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Review

Liquid Biopsy – Possibilities for Monitoring the Therapeutic Response in Non-Small Cell Lung Cancer

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Abstract

Lung cancer is the leading cause of death from malignancy worldwide. Its heterogeneity and tumour biology make treatment considerably more difficult. The introduction of target molecules heralded the beginning of the personalized medicine which tailors medical treatments to the molecular and genetic profile of a patient. Liquid biopsy is an innovative, non-invasive method which is used both for diagnostic purposes and for therapeutic monitoring. Liquid biopsy has the potential to help manage non-small cell lung cancer throughout all stages of this cancer: screening, detection of minimal residual disease to guide adjuvant treatment, early detection of relapse, systemic treatment initiation, monitoring of response to targeted or immune therapy, and the emergence of resistance to applied treatment. At present, the study of circulating tumour DNA is used in clinical practice, but circulating tumour cells, miRNAs, exosomes, and platelets formed in the tumour also show promising results.

Keywords

cfDNA/ctDNA, genomic profiling, personalized medicine

INTRODUCTION

Lung cancer ranks first as the leading cause of death from malignancy in 2020, with an estimated 1.79 million deaths.[1] Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers. Over 80% of these patients are in stages III and IV at the time of diagnosis, and despite the therapeutic progress, the prognosis is extremely poor. The five-year overall survival for all stages is only 25%. NSCLC is characterized by a high frequency of metastases in the central nervous system. Brain metastases develop in 25%–50% of patients during the course of the disease, and 10%–20% have brain lesions at the time of diagnosis.[2] Most often, these are tumours with oncogenic drivers (activating EGFR mutations or rearrangements in ALK). Due to the limited penetration of target molecules across the blood-brain barrier, radiation oncology plays a central role in the treatment of these patients. The aim is to influence the neurological symptoms, stabilize the general condition and improve the quality of life.[3,4]

The statistics prove the need for more precise methods for timely diagnosis and optimization of the therapeutic approach. Lung cancer is among the tumours with the highest number of described genetic aberrations. Currently, the "gold standard" for histological verification and subsequent molecular genetic analysis of the tumour is tissue biopsy.

This procedure is invasive and involves certain risks for the patient. Very often, the samples taken are insufficient for examination. In addition, the biopsy does not always provide comprehensive information given the heterogeneity of the tumour. In the course of therapy with target molecules, other genetic changes associated with acquired resistance occur, which requires re-examination.

Liquid biopsy is a minimally invasive method that can be used for early diagnosis, detection of minimal residual disease, and therapeutic monitoring. A liquid biopsy involves the analysis of cell-free nucleic acids, mainly circulating free DNA (cfDNA), in the body fluids such as blood. Sampling is easier for patients and less invasive than tissue biopsy, but this technique also has certain limitations.[5] Specific molecular changes that stimulate tumour growth and represent therapeutic targets are best studied in adenocarcinoma. There is also a growing interest in squamous cell lung cancer to identify new potential treatment targets.[6]

Circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA), the most widely studied substrates in the field of NSCLC, have various advantages and disadvantages and can complement each other.[6] Circulating exosomes, miRNAs, and tumour-forming platelets are other attractive but as yet unproven approaches to tumour genotyping[7,8] **(Table 1)**.

Improvements in the understanding of multilevel molecular changes (genetic, epigenetic, protein expression) and their functional significance have the potential to influence the diagnosis, prognosis, and treatment of lung cancer.

Circulating tumour cells

The circulating tumour cells (CTCs) are shed either from the primary tumour or its metastases into the peripheral blood. They are relatively rare (in the rate of one to ten CTCs per 1 ml of blood). Groups with more than two or three CTCs originate from clusters, also known as circulating micrometastases, circulating tumour microemboli, or circulating tumour aggregates. Individual CTCs have a longer life (several hours) than the CTC clusters, which can be blocked by small vessels.[9] They are very diverse and, thanks to their biological and physical properties, can be detected by different techniques. CTCs contain information about the genetic and molecular characteristics of the tumour, as well as information about acquired resistance to previous therapies. The fact that CTCs can originate from both the tumour and its metastases gives a complete picture of the overall picture of the disease compared to tissue biopsies.[10] Therefore, the isolation of CTCs from peripheral blood or the so-called liquid biopsy is an alternative non-invasive method for tumour analysis.

There are many methods developed for CTC detection. CellSearch (Veridex LLC) is currently the only FDA-approved system for isolating and quantifying CTCs as a prognostic marker in patients with metastatic colorectal cancer, breast cancer, or prostate cancer. It uses antibodies bound to ferromagnetic particles, specific against the EpCAM-antigen which is expressed on the surface of epithelial cells. Separation of CTC from other cells is performed by immunomagnetic separation. The identification and enumeration of CTCs are obtained by adding fluorescent reagents that include anti-CK-phycoerythrin (PE), cytokeratin, and anti-CD45 allophycocyanin (APC), specific for intracellular proteins, epithelial cells, and leukocytes.[11] This method is suitable for determining the number of CTC.

Alternative methods for isolating CTC use specific labelling and separation of normal cells from tumour cells by flow cytometry, laser scanning cytometry, and a few others, or provide cell division using microfilters, as in the method of isolation by size epithelial tumour cells (ISET). Unlike CellSearch, the filtration-based technique allows the collection of EpCAM negative cells but cannot detect a very small size CTC.[12]

In one study, Farace et al. compared CellSearch and ISET techniques to detect CTC in 60 patients with metastatic lung, breast, or prostate cancer, noting some differences in the number of CTCs found in the two analyses. In fact, by applying CellSearch and ISET tests, 18 (30%) in one and three (5%) patients in the other method, respectively, had a negative result. The main limitations of CellSearch have been observed in patients with NSCLC and prostate cancer. The lower number of CTCs reported in these tumours can be explained by the epithelial-mesenchymal transition (EMT) of cancer cells. The latter lose the expression of epithelial markers and gain the expression of

Table 1. Pros and cons of tissue biopsy and liquid biopsy

Tissue Biopsy	Liquid Biopsy
Clinically validated	Clinical practice rules are not yet established
Invasive and risky	Non-invasive
Difficult to repeat	Easily repeated
Failure to reflect tumour heterogeneity	Potential to reveal spatial and temporal tumour heterogeneity
Failure to detect metastasis at distant sites	Offers a more comprehensive picture of the disease
Impractical for periodic monitoring of treatment response	Real-time monitoring for drug response and resistance

Source: Lim M, Kim CJ, Sunkara V, et al. Liquid biopsy in lung cancer: clinical applications of circulating biomarkers (CTCs and ctDNA). Micromachines 2018; 9(3):100. Table 1.

cytoplasmic mesenchymal markers that are not detected by CellSearch.[13]

To develop a method capable of detecting a greater number of CTCs in patients with NSCLC, Sharpenseel et al.[14] evaluated the expression of various surface markers (EGFR and HER3). These markers were found to be regulated to a greater extent in the metastatic tissue and in CTCs than in the primary tumour. The authors summarize that the combination of EGFR/HER3 enrichment with the EpCAM-based CellSearch technique can detect a significantly higher number of CTCs in patients with NSCLC by liquid biopsy.[14] The number of CTCs can be used as a reliable prognostic biomarker in several metastatic solid tumours such as breast cancer, NSCLC, colorectal cancer, and prostate cancer. A number of studies have shown that the presence of >5 CTCs in a 7.5-ml blood sample is associated with a poor prognosis.

A study with 101 chemotherapeutic patients with advanced NSCLC demonstrated the prognostic value of CTCs obtained with CellSearch. Blood samples were taken at baseline and after one cycle of standard chemotherapy. Progression-free survival (PFS) and overall survival (OS) were significantly longer in patients with less than five CTCs compared to patients with five or more CTCs (6.8 vs. 2.4 months, $p<0.001$, and 8.1 vs. 4.3 months, $p<0.001$, respectively). In the multivariate analysis, the number of CTCs is the strongest predictor of OS ($p<0.001$).[15]

In addition to prognostic value, changes in CTC levels during therapy can be used to predict response to it.[16] Cristofanilli et al.[17] use the commercial CellSearch kit to detect and quantify CTCs in patients with metastatic breast cancer. Their hypothesis is based on the assumption that CTC is characteristic of a more aggressive type of disease (i.e., they have a prognostic value) and provide information on the efficacy of a given type of therapy at an earlier stage (i.e., they have a predictive value). They found that the presence of >5 CTCs in 7.5 ml of blood in patients before treatment was a predictive factor for a poor response to the response of patients without CTCs, and they also had lower overall survival.[17]

Given the shortcomings of liquid biopsy, CTC analysis is unlikely to completely replace tissue biopsy in molecular pathology. However, the CTC test can be used as an accompanying diagnostic test when the tissue source material is insufficient or when taking a biopsy is difficult. In addition, CTC analysis may help to identify the mechanisms of drug resistance **(Table 2)**.

Circulating cell-free DNA

Extracellular DNA fragments, called cell-free DNA (cfDNA), are found in various body fluids in both healthy and diseased individuals. CfDNA fragments are 150 to 200 bp long; have a short half-life (about two and a half hours), which makes them suitable for analysis of tumour mutations, monitoring of treatment response, and minimal residual disease.[18,19] The amount of plasma cfDNA increases in patients with solid tumours due to increased cell turnover. The DNA of cancer cells contributes to the concentration of cfDNA in the plasma and is called ctDNA. In lung cancer, the higher amount of ctDNA is associated with locally advanced or metastatic disease. CtDNA levels increase as the disease progresses.[20,21]

In clinical practice, liquid biopsy is primarily used to detect ctDNA in blood plasma. CtDNA analysis can identify active tumour mutations at the time of diagnosis and/or recurrence in patients with advanced or metastatic lung cancer.[22] CtDNA testing is a useful method for genotyp-

Table 2. Comparison of CTC and circulating tumour (ct) DNA

	Advantages	Limitations
CTC	Immunohistochemical assessment of cell biomarkers, such as ER, HER2 Detection of key target mutations or markers of resistance Full genome analysis Gene / protein expression analysis Potential to detect heterogeneity and specific clones Development of cell lines for drug sensitivity testing / other PGx analysis Levels may correlate with response to therapy	Scarcity in the bloodstream, difficulty of capture Heterogeneity of cells and thus potential to capture only a subset It may be less sensitive for detecting specific mutations, e.g. EGFR mutation in lung Adenocarcinoma
ctDNA	Detection of key target mutations or markers of resistance Potentially more sensitive the detector of targetable mutations than CTC, especially the latest generation detectors Potential to detect some heterogeneity	Difficulty of capture Unable to assess gene expression or signalling pathway activity Unable to develop cell lines

Source: Hart CD, Galardi F, Pestrin M, et al. Using CTCs for pharmacogenomic analysis. Pharmacological research 2016; 106:92–100. Table 1.

ing patients with NSCLC. Various studies using ctDNA tests have demonstrated a sensitivity of 75%–90% and high mutational compatibility with tissue biopsies.[18] Mutations and driver mutations leading to resistance to targeted therapies have been found in 20%–30% of patients tested. Active biomarkers have been identified in 10%–20% of patients where no tissue biopsy material is available.[23]

EGFR mutations

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are the recommended first-line therapy in patients with advanced NSCLC. Most patients treated with first- or second-generation EGFR-TKI eventually develop resistance, and the T790M mutation is detected in approximately 50% of cases of resistance.[24] The FLAURA study for selection of first-line therapy with osimertinib in patients with EGFR-TKI-sensitizing mutant (EGFRm) advanced or metastatic NSCLC showed that the lack of EGFRm in plasma was associated with progression-free survival (PFS) compared to patients in whom plasma EGFRm was detected. These data underscore the predictive value of ctDNA analysis in EGFR-targeted therapy.[25,26]

A recent meta-analysis performed by Passiglia et al.[27] demonstrated the diagnostic accuracy of circulating tumour DNA for the detection of the EGFR-T790M mutation in NSCLC. A global sensitivity for ctDNA analysis of 0.67 (95% CI: 0.64–0.70) was demonstrated, with a generalized specificity of 0.80 (95% CI: 0.77–0.83). The global PPV is 0.85 (95% CI: 0.82–0.87) and the negative prognostic value (NPV) is 0.60 (95% CI: 0.56–0.63). Their data indicate that ctDNA analysis is a promising, non-invasive method for detecting and monitoring the T790M mutation in patients with NSCLC.[27]

ALK rearrangements

Anaplastic lymphoma kinase (ALK) gene rearrangements were observed in 3%–7% of patients with NSCLC. Cheol-Kyu Park et al.[28] used reverse transcription polymerase chain reaction (RT-PCR) to assess ALK status in 33 FISH-positive and 28 FISH-negative patients with lung cancer. RNA is extracted from plasma, platelets, and tumour tissue. Liquid biopsy results showed higher sensitivity (78.8%), specificity (89.3%), and accuracy (83.6%) for detecting ALK rearrangements compared to formalin-fixed paraffin-embedded (FFPE) analysis of tissues, with 54.5% sensitivity, 78.6% specificity, and 75.5% accuracy. 2219 patients with advanced (stage IIIB or IV) NSCLC underwent FoundationOne® Liquid CDx analysis in the BFAST study. ALK rearrangement was found in 119 patients (5.4%), and 87 of 119 received alectinib treatment. This subgroup showed an objective response rate of 87.4% (95% CI: 78.5-93.5%), with a response rate of 75.9% (95% CI: 63.6-88.2%) at 12 months and a median investigator-assessed PFS of 78.4% (95% CI: 69.1–87.7%).[29,30] Data show that liquid biopsy can be used to diagnose ALK-positive NSCLC and be useful for predicting the results of treatment with ALK inhibitors.

MET

Mutations such as amplification and structural changes (e.g., events skipping Exon 14) in the MET gene, which encodes a receptor tyrosine kinase, are of particular clinical interest. These aberrations can be detected by ctDNA analysis. MET amplification has been identified as both a major targeting engine and a mechanism of acquired resistance in 5% to 10% of EGFR-mutated NSCLCs. Some of them are potential targets for drug therapy.[31] Mondelo-Macía et al.[32] studied MET mutations in ctDNA in 174 patients with various solid tumours by droplet digital PCR (ddPCR). They report a sensitivity of 85.71% compared to tissue analysis and a specificity of 100%.

A recent study analysed mutations in the MET gene in ctDNA by next-generation sequencing (NGS) in 438 patients with solid tumours, and 263 tissue samples from these patients were examined. Thirty-one patients (7.1%) showed changes in MET. It was found that the shorter mean time to tumour metastasis or recurrence was associated with changes in MET (1.0 vs. 10.4 months, p=0.044), as well as shorter overall survival (30.6 vs. 58.4 months, p=0.013). Tissue biopsies were examined for changes in MET in only 18 of 31 patients. Mutations were found in only two tissue biopsies, so the authors concluded that ctDNA was more sensitive to detect alterations in MET and was associated with a worse prognosis in the analyzed population.[33]

These studies highlight the usefulness of ctDNA testing to detect primary drivers and acquired mutations leading to resistance to targeted therapy.

KRAS

Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) mutations occur in 25%–30% of patients with NSCLC. They are negative prognostic biomarkers and active treatment targets. Nacchio M et al.[34] examined plasma from 194 liquid biopsies using a NGS (SiRe®). KRAS mutations were identified in 36 (18.6%) samples. Exon 2 mutation p.G12C, which is currently targeted for treatment, is the most common mutation detected (13/36, 36.1%).[34]

Michaelidou et al.[35] evaluated KRAS G12/G13 mutations in cfDNA by ddPCR in 114 patients with advanced NSCLC. The results show that these mutations are associated with poor clinical outcomes in terms of disease control, PFS, and OS among patients with NSCLC treated with first-line systemic therapy, outlining the prognostic value of plasma KRAS mutations in NSCLC. The KRAS G12/G13 plasma mutation load is an independent predictor of poor prognosis in these patients. The KRAS G12/G13 mutation load study in cfDNA has a great potential

to assist clinicians not only in predicting patient outcomes but also in the therapeutic monitoring of patients with NSCLC.[35]

RET

Rearranged during transfection (RET) gene fusions are other mutations identified as targets for anticancer therapy.[36] In the largest series to date describing the genomic characteristics of advanced cancers containing activating somatic RET changes, Rich et al.[36] studied 32989 patients with various solid tumours in stage III-IV. They identified 176 different somatic changes in RET that were predicted to be oncogenic in cfDNA in 170 (0.5%) patients, and another 529 patients had only variants of uncertain significance. Among the 170 RET-positive patients, 125 had NSCLC.[36]

Analysis of ctDNA by NGS may be useful in identifying less common but potentially significant alterations, e.g., activating RET fusions. Numerous mechanisms of resistance have been identified in different tumour populations.

ROS1

ROS1 rearrangement can be detected in approximately 1% of patients with NSCLC, but data on the clinical significance of ROS1 alterations are scarce. In a recent study, Mezquita et al.[37] analyzed cfDNA by NGS method in 128 patients with known alterations in ALK or ROS1. Of the 128 patients enrolled in the study, 101 were positive for ALK and 27 for ROS1 alterations. The sensitivity was 67%. The absence of mutations in circulating tumour DNA during failed TKI treatment was associated with prolonged mean overall survival (105.7 months).[37] Their results demonstrated the clinical benefit of cfDNA analysis at both diagnosis and disease progression to detect resistance to therapy due to emerging mutations. Liquid biopsy in TKI-treated patients can detect tumour heterogeneity or the causes of TKI resistance, proving its role in therapeutic behavior.

Circulating miRNAs

MiRNAs are short non-coding single-stranded RNA molecules (19 to 22 nucleotides in length) that target complementary mRNA sequences. They are important regulators of gene expression. Genes encoding miRNAs are often located in "fragile" regions of chromosomes, in very small regions of heterozygosity loss and "breakpoint" regions, indicating that they are probably a new class of genes that play a role in tumourigenesis. MiRNAs can function as both tumour suppressors and oncogenes, and disruption of their expression can ultimately lead to tumour formation, cell proliferation, invasion, cell death, and angiogenesis.[38]

Circulating miRNAs can be found in biological fluids (serum, plasma, saliva, urine) of cancer patients and can transmit signals between cells, affecting the expression of various genes. A number of studies have shown that circulating miRNAs are extremely stable and can be easily isolated and detected. They have the potential to be used as prognostic, diagnostic, and predictive biomarkers in various types of tumours.[39]

Exosomes

Exosomes are the smallest extracellular vesicles, 40–100 nm in size. They originate from an endosomal system carrying DNA, mRNA, non-coding miRNAs, proteins, and lipids.[40] Exosomes have been identified as mediators of intercellular communication by transferring bioactive molecules such as nucleic acids, proteins, and lipids to recipient cells. Intercellular communication mediated by exosomes is not only involved in the regulation of normal physiological processes but also in the pathological processes of many diseases, including cancer. They are secreted by most cell types and released into body fluids such as urine, plasma, saliva, and breast milk.[41,42]

Due to their presence and stability in most body fluids and the similarity of their content with parental cells, exosomes have great potential to serve as a tool for liquid biopsy in various diseases.[43] In particular, exosomes isolated from carcinomas are likely to serve as a biomarker for early detection of cancer because they carry a load that reflects genetic or signalling changes in the initially occurring cancer cells.[44]

Tumour educated platelets

Platelets are isolated from blood by a two-step room temperature centrifugation protocol, which makes their isolation a very simple procedure.[45,46] Platelets play a leading role in the coagulation process; and are known to regulate tumour angiogenesis.[47] Tumour cells produce growth factors such as granulocyte-macrophage colony-stimulating factor, granulocyte-colon-stimulating factor, and cytokines (interleukin-1 and interleukin-6) capable of stimulating its thrombocytosis.[48] Interaction between platelets, tumour and its microenvironment leads to the formation of tumour educated platelets (TEP). Various studies have confirmed that the number and size of platelets can provide clinically relevant information about the presence of cancer.[49] High platelet counts have been associated with increased mortality in various types of carcinomas such as malignant mesothelioma, gynecological malignancies, and carcinomas of the lung, kidney, stomach, colon, and breast. Tumour-derived platelet factor 4 (PF4, CXCL4) has been shown to support bone marrow-mediated megakaryocyte platelet production in patients with NSCLC.[50]

Sheng et al. analyzed TEP RNA sequencing data in 402 patients with NSCLC and 231 samples from healthy controls. The authors identified 48 genes that play a key role in tumourigenesis and cancer progression and can accurately demonstrate NSCLC. This confirms the thesis that TEPs may be useful for the early detection of NSCLC.[51]

CONCLUSIONS

Currently, the detection and analysis of ctDNA in clinical practice are largely limited to the diagnostic testing of recurrent and/or metastatic disease. It can also be used to select targeted therapies when tissue biopsy is not possible, or tissue samples are insufficient for examination. The ctDNA assay has the potential to become an essential part of genomic profiling. It includes dynamic monitoring of NSCLC, allowing the characterization of genetic heterogeneity and clonal evolution that occur during tumour progression and as an adaptive response to targeted cancer therapy. Compared to tissue biopsy, minimally invasive and low-cost liquid biopsy allows repeated examination of cancer patients – a useful and modern method in routine clinical practice.

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Жидкая биопсия - возможности мониторинга терапевтического ответа при немелкоклеточном раке лёгкого

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Резюме

Рак лёгких - основная причина смерти от злокачественных заболеваний во всём мире. Его неоднородность и биология опухоли значительно затрудняют лечение. Введение целевых молекул положило начало персонализированной медицине, которая адаптировала лечение к молекулярному и генетическому профилю пациента. Жидкая биопсия - это инновационный неинвазивный метод, который используется как для диагностических целей, так и для терапевтического наблюдения. Жидкая биопсия может контролировать немелкоклеточный рак лёгкого на всех его стадиях: от скрининга, выявления минимальной остаточной болезни до адъювантной терапии, раннего выявления рецидива, начала системного лечения, мониторинга ответа на таргетную иммунную терапию и возникновения устойчивости к применяемому лечению. В настоящее время исследование циркулирующей опухолевой ДНК используется в клинической практике, но циркулирующие опухолевые клетки, мРНК, аксосомы и тромбоциты, образующиеся в опухоли, также имеют многообещающие результаты.

Ключевые слова

cfDNA / ctDNA, геномное профилирование, персонализированная медицина