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The role of liquid biopsy in lung cancer – from detection to disease control

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ABSTRACT

Lung cancer is one of the dominant causes of all cancer deaths. In 2020 worldwide there were 2.2 million patients diagnosed with lung cancer, and 1.8 million patients died at the same time. Based on the data from the European Cancer Information System, 318,327 patients were diagnosed with lung cancer in Europe in 2020.

Lung cancer symptoms, e.g. persistent cough and shortness of breath often fail to be recognised properly, which delays a visit to a specialist. Finally, most symptoms are diagnosed in stage IV of the disease, where therapeutic options are rather limited. Surely, there is an urgent clinical need in this area waiting to be recognised. Scientists all over the world are trying to find various solutions to better detect, predict forecast and avoid relapse of disease using novel techniques called "liquid biopsy". Circulating tumour DNA (ctDNA) is a biomarker that holds promising potential. One of the fractions of all Circulating Cell Free DNA (cfDNA), circulating tumour DNA is degraded and fragments of the DNA are released passively from apoptotic and necrotic cancer cells. Moreover, it has been discovered that cancer cells can also actively release ctDNA owing to the mechanism of producing extracellular vesicles (EVs).

The biology and the methods of the ctDNA identification will be presented in this narrative review. Further-more, it will be focused on presenting the role of ctDNA in screening and early diagnosis as well as on the effect of different concentrations of ctDNA on prognosis, staging, patients' stratification and detection of MRD (Minimal Residual Disease) for the group of lung cancer patients. It will be also highlighted that the ctDNA can be considered biomarker in terms of immunotherapy usage in different subgroups of patients. At the end, the review will focus on the future directions with innovative approaches that can potentially improve detection, therapy and finally make it possible to accurately predict and monitor the disease.

INTRODUCTION

CHARACTERISTICS OF LUNG CANCER

Based on the newest estimations provided by GLOBOCAN 2020, there were 19.3 million patients with a diagnosis of cancer, nearly 10 million of whom died. Lung cancer was responsible for 2.2 million cases and 1.8 million deaths. It is worth mentioning that lung cancer is now the second most frequent diagnosed cancer with the highest mortality in men, however, in women it is third most common cancer preceded only by colorectal and breast cancers (Sung, 2021). In Europe, lung cancer is the second most common cancer in males, preceded only by prostate cancer. As far as females are concerned, the incidence in Europe is exactly the same as globally. In 2020, there were about 320,000 patients diagnosed with lung cancer in the EU with over 257,000 deaths accordingly (OECD, 2020).

It is common knowledge that main risk factors for developing lung cancer remain unchanged and include tobacco smoking and environmental factors such as: air pollution, arsenic in drinking water, and exposure to asbestos. Increasing cases of the disease can be broadly prevented by implementing tobacco control policies and regulations (Carioli, 2021). Unfortunately, survival rates of patients diagnosed with lung cancer after 5 years are extremely low ranging from 10% to 20%. Survival rates are slightly higher in Japan (33%), Israel (27%) and the Republic of Korea (25%) (Allemani, 2018).

Lung cancer can be divided into two main groups: Non-Small Cell Lung Cancer (≈85% cases) and Small Cell Lung Cancer (≈15 % cases). The former (NSCLC) has different origins and is being categorized into three subtypes: Adenocarcinoma (LUAD, alveolar type II epithelial cell), Squamous Cell Carcinoma (LUSC, basal epithelial cell) and Large Cell Carcinoma (LCC); various epithelial cells. The later (SCLC) has its origin in neuroendocrine cell linage (Sánchez-Ortega, 2021).

It is important to note that survival rates depend on several factors, including the stage of the disease, histology type, and genetic alterations. In general, the prognosis for lung cancer patients remain poor. The 5-year survival rate for NSCLC patients is very low (about 16% at 5 years). In case of SCLC patients, the 5-year survival rate for people with SCLC is currently

7% (Sánchez-Ortega, 2021). It has been proved in multiple international randomized clinical trials that the introduction of annual low-dose computed tomography may significantly improve early diagnosis and reduce lung cancer mortality (Aberle, 2019).

Currently, we are witnessing a rapid development in precision oncology. These achievements greatly improve patients' outcomes, however, as indicated above there is a lot of space for improvement. Nowadays, we can describe a few examples of various genetic NSCLC (adenocarcinomas) growth drivers, which have contributed to developements in targeted therapies and immunotherapies. Currently, patients suffering from advanced lung cancer other than squamous cell carcinoma (SCC) should undergo genetic tests in clinical practice for the presence of EGFR, ALK and ROS1. The presence of these mutations is an important predictive factor for targeted therapies in terms of using inhibitors of EGFR (e.g. afatinib, erlotinib, gefitnib, osimertinib) and ALK or ROS1 inhibitors (e.g. crizotinib) (Lindeman, 2018). It was established that lung cancer, along with other cancer types, demonstrates a high genomic instability with the progressive acquisition of genetic alterations considered as: point mutations, epigenetic alterations etc. resulting in an expansion of complex diverse disease (Di Capua, 2021). Therefore, future directions will be focused on the development of various targeted therapies along with the evolution of standardized techniques of detecting biomarkers, which proves helpful in the disease control. Detection of circulating tumour DNA (ctDNA) has such a potential due to the fact that serum biomarkers are not generally used and surveillance is only based on clinical and radiological examinations (Schneider, 2020). Tumour originated DNA is released into various body fluids from: the primary tumour, metastatic lesions, circulating tumour cells (CTCs), minimal residual disease (MRD) (Li, 2020).

PURPOSE OF THE REVIEW

The main purpose of this review, based on the available literature, is an update and revision of current knowledge on the subject of the usage of detecting ctDNA in lung cancer patients. Detecting ctDNA, especially in case of non-small cell lung cancer (NSCLC), holds a big potential in early screening and disease control such as: detection of MRD, and further impact of next therapeutic options.

SEARCH STRATEGY AND SELECTION CRITERIA

To research the subject, the PubMed/MEDLINE, European Cancer Organization, Science Direct, Scopus databases were searched in a non-systematic manner for relevant publications. Articles that were published until August 2021 were included. Retrieved articles were filtered to remove duplicates and irrelevant results. The reference lists of the selected articles were checked for any other publications pertinent to this manuscript. The following key words were used: ("lung cancer" OR "non-small cell lung carcinoma") and ("ctDNA" OR "cfDNA" OR "liquid biopsy"). The research was limited to the available English reports (abstracts or full texts). All reports were selected (original and review articles) focusing on the ctDNA in the context of lung cancer diagnosis and therapy.

REVIEW

BIOLOGY OF "CFDNA – CTDNA" AND THE TECHNOLOGY OF DETECTION

Looking back on a timeframe, the first records of detection of cell free DNA (cfDNA) was date back to 1948, to the work of Mandel and co-researchers (Mandel, 1948). In the early 80's (1983) Shapiro with the team proved that there is a clear correlation between malignant and benign tissue and corresponding cfDNA concentrations (Shapiro, 1983). Cell-free DNA is released mostly from cells through mechanisms of apoptosis, necrosis, and also through active secretion in the process of forming extracellular vesicles (EVs). Apart from blood draw, the cfDNA can be detected in various body fluids such as: cerebrospinal fluid, urine, saliva and pleura (Wan, 2017). Elevated levels of cfDNA are described in various pathological conditions e.g. cancer, sepsis, autoimmune diseases and in particular, in physiological conditions such as pregnancy or intense physical exercise. As a result, the cfDNA level is studied as a potential biomarker for early diagnosis as well as for the diagnosis and prognosis (Khier, 2018). Unfortunately, there is insufficient evidence to support the pathophysiological role of cfDNA. Pre-

sumably, this is a byproduct of cellular processes/cellular stress (nonspecific biomarker of tissue damage). The role of cfDNA when released from healthy tissues is unclear.

Usually cfDNA are nucleosome protected 150-200 bp sized fragments, and have 2-hour half-life time (Perez-Barrios, 2016). However, in non-small cell lung cancer it has been determined that a molecule lasts for 35 minutes. (Chen, 2019). Different lengths of released fragments depend on the caspase endonuclease, which specifically splits DNA fragments. The newest studies have shown that the fragmentation pattern understood as length of released DNA is strictly dependent on tissue origin (Snyder, 2016). It important to highlight that ctDNA is one of the fractions of all cfDNA, no matter if it comes from healthy or cancerous tissue. The concentration of cfDNA in blood plasma is generally low ranging from 5-10 ng/mL, whereeas the fraction of ctDNA varies from 0.1% to 30% of the total cfDNA and is strictly dependent on the tumour size and disease stage (Crowley, 2013).

Taking into consideration low concentrations of cfDNA for liquid biopsy, samples require strictly defined conditions. Plasma is preferred over serum, due to a high risk of lymphoid cells lysis that lead to the deafening of ctDNA, finally giving false positive results. To avoid undesirable process of blood cells lysis, samples should be processed maximally within 4 hours from the collection in room temperature or within 24 hours at 4°C. To prolong the stability of the samples, there are also available commercially produced collection tubes (e.g. Roche Diagnostics, Qiagen). Those tubes contain leukocyte stabilizing agents that are capable of keeping the integrity of samples for at least 48 hours or even a week in a room temperature (Nikolaev, 2018).

Detection of ctDNA has been improved over the years, which has increased sensitivity and improved correlation with the results of tumour biopsy specimens. Detection of ctDNA requires the presence of mutations that can be detected by various sequencing techniques (Filipska, 2021). The biggest challenge in performing the analysis is to detect ctDNA in the background of cfDNA originating from healthy tissue, where allelic copies of mutated genes are pretty low (Crowley, 2013). In targeted methods of detection, better sensitivity scores can be observed, together with reduced scope in the genome (Ross, 2011). For example, the sensitivity of ddPCR technique varies from 74% to 82% with the specificity from 63 to 100% (Sacher, 2016). NGS techniques have even higher sensitivity and specificity, respectively ranging from 79% to 100% and 94-100% (Paweletz, 2016). Unfortunately, there is currently no "golden standard" in the detection of ctDNA. In general, the approach should fit the stage of the disease (fig. 1) (Riva, 2016). For example, PCR-based methods are quite sensitive and cost-effective, nevertheless, they also hold serious limitations. In the setting of NSCLC they are not recommended for detecting ALK and ROS rearrangements in ctDNA. Moreover, methods based on PCR are able to interrogate discreet and genetic mutations, and are limited in terms of the number of genetic targets that assays can detect (Rolfo, 2018).

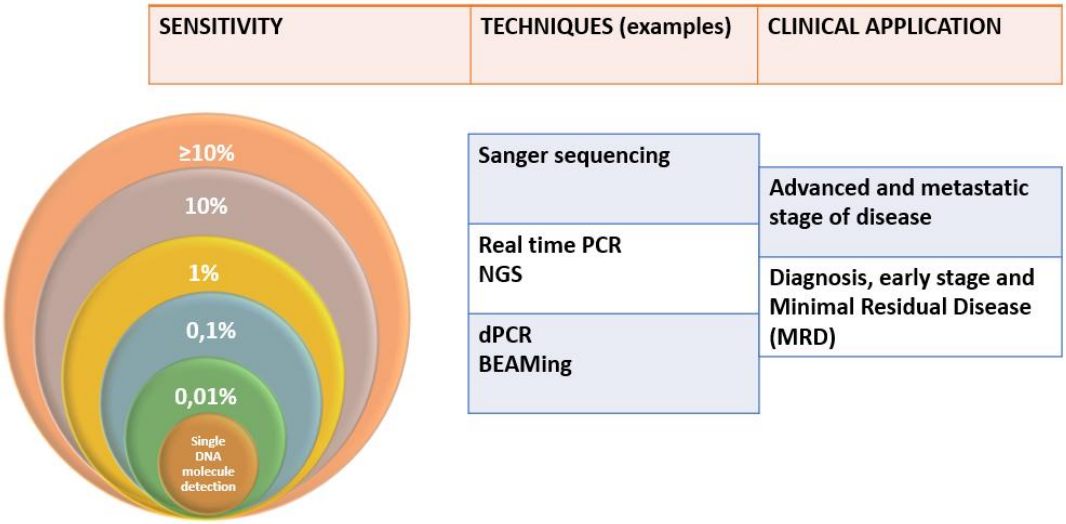

Figure 1. Different ctDNA detection techniques and their sensitivity versus clinical application (based on Riva, 2016)

Researches have already described a number of various molecular changes that cause lung cancer. Due to these findings patients' outcomes have significantly improved over the years, however, poor disease prognosis in late stages (III, IV) remain unchanged. The first symptoms of the disease, such as: shortness of breath, persistent cough, unintended weight loss, generalized weakness, are ignored by the patients (Gridelli, 2015). Thus, there is a reasonable unmet clinical need to develop noninvasive biomarkers such as ctDNA.

It has been proved that ctDNA genotyping can be a successful method for the early diagnosis of lung cancer (Li, 2020). It is important to highlight that a quick diagnosis allows early intervention and significantly improves survival rates (Cassim, 2019).

In trials conducted for various cancer types, there was approximately 82% sensitivity to a ctDNA detection in stage IV of the disease, however, in stage I of the disease, the sensitivity dopped to 47% (Bettegowda, 2014). Another trial involved female NSCLC patients who were tested for the presence of ctDNA. Plasma ctDNA, blood cell ctDNA, pleural effusion DNA samples where collected from these patients and the analysis justified that Next Generation Sequencing, droplet digital Polymerase Chain Reaction (ddPCR) was more sensitive and reliable than the usage of amplification refractory mutation system (ARMS) in terms of detecting ctDNA Epidermal Growth Factor Receptor (EGFR) mutations, specifically L858R and T790M mutations of early stage NSCLC. The detection of these alterations is important in terms of treatment with Tyrosine Kinase Inhibitors (TKI) (Rebuzzi, 2019). Moreover, in 2018 Cohen with the team developed CancerSEEK (Cohen, 2018). The test can detect eight different human cancer types by measuring concertation of circulating polypeptides and mutations in ctDNA. In case of lung cancer, detection rate reached 75%. It appears that the combination of detection levels of eight proteins and the presence of mutations in 1,933 distinct genomic positions can be considered to be an opportunity to diagnose lung cancer before the presence of radiological lesions, however, further studies on a bigger cohort of patients should be performed.

Advantages in the abovementioned techniques are undeniable, however, it is important to highlight the presence of the phenomenon called clonal hematopoiesis (CH). This condition, in some of cases, can be a source of false-positive results. It is considered that detectable mutations of cfDNA/ctDNA can lead to overdiagnosis. Clonal hematopoiesis (CH) is a typical age-related spread of white blood cells that carry somatic mutations, which is linked with an increased risk of hematological malignancies, cardiovascular diseases and other all-cause mortality (Silver, 2021). CH is caused by point mutations in genes associated with myeloid malignancies, chromosomal changes and loss of heterozygosity, whereby nonmalignant progenitor and hematopoietic cells acquire genetic alterations and may involve canonical CH genes. These genes include: tet methylocytosine dioxygenase 2 (TET2), DNA methyltransferase 3 alpha (DNMT3A), ASXL transcriptional regulator 1 (ASXL1), Janus kinase (JAK), tumourigenesis drivers, such as phosphatidylinositol 3-kinase (PI3KCA) and the abovementioned epidermal growth factor receptor (EGFR) (Liu, 2018; Hu,2019). The incidence of CH is ambiguous, but raises to 10-20% in individuals above the age of 70 (Silver, 2021).

The majority of cfDNA originates in hematopoietic cells, but those clonal hematopoiesis (CH) alterations in cfDNA are widely detected in serum and plasma. It means that with inappropriate controls, those mutations can be considered tumour-originated, causing misdiagnosis. High sensitivity analyses of cfDNA unveiled approximately 60-90% of CH mutations in patients without cancer, which proved that this condition is age-related (Liu, 2018). To ward off those false-positive results, there will be a need for introducing a procedure of white blood cells' control, that will include analyses of fragment length discrimination and deep error controlled sequencing (Filipska, 2021). Fortunately, this method is technically achievable and simple to conduct, although it doubles the costs and significantly reduces cost effectiveness (Chabon, 2020).

THE SIGNIFICANCE FOR PROGNOSIS PREDICTION AND TREATMENT CONTROL

As it has been described, a lung cancer patient has significantly higher total concentrations of cfDNA than healthy individuals. The total amount of cfDNA and ctDNA is correlated with tumour progression and the number of metastatic sites. Patients with different concentrations of ctDNA can be stratified into two groups: low-ctDNA concentration and high ctDNA concertation, which clearly guides the prognosis (Li, 2020). For example, the status of EGFR mutation significantly impacts patient outcomes. Alteration of EGFR gene leads to a dysregulation of cellular growth and proliferation. The frequency of EGFR mutations in NSCLC varies from 13% to 22%, depending on patient's ethnicity with a higher incidence in Asians (Antonoff, 2012). The status of EGFR ctDNA mutations has been checked in various clinical studies. One of them proved that the presence of EGFR ctDNA in patients with metastatic disease significantly reduces the time of progression free survival (PFS) and overall survival (OS) (Kim, 2019). There were several other clinical studies supporting the argument that detection of EGFR ctDNA shortens patients' outcomes measured as PFS and OS (Mok, 2015; Lee, 2016). Nonetheless, other studies showed contradictory correlation between the ctDNA EGFR presence and its impact on PFS, OS (Fan, 2017; Mao, 2015).

Fan et al. published metanalysis that reported PFS and OS stratified by the presence of EGFR and KRAS mutations in ctDNA in advanced NSCLC lung. The pooled analysis showed that EGFR mutations in ctDNA were significantly improving the PFS (HR = 0.64, 95% CI [0.51-0.81], I^2 = 0%, p < 0.001) in patients treated with EGFR-TKI. A trend was also observed of prolonged OS in terms of EGFR ctDNA presence. On the other hand, the presence of KRAS mutations in ctDNA was correlated with a prediction of worse PFS (HR = 1.83, 95% CI [1.40-2.40], p < 0.001) and OS (HR = 2.07, 95%CI [1.54-2.78], p < 0.001) in advanced NSCLC patients treated with chemotherapy. The abovementioned conflicting results may be due to the application of different inclusion/ exclusion criteria, as well as various generations of TKI (currently three generations are used in treatment) (Zhang, 2016), and finally due to small sample sizes.

Undoubtedly, globally performed clinical studies with improved stratification of patients in terms of ctDNA detection will facilitate proper direction. Presently, there are two assay kits approved for the EGFR testing in liquid biopsies, TheraScreen EGFR RGQ PCR Kit (Qiagen) and Cobas EGFR Mutation test v2 (Roche Diagnostics). Generally, researchers aim to develop finelytuned approach to guiding lung cancer patients that will be approved by regulatory authorities for usage in clinical practice.

MINIMAL RESIDUAL DISEASE (MRD)

ctDNA is also under investigation for usage as a marker for detecting minimal residual disease (MRD) in patients who have undergone therapy for NSCLC. The intent of applying this approach comes from disadvantages of standard imaging. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) feature limited sensitivity to detecting micrometastases compared to overt metastases (Li, 2020). There was a demonstrable decrease in ctDNA in patients that have undergone resection of NSCLC (Guo, 2016). In one study, over 50% of patients showed detectable ctDNA in the post treatment period, while 72% of patients had detectable ctDNA prior to the relapse of the disease. Moreover, the detection of ctDNA anticipated a radiological relapse by a median of 5.3 months (Chadhuri, 2017). Another study evaluated the dynamics of ctDNA testing. The presence of ctDNA one day after the surgery was not impacting the outcomes for patients understood to have had relapse free survival (RFS) and OS. How-ever, the presence of ctDNA 3 and 30 days after surgery was associated with poor outcomes (Chen, 2019). Surely, the introduction of detecting ctDNA defined as moni-toring the presence of MRD into regular clinical practice e.g. performing "liquid biopsy" during follow-up visits can significantly improve patients' outcomes. Implementing targeted treatment before the occurrence of detectable metastases can unquestionably prolong life expectancy.

CTDNA – BIOMARKER FOR IMMUNOTHERAPY

The introduction of immunotherapy into treatments of different types of cancer has showed promising responses depending of the subgroup of patients. In terms of these results, there is a need for searching biomarkers which will better stratify the patients (Filipska, 2021). One of the

recently launched clinical trials can change current clinical practices. BESPOKE study (NCT04761783 – May 2021) will examine the impact of SIGNATERA™ (personalized, tumour informed 16-plex Next Generation Sequencing assay for the detection of ctDNA) on treatment decisions on tumour assessment (NSCLC, Melanoma, Colorectal Cancer) and timepoints after the initiation of immunotherapy. Furthermore, researchers detect Histone-Lysine N-Methyltransferase SETD2 mutation in fusion drived NSCLC, which is not detected in patients with BRAF, KRAS, EGFR or MET mutations. These findings are important because ALK, ROS1 fusion mutations showed no clinical response to the treatment with immune checkpoint inhibitors e.g. antibodies targeting anti-PD-1 (nivolumab and pembrolizumab) and anti-PD-L1 (atezolizumab). Moreover, the PD-1/PD-L1 expression level is correlated with EGFR, ALK, and the treatment of patients with TKIs. Several clinical studies have shown that the level of expression of PD-L1 protein is upregulated in NSCLC cell lines that express an EGFR and the EML4-ALK fusion protein that are responsible for the progression of tumour (Zhu, 2020).

Although the development antibodies for the treatment of cancer is indisputable, not all NSCLC respond to the treatment. There is a need to validate the feasibility of ctDNA detection in large cohort clinical trials to maximize the outcomes for patients treated with immunotherapies (Yang, 2021).

CONCLUSION

It is certain that ctDNA-based liquid biopsies may be a forceful tool for diagnosing cancer, monitoring the disease, making predictions and obviously changing the current procedures in managing the patients with different types of cancer (fig. 2). In authors' opinion these techniques will provide detailed molecular information and may reduce a need for performing high risk invasive procedures as well as the execution of pathological assessment.

However, introducing ctDNA testing for regular practice in each and every case faces several barriers. The biggest obstacle is the requirement for proper specificity and sensitivity. Moreover, there is a need for lowering the testing costs. This problem stems from very low concentrations of ctDNA, especially in early stage of the disease and is also strictly dependant on tumour biology. The number of patients enrolled for ctDNA research is much lower than the respective number in other clinical studies. Due to the aforementioned reasons clinical interpretations of given results are presently rather hobbled. It will be crucial to test composite gene panels with well indicated endpoints to prove their clinical utility.

Certainly, further studies will deliver more "real-world" data which should and can improve all stages of ctDNA analysis starting from the isolation and finishing on the interpretation of received results. A combination of standard of care approaches with the abovementioned novel techniques can significantly change lives of patients suffering from lung cancer and also other types of cancer.

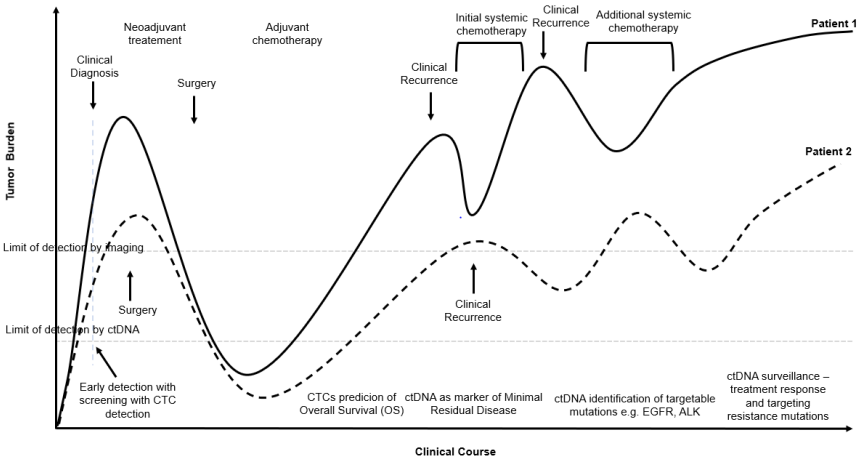

Figure 2. Graph indicating possible roles of ctDNA along with CTCs in the clinical course of patients suffering from Non-Small Cell Lung Cancer in comparison with standard of care (SoC) approaches. Patient 1 (solid line) demonstrates a standard "journey", but Patient 2 (dotted line) goes with the approach including the usage of ctDNA and CTCs in NSCLC treatment (based on Di Capua, 2021)

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