



# The potential of liquid biopsies

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## 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.

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**Curr Opin Oncol** 2016, 28:130–134

DOI:10.1097/CCO.000000000000267

## 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<sup>11</sup>].

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<sup>11</sup>].

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 theascreen) 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 theascreen, 90% for ddPCR, and 100% for BEAMing dPCR; specificity was 100% for cobas, 100% for theascreen, 100% for ddPCR, and 93% for BEAMing dPCR. More importantly, for the T790M resistance mutation, the sensitivity was 41% for cobas, 29% for theascreen, 71% for ddPCR, and 71% for BEAMing dPCR; specificity was 100% for cobas, 100% for theascreen, 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<sup>11</sup>,13<sup>11</sup>]. 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<sup>11</sup>,13<sup>11</sup>].

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<sup>11</sup>]. 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.

## Acknowledgements

None.

## Financial support and sponsorship

None.

## Conflicts of interest

*M.F. reported consultancy for AstraZeneca, Boehringer Ingelheim, Merck Sharp & Dohme and payment for lectures of AstraZeneca, Boehringer Ingelheim, Merck Sharp & Dohme, Novartis, Pfizer, and Roche.*

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