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Liquid biopsy – a diagnostic and therapeutic tool in the treatment of non-small cell lung cancer

Tekočinska biopsija – diagnostični in terapevtski pripomoček pri zdravljenju nedrobnoceličnega raka pljuč

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Abstract

Lung cancer is one of the most prevalent cancers and the leading cause of cancer mortality worldwide. The past decade has brought important progress in drug treatments by discovering the driver mutations. The evolution of targeted oncological treatments directed to the biological properties of lung cancer in the individual patient has led to a significant increase in survival. During treatment, new mutations accumulate in the tumour, which prevents the long-term success of the therapy. Liquid biopsy is a method that has established itself in recent years as a less invasive diagnostic procedure that allows monitoring the response to treatment and identifying the mechanisms of resistance. The circulating tumor DNA is the most prevalent biomarker in lung cancer, but research on other biomarkers is also active. In this review article, we present the use of liquid biopsy in the clinical treatment of patients with non-small cell lung cancer. Its use is increasingly recognized in early detection of lung cancer, identifying resistant mutations, potential assessment of disease burden, and longitudinal monitoring.

Izvleček

Rak pljuč je ena najbolj pogostih in smrtonosnih rakavih bolezni na svetu. V zadnjih 10 letih je z odkritjem vodilnih mutacij prišlo do velikega napredka pri zdravljenju. Zdravljenje postaja vse bolj prilagojeno bolniku oz. biološkim lastnostim raka, proti katerim so usmerjena tarčna onkološka zdravila, ki so bolnikom z napredovalo rakavo boleznijo pomembno podaljšala preživetje. Med tarčnim zdravljenjem se v tumorju razvijajo nove genetske spremembe, ki vodijo v odpornost tarč v rakavem tkivu, s čimer dolgoročno omejujejo uspešnost zdravljenja. Tekočinska biopsija (*angl.* liquid biopsy) je metoda, ki se je v zadnjih letih uveljavila kot manj invazivni diagnostični postopek, ki omogoča spremljanje odziva na zdravljenje in prepoznavanje mehanizmov odpornosti. Pri raku pljuč se najbolj uveljavlja vloga cirkulirajoče tumorske DNK (ctDNK), aktivne pa so raziskave tudi drugih bioloških označevalcev. V preglednem članku predstavljamo uporabo

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Ključne besede: rak pljuč; tekočinska biopsija; cirkulirajoča tumorska DNK; PCR; NGS

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tekočinske biopsije pri klinični obravnavi bolnikov z nedrobnoceličnim rakom pljuč, ki se kaže v možnosti zgodnjega odkrivanja raka pljuč, identifikaciji vodilnih in odpornih mutacij, potencialni oceni bremena bolezni ter dolgoročnega spremljanja bolnika.

1 Introduction

In recent years, the development of medicine has been directed towards patient-focused, personalized treatment. It is an individual approach to disease prevention and treatment, based on genetic and molecular diversity and the influence of the environment on the individual's health (1). The greatest progress can be seen in the field of oncology, which has been one of the most active branches of medicine in terms of research in the last decade. Cancer is a genetic disease that contains a patient-specific and unique mutation profile. With the development of increasingly sensitive, specific and affordable technologies for the isolation and analysis of a tumour's genetic material, the role of personalized treatment is increasingly included in oncology treatment guidelines worldwide. With the discovery of mutations and thus new possibilities for effective targeted drugs, even in the treatment of patients with lung cancer, the successes of the personalized approach are already visible with prolongation of survival (2).

2 Lung cancer

Lung cancer is one of the most common and deadly cancers worldwide (3). In Slovenia, lung cancer ranks 3rd among cancer types in men and 4th in women, with the proportion of female cancer patients still increasing (4). It is important to suspect and diagnose lung cancer as soon as possible (5). Despite medical progress, in most cases, the disease is detected at an advanced stage with subsequent poor survival and limited curative treatment options. In the last 10 years, with the discovery of driver mutations, significant progress has been made with treatment, which is becoming more and more tailored to the biological properties of the patient's cancer (6,7).

Lung cancer is a heterogeneous disease with several histological subtypes, according to the World Health Organization classification (8). Due to differences in treatment methods, the division into small cell and non-small cell lung cancer, which includes adenocarcinoma and squamous cell carcinoma, is particularly important. Due to the emergence of many targeted treatments, an active and targeted search for driver mutations is recommended in lung adenocarcinoma. In patients with small cell or squamous cell carcinoma, specific targeted therapy is not available, so routine identification of driver mutations is not necessary (9).

2.1 Lung adenocarcinoma

The most studied and common mutation in non-small cell lung cancer is a mutation in the epidermal growth factor receptor (EGFR) gene; other types of mutations, such as ALK (anaplastic lymphoma kinase) rearrangements, V600E point mutation in the BRAF gene (v-raf murine sarcoma viral oncogene homolog B1), rearrangements of ROS1 (c-ros oncogene 1), RET (rearranged during transfection), NTRK (neurotrophic tyrosine receptor kinase), and a skipping mutation in exon 14 of the MET gene are rarer (6,7,10). When patients are diagnosed with lung adenocarcinoma in Slovenia, EGFR, KRAS (Kirsten rat sarcoma viral oncogene homolog), and BRAF mutations and ALK, ROS 1, and NTRK rearrangements are determined in the tumour sample (9). As a result of targeted therapy, the survival of patients with advanced cancer and their quality of life have significantly improved (2). During targeted therapy, new genetic changes which prevent the long-term success of this treatment develop in the tumour. Extensive research is being conducted around the world to identify the mechanisms of treatment resistance as precisely and quickly as possible with minimal invasiveness. The need for repeat tumour biopsies represents a limitation; a new method, the liquid biopsy, is becoming available for this purpose (11).

2.2 Squamous cell lung carcinoma

At the oncologist's request, the aforementioned mutations, though rarely present, are also tested in selected younger and non-smoking patients with squamous cell carcinoma and in other rare histological types of non-small cell lung cancer. As a rule, in patients with squamous cell carcinoma (and adenocarcinoma with negative markers), due to the possibility of immunotherapy, only immunohistochemical staining for PD-L1 (programmed death-ligand 1) is performed (9).

Table 1: Comparison of tissue and liquid biopsy in lung cancer (13-26).

	Tkivna biopsija	Tekočinska biopsija
Sample type	tumour tissue biopsy (bronchoscopy with biopsy, CT-guided needle biopsy, surgical biopsy)	blood sample (less frequently pleural effusion, saliva, urine, ascites, cerebrospinal fluid)
Procedure invasiveness	Invasive	Minimally invasive, non-invasive
Procedure difficulty	Difficult	Less difficult
Complications	Rare, potentially life-threatening	Very rare, less dangerous
Tumour heterogeneity detection	No	Yes
Patient burden	Yes	No
Longitudinal follow-up of tumour characteristics	No	Yes
Monitoring treatment response	No	Yes
Sensitivity	High	High, but it differs depending on the authors and the type of method used

Legend: CT – computed tomography.

3 Liquid biopsy

3.1 A comparison of liquid and tissue biopsy

A liquid biopsy is a procedure for the detection of cancer and molecular tumour characteristics in body fluids (12). Unlike traditional tissue biopsy using bronchoscopy or other minimally invasive procedures, a liquid biopsy is much less burdensome for patients. Around the world, different types of body fluids are used for analysis (e.g. pleural effusion, urine, sputum); most frequently, samples of peripheral blood or plasma are used (13-16). The role of liquid biopsy in the treatment of cancer is mainly in the identification of driver mutations, which allow for targeted treatment, monitoring the treatment response and success, as well as early detection of disease recurrence (17).

Tissue biopsy is still the gold standard for cancer diagnosis, defining the tumour type and its characteristics. By tumour tissue sampling and processing, driver mutations have been discovered in recent decades, allowing for the development of targeted therapy. By taking tissue samples before and after the appearance of resistance to targeted therapy, researchers identified the mechanisms of new, secondary mutations. Despite the advantages offered by tissue biopsy, it is still a time-consuming invasive method that requires an experienced team of specialists to perform the procedure and process the samples. There are several contraindications for invasive tissue biopsy, limiting its use in patients with advanced cancer. Complications are also possible (18). Time required for the biopsy and intra- and intertumour heterogeneity are the most significant limitations. Accurate identification of mutations with a tissue biopsy is frequently not possible at all, as due to tumour heterogeneity, the biopsy might not capture the entire set of mutations (19). Additionally, tissue biopsy does not allow for monitoring changes in the tumour's genetic profile over time and the possible discovery of new mutations that pose an increased risk of treatment resistance (20).

On the other hand, as a newer method, liquid biopsy is more accessible, reproducible, easier and faster to perform, either minimally or even non-invasively, and less burdensome for the patient. Compared to tissue biopsy, the risk of complications is negligible (18). Repeat sample collection is possible, giving immediate insight into the tumour's changes and allowing longitudinal follow-up (21,22). Despite the many advantages that place liquid biopsy alongside the established tissue biopsy (Table 1), its use in everyday practice is gaining ground slowly, mainly due to dilemmas regarding the reliability of the test to prove the presence of a mutation and current high costs when using highly sensitive techniques. Liquid biopsy has already found a place in routine clinical practice in non-small cell lung cancer. In international recommendations (23), liquid biopsy is already the method of choice for the detection of

treatment-resistant T790M mutation in patients with EGFR-mutated lung cancer treated with EGFR tyrosine kinase inhibitors. The latest recommendations of the IASLC (International Association for the Study of Lung Cancer) include an expanded use of liquid biopsy in patients with lung cancer (24). The test's sensitivity and specificity undoubtedly depend on the size of the tumour mass, the presence of metastases and the type of method used to prove or determine mutations (25,26).

4 Types of liquid biopsy

Liquid biopsy is based on the knowledge that cancer cells present in the bloodstream are identical to primary tumour cells, which was first demonstrated by Australian scientist Thomas R. Ashworth in 1869 (27). It is estimated that thousands of cells are released into the circulation daily with an average half-life of 1–2.5 hours. As early as 1948, researchers Mandel and Metais described circulating free DNA fragments (cfDNA) in plasma, but the association between cfDNA concentration and disease states was not proven until many years later (28). cfDNA is the total freely circulating DNA originating from all cells in the organism, healthy and diseased. With the improvement of techniques for the isolation of genetic material, it is now possible to detect and monitor various types of mutations that serve as specific biological markers for the presence of cancer in the bloodstream.

4.1 Molecular technologies

In liquid biopsy, several types of molecular techniques are used, which are roughly divided into two groups. The first, the target group, represents a fast, more affordable, highly sensitive and specific technique that can identify even a small number of point mutations in one gene. This group includes methods based on the use of polymerase chain reaction (PCR). As narrowly focused methods, prior knowledge about the type of tumour and mutation is required. The second group includes high-performance methods that identify mutations in several genes (next-generation sequencing, NGS). Since they are aimed at identifying a large part of the genome and can identify both specific and non-specific mutations, prior knowledge about the type of tumour and mutation is not required. Their biggest limitations are high cost, long procedure time, increased technology demands, and analysis (23,29). According to data from the literature, the sensitivity of cfDNA detection methods using NGS is between 75% and 90% with high tissue biopsy concordance (30).

4.2 Types of biological markers

Liquid biopsy involves different methods of circulating marker detection. From a clinical point of view, among these markers, cfDNA, of which tumour DNA is a component, has been by far the most widely researched and used for genotyping solid cancer types, including lung cancer (mainly non-small cell lung cancer), where it is already included in routine management (24). There are several other biomarkers, but their clinical use in lung cancer is rare and currently limited to research.

4.2.1 Circulating tumour DNA

Circulating tumour DNA (ctDNA) is a subtype of cfDNA that is released from tumour cells and contains a transcript of extracellular tumour DNA (double-stranded or mitochondrial). It represents only a part of the total cfDNA, which also originates from non-tumour cells. It is found in body fluids (most often in blood), into which it is released through various mechanisms, such as necrosis, apoptosis, active secretion, or cancer cell senescence (31). The concentration of ctDNA in body fluids is not constant. Its amount depends on several factors, mainly on the size of the tumour. The half-life of ctDNA is short (from a few minutes to two hours), allowing monitoring the tumour's current state. Its concentration is unfortunately low, therefore highly sensitive and advanced isolation and detection molecular techniques are needed to identify ctDNA (32).

An additional obstacle with peripheral blood analysis is cfDNA of non-cancerous origin, released from leukocytes. It dilutes the ctDNA present, further reducing the detection method's sensitivity. In practice, adapted test tubes for sample collection and centrifuging the sample as soon as possible after collection are used to solve this problem (33). In addition to false negative results, record false positive results of liquid biopsy with ctDNA are also possible. In healthy subjects, cancer-unrelated somatic mutations can arise from haematopoietic cells through the process of clonal haematopoiesis, which complicates the interpretation of liquid biopsy results. These mutations most frequently arise from genes associated with haematological malignancies, but less frequently, mutations associated with lung cancer can also arise through this

process, e.g. KRAS mutations. In patients undergoing liquid biopsy for suspected lung cancer, a KRAS mutation can thus be detected despite the absence of lung cancer (34,35). Some specific tumour mutations, e.g. EGRF and BRAF, do not have a haematopoietic origin, so detection of a false mutation due to clonal haematopoiesis is not possible.

Despite excellent prospects, before the expected wider clinical use of ctDNA in oncology, the result's credibility will require a precise standardization of the entire procedure from sample collection to final analysis (12). In the literature, studies that use targeted and even more affordable PCR methods are introducing ctDNA into clinical practice. Due to increasing sensitivity and gradually more affordable NGS methods, the use of these methods in clinical practice is expected to expand in the future.

4.2.2 Circulating tumour cells

Circulating tumour cells (CTCs) are individual tumour cells that leave their site of origin (primary tumour or metastasis). They travel throughout the body to distant locations via the bloodstream (36). Blood is therefore a suitable sample for their identification, cancer diagnosis and cancer monitoring. CTCs already play an important role in the diagnosis of metastatic breast, prostate, and colon cancer, and they already have a possible role in lung cancer management (37-39). Similar to ctDNA, their blood concentration is unfortunately low (0.1-10 CTC/mL whole blood), and the half-life is short (1-2.4 hours). Detection requires highly sensitive techniques that distinguish CTCs from other circulating cells based on their physical, morphological, and biological characteristics (40).

4.2.3 Other biological markers

There are more types of biomarkers, but compared to ctDNA, they are currently used in lung cancer only for research purposes. Due to the extensive research on their possible use and predicted important future role in the treatment of lung cancer, they are only mentioned in this article. Since they are not currently used in clinical practice, a detailed presentation is beyond the scope of this article.

Various biological markers with possible roles in cancer development are used for research purposes. Exomes are small, 30–100 nm membrane-bound vesicles that are produced by endocytosis or release from cells. They contain proteins and nucleic acids that can be transported between cells. Due to their lipid membrane, they are protected from degradation, can pass through the membrane, and persist in the bloodstream for longer periods. They play many different roles in the development of cancer: in carcinogenesis, the formation of metastases and the transfer of oncogenic factors between cells. The use of TEP (tumour educated platelets), tumour-associated autoantibodies (TA-Ab) and microRNA is also mentioned (36,41).

5 Use of liquid biopsy in patients with lung cancer in clinical practice

Liquid biopsy has established itself in clinical practice in patients with lung cancer. With the discovery of critical molecular and cellular mechanisms of lung cancer development and the emergence of new targeted therapy methods, new techniques have been developed and are being used, particularly in the treatment of non-small cell lung cancer. Liquid biopsy has established itself as a method to identify specific EGFR mutations in patients who develop targeted treatment resistance. Due to its clinical availability and cost-effectiveness, the current most widely used method is the detection of ctDNA with PCR, hence the reason for this article's focus upon it. However, it is important to emphasize that in recent years, the number of scientific publications investigating its use in patients with cancer other than lung cancer is rapidly increasing (Figure 1). We expect that in the coming years, the field of liquid biopsy will advance professionally and technologically, particularly with the expansion of the NGS method.

5.1 Early lung cancer detection

Due to its non-invasiveness and potential low cost, liquid biopsy has shown its potential for use in screening and early detection of lung cancer. Currently, the only recognized and recommended method for lung cancer screening is the use of low-dose computed tomography (CT) with confirmatory tissue biopsy. Due to the low sensitivity and specificity for early cancer detection, ideas on improving screening with a combination of CT imaging and a blood test (liquid biopsy) have appeared in the literature (42). According to data from the TRACERx study, due to early genomic and chemical changes, ctDNA should be present in the bloodstream 6-12 months before the first radiological signs of lung cancer, and the concentration of cfDNA is higher in cancer patients compared to healthy controls

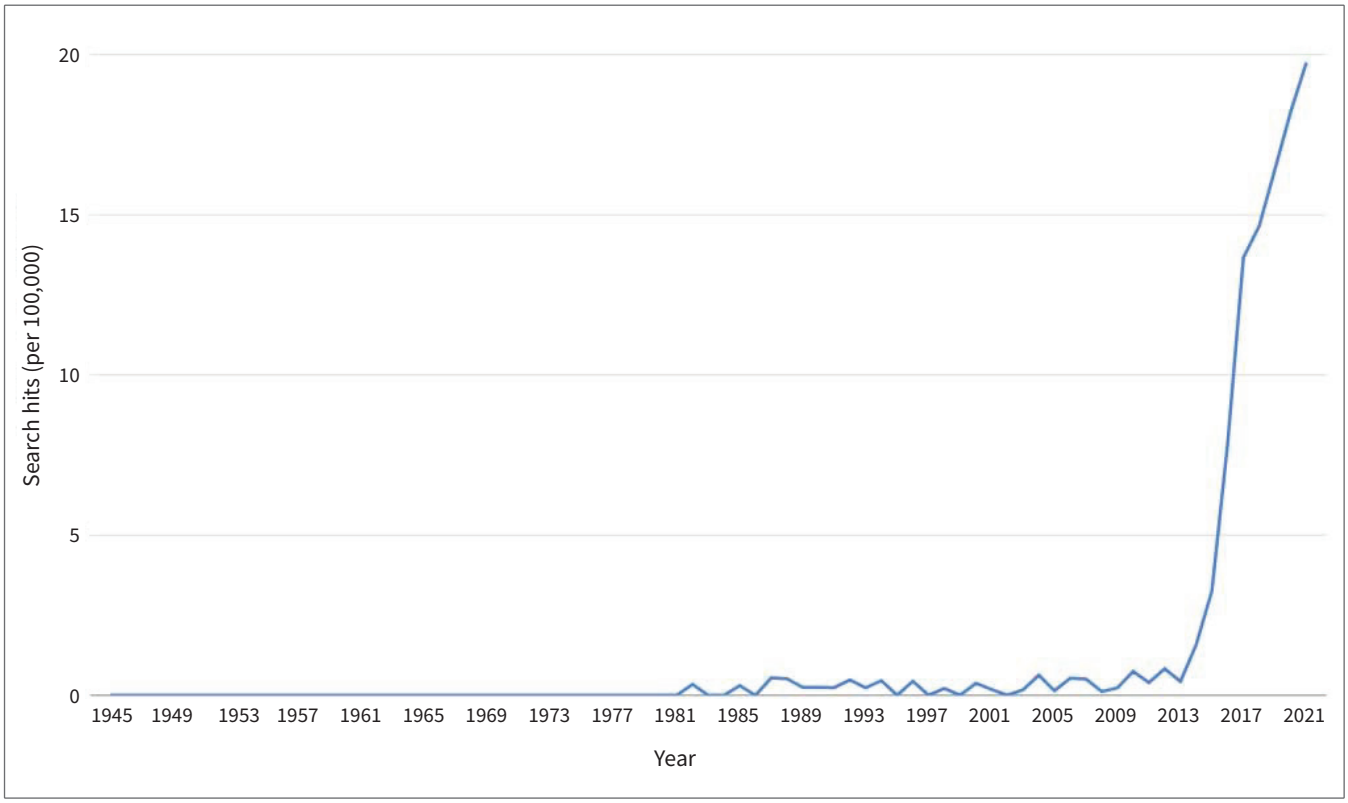

Figure 1: Graphic representation of the temporal distribution of search results in the PubMed digital database up to 2021. The search keywords used were »lung cancer« and »liquid biopsy« with synonyms.

and patients with benign lung changes (43,44). Many studies in recent years have been concerned with obtaining the most favourable biological markers for screening use, e.g. ctDNA, CTC, microRNA, exomes, proteins, etc. (45-47). One of the first studies, conducted in 2014, to isolate CTCs from patients with chronic obstructive pulmonary disease, who represent a group at higher risk of developing lung cancer, was the first to show promising results for detecting patients at high risk of developing lung cancer with a normal CT scan result (48). Some similar follow-up studies have failed to demonstrate similar statistical reliability for predicting lung cancer risk (49,50). Currently, the combination of a low-dose CT and liquid biopsy with CTC and/or ctDNA cells is an important research topic for the early detection of lung cancer (studies by Spira's group, Lecia et al) (51). A recent larger study by Chen et al, which included more than 120,000 healthy, asymptomatic individuals, highlighted the potential for early detection of multiple specific ctDNA methylations in 477 regions, which research suggests may be specific to certain solid tumours, including lung cancer. They reported correct identification of 88% of solid tumours (including lung cancer) demonstrated over a 4-year follow-up and 95% specificity for identifying the type of cancer (52). Despite high expectations, a problem arises when using ctDNA as a biological marker due to the low frequency of mutated alleles in early, curable forms of lung cancer, as their detection exceeds the sensitivity of the currently available methods (53).

Today, liquid biopsy as a screening method does not yet seem viable from an organizational and cost point of view. False-negative tests are an additional problem; currently, it would be reasonable to use the test only in patients with risk factors for the development of lung cancer. Considering the extensive research into liquid biopsy, it is expected that the method will establish itself as a new approach to disease management in the future.

5.2 Identifying mutations and treatment resistance

Several different types of mutations have been identified that participate in lung cancer development. Identifying mutations in cancer cells is key to selecting an appropriate and effective targeted therapy. According to the recommendations of the European and

American professional guidelines, treatment should be directed according to the detected resistance mechanism; before first-line treatment, a tissue biopsy is still in place today (54,55). It is particularly important to identify the mutations for which targeted treatment is available. The biggest limitation in the use of targeted therapy are secondary mutations resulting in resistance to primary treatment, which require a repeat biopsy to change treatment. According to data from Nosaki et al, a repeat lung tumour biopsy is not feasible in as many as 20% of patients with treatment resistance, and many studies also report insufficient or unrepresentative tumour tissue samples (56,57).

Based on the favourable study results and the aforementioned problems with repeat tissue biopsies in the event of resistance, the European Medicines Agency (EMA) was in 2015 the first to issue an approval for the use of plasma ctDNA in lung cancer; a year later, the US Food and Drug Administration (FDA) did the same (58,59). Its use is currently limited for EGFR status determination in patients with newly diagnosed non-small cell lung cancer where tissue biopsy is not possible or in the event of resistance during treatment with primary or second-generation tyrosine kinase inhibitors (TKIs) for the detection of the point mutation in exon 20 T790M. This is the most common treatment-resistant mutation (proven in approximately 50% of patients) and is an indication for treatment with third-generation TKIs. Two standardized, highly sensitive and specific mutation tests (Cobas EGFR mutations Test v2, Roche; Therascreen, Qiagen) based on the use of qPCR have been approved for detection (20,60-62).

However, according to the AURA study, caution is required when interpreting the results of T790M mutation detection (63). In the study, they found inconsistencies between the presence of the mutation in tissue and liquid samples as a consequence of tumour heterogeneity. In case of a positive result (i.e. proven resistance mutation), the type of treatment should be changed. Additionally, due to low method sensitivity, it is necessary to take into account the possibility of false negative results, so another sample should be taken in the event of a negative result (64-66).

In the case of EGFR-independent mutations, cancer cells acquire an EGFR-independent alternative pathway for cancer cell survival and proliferation by acquiring new mutations, e.g. KRAS, HER2, MET, PI3KCA, etc. Transformation from non-small cell to small cell lung cancer is rarely seen. With the expansion of advanced techniques in everyday clinical practice, ctDNA analysis with NGS with which we can identify a wider range of cancer cell mutations is becoming more and more popular. Since the publication of the latest IASLC recommendations, a number of additional studies have emerged to support the use of broad-spectrum NGS analysis (24). It can be used to demonstrate the presence of other important mutations for which we have available targeted treatments, e.g. ALK (67) and ROS 1 (68) rearrangements, BRAF mutations (69), etc. In 2020, the ESMO expert group already recommended NGS for the detection of genetic alterations (EGFR, ALK, etc.) in advanced lung cancer in regular clinical practice (70). At the end of 2020, the FDA was the first to issue approval for two commercial NGS tests (Guardant360, FoundationOne Liquid CDx) (24).

So far, the use of NGS worldwide is still limited compared to PCR methods. However, its role will undoubtedly increase significantly in the future with lower costs and better availability. Despite the increasing role of liquid biopsy compared to tissue biopsy, it should be taken into account that ctDNA analysis does not detect the emergence of some other resistance mechanisms, e.g. histological transformations of cancer tissue (71).

5.3 Assessment of disease burden and long-term monitoring of treatment response

With a liquid biopsy, adequate treatment response, disease progression, and assessment of patient survival could be determined early. Studies examining liquid biopsy as a method for quantitative and qualitative monitoring of tumour burden and molecular profile are emerging in the literature. The concentration of ctDNA in plasma changes, and depends on the type of cancer, its location, size, and tumour blood supply, ergo monitoring trends and not the absolute concentration is more appropriate (72). Due to this variability, different concentrations of ctDNA can be found in patients with the same tumour type and the same disease stage. Despite the variability, in one large Chinese multicentre prospective cohort study from 2020, significantly shorter non-small cell lung cancer survival was reported in patients with higher pre-treatment ctDNA concentrations and mutations (73,74). This was associated with a higher baseline disease burden. However, on the other hand, data can be found in the literature that baseline ctDNA concentrations do not predict response to treatment (75).

With the introduction of a new drug, sequential

blood analyses could be used to monitor the response to treatment and rate of decline in ctDNA concentrations to assess treatment effectiveness. In their 2016 study, Lee et al used regular plasma samples to monitor the presence of EGFR mutation in patients from the initiation of EGFR TKI therapy and compared this with the clinical response. They found a statistically significant difference in median progression-free survival in patients with undetectable EGFR mutation after EGFR TKI treatment compared with those with recurrence within two months of starting treatment (10.1 and 6.3 months, respectively, P=0.006) (76). Song et al also found a longer time to disease progression and increased overall survival in patients with increased ctDNA clearance, regardless of the treatment type and regimen.

In addition to evaluating the effectiveness of the treatment type, monitoring mutation levels would also allow early detection of treatment resistance emergence (24). In a Slovenian study that was conducted between 2014 and 2017, Kern et al studied the level of EGFR mutations in patients with non-small cell lung cancer who were treated with first-generation EGFR TKIs and compared changes in levels with clinical and radiological signs of disease progression. Patients were followed up with imaging after the first eight weeks of treatment and then every 16 weeks or earlier, if signs of disease progression were noted. Blood samples for EGFR mutation testing in peripheral blood were collected at each follow-up visit and processed with a standardized qPCR Cobas EGFR assay. In 12 out of 35 patients, an increase in EGFR mutation levels was observed an average of 10 weeks before clinical and radiological signs of disease progression (77).

According to recent data, the speed of response to treatment is a possible indicator of the long-term treatment success and indirectly affects the disease outcome. A recent Italian study reported a worse outcome in patients with a KRAS mutation who maintained high plasma ctDNA concentrations 3–4 weeks after treatment initiation. When ctDNA concentrations had increased, the risk for disease progression increased by as much as sevenfold (78). By identifying these patients, it could be possible to replace treatment with a more appropriate and effective faster (e.g. immunotherapy, chemotherapy).

6 Conclusion

Liquid biopsy is a method that is slowly taking an important place in diagnosis and monitoring the response to lung cancer treatment. Although its role in the treatment of lung cancer is currently still limited, it is gradually establishing itself in global guidelines (55,56). Currently, ctDNA is already used in clinical practice in patients with non-small cell lung cancer for the primary detection of driver mutations (mainly EGFR) and resistance mechanisms during targeted therapy. The method also shows great promise in the early detection of cancer and in monitoring the response to treatment (24,55). Due to the increasing sensitivity and more affordable methods, the use of the NGS molecular method is expected to expand into clinical practice, and other types of biological markers also show great potential in research (24). Further technological advancements and research are needed, to improve the reliability and applicability of the method in regular clinical practice, and above all, the standardization of the entire procedure is essential - from sample collection to reporting of results and their evaluation.

Conflict of interest

None declared.

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