The potential of liquid biopsies

Anna Buder, Christian Tomuta, and Martin Filipits

Purpose of review
This article discusses the current status and applications of liquid biopsy in nonsmall cell lung cancer (NSCLC).

Recent findings
The discovery of genetic alterations which are responsible for the development and progression of NSCLC led to the identification of a new generation of molecular biomarkers. However, in NSCLC, it is often difficult in clinical practice to obtain sufficient tumor material for genetic analyses. Therefore, analyses of tumor-specific genetic alterations in the serum or plasma of the patients are particularly valuable because they can provide temporal measurements of the total tumor burden as well as identify specific mutations that arise during therapy. The procedure of taking blood samples to detect tumor-specific genetic alterations is termed 'liquid biopsy'. In particular, it can be used for a variety of clinical and research applications, including response assessment in epidermal growth factor receptor (*EGFR*)-mutated NSCLC patients receiving EGFR tyrosine kinase inhibitor therapy. It has been demonstrated that liquid biopsy is a fast and easy way to obtain information on tumor burden and assess the changes of the molecular nature of a tumor during the course of therapy. However, because of the limited amount of tumor material in the blood and yet insufficient knowledge of specific cancer biomarkers, extensive research has to be continued in this field to implement this method into clinical routine.

Summary
In this review, we highlight the opportunities and clinical as well as research applications of liquid biopsy in NSCLC patients.

Keywords
circulating cell-free tumor DNA, circulating tumor cells, exosomes, resistance monitoring, therapy monitoring

INTRODUCTION

Although tumor tissue is still the gold standard source for clinical genetic analyses, cancer-derived material circulating in the bloodstream has become a promising target for tumor genotyping. The procedure of taking blood samples to detect tumor-specific genetic alterations is termed 'liquid biopsy'. There are several sources of tumor DNA that can be noninvasively assessed in the blood of the cancer patients by liquid biopsy: circulating cell-free DNA (cfDNA) [1], circulating tumor cells (CTCs) [2], and exosomes [3]. cfDNA is composed of small fragments of nucleic acids that are not associated with cells or cell fragments, whereas CTCs represent intact, viable cells that can be purified from blood. Exosomes are extracellular vesicles that contain RNA, DNA fragments, proteins, and metabolites that may also serve as molecular biomarkers.

Although tissue biopsies are not always possible because of the poor performance status of many advanced nonsmall cell lung cancer (NSCLC) patients, blood samples are easily obtainable and can be taken repeatedly even in short time intervals. In addition, genetic heterogeneity of the progressing tumor may lead to an incomplete picture of the tumor genome if only single tissue biopsies are used. Furthermore, blood-based analytic approaches may allow real-time monitoring of the total tumor burden and the detection of upcoming mutations that arise during clinical treatment through serial blood sampling and analysis. Blood samples can be collected during routine care at the time of diagnosis, before first-line therapy, and at subsequent time points when the tumor is progressing on therapy.

Institute of Cancer Research, Department of Medicine I, Comprehensive Cancer Center Vienna, Medical University of Vienna, Vienna, Austria

Correspondence to Martin Filipits, PhD, Institute of Cancer Research, Department of Medicine I, Comprehensive Cancer Center Vienna, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria. Tel: +43 1 40160 57528; e-mail: martin.filipits@meduniwien.ac.at

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KEY POINTS

- Liquid biopsy comprises a set of blood-based analyses to assess tumor-specific genetic alterations, therapy response, and resistance development.
- cfDNA consists of small fragments of nucleic acids that are not associated with cells or cell fragments.
- CTCs represent intact, viable tumor cells that can be purified from blood.
- Exosomes are extracellular vesicles that contain nucleic acids, proteins, and metabolites.

CIRCULATING CELL-FREE DNA

cfDNA consists of DNA fragments originating from apoptotic and necrotic tumor cells but also normal cells that are released into the bloodstream [4]. Although the amount of cfDNA in the blood can vary, cancer patients tend to have higher levels of cfDNA [5].

The detection of tumor-derived, circulating cfDNA is particularly technically challenging because tumor-derived cfDNA levels are often extremely low, and the discrimination of mutated cfDNA from wild-type cfDNA demands very high sensitivity and accurate quantification of these mutated fragments. Although for various solid tumors levels of tumor-derived cfDNA were shown to correlate with tumor stage and are frequently elevated in patients with metastatic cancers, the variability of the fraction of tumor-derived cfDNA within background levels of normal cfDNA can be very high [6▪▪].

Several detection methods can be used for the analysis of tumor-derived cfDNA, including digital PCR (dPCR), BEAMing (beads, emulsion, amplification, and magnetics), pyrophosphorolysis-activated polymerization, and tagged-amplicon deep sequencing. In general, digital genomic approaches are more sensitive than nondigital approaches and will most likely be used for the analyses of cfDNA in the future. The sensitivity of these techniques is limited by the low number of tumor-derived cfDNA copies and error rates of the polymerases used for PCR. However, in particular for patients with advanced disease, sensitivity ranges from 74 to 100% for the detection of the L858R mutation and from 29 to 71% for the detection of the T790M mutation – depending on the detection method [7,8,9▪].

Recently, a comparison of several platforms for the detection of epidermal growth factor receptor (*EGFR*) mutations in circulating cfDNA in advanced NSCLC patients was performed [8]. Circulating cfDNA samples from 38 NSCLC patients treated within the AURA trial program were analyzed using two nondigital platforms (cobas and therascreen) and two digital platforms [droplet digital PCR (ddPCR) and BEAMing dPCR] and compared with the results obtained using tumor tissue. For the L858R mutation, the sensitivity was 90% for cobas, 78% for therascreen, 90% for ddPCR, and 100% for BEAMing dPCR; specificity was 100% for cobas, 100% for therascreen, 100% for ddPCR, and 93% for BEAMing dPCR. More importantly, for the T790M resistance mutation, the sensitivity was 41% for cobas, 29% for therascreen, 71% for ddPCR, and 71% for BEAMing dPCR; specificity was 100% for cobas, 100% for therascreen, 83% for ddPCR, and 67% for BEAMing dPCR. Thus in this study the digital platforms outperformed the nondigital platforms [8].

Tumor-derived circulating cfDNA may provide a valuable source of material for monitoring the therapeutic response of NSCLC patients. *EGFR* mutations are detected in approximately 10–15% of NSCLCs from Caucasian patients. *EGFR*-mutated NSCLCs depend on EGFR signaling for growth and survival and are sensitive to treatment with EGFR tyrosine kinase inhibitors (TKIs). Among patients with advanced *EGFR*-mutated NSCLC, treatment with EGFR TKIs (e.g., gefitinib, erlotinib, and afatinib) is associated with response rates of 56–74% and a median progression-free survival (PFS) of approximately 12 months.

Despite initial responses to EGFR TKIs, the majority of patients will relapse within 1–2 years (acquired resistance). In approximately two-thirds of these patients, the mechanism of acquired resistance is the development of a resistance mutation, the *EGFR* T790M mutation [10,11]. This mutation leads to an enhanced affinity for ATP, thus reducing the ability of ATP-competitive reversible EGFR TKIs to bind to the tyrosine kinase domain of EGFR. One strategy to overcome this mechanism of resistance is through the use of irreversible third-generation EGFR inhibitors such as AZD9291 and rociletinib [12▪,13▪]. Recent clinical trials showed that these third-generation EGFR TKIs are highly active in patients with advanced *EGFR*-mutated NSCLC who relapsed after treatment with EGFR inhibitors [12▪,13▪].

A major limitation in the clinical implementation of these third-generation EGFR TKIs in patients with NSCLC is the challenge of tumor rebiopsy to detect the T790M mutation. Analysis of circulating cfDNA has the potential to enable noninvasive assessment of the *EGFR* mutation status in patients with advanced NSCLC. Continuous monitoring of the tumor genotype would also be important for

the early identification of emerging changes in tumor biology leading to acquired resistances against initially effective TKIs. Several studies have now suggested that highly sensitive genotyping assays such as ddPCR can indeed detect *EGFR* sensitizing and resistance mutations in circulating cfDNA from patients with *EGFR*-mutated advanced NSCLC [7,9▪,14]. A correlation between the plasma concentration of exon 19 deletions and L858R mutation in pretreatment plasma samples assessed by dPCR and response to treatment was observed [14]. Decreased concentrations were observed in all patients with partial or complete clinical remission, whereas persistence of mutation was observed in one patient with cancer progression [14].

More recently, in 62 advanced NSCLC patients with *EGFR* activating mutations, pretreatment plasma used for *EGFR* mutation testing showed a sensitivity of 59.7% and a specificity of 100% [7]. Detection sensitivity correlated significantly with stage of the disease (78.0% in stage IV-M1b versus 23.8% in stage IIIb or IV-M1a, $P < 0.001$). These patients received first-line EGFR TKI therapy. Detection of *EGFR* mutations in the plasma after EGFR TKI treatment was significantly associated with a lower disease control rate [odds ratio 5.26, 95% confidence interval (CI) 1.13–24.44; $P = 0.034$], shorter PFS (hazard ratio 1.97, 95% CI 1.33–2.91; $P = 0.001$), and shorter overall survival (hazard ratio 1.82, 95% CI 1.04–3.18; $P = 0.036$) [7].

Oxnard *et al.* [9▪] showed that plasma monitoring and analysis of circulating cfDNA can serve as a diagnostic biomarker for T790M-mediated resistance. Plasma monitoring allows prediction of therapy resistance several weeks before radiographic progression in advanced NSCLC patients receiving first-line erlotinib treatment. Applying ddPCR, a plasma response to first-line erlotinib treatment could be monitored with plasma levels of the *EGFR* sensitizing mutation decreasing or even approaching zero in six out of nine patients. In addition, the T790M resistance mutation emerged simultaneously within 4–14 weeks before radiographic progression was detectable in these patients [9▪].

Baseline cfDNA concentration in plasma of NSCLC patients treated with chemotherapy was shown to have independent prognostic value. Patients with high cfDNA concentrations at baseline have a significantly shorter overall survival compared with patients with lower concentrations (median 10 versus 14.2 months, $P = 0.001$). However, no association of cfDNA concentrations and response to chemotherapy could be shown [15].

These findings indicate the potential benefit of employing liquid biopsies in clinical routine for the evaluation of early treatment response, monitoring of minimal residual disease, and assessment of evolution of resistance in real time, particularly in NSCLC patients treated with EGFR TKIs.

Nevertheless, large prospective studies are needed to implement standards for preanalytical processing of blood samples and to validate the analysis of circulating cfDNA.

CIRCULATING TUMOR CELLS

CTCs are relatively easily accessible because of their presence in blood but, in most cases, they are rare against a large background of normal cells and have to be enriched for further investigations. CTCs can be present throughout the course of the disease, from very early to advanced stages, enabling the researcher to assess disease progression [2]. Particularly after curative treatment of cancer patients, continuous monitoring would allow the detection of residual tumor cells that may cause recurrence of the cancer. Probably, the most convincing argument for using CTCs is the short time of analysis: progression during chemotherapy may be followed by the amount of CTCs in the blood in real time and the treatment may also be adjusted accordingly [2].

First trials have been performed, indicating promising advancements for clinical application.

A study of Ilie *et al.* [16] demonstrated that CTCs can be detected in patients with chronic obstructive pulmonary disease without clinically detectable lung cancer. As a consequence of early detection by blood screening, early surgical removal of the tumor decreased the risk of tumor recurrence [16].

Analysis of CTCs in patients who had been treated with an EGFR TKI revealed the expected *EGFR*-activating mutation in CTCs from 11 of 12 patients (92%) [17]. In addition, the T790M resistance mutation was detected. Interestingly, serial analysis of CTCs showed that a reduction in the number of captured cells was associated with response to EGFR TKI treatment, and an increase in the number of cells was associated with tumor progression [17].

Monitoring of CTCs was performed in patients with advanced NSCLC who were enrolled in a phase II clinical trial of pertuzumab and erlotinib. Decreased CTC count after treatment was significantly associated with higher response rates ($P = 0.0019$) and longer PFS ($P = 0.05$) [18].

However, there are several drawbacks in the analysis of CTCs. Generally, CTC count in the blood is low even in late-stage cancers with approximately one CTC among 100 million blood cells [19]. In NSCLC patients, high tumor burden does not seem to be clearly correlated with the amount of CTCs in

the bloodstream. Moreover, the influence of other tumor characteristics like invasiveness and vascularity on the quantity of CTCs is not yet clarified [17]. To separate CTCs from normal cells, molecular biomarkers that are exclusively expressed in tumor cells have to be chosen. Currently, epithelial cell adhesion molecule (EpCAM) expression is the most commonly used biomarker for distinction and subsequent purification of CTCs. However, epithelial-to-mesenchymal transition may cause downregulation of epithelial markers such as EpCAM and, therefore, EpCAM-based detection may be impossible [20]. In addition, the equipment that needs to be acquired for detection and purification of CTCs is expensive and no standardized protocols for their isolation exist. Despite these shortcomings, the analysis of CTCs may provide an excellent, minimally invasive method for staging and response assessment [21]. However, the extraction of cfDNA from plasma is easier compared with the capture of CTCs and requires no specific instrumental facilities [6▪▪]. Especially in NSCLC, monitoring of evolving mutations in the tumor genotype seems to be reflected in cfDNA in the blood. Hence, non-invasive genotyping of cfDNA provides an important tool to observe therapy response prior to tumor progression.

EXOSOMES

Only recently the potential of exosomes isolated from the bloodstream of cancer patients as novel biomarkers was identified [3]. Exosomes are membrane-encapsulated vesicles containing different types of nucleic acids and proteins from the cell they originate from, thereby operating as information carriers between cells. Owing to the fact that exosomes deliver information both to their close environment and to distant organs, they are detectable in many biological fluids, for example, blood, thus making them easily accessible for research.

Most importantly, exosomes reflect protein expression and DNA mutations of their origin. Studies revealed that, for example, mutated, activated receptors of the EGFR family in NSCLC and cell adhesion molecules such as EpCAM in epithelial tumors can be detected in exosomes [22].

However, considering that exosome release is not limited to cancer cells, a generalized biomarker for the characterization of malignant diseases or their treatment is complex [23,24]. Nonetheless, studies have shown that cancer cells secrete increased amounts of specific microRNAs into exosomes, for example, for stimulation of macrophages leading to tumor dissemination in the case of lung cancer and that in certain other cancer types, microRNA signatures are different in localized and metastatic cancer [25]. Thus, it can be assumed that each subtype of lung cancer has its own RNA, DNA and/or protein signature in exosomes, dependent on its stage, its inclination to metastasize, and possibly even influenced by drugs, making exosomes ideal candidates for analysis of malignant diseases. Furthermore, exosomes could be comprised in cancer treatment, for example, by elimination of those that are involved in malignant signaling, by reprogramming their content, or by loading them with drugs.

CONCLUSION

A major progress in the treatment of NSCLC patients is the continuous discovery and development of novel molecular biomarkers. Liquid biopsies represent a new generation of biomarkers. The applications of liquid biopsy are very promising and include early detection, assessment of molecular heterogeneity of the tumor, monitoring of tumor dynamics, identification of genetic determinants for targeted therapy, evaluation of early treatment response, and monitoring of minimal residual disease to assessment of evolution of resistance in real time. Because blood-based biopsy techniques are minimally invasive, regular sampling can be performed without considerable morbidity burden for patients. Although the liquid biopsy approach is very promising, its sensitivity and specificity with respect to conventional tumor biopsies have to be evaluated in large, clinically relevant cohorts before widespread use in clinical routine.

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REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

1. Heitzer E. Clinical utility of circulating tumor DNA in human cancers. Memo 2015; DOI 10.1007/s12254-015-0217-5.
2. Hamilton G. Clinical relevance of circulating tumor cells in cancer patients. Memo 2015; DOI 10.1007/s12254-015-0237-1.

3. Senfter D, Mader RM. Exosomes as novel biomarkers in anticancer therapy. Memo 2015; DOI 10.1007/s12254-015-0245-1.
4. Jahr S, Hentze H, Englisch S, *et al.* DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001; 61:1659–1665.
5. Leon SA, Shapiro B, Sklaroff DM, *et al.* Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977; 37:646–650.
6. ■■ Bettegowda C, Sausen M, Leary RJ, *et al.* Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014; 6:224ra224.

The article highlights that cfDNA is a broadly applicable, sensitive, and specific biomarker that can be used for a variety of clinical and research purposes in patients with different types of cancer, including NSCLC.

7. Tseng JS, Yang TY, Tsai CR, *et al.* Dynamic plasma EGFR mutation status as a predictor of EGFR-TKI efficacy in patients with EGFR-mutant lung adenocarcinoma. J Thorac Oncol 2015; 10:603–610.
8. Thress KS, Brant R, Carr TH, *et al.* EGFR mutation detection in ctDNA from NSCLC patient plasma: a cross-platform comparison of leading technologies to support the clinical development of AZD9291. Lung Cancer 2015; 90:509–515.
9. ■ Oxnard GR, Paweletz CP, Kuang Y, *et al.* Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res 2014; 20:1698–1705.

Serial quantification of plasma genotype using ddPCR allows noninvasive assessment of response and resistance in *EGFR*-mutated NSCLC.

10. Kobayashi S, Boggon TJ, Dayaram T, *et al.* EGFR mutation and resistance of nonsmall-cell lung cancer to gefitinib. N Engl J Med 2005; 352:786–792.
11. Gainor JF, Shaw AT. Emerging paradigms in the development of resistance to tyrosine kinase inhibitors in lung cancer. J Clin Oncol 2013; 31:3987–3996.
12. ■ Janne PA, Yang JC, Kim DW, *et al.* AZD9291 in EGFR inhibitor-resistant nonsmall-cell lung cancer. N Engl J Med 2015; 372:1689–1699.

A first clinical trial to demonstrate the efficacy of AZD9291 in EGFR TKI-resistant NSCLC. This trial highlights the importance of T790M resistance mutation testing.

13. ■ Sequist LV, Soria JC, Goldman JW, *et al.* Rociletinib in EGFR-mutated nonsmall-cell lung cancer. N Engl J Med 2015; 372:1700–1709.

The first clinical trial to demonstrate the efficacy of rociletinib in EGFR TKI-resistant NSCLC. This trial highlights the importance of T790M resistance mutation testing.

14. Yung TK, Chan KC, Mok TS, *et al.* Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in nonsmall cell lung cancer patients. Clin Cancer Res 2009; 15:2076–2084.
15. Tissot C, Toffart AC, Villar S, *et al.* Circulating free DNA concentration is an independent prognostic biomarker in lung cancer. Eur Respir J 2015; 46:1773–1780.
16. Ilie M, Hofman V, Long-Mira E, *et al.* 'Sentinel' circulating tumor cells allow early diagnosis of lung cancer in patients with chronic obstructive pulmonary disease. PLoS One 2014; 9:e111597.
17. Maheswaran S, Sequist LV, Nagrath S, *et al.* Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008; 359:366–377.
18. Punnoose EA, Atwal S, Liu W, *et al.* Evaluation of circulating tumor cells and circulating tumor DNA in nonsmall cell lung cancer: association with clinical endpoints in a phase II clinical trial of pertuzumab and erlotinib. Clin Cancer Res 2012; 18:2391–2401.
19. Nelson NJ. Circulating tumor cells: will they be clinically useful? J Natl Cancer Inst 2010; 102:146–148.
20. Yu M, Bardia A, Wittner BS, *et al.* Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 2013; 339:580–584.
21. Pantel K, Speicher MR. The biology of circulating tumor cells. Oncogene 2015. [Epub ahead of print]
22. Thakur BK, Zhang H, Becker A, *et al.* Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res 2014; 24:766–769.
23. Yanez-Mo M, Siljander PR, Andreu Z, *et al.* Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 2015; 4:27066.
24. Camussi G, Deregibus MC, Bruno S, *et al.* Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int 2010; 78:838–848.
25. Rabinowits G, Gercel-Taylor C, Day JM, *et al.* Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer 2009; 10:42–46.