], 2011	Mutant-enriched PCR	Exon 19 del, L858R	22.6 (7/31)	31 plasma	58.1 (18/31)	38.9 (7/18)	84.6 (11/13)
Jiang et al. [72], 2011	Mutant-enriched PCR	Exon 19 del, L858R	24.1 (14/58)	58 serum	93.1 (54/58)	77.8 (14/18)	100.0 (40/40)
Sriram et al. [73], 2011	Mutant-enriched PCR	Exon 19 del, L858R	4.7 (3/64)	64 serum	95.3 (61/64)	50.0 (3/6)	100.0 (58/58)
Goto et al. [20], 2012	ARMS	Exon 19 del, L858R	25.6 (22/86)	86 serum	66.3 (57/86)	43.1 (22/51)	100.0 (35/35)
Xu et al. [74], 2012	ARMS	Exon 19 del, L858R	29.4 (15/51)	34 plasma	88.2 (30/34) [L858R]	50.0 (4/8) [L858R]	100.0 (26/26) [L858R]
Huang et al. [75], 2012	DHPLC	Exon 19 del, L858R	32.1 (270/822)	822 plasma	77.0 (633/822)	63.5 (188/296)	84.6 (445/526)
Zhang et al. [76], 2013	Liquidchip technology	Exon 19 del, L858R	26.7 (23/86)	86 plasma	91.9 (79/86) [exon 19 del]	68.2 (15/22) [exon 19 del]	100.0 (64/64) [exon 19 del]
Liu et al. [77], 2013	ARMS	Exon 19 del, L858R	31.4 (27/86)	86 plasma	84.9 (73/86)	67.5 (27/40)	100.0 (46/46)
Kim et al. [78], 2013	PNA-PCR	Exons 18 to 21 mutations or amplifications	14.0 (8/57)	57 serum	87.7 (50/57)	66.7 (8/12)	93.3 (42/45)
Kim et al. [79], 2013	PNA-PCR	Exon 19 del, L858R	16.7 (10/60)	40 plasma	27.5 (11/40)	17.1 (6/35)	100.0 (5/5)
Zhao et al. [80], 2013	Mutant-enriched PCR	Exon 19 del, L858R	14.4 (16/111)	111 plasma	71.2 (79/111)	35.6 (16/45)	95.5 (63/66)
Doulliard et al. [14], 2014	ARMS	Exon 19 del, L858R,	10.6 (69/652)	652 plasma	94.3 (615/652)	65.7 (69/105)	99.8 (546/547)
Wang et al. [81], 2014	ARMS	Exon 19 del, L858R	12.7 (17/134)	134 plasma	59.0 (79/134)	22.1 (15/68)	97.0 (64/66)
Jing et al. [82], 2014	High resolution melting analysis	Exon 19 del, L858R	37.5 (45/120)	120 plasma	85.0 (102/120)	66.4 (29/45)	97.3 (73/75)
Weber et al. [83], 2014	cobas *EGFR* tissue test	Exon 19 del, L858R	12.1 (24/199)	196 plasma	91.3 (179/196)	60.7 (17/28)	96.4 (162/168)
Sequist et al. [31], 2015	BEAMing	T790M	77.0 (190/247)	227 plasma	73.6 (167/227)	80.7 (155/192)	34.3 (12/35)
Husain et al. [32], 2015	MiSeq	T790M	68.1 (15/22)	22 urine	NR	66.7 (10/15)	NR

Abbreviations: ARMS, amplification refractory mutation system; cfDNA, cell-free DNA; DHPLC, denaturing high-performance liquid chromatography; NSCLC, non-small cell lung cancer; NR, not reported; PCR, polymerase chain reaction; PNA, peptide nucleic acid; TAM-Seq, tagged-amplicon deep sequencing.

Genetic interrogation of cfDNA via NGS has revealed additional mutations beyond *EGFR*. In one study, plasma-based NGS genotyped 8 of 11 patients and accurately identified two *ALK* rearrangements, one *ROS1* rearrangement, one *RET* rearrangement, one *EGFR* G719A mutation, one *KRAS* G12C, and one combined *KRAS* G12C/PIK3CA [17]. In a second study, 42 of 54 patients evaluated using plasma NGS had at least one identifiable alteration, with 7 patients linked to a U.S. Food and Drug Administration-approved drug and 17 eligible for a targeted therapy approved for another disease type [18]. Finally, using a plasma-based digital NGS platform with the capability of capturing point mutations, insertions/deletions,

fusion, and amplifications, Mack et al. retrospectively evaluated 978 patients with advanced NSCLC and identified actionable mutations in 412 patients (42%) [19]. These included activation *EGFR* mutations ($n = 116$), *ALK* fusions ($n = 6$), *RET* fusions ($n = 9$), *HER2* exon 20 insertions ($n = 9$), *BRAF* mutations ($n = 26$), *MET* exon 14 skipping mutation ($n = 8$), met amplifications ($n = 18$), *EGFR* exon 20 insertions ($n = 7$), and *KRAS/NF1/MEK* mutations ($n = 213$). Although tissue was not available for concordance rates, this technology remains promising.

Predictive and Prognostic Utility of *EGFR* Mutations Identified by cfDNA

The clinical promise of cfDNA is rooted in its ability to serve as a predictive biomarker for targeted therapy. In the aforementioned phase IV gefitinib study, patients with *EGFR* mutation-positive cfDNA, regardless of mutation subtype, had a similar overall response rate (ORR) as that of patients with tissue *EGFR* mutation-positive tumors (76.9% and 69.8%, respectively), suggesting that the blood-based EGFR test might be as predictive to TKI treatment as tissue [14]. The ORRs were higher in patients with matched samples who harbored mutations in both plasma and tissue (76.9%; 95% CI, 65.4–85.5) compared with patients with only mutation-positive tumor tissue (59.5%; 95% CI, 43.5–73.7). Similar results were demonstrated in an exploratory analysis of 233 patients enrolled in the Iressa Pan-Asia Study (IPASS) comparing gefitinib and carboplatin-paclitaxel in 1217 treatment-naïve patients clinically enriched for *EGFR* mutations [20]. Of 194 patients who provided pretreatment serum samples, 46 (23.7%) had cfDNA *EGFR* mutations identified by Scorpion ARMS. Although the improvement in ORR in patients treated with gefitinib ($n = 24$) compared with chemotherapy ($n = 22$) was not significant (ORR, 75% vs. 64%; $p = .40$), statistically significant improvement was found in progression-free survival (PFS) in the cfDNA *EGFR* subgroup (hazard ratio [HR], 0.29; 95% CI, 0.14–0.60; $p < .001$). A significant interaction between cfDNA *EGFR* mutation status and treatment was evident for PFS (interaction test, $p = .045$). Multiple other studies have demonstrated *EGFR* mutations identified in cfDNA to be a reliable predictive biomarker of TKI treatment [13, 21–25].

Recent studies have also demonstrated the predictive value of quantitative changes in *EGFR* mutations in cfDNA at different time points during TKI treatment. Tseng et al. prospectively evaluated matched serum and tissue samples from 62 advanced-stage EGFR-positive patients who had received gefitinib [26]. Evaluating cfDNA using the peptide nucleic acid-zip nucleic acid PCR clamp method at 10 weeks and on progression of disease, the study demonstrated that failure to clear plasma *EGFR* mutations was an independent predictor of a lower disease control rate (odds ratio [OR], 5.26; 95% CI, 1.13–24.44; $p = .034$), shorter PFS (HR, 1.97; 95% CI, 1.33–2.91; $p = .001$), and decreased overall survival (OS; HR, 1.82; 95% CI, 1.04–3.18; $p = .036$). Mok et al. used a cobas test (Roche Molecular Diagnostics, Pleasanton, CA, http://www.molecular.roche.com) to evaluate serum/plasma *EGFR* mutations in patients from a phase III study randomized to receive six cycles of gemcitabine/platinum plus sequential erlotinib or placebo [25]. For patients treated in the erlotinib arm who were EGFR positive by cfDNA at baseline, the disappearance of cfDNA at cycle 3 was associated with significantly improved PFS (HR, 0.38; $p = .0083$) and longer OS (HR, 0.45; $p = .0831$) compared with patients with persistence of cfDNA *EGFR*. Most recently, Marchetti et al. performed PCR and ultra-deep NGS on serial plasma samples of advanced-stage patients ($n = 20$) with known tissue and plasma *EGFR* mutations before TKI treatment. Patients who had a 50% decrease in plasma *EGFR* copy number at 14 days (rapid responders; $n = 14$) had a greater mean percentage of tumor shrinkage than that of slow responders ($n = 6$) who had not achieved this change (70% vs. 30%; $p < .0001$) [27]. Finally, using a deep sequencing method (cancer personalized profiling by deep sequencing) that quantifies cancer-specific genomic alterations, Newman et al. was able to demonstrate that plasma ctDNA levels isolated from longitudinal samples in advanced-stage patients ($n = 3$) receiving either chemotherapy or targeted therapy highly correlated with the tumor volumes during therapy [28]. In sum, the real-time pharmacodynamic monitoring of mutations, most notably *EGFR*, during treatment in these studies highlights the potential of this platform to serve as an early predictor of response or resistance to therapy that could inform treatment decisions.

The prognostic utility of cfDNA has been studied by the Spanish Lung Cancer Group, which reported on a prespecified analysis from the erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced *EGFR* mutation-positive non-small cell lung cancer (EURTAC) study of 76 patients with identifiable cfDNA *EGFR* mutations [29]. Evaluating subtypes of *EGFR* mutations in cfDNA using the TaqMan assay, the study demonstrated a shorter median OS in patients with the L858R mutation than in those with the exon 19 deletion (13.7 months; 95% CI, 7.1–17.7; vs. 30.0 months; 95% CI, 19.3–37.7; $p < .001$). Among the 41 patients with the L858R mutation in tissue, those in whom the L858R mutation was also detected in cfDNA had notably shorter median survival than those in whom the mutation was not detected in cfDNA (13.7 vs. 27.7 months; HR, 2.22; $p = .03$), suggesting a prognostic value of plasma cfDNA L858R mutations.

Identification of T790M in TKI-Resistant and -Naïve Patients

Several studies have identified resistant T790M mutations in cfDNA for both TKI-naïve and -resistant patients. Kuang et al. detected *EGFR* T790M in cfDNA from plasma via ARMS and/or the WAVE/Surveyor method in 15 of 28 patients with a previous clinical response to an *EGFR* TKI but only 4 of 14 with previous stable disease. *EGFR* T790M mutations detected in plasma DNA were strongly associated with a previous clinical response to TKI ($p = .004$) [30]. More recently, plasma BEAMing was used in a phase I/II trial evaluating rociletinib, a TKI selectively targeting both activating and T790M *EGFR* mutations in EGFR-positive, advanced-stage NSCLC patients who experienced disease progression during treatment with EGFR-directed therapy [31]. With tissue as the reference standard, the positive percentage of agreement for plasma T790M was 81% (155 of 192). Although the unconfirmed response rate to rociletinib in both plasma-positive and tissue-positive T790M patients was 53%, all plasma-positive patients were also tissue positive for T790M. Similar results have been demonstrated with osimertinib, another third-generation TKI, in

patients with documented plasma- or tissue-positive T790M disease. Finally, using a PCR method coupled with NGS, Husain et al. identified T790M mutation in the urine of 15 of 22 EGFR-positive patients (68%) receiving TKI treatment. Of the 15 patients positive for T790M by urine, 10 patients had T790M mutation in the tissue biopsy using the Clinical Laboratory Improvement Amendments test [32].

T790M identification in cfDNA has also been clinically detected in treatment-naïve patients and for monitoring the real-time response. Wang et al. evaluated 135 patients with advanced-stage NSCLC who had clinical benefit of 6 months or more (PFS >6 months) with first- or second-line TKI treatment and retrospectively analyzed *EGFR*-sensitizing mutations and T790M mutation in matched pre- and post-TKI plasma via multiple assays, including D-PCR [33]. In the cohort of 83 patients with known *EGFR* mutations by tissue, the patients were subdivided into three groups according to the quantity of T790M in pre-TKI plasma samples by D-PCR (high, >5%, $n = 7$; low, 0%–5%, $n = 20$; and nil, 0%, $n = 56$). The median PFS was 7.1, 9.5, and 12.8 months ($p = .001$) and the median OS was 18.2, 21.2, and 32.5 months ($p = .005$) for the high, low, and nil groups, respectively, suggesting that a high pretreatment T790M mutational load might define patients less likely to benefit from TKI therapy. Sorensen et al. used allele-specific PCR to identify both sensitizing and resistant *EGFR* mutations from plasma in 23 patients with advanced adenocarcinoma receiving second-line TKI therapy as part of larger clinical trial [34]. cfDNA was evaluated at multiple time points during treatment and revealed T790M mutations in 9 patients up to 344 days before disease progression using the Response Evaluation Criteria In Solid Tumors, illustrating the potential to identify resistant disease before routine imaging. In the two aforementioned studies by Wang et al. and Sorenson et al., T790M was not interrogated in tissue samples; thus, concordance rates could not be established. It remains unclear whether identification of plasma or tissue T790M, either de novo or before clinical progression with TKI therapy, should guide treatment decisions with T790M-directed therapies.

Proteins

A number of proteins originating from the tumor and tumor microenvironment have been studied as biomarkers for NSCLC. Historically, protein biomarkers have included carcinoembryonic antigen, cytokeratin-19 fragment, and squamous cell carcinoma antigen. Although several studies have demonstrated the prognostic and predictive value of these markers, they have generally had limited clinical utility in lung cancer detection or as surrogates for response, given their relatively low sensitivity and specificity. More recently, a mass spectrometry (e.g., matrix-assisted laser desorption ionization) classifier has been used to identify proteomic signatures. These signatures are the basis of an algorithm that has resulted in a commercially available test that classifies advanced-stage patients into "poor" or "good" groups compared with a reference set. This test has served in both a predictive and a prognostic capacity for advanced-stage NSCLC patients receiving second-line therapy with either docetaxel or erlotinib [35]. Several studies have also highlighted the prognostic utility of this platform in advanced NSCLC patients receiving first-line therapy, independent of other common prognostic factors [35–38].

mRNA and miRNA

miRNAs are small noncoding RNAs predicted to regulate 10%–30% of the protein-coding genes in the human genome. They have been shown to play important roles in tumor initiation, invasion, and metastasis [39]. Circulating miRNAs are considered attractive cancer biomarkers owing to their stability and quantifiability using quantitative reverse transcription PCR (qRT-PCR) [40] (Table 3). For NSCLC, circulating miRNAs have been investigated for their potential in diagnosis, prognostic value, and predictive response to treatment. A number of miRNA signature profiles have been identified for NSCLC.

Diagnosis and Screening of Lung Cancer

Multiple studies have reported on the potential utility of miRNAs to diagnosis NSCLC. Shen et al. determined that a panel of four miRNAs in plasma (miRNA-21, miRNA-126, miRNA-210, and miRNA-486-5p) were significantly elevated in NSCLC patients ($n = 58$) compared with healthy individuals ($n = 29$), with 86% sensitivity and 97% specificity [41]. In an exploratory phase I/II biomarker study, Hennessey et al. identified a miRNA pair (miRNA-15b and miRNA-27b) able to distinguish between NSCLC ($n = 55$) and cancer-free patients ($n = 75$), with a specificity of 84% (95% CI, 0.73–0.91) and sensitivity of 100% (95% CI,0.93–1.0) [42]. Leidinger et al. used qRT-PCR analysis to identify a set of five markers able to separate lung cancer patients ($n = 74$) from healthy controls ($n = 20$), with a specificity of 98% (95% CI, 0.95–1.0) and sensitivity of 91% (95% CI, 0.87–0.94) [43]. In addition, they were able to differentiate NSCLC patients ($n = 74$) from patients with chronic obstructive pulmonary disease ($n = 26$), with a specificity of 81% and sensitivity of 86% using 10 miRNA markers.

miRNAs have also been used to screen for individuals at risk of developing NSCLC. After identifying a set of 10 miRNAs that were differentially expressed in the serum of NSCLC patients compared with cancer-free controls, Chen et al. used the miRNA set to retrospectively analyze the serum samples of 7 NSCLC patients before and after their diagnosis [44]. They found that their miRNA profile could identify cancer up to 33 months ahead of the clinical diagnosis. Boeri et al. identified specific plasma miRNA signatures associated with lung cancer among patients enrolled in two different spiral-CT screening trials [45]. The miRNA signature classifier (MSC) algorithm was then validated in a study retrospectively evaluating 939 plasma samples (69 with lung cancer and 870 without) from the Multicenter Italian Lung Detection (MILD) clinical trial comparing LDCT ($n = 652$) and observation ($n = 287$) [46]. The MSC had a sensitivity of 87% and specificity of 81% across both arms; LDCT had a sensitivity of 79% and specificity of 81%. Using MSC and LDCT together resulted in a fivefold reduction of the LDCT false-positive rate (from 19.4% to 3.7%). The investigators calculated that the screening sensitivity could be increased to 98% using both tests [46]. Bianchi et al. compared serum samples from 93 patients enrolled in a spiral CT screening study with a diagnosis of NSCLC with serum samples from 60 individuals in the same study without cancer [47]. They found a 34-miRNA signature that could differentiate asymptomatic high-risk individuals with early-stage lung cancer ($n = 34$) from

Table 3. Studies reporting circulating miRNAs as biomarkers for NSCLC

Study	Study design	miRNAs of interest	Comparison	Results
Hu et al. [48], 2010	Long vs. short survival	miR-486, miR-30d, miR-1, and miR-499	123 patients split between low- and high-risk groups	for 4 high-risk miRNA carriers, HR, 34.13 (95% CI, 16.28–71.56; $p < .001$)
Boeri et al. [45], 2011	Risk of developing of cancer	miR-660, miR-140-5p, miR-451, miR-28-3p, miR-30c, and miR-92a	15 patients vs. 10 controls	AUC, 0.85 (sensitivity, 80%; specificity, 90%, $p < .0001$)
Zheng et al. [49], 2011	Cancer vs. control	miR-155, miR-197, and miR-182	74 patients vs. 68 controls	AUC, 0.9012 (95% CI, 0.8511–0.9513; sensitivity, 81.33%; specificity, 86.76%)
Shen et al. [41], 2011	Cancer vs. control	miR-21, miR-126, miR-210, and miR-486-5p	58 NSCLC patients vs. 29 controls	AUC, 0.926 (sensitivity, 82.22%; specificity, 96.55%)
Yuxia et al. [84], 2012	Cancer vs. control	miR-125b	193 NSCLC patients vs. 110 controls	HR, 2.46 (95% CI, 1.80–3.38; $p < .0001$)
Le et al. [85], 2012	Cancer vs. control	miR-24	82 patients vs. 50 controls	AUC, 0.855 (95% CI, 0.768–0.918; sensitivity, 82.6%; specificity, 80.0%)
Liu et al. [86], 2012	Poor vs. good prognosis	miR-21, miR-200c	70 NSCLC patients	HR, 4.316 (95% CI, 1.265–19.206; $p = .046$)
Tang et al. [87], 2013	Cancer vs. control	miR-21, miR-145, and miR-155	34 patients vs. 30 patients with benign pulmonary nodules vs. 32 healthy smokers	AUC, 0.872 (sensitivity, 76.5%; specificity, 81.3%)
Li et al. [88], 2014	Cancer vs. control	miR-499	514 NSCLC patients vs. 54 controls	AUC, 0.906 (95% CI, 0.879–0.929; sensitivity, 73.7%; specificity, 92.7%)
Yu et al. [89], 2014	Poor vs. good prognosis	miR-375	53 NSCLC patients	HR, 2.760 (95% CI, 1.418–5.375; $p = .003$)
Geng et al. [90], 2014	Cancer vs. control	miR-223	126 NSCLC patients vs. 42 NCPD patients vs. 60 controls	AUC, 0.96 (95% CI, 0.94–0.98; sensitivity, 87%; specificity, 86%)
Tejero et al. [91], 2014	High vs. intermediate vs. low risk	miR-141, miR-200c	73 adenocarcinoma patients	5-year OS: 49.4% for high-risk patients, 66.7% for intermediate-risk patients, 100% for low-risk patients ($p = .002$)
Powrózek et al. [92], 2015	Cancer vs. control	miR-944, miR-3662	60 NSCLC and 30 SCLC patients vs. 85 controls	AUC, 0.909 (95% CI, 0.830–0.959; sensitivity, 81.5%; specificity, 92%)
Wozniak et al. [93], 2015	Cancer vs. control	24 miRNA panel	100 stage I–IIIa NSCLC patients vs. 100 controls	AUC, 0.92 (95% CI, 0.87–0.95; sensitivity, 83%; specificity, 80%)
Powrózek et al. [94], 2015	Cancer vs. control	miR-448, miR-4478	65 NSCLC and 35 SCLC vs. 85 controls	AUC, 0.896 (95% CI, 0.778–0.961; sensitivity, 90%; specificity, 76.3%)
Wang et al. [95], 2015	Cancer vs. control	miR-145	70 NSCLC patients vs. 70 controls	AUC, 0.84 (95% CI, 0.78–0.91; sensitivity, 92.75%; specificity, 61.43%)
Yang et al. [96], 2015	Cancer vs. control	miR-148a, miR-148b, miR-152, miR-21	152 NSCLC patients vs. 300 controls	AUC, 0.98 (95% CI, 0.95–0.99; sensitivity, 96%; specificity, 91%)
Leidinger et al. [43], 2015	Cancer vs. control	hsa-miR-20b-5p, hsa-miR-20a-5p, hsa-miR-17-5p, and hsa-miR-106a-5p	74 NSCLC patients, 26 COPD patients without lung cancer, and 20 controls	AUC, 0.978; sensitivity, 91% (95% CI, 0.875–0.945); specificity, 98% (95% CI, 0.957–1.00)

Abbreviations: AUC, area under the curve; CI, confidence interval; COPD, chronic obstructive pulmonary disease; HR, hazard ratio; miR, microRNA; NCPD, noncancerous pulmonary disease; NSCLC, non-small cell lung cancer; OS, overall survival; SCLC, small cell lung cancer.

individuals with no cancer ($n = 30$), with a sensitivity of 71% and specificity of 90%. Evaluating 13 patients before and after they developed lung cancer, the investigators also applied a risk predictor algorithm based on the miRNA signature that showed a significantly increased risk index in the serum after the onset of the disease ($p < .001$, paired t test).

Prognosis and Predictive Response to Treatment

Using genome-wide sequencing, Hu et al. found 11 miRNAs were altered more than fivefold in the serum of lung cancer patients who survived for more than 30 months compared with those who had survived for less than 25 months [48]. Of the 11 miRNAs, 4 (miR-486, miR-30d, miR-1, and miR-499) were significantly associated with overall survival. Patients carrying two or more high-risk miRNAs had a significantly increased risk of cancer death compared with patients with one or no high-risk miRNA (log-rank test, $p < .0001$; HR, 3.14; 95% CI, 1.65–5.97, for two high-risk miRNA carriers; HR, 16.52; 95% CI, 8.62–31.68, for three high-risk miRNA carriers; HR, 34.13; 95% CI, 16.28–71.56, for four high-risk miRNA carriers). miR-155 and miR-197 levels have been reported to be higher in lung cancer patients with metastasis ($p < .05$) and to be significantly decreased in patients with a response to chemotherapy ($p < .001$) [49].

In addition to miRNA, messenger RNAs (mRNAs) have also been evaluated in early- and late-stage lung cancer patients. Cancer/testis antigens (CTAs) are protein antigens normally

expressed in the testis but frequently expressed in lung cancer. Gumireddy et al. used a nested PCR assay to determine whether the mRNA levels of 116 *CTA* genes in the peripheral blood mononuclear cells of NSCLC lung cancer patients were differentially expressed compared with individuals with smoking-related benign diseases [50]. The expression of one gene, *AKAP4*, was able to distinguish between NSCLC patients ($n = 264$) and individuals with benign disease ($n = 135$), as well as those with benign nodules, with an area under the curve of 0.9714 (95% CI, 0.956–0.986) and 0.9825 (95% CI, 0.969–0.995), respectively. Similar to cfDNA, the investigators found that AKAP4 increased with cancer stage, with AKAP4 mRNA expression levels in stage IV NSCLC patients 3,254-fold higher than that in patients with stage I NSCLC. AKAP4 mRNA expression did not have a significant association with tobacco use, which might allow AKAP4 to be used as a screening tool for both smokers and nonsmokers.

Given that only a subpopulation of NSCLC CTCs undergo epithelial-to-mesenchymal transition and downregulate their epithelial markers, the detection rates using EpCAM-based methods have been lower.

CTCs

CTCs are tumor cells shed into the blood by solid neoplasms. They are exceedingly rare, with as few as one in every 10^9 blood cells in patients with metastatic disease [51]. A number of methods have been developed to detect CTCs, including the CellSearch system (Janssen Diagnostics, Raritan, NJ, http://www.janssen.com), the Intelligent System Emulation Technology (ISET) system (Aligent Technologies, Santa Clara, CA, http://www.aligent.com), flow cytometry, laser scanning cytometry, PCR-based approaches, and CTC microchip technology [52–55]. Following CTC isolation, DNA can then be extracted and analyzed in the same manner as cfDNA, which has revealed *EGFR* mutations [56, 57], *ALK* rearrangements [58, 59], and *ROS1* rearrangements [60]. Although CTCs have been identified in up to 85% of small cell lung cancer patients, the reported detection rates in NSCLC using epithelial cell adhesion molecule (EpCAM)-based methods have been significantly lower [61]. Given that only a subpopulation of NSCLC CTCs undergo epithelial-to-mesenchymal transition and downregulate their epithelial markers, the detection rates using EpCAM-based methods have been lower. Although higher CTC detection rates have been reported using non-EpCAM-based methods, these methods need to be validated further in independent and large multicenter studies.

Prognosis and Predictive Response to Treatment

CTCs have been shown to have prognostic significance in metastatic NSCLC [62, 63]. Using ISET, Hofman et al. isolated CTCs from 208 NSCLC patients and found that a cutoff value of >50 corresponded to shorter OS and PFS. In a global meta-analysis, the appearance of pretreatment CTCs in patients with different stages of lung cancer correlated with lymph node status, distant metastasis, and TNM staging [64]. Longitudinal CTC monitoring has also shown that patients with more than one time point positive for CTCs had worse survival than those with conversion from CTC positive to CTC negative or persistently negative [65].

Presence of *EGFR* Mutations and Disease Monitoring

Breitenbuecher et al. used combined RT-PCR and melting curve analysis to show that an increase in *EGFR*-mutant CTCs might be an indicator for disease relapse and EGFR-TKI resistance [56]. Additionally, just as in cfDNA, T790M identification in CTCs has been used in TKI-resistant patients and to monitor the real-time response. In NSCLC patients treated with EGFR-TKIs, Maheswaran et al. found T790M mutations in CTCs from 9 of 14 patients (64%) with clinical progression, and the presence of the mutation correlated with reduced PFS (7.7 months vs. 16.5 months; $p < .001$) [57].

Discussion

Despite recent therapeutic advances in NSCLC, significant diagnostic challenges remain that can bottleneck treatment decisions and negatively affect clinical care. The liquid diagnostic platforms we have reviewed have the potential to overcome many of these barriers. Perhaps the greatest immediate clinical effect will be the routine use of cfDNA to identify de novo and resistant genetic alterations in patients with advanced-stage disease (i.e., plasma genotyping). The limitations of tumor biopsies have recently been supported by a study that demonstrated up to 30% of patients at a community-based academic center did not undergo guideline-recommended molecular testing, despite an institutional reflex testing policy for tissue [66]. In addition, repeat tissue acquisition poses barriers to clinical trial enrollment, as demonstrated by a recent study retrospectively evaluating patient enrollment to 55 clinical trials at a single Canadian institution in which fewer patients received study treatment in trials mandating tissue specimens compared with trials that did not (55% vs. 83%; $p < .001$) [67]. Plasma cfDNA platforms offer promise in overcoming these challenges by rapidly genotyping treatment-naïve and -refractory patients and might eventually obviate the need for the repeat biopsies mandated by many clinical trials.

In addition to plasma genotyping, liquid platforms have tremendous potential to improve screening in patients at high risk of both first cancers and recurrences. The goal to identify early stage or minimal residual disease before or in combination with routine imaging remains a challenge. Although the U.S. National Lung Screening Trial (NLST) demonstrated a 20% reduction in lung cancer mortality with LDCT scans, the high false-positive rate with this modality warrants further enrichment strategies. The accuracy of mRNA and miRNA platforms in delineating benign from malignant nodules is promising, with the potential to enhance the sensitivity and specificity of screening techniques. These platforms might also contribute to surveillance strategies after curative intent therapy. A recent study of colorectal cancer patients undergoing curative-intent surgery reported cfDNA identified after resection in all patients who experienced eventual recurrence but not in the patients who remained disease free [10].

Plasma cfDNA platforms offer promise in overcoming these challenges by rapidly genotyping treatment-naïve and refractory patients and might eventually obviate the need for the repeat biopsies mandated by many clinical trials.

Despite the promise of liquid diagnostics in lung cancer, significant challenges remain. In the presence of tumor heterogeneity, it is uncertain whether cfDNA sampling provides a reliable molecular proxy of overall tumor biology and whether plasma or tissue is the true reference standard for molecular characterization of disease. Further studies evaluating the predictive utility of genetic alterations identified in cfDNA independent of those identified by tissue interrogation are urgently needed. Additionally, currently, no widely accepted standards are available for processing samples, which might contribute to the disparate test results. Performance verification and method development, including optimal preparation strategies (collecting, isolating, and storing cfDNA), and downstream analysis need to be carefully formalized. Coordination and communication between laboratories of competing platforms would help facilitate this process. Finally, given the wide range of reported sensitivities and specificities of the different platforms, it will be essential for prospective therapeutic trials to mandate the collection of plasma and/or serum to establish reproducible concordance rates with tissue for clinical validation. Special attention should be devoted to the impact of both clinical (tumor burden) and sampling variables on the accuracy and reproducibility of these platforms. It is important to note that, to date, very few, if any, published series have evaluated the accuracy of specific commercially available platforms or their concordance with paired tissue. Thus, caution should be used when interpreting the results. In addition, many of these assays' threshold for detection have been set arbitrarily, with different internal cutoff levels, making the interpretation of some results challenging.

In addition to technical considerations, the application of liquid platforms might create new therapeutic dilemmas. For instance, whether pharmacodynamic monitoring of either *EGFR* or T790M in cfDNA should be performed and guide therapeutic decision making before clinical or radiographic progression for patients receiving TKI therapy remains unclear. Likewise, whether treatment of minimal residual disease identified in plasma only would improve survival for patients after curative intent therapy is also unknown. Prospective trials evaluating treatment decisions triggered by plasma tests, independent of the clinical or radiographic findings, for both early- and advanced-stage patients should be pursued. We look forward to the further development and refinement of liquid platforms and their routine use in accurately identifying recurrent and de novo disease and genotyping patients with all stages of NSCLC.

Author Contributions

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Collection and/or assembly of data: Benjamin Levy, Zishuo I. Hu, Kristen N. Cordova, Sandra Close, Karen Lee, Daniel Becker
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Disclosures

Benjamin Levy: Celgene, AstraZeneca, Pfizer, Genentech, Biodesix, Eli Lilly, Merck (C/A), Genetech, Eli Lilly (H); **Kristen N. Cordova:** Biodesix (Medical Affairs) (E, former); **Sandra Close:** Biodesix (C/A); **Karen Lee:** Pfizer, Bristol-Myers Squibb, Merck (H). The other authors indicated no financial relationships.

(C/A) Consulting/advisory relationship; (RF) Research funding; (E) Employment; (ET) Expert testimony; (H) Honoraria received; (OI) Ownership interests; (IP) Intellectual property rights/inventor/patent holder; (SAB) Scientific advisory board

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