Chapter

Liquid Biopsy: A New Diagnostic Strategy and Not Only for Lung Cancer?

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Abstract

Targeted molecular therapies have significantly improved the therapeutic management of advanced lung cancer. The possibility of detecting lung cancer at an early stage is surely an important event in order to improve patient survival. Liquid biopsy has recently demonstrated its clinical utility in advanced non-small cell lung cancer (NSCLC) as a possible alternative to tissue biopsy for non-invasive evaluation of specific genomic alterations, thus providing prognostic and predictive information when the tissue is difficult to find or the material is not sufficient for the numerous investigations to be carried out. Several biosources from liquid biopsy, including free circulating tumor DNA (ctDNA) and RNA (ctRNA), circulating tumor cells (CTCs), exosomes and tumor-educated platelets (TEPs), have been extensively studied for their potential role in the diagnosis of lung cancer. This chapter proposes an overview of the circulating biomarkers assessed for the detention and monitoring of disease evolution with a particular focus on cell-free DNA, on the techniques developed to perform the evaluation and on the results of the most recent studies. The text will analyze in greater depth the liquid biopsy applied to the clinical practice of the management of NSCLC.

Keywords: liquid biopsy, NSCLC, cfDNA, ctDNA, biomarkers

1. Introduction

In the era of precision medicine, the management of cancer patients has dramatically changed. An increasing number of prognostic and predictive biomarkers are being implemented in order to ensure the best treatment option for advanced stage cancer patients and pathologists have learned to refine their reports [1]. To date, the analysis epidermal growth factor receptor (EGFR) gene mutations, anaplastic lymphoma kinase (ALK), and ROS 1 proto-oncogene (ROS1) rearrangements and schedules the state of death-ligand 1 (PD-L1) is capital for clinical decision making [2, 3]. In addition to these biomarkers, other activating mutations harbored by other clinically relevant genes are under investigation in clinical trials. However, despite the rapid development of this field in terms of discovered predictive biomarkers and platforms, tissue still remains an issue. To date, tissue samples represent the "gold standard" starting material to obtain nucleic acids (DNA and RNA) for molecular purposes and the only matherial available for morphological diagnosis and molecular analisys is often represented by paucicellular samples

(cytological speciments or small tissue histological biopsies) [4]. However, a not negligible percentage, about 30%, of advanced stage NSCLC patients, cannot be tested because tissue sample is not adequate both for anatomo pathological revision and for molecular analysis [5]. In addition, even in high expertise centers, the percentage of inadequate molecular results, in particular when small tissue samples are adopted, may be significant [6]. In this setting, and in order to avoid to leave patients behind, liquid biopsy represents a valid option as a rapid, noninvasive and accurate clinical option. Several biosources from liquid biopsy, including free circulating DNA (cfDNA) and RNA (cfRNA), circulating tumor cells (CTCs), exosomes and tumor-educated platelets (TEPs), can be isolated. While each of these modalities has the potential to provide new diagnostic information and their exploration is highly encouraged, ctDNA certainly represents the most mature example of the survey on liquid biopsy in clinical practice for lung cancer patients. Therefore, this chapter will mainly focus on the clinical value of the free circulating tumor DNA (ctDNA), a small fraction of cfDNA extracted from plasma samples and we will review the available data that suggest the role of liquid biopsy in the management of NSCLC.

To date, Food and Drug Administration (FDA) has approved the analysis of ctDNA in two different NSCLC patients settings: naïve advanced stage NSCLC (basal setting), when tissue is not available or inadequate for molecular analysis of the Epidermal Growth Factor Receptor (EGFR) in order to select patients for first or second generation EGFR tyrosine kinase inhibitor (TKI) administration; acquisition of somatic resistance mechanism after first or second EGFR TKI administration (resistance setting), in order to detect the EGFR exon 20 p.T790M resistance point mutation and select patients for third generation EGFR TKIs [7].

Here we will report the potential of liquid biopsy to help manage NSCLC throughout all stages: cancer screening, minimal residual disease detection to guide adjuvant treatment, early detection of relapse, systemic treatment initiation and monitoring of response (targeted or immune therapy), and resistance genotyping. Moreover, we will also carefully analyze each step of the pre-analytical management of liquid biopsy specimen, (sample collection, ctDNA extraction, and molecular analysis) and the advantages and disadvantages found in the use of liquid biopsy respect the adoption of gold standard tissue sample in the context of clinical practice.

2. Liquid biopsy: definition

The term liquid biopsy refers to the use of biological fluids as a surrogate for neoplastic tissue to obtain information useful for diagnostic, prognostic purposes or to predict the response to therapy with specific anticancer drugs. In the biological fluids (blood, urine, saliva, cerebrospinal fluid, pleural effusions, ascites or cytology specimen-derived supernatant [8–10] of tumor patients are contained cell-free DNA (cfDNA), circulating tumor DNA (ctDNA)circulating tumor cells, circulating RNA, microRNAs (miRNA), platelets and exosomes, which can be a valuable source of information about molecular assessment of cancer. Analysis of ctDNA contained in the free circulating DNA (cfDNA) that can be isolated from peripheral blood, represents to date the main liquid biopsy approach employed in clinical practice. In healthy patients, cfDNA is released in low quantity from normal cells during cellular turnover and is represented by small DNA fragments (150–200 base pairs). On the otherwise cancer patients show an increased levels of cfDNA [11, 12] with a consistent release of ctDNA generally represented, by more small fragment, with sizes ranging from 90 to 150 base pairs [13]. The amount of ctDNA is variable

in cancer patients, ranging from less than 0.1% to more than 90% [11, 12]. The cf./ctDNA ratio can depends on the time of sample collection and clinical condition of the patient and is influenced by total tumor burden, location and extent of metastases, proliferation rate, apoptotic potential and genome instability [14]. ctDNA can enter the bloodstream through two different mechanisms: by a passive mechanism derived from apoptosis and necrosis or by an active mechanism derived from a spontaneous release of DNA fragment from primary tumor tissues or from circulating tumor cells or tumor-associated macrophages [15, 16].

2.1 Liquid biopsy versus traditional biopsy

The new College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC)/Association for Molecular Pathology (AMP) guideline for molecular testing of patients with NSCLC, although did not recommend the performance of molecular analysis when tissue is available, they strongly suggest liquid biopsy in those cases where tissue sample is not adequate to perform molecular analysis [17] In fact, a significant subgroup of patients cannot undergo a biopsy or rebiopsy for several reasons such as unsuitable clinical conditions or an unfavorable tumor site such as bone or central nervous system or multiple small pulmonary nodules that are not safely amenable to biopsy [18].

Let us see what are the advantages and disadvantages of using liquid biopsy.
Liquid biopsy shows several advantages over that traditional biopsy:

- the procedure is not invasive, as it is a simple blood sampling and than may spare the patient an invasive procedure with the incumbent risks of major complications. Often occurs that patient need a rebiopsy due to the scant tumor tissue to obtain new tissue for further tests and to performing all the required analyses;

- the ability to represent more comprehensively than tissue biopsy the molecular heterogeneity of the disease. Several studies have compared mutational profiles of different sites of metastatic disease from the same patient and have established the intratumoral heterogeneity and spatial and temporal tumor heterogeneity of solid tumors [19, 20]. While tissue only offers a snapshot of the tumor at a given time and location, may not represent the complex genetic profile of a patient's tumor and can introduce the hypothesis of false-negative results, ctDNA that is shed into the periphery from multiple sites of disease has the potential to overcome both spatial and temporal tumor heterogeneity. Circulating markers are more effective in depicting the emergent biology in actively growing metastatic lesions which may be missed [21, 22].

- can follow subclonal evolution through iterative noninvasively blood draws taking into account different clones present within all metastatic sites as a surrogate for more invasive tissue biopsies. It can be usefull to monitor molecular evolution of the disease, although there is no evidence to date that addresses a modify the therapeutic choice, in the absence of clinical progression of disease;

- as a cost-effective alternative for patients considering that performing a tissue biopsy is considerably more expensive than a blood draw;

- turnaround time may be shorter considering the scheduling time involved [23].

- However, ctDNA suffers of several limitations that may impact on the adoption in routine predictive laboratory practice:

- the amount of ctDNA in the context of cfDNA is often extremely limited, (<0.5% of the total circulating cell free DNA) [24] depending on both the volume and the localization of the disease and this can lead to misinterpret molecular results (false negatives);

- ctDNA has a very low half-life (about 15 minutes) and this is a crucial limitation [24]. This aspect lead to reduce of 50% cfDNA molecules each 15 minutes from the withdrawn.

The International Association for the Study of Lung Cancer (IASLC) established in a statement paper the guideline when considering to adopt liquid biopsy samples for molecular analysis [7].

3. What are the methods for ctDNA analysis? Pre-analytical consideration: from blood collection to ctDNA

CtDNA can be extracted from various biological fluids. However, the procedures more standardized in clinical practice concern isolation of ctDNA from peripheral blood. ctDNA can be isolated from serum or plasma. Several studies compare cfDNA levels in plasma and serum samples and have shown significantly higher cfDNA concentrations in serum [25, 26] but shown that the use of plasma is preferable to serum. In the latter, in fact, the coagulation process causes the release of genomic DNA deriving from leukocytes and leads to a contamination of germline DNA that causes a ctDNA dilution.

Isolation and enrichment of ctDNA is a great challenge given the high degree of cfDNA fragmentation and its low concentration in the bloodstream to the extent of a few ng/ml, of which the ctDNA is alone a fraction [15]. Several factors affect the quality and quantity of ctDNA including: the burden of disease, the rate of release of the ctDNA in the bloodstream and the levels of DNA released by non- transformed cells. Therefore, for these reasons the pre-analytical phase must be carefully checked. to achieve superior quality results.

3.1 Sample collection

A first problem that can affect the quality of the sample is constituted from hemolysis that can occur during phlebotomy. It is necessary, therefore, that blood sampling is carried out by highly qualified personnel.

The sample can be collected in standard EDTA tubes or using tubes containing special fixatives, capable of stabilizing the blood and the cfDNA for several days. If the sampling is carried out using standard tubes, one important factor must be taken into account. cfDNA has a short half-life, estimated at around 2.5 hours therefore tubes can be stored before plasma isolation for a maximum of 3 hours at room temperature (**Figure 1**). Several studies have shown that after three hours of sampling, the wild-type cfDNA concentration could increase due to lysis of hematological cells thus reduce the relative percentage of tumor-specific ctDNA [27]. The storage of blood at a temperature of 4°C it does not prevent leukocyte lysis. Operators dealing with blood collection, handling, and eventual shipping should be cognizant of these time constraints. Blood should never be frozen before plasma extraction.

3.2 Extraction, quantification and storage of cfDNA

There are many methods for extracting cfDNA, which include both the use of commercial kits and protocols developed by laboratories.

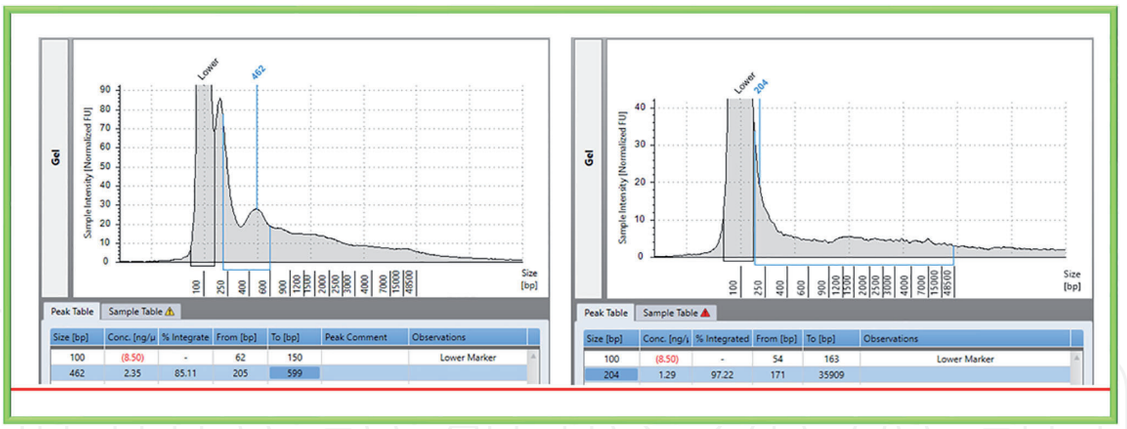

Figure 1.
Incidence of time withdrawn in the managment of liquid biopsy specimen. The figure shows the comparison between electhropherograms performed by nanofluidic platform Tapestation 4200, (Agilent, Santa Clara, California, USA) on a sample immediately and after 1 h processed to isolate cfDNA respect time of withdrawn. In the left profile, ctDNA peak of 180–200 bp is inspected; on the right profile the peak is not detected.

The extraction method must be very reliable and must generate as much possible DNA of the sample under examination. For the extraction and purification of cfDNA from plasma they are now available various commercial kits dedicated to this specific use. These kits are generally based on the use of columns equipped with silica membranes in association with a vacuum pump, or on the use of magnetic balls for the capture of nucleic acids.

Once extracted, the cfDNA must undergo quantification in order to optimize the amplification process and to know if the subsequent molecular analyzes may be possible starting from the cfDNA extract [28].

Optimal storage of cfDNA is very important as it allows its use also a time to carry out further molecular investigations, The process takes adequate equipment, including –20° C/ -80° C freezers, devices graphic temperature control, acoustic alarm systems, quality controls of the biological material stored [28].

4. Analytical methods for detecting ctDNA

Until recently, the available technology was not sensitive enough to detect ctDNA and use it in a meaningful way but in recent years highly sensitive blood-based assays have been developed to test cfDNA at very low concentrations for most genomic abnormalities and advances in pre-analytical processes and purification methods have enabled the capture, amplification and sequencing of ctDNA to be successful. A good molecular ctDNA test should retain an acceptable concordance to molecular testing in the tumor tissue. However, even with the increased sensitivity, a negative result from ctDNA analisys is not sufficient to exclude the potential existence of a driver alteration; therefore, in these circumstances tissue analysis should be performed.

The methods currently used to detect or measure ctDNA are numerous and can be divided into two categories: polymerase chain reaction (PCR)-based techniques and next-generation sequencing (NGS) technologies.

4.1 Polymerase chain reaction (PCR)-based techniques

Within the PCR-based techniques are included real time PCR, digital PCR (dPCR), droplet digital PCR (ddPCR), peptide nucleid acid (PNA) clamp-based PCR assay (Taqman assay), beads, emulsions, amplification and magnetics

(BEAMing). PCR-based approaches, can detect mutations in cfDNA at allele frequencies of 0.01% or lower. Although there are numerous platforms currently for ctDNA evaluation, the gold standards in pcr-based technologies are basically quantitative PCR (qPCR) and digital PCR.

4.1.1 PCR real time

PCR-based tests generally have faster response times and are less expensive, but can typically evaluate only one or a few specific mutations at a time. The analysis of point mutations or small insertions/deletions on ctDNA it can be often conducted through the use of Real Time technologies PCR, often modified to increase the sensitivity of the test. There are commercially available ctDNA kits based on different amplification technology (e.g. Refractory Mutation System ARMS/SCORPION) which detect mutations against EGFR exons 19, 20 and 21. These kits allow the co-amplification of one or more mutated alleles and of an endogenous control gene. The analysis with these kits allows to detect low percentages of mutated allele in the presence of high amounts of wild-type genomic DNA and can reach an even lower limit of detection (LOD) 0.5%. The main limitation related to the adoption of this approach is related to the annealing step where probes may not able to target corresponding genomic region (**Figure 2**).

4.1.2 Digital PCR (dPCR)

Digital PCR (dPCR) is a next-generation evolution of PCR of which there are two technological platforms: "digital droplet PCR - ddPCR" and "BEAMing dPCR" (Beam, Emulsion, Amplification, Magnetics).

Both methods, utilize emulsion technology to quantify the amount of mutant cfDNA in patient plasma and are based on the limiting dilution of DNA with a distribution of the sample in thousands of homogeneous "droplets" in an oil–water

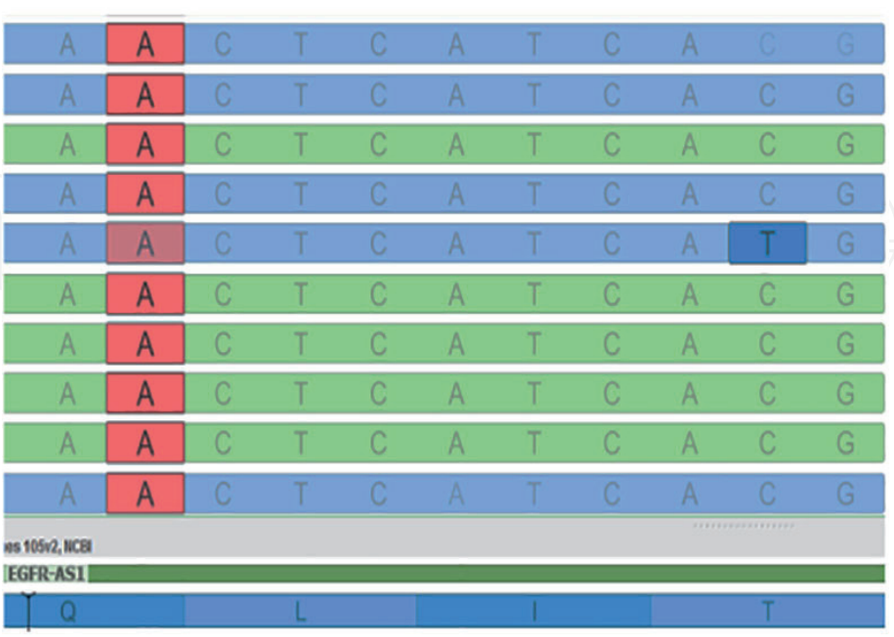

Figure 2.
Visual inspection of p.T790M EGFR acquired resistance mutations by using NGS system. The figure shows the manual count of aligned reads generated by Golden helix genome browse 1.1.2 (Golden helix genome browse Inc). In the red box is reported a polymorphism in the genomic region near p.T790M base change (blue box). Although the presence of this polymorphic alteration, NGS platform is able to p.T790M resistance mutation while RT-qPCR based approaches report a false negative result.

emulsion. These characteristics are of great importance in the context of the analysis of ctDNA, where it is necessary to search for and amplify rare molecules of tumor DNA in the presence of a large excess of wildtype germline DNA.

In fact, the breakdown of the sample into droplets has the function of reduce competition between mutated tumor DNA and wild-type DNA increasing the specificity and sensitivity of the analysis.

Both of these experimental approach is very useful for the identification of rare variants since only a small concentration of template is required for the analysis.

The sensibility and the specificity of the tests with ddPCR and BEAMing dPCR are, respectively 0.1% and 0.01%.

Several studies have compared multiple platforms for detecting EGFR mutations in plasma ctDNA. In one study, two non-digital platforms (cobas®EGFR mutation test and therascreen EGFR amplification refractory mutation system test) and two digital platforms (Droplet Digital PCR and BEAMing digital PCR) were compared in their ability to identify sensitizing mutations and the results support the potential use of both platforms in a clinical development program [29].

The limit of these technologies is that of their limited ability to detect complex genomic alterations and perform multiplex testing.

4.2 NGS in liquid biopsy

In contrast to PCR-based methods, NGS is a fascinating technology able to analyze different biomarkers for different patients, simultaneously [30] and detect rare and previously uncharacterized alterations. NGS are capable of detection of mutations, indels, copy number variations and genomic rearrangements such as oncogenic fusions. Another advantage of an NGS approach is the possibility to quantify the amount of DNA that brings a particular alteration.

This technology, based on massive and parallel sequencing, ensures a high sequencing throughput, by generating from hundreds of thousands to millions of sequences (reads) [30]. To overcome the limitations of ctDNA, next generation sequencing (NGS) may be a viable option. The use of NGS for liquid biopsy requires changes to the protocols normally used for blood and tissue analysis. Operational protocols dedicated to liquid biopsy.

Different NGS platforms are commercially available and validated on liquid biopsy samples [31]. Among these, Illumina (San Diego, California) platform adopts a sequencing-by-synthesis chemistry able to identify DNA bases, while introducing them into a nucleic acid strand, by adopting a system of fluorescently labeled nucleotides; Ion Torrent (ThermoFisher Scientifics, Waltham, Massachusetts) platform adopts a sequencing-by-synthesis chemistry able to identify DNA bases, while introducing them into a nucleic acid strand, by adopting a semiconductor system useful to measure a change in pH due to the release of an H+ ion [32]. Important differences exist between these various platforms with respect to the number of genomic alterations included in a single panel, the ability to multiplex these assays, the turnaround time of the test, and its ability to detect complex genomic alterations.

Different NGS gene panels have been adopted for ctDNA analysis and numerous studies have been carried out in recent years to validate concordance to molecular testing in the tumor tissue. A higher sensitivity and specificity was obtained by Reckampet al by using a short footprint mutation enrichment NGS assay to analyze ctDNA samples extracted from plasma of NSCLC patients [8]. On the overall, taking into account the results obtained on matched tissue samples, a sensitivity of 93, 100, and 87% for EGFR exon 20 p.T790M, EGFR exon 21 p.L858R, and EGFR exon 19 deletions, was reached. The specificity, for the same mutations was 94, 100,

and 96% [8]. In the experience of Paweletz et al. a high sensitivity (86% and 79%) and specificity (100% and 100%) was obtained by using a targeted NGS approach on NSCLC patients for EGFR and KRAS mutations [33]. Another valid approach is represented by the use of narrow gene panels. In the experience of the Predictive Molecular Pathology Laboratory at the University of Naples Federico II a custom, narrow NGS gene panel (named SiRe®), that cover 568 clinical relevant mutations in six genes (EGFR, KRAS, BRAF, NRAS, KIT and PDGFRA), was routinely employed for both tissue and liquid biopsy testing [34, 35]. In the validation study on different tumor types, a sensitivity of 90.5% and a specificity of 100% was reached by comparing results obtained on ctDNA extracted from serum and plasma with those obtained on matched tissue samples. In addition, this panel may be useful to detect EGFR and KRAS actionable mutations in basal setting NSCLC patients [35].

Ultra-sensitive NGS techniques dedicated to analysis have been developed of the ctDNA. The characterizing element of Cancer custom profiling deep sequencing (Capp-Seq), its is a selector that identifies different classes of somatic mutations with sensitivity and specificity greater than 90%. Similar results in terms of sensitivity and specificity were achieved with Tagged-amplicon deep sequencing (Tam-Seq) and Safe-Sequencing techniques System (Safe-SeqS).

What was once a limitation of ngs technology, the turnaround time is now acceptable for clinical management — approximately 13 days — and costs have been significantly reduced [36]. Also the difficulty of analyzing the numerous information deriving from the multigene panels has also been overcome since a variety of publically available and proprietary bioinformatics tools have been developed to assist in these calculations.

4.3 New emerging technologies to the study of liquid biopsy

Several studies today aim to overcome the current limits of sensitivity for liquid biopsy, to support extensive research and clinics applications. Although for the evaluation of the ctDNA the gold standards are basically quantitative PCR (qPCR), digital PCR and NGS to these have been added many technologies such as whole genome sequencing (WGS) [37], Rare Ep iAlleles by Melt qPCR (DREAMing) [38] and bidirectional pyrophosphorolysis activated polymerization (bi-PAP).

Moreover, of great interest in biomedicine applied to the study of liquid biopsy are the PCR-free methods [39]. Several articles have been published dealing with PCR-free methods for the detection of point mutations [40]. These methods applied alternative isothermal-amplification methods whitch do not require thermal cycling to avoid heating and cooling steps. About the smetods include those based on nucleic acid sequences polymerization (NASBA), loop-mediated amplification (LAMP, helicase-dependent amplification (HAD), rolling - circle amplification (RCA), recombinase-polymerase amplification (RPA) and multi-displacement amplification (MDA), isothermal displacement of the circular filament polymerization (ICSPD).

Among these methods of sure interest is surface plasmon resonance imaging (SPR-I). This tecnology are able to detected Attomolar concentrations of target genomic DNA, demonstrating the ultra-sensitivity of the new method [39].

Thanks to these new ultra-sensitive technologies, several authors are pushing towards new horizons. In some studies differences in fragment lengths of circulating DNA could be exploited to enhance sensitivity for detecting the presence of ctDNA and for noninvasive genomic analysis of cancer. Mouliere et al. [13] argue that cfDNA fragment size analysis improved the discrimination between samples from patients with cancer and those from healthy individuals.

All these technologies are now supported by different platforms and have already been approved for clinical use [41]. However, the lack of standardization limit the clinical implementation for most of these methodologies.

5. Liquid biopsy in clinical practice

There are two main scenarios in which the liquid biopsy might confer an advantage to NSCLC advanced patients:at initial molecular profiling and at progression during targeted therapy. To date, Food and Drug Administration (FDA) has approved the analysis of ctDNA extracted from plasma samples in two different patient settings: in treatment naïve advanced stage NSCLC (basal setting), when tissue is not available or inadequate for molecular purposes, for the Epidermal Growth Factor Receptor (EGFR) gene assessment in order to select patients for first or second generation EGFR tyrosine kinase inhibitor (TKI) treatments and in advanced stage NSCLC resistant to a first or second EGFR TKI (resistance setting), in order to detect the EGFR exon 20 p.T790M resistance point mutation and select patients for third generation EGFR TKIs [7].

5.1 Tumor molecular profiling of naïve patients (basal setting)

Liquid biopsy should be taken into consideration at the time of initial diagnosis in all patients who need tumor molecular profiling, but:

- tumor tissue is insufficient or unavailable;
- in patients for whom invasive procedures may be risky or contraindicated;
- in those cases where the biopsy scheduling time exceeds two weeks causing a serious delay in diagnosis;
- in the case of bone biopsies where the decalcification processes of the sample irreversibly damage the nucleic acids and therefore invalidate any molecular investigation;
- moreover, indications for choosing treatment-naive patients for ctDNA molecular testing are all patients with advanced/metastatic nonsquamous NSCLC and patients with squamous NSCLC if never smoker and/or younger age.

In clinical practice, liquid biopsy is currently mainly used for analysis of mutational status of the epidermal growth factor receptor (EGFR) in advanced NSCLC patients. Several studies and meta-analyzes [42, 43] have evaluated the diagnostic accuracy of ctDNA analysis for the identification of the most frequent activating mutations of the EGFR gene (deletions of exon 19, L858R of exon 21) in patients naïve with advanced NSCLC proving a good specificity greater than 90%. Sensitivity results instead be lower, with fluctuations between 50% and 80% depending on the technology used. On the basis of these evidences the evaluation of the mutational status of the EGFR gene on liquid biopsy is currently recommended as a possible alternative to analysis on tumor tissue.

A positive finding of an actionable mutation in ctDNA, if using a validated assay, is sufficient to initiate targeted treatment. However, a negative result it cannot be trusted and it should be followed up with a secondary test or conventional tumor testing. A negative result can be negative for several factors. The amounts

of DNA into peripheral circulation of patients with indolent slow-growing tumors is often insufficient for detection and these patient may be at more risk of a false negative compared to patients with a more disseminated cancer. Therefore, it is imperative that operators are aware of the possibility of a false negative result from the liquid biopsy.

For this reason the liquid biopsy at diagnosis is far from being able to replace the traditional analysis of the tissue which therefore remains the standard goal in patient's diagnosis.

5.1.1 Other oncogenic drivers in NSCLC have been detected in ctDNA isolated from plasma

In addition to EGFR mutations a wide range of potentially actionable alterations are of particular interest clinically and are detectable in patients with NSCLC, including ROS1, fibroblast growth factor receptor 3 (FGFR3), and neurotrophic receptor tyrosine kinase (NTRK) rearrangements and ALK, MEK, AKT, BRAF, HER2, MEK1/2, NRAS, KRAS mutations and MET receptor tyrosine kinase mutations and amplification. All of these abnormalities have been detected in ctDNA isolated from plasma and could therefore be used for screening naive patients.

As regards the study of rearrangements, which is carried out at the ctRNA level, some technical difficulties may arise due to the low stability of the circulating plasma RNA which easily undergoes degradation. The most commonly used techniques to identified ALK at the time of diagnosis, are qPCR and digital PCR or next-generation sequencing (NGS) approaches [44, 45].

The sensitivity of the techniques for studying this alteration is less high than those that can normally be used on the tissue (e.g., immunohistochemistry or Fluorescent in situ Hybridization (FISH).

5.1.2 The liquid biopsy in other fluids

Although plasma is the most widely used fluid sample for liquid biopsy, other samples are available such as urine, sputum, cerebrospinal fluid or pleural effusion as a source of ctDNA.

Sputum is an important source of nucleic acids and different studies investigated EGFR status in NSCLC patients. Wu et al. [46] identified a high concordance between sputum and tissue samples (74%). In a recent study it has been identified the potential role as a diagnostic biomarker for NSCLC of P16INK4 gene promoter methylation in both bronchoalveolar lavage (BAL) and sputum [47]. A recent study showed that sensitivity of EGFR mutation detection in the urine is comparable to that found in the plasma of the same patient [48] and concordance of mutations in the driver gene may increase when compared to plasma alone to combination of plasma, urine and sputum [49].

The ability to use ctDNA obtained from cerebrospinal fluid (CSF) to study genetic alterations and monitor response to treatment is very important as brain metastases are difficult to reach.

5.2 Monitoring therapeutic resistance

Resistance inevitably arises in almost all patients undergoing treatment for metastatic disease [50]. The ability to detect the presence of multiple resistance mechanisms is critical. Acquired resistance to therapy is often driven by presence of one or more tumor subclones that harbor resistance alterations [51]. These

subclones drive disease progression and may reside in the same tumor lesion or in different metastatic lesions [52]. Therefore, a standard tumor biopsy of a single lesion at the time of disease progression may fail to capture resistance mechanisms present in tumor cells outside of the biopsied region [53]. This is where the liquid biopsy comes into play overcomes the limit of tumor heterogeneity.

p.T790M gatekeeper resistance identification in liquid biopsy is currently employed as a first diagnostic approach in all patients with EGFR-positive advanced NSCLC in progression after EGFR-TKI treatment. However, due to the risk of "false negatives" associated with such method, all patients in whom the mutational analysis on ctDNA results "negative" and even the initial sensitizing mutation is not detected must be subjected to test tumor tissue taken by re-biopsy, in order to define the best therapeutic strategy. However before proceeding with the tissue biopsy, it would be advisable to repeat liquid biopsy. In the absence of such a mutation, the test should be considered non-informative as the sample contains no sufficient ctDNA. In this regard, multiple studies, and a recent meta-analysis, have clearly highlighted as the site of metastases cancer significantly affects the diagnostic accuracy of the mutation analysis of the EGFR gene performed on ctDNA. The sensitivity of that method in determining both activating and p.T790M mutation can in fact vary from 80% in presence of extra-thoracic metastases at 50% in the presence of exclusively intra-thoracic localizations [28]. In order to increase the chances of success of the liquid biopsy, the test should be performed at the time of obvious progression of disease, when the probability that the tumor DNA is released into the circulation is higher.

Liquid biopsy is also utilized to detected additional coexisting resistance alterations, such as MET amplification that predicts decreased benefit from subsequent therapy with third-generation EGFR inhibitors and EGFR p.C797S mutation following therapy with the third-generation EGFR inhibitor osimertinib [52].

6. How should the results of liquid biopsy be reported? The reporting

European Society of Pathology Task Force on Quality Assurance in Molecular Pathology and the Royal College of Pathologists published standards of molecular diagnostics reporting [54].

Reporting is an integral part of the diagnostic procedure and should contain the following information: patient identifiers, specimen type, assay methodology and the platform used including sensitivity and limit of detection, date of collection of the material used for the analysis and date of arrival of the sample in the laboratory that performs the analysis, methods of conservation of the sample investigated mutations, results of the test, data interpretation and an overall evaluation of the analysis specifying whether a detected alteration is clinically relevant.

The report must be completed on a predetermined form, dated and signed by the service manager.

7. Emerging application and future direction of liquid biopsy

Liquid biopsy is a rapidly growing field in oncology and ctDNA analysis is considered to be very promising as a biomarker for early stage detection, identification and monitoring of minimal residual disease (MRD), immuno-oncology, assessment of treatment response, and monitoring tumor evolution. Currently, several trials are ongoing in this field.

7.1 Potential applications in early stage NSCLC: screening

Detection of lung cancer at an earlier stage of disease, potentially susceptible of curative resection, can be critical to improve patients survival. Current screening and diagnostic tests, such as computed tomography (CT) scans and cytological/histological analyzes could be supplemented by the specificity of cfDNA [55]. The evaluation of genomic anomalies, including specific mutations, in cfDNA could offer a promising, non-invasive approach for the early diagnosis of lung cancer, considering the high specificity of these related tumors alterations. A limitation is due to the low frequency of some mutations and the fact that the mutant cfDNA can be obscured by an excess of background wild-type DNA. However, highly sensitive approaches have been developed to analyze low-level mutants cfDNA, including NGS-based options.

Many studies have focused on assessing the quantities of cfDNA. In a study carried out on smoking patients it was shown that the concentration in cfDNA, of the human telomerase reverse transcription (hTERT) gene was eight times higher in lung cancer patients than in controls and correlate with a strong risk factor for the development of lung cancer [56]. In other studies it has been shown that a greater amount of cfDNA during surgery correlates with a worse prognosis at 5 years survival, selecting a more aggressive disease [57]. Using a very sensitive quantitative test [57] it was demonstrated the possibility of discriminate between healthy subjects and NSCLC patients by measuring the quantities of cfDNA [58, 59]. cfDNA were significantly higher in NSCLC patients compared to healthy controls.

Methylation is an early and frequent epigenetic alteration that can be detected in cfDNA, including in plasma [60, 61]. Epigenetic modifications, regulate the expression of a large number of genes involved in malignant transformation and carcinogenesis. DNA alterations in methylation occur early in carcinogenesis and are remarkably more stable than other potentials diagnostic biomarkers.

Several studies have evaluated the methylation levels of single or panels of tumor suppressor genes in plasma or serum from lung cancer patients. Several genes have been found to be differentially methylated in cfDNA between patients with lung cancer and controls including MGMT, p16, RASSF1A, DAPK, RAR-β, DCLK1, SHOX2 and septin9 [62, 63]. In a recent study, a methylation panel of six genes showed a sensitivity of 72% for the detection of stage Ia NSCLC [64].

However though, methylation changes can also occur in the DNA of peritumor normal tissue which can therefore interfere with the analysis of tumor DNA generating a false positive result [65]. Considering together the fact that ctDNA is absent or very low in the early stages, the possibility to detect mutations in cfDNA derived from non tumor cells and the lack of standardized methods and large validation studies the analysis of ctDNA has not been introduced in the clinic screening yet.

7.2 Identification and monitoring residual disease

Measurement of residual disease after primary tumor treatment is an area of active investigation.

Efficient methods of identifying MRD in patients treated with surgery or adjuvant chemotherapy are currently lacking. Mutation monitoring can be a significant predictor of early relapses and MRD. Several studies have focused on detecting these mutations in ctDNA as an early indicator of relapse and a potential marker of residual disease.

Monitoring of ctDNA for residual disease has been used in several studies in lung cancer. Presence of ctDNA after local treatment was highly predictive of disease recurrence in a small cohort of lung cancer patients with stage I-III lung undergoing local treatment with radiation, surgery, or both [66].

A prospective trial is ongoing to investigate the ability of ctDNA mutations and methylation monitoring to detect MRD after surgery for stage Ia–III NSCLC [67].

The limit of the use of ctDNA in detect MRD in clinical practice beyond is the lack of circulating tumor specific alterations.

7.3 Monitoring treatment response and cancer progression

CtDNA analysis could be a way to monitor cancer status in real time. Methods for monitoring treatment response and changes in tumor burden with cfDNA involve the identification of genomic alterations specific to an individual patient's tumor and the relationship between changes in ctDNA levels, that can be monitored in real time during therapy (**Figure 3**).

In one study, long-term monitoring of a patient with NSCLC for analysis of EGFR mutations (p.L858R and p.T790M) using ctDNA isolated from serum and plasma a close correlation with the onset of metastasis was identified. In another study carried out with NGS technology on plasma-derived ctDNA from 168 patients with different types of cancer, the molecular alterations identified correlated to which of tissue [68].

ctDNA levels drop dramatically after one to two weeks in patients responding to treatment [69, 70]. Some authors suggested that a rise in ctDNA levels may precede radiographic progression.

Data obtained by several studies, conducted on different tumor type [71] using very sensitive new technologies involving multiplexed mutation-specific PCR and NGS suggest that the additionof ctDNA monitoring into clinical care could be a valuable tool to more accurately predict patient response and detect progression.

7.4 Liquid biopsy and immuno-oncology

Cancer immunotherapy is certainly the most profound innovation recorded in recent years in the fight against cancer. The recent use of anti-programmed death

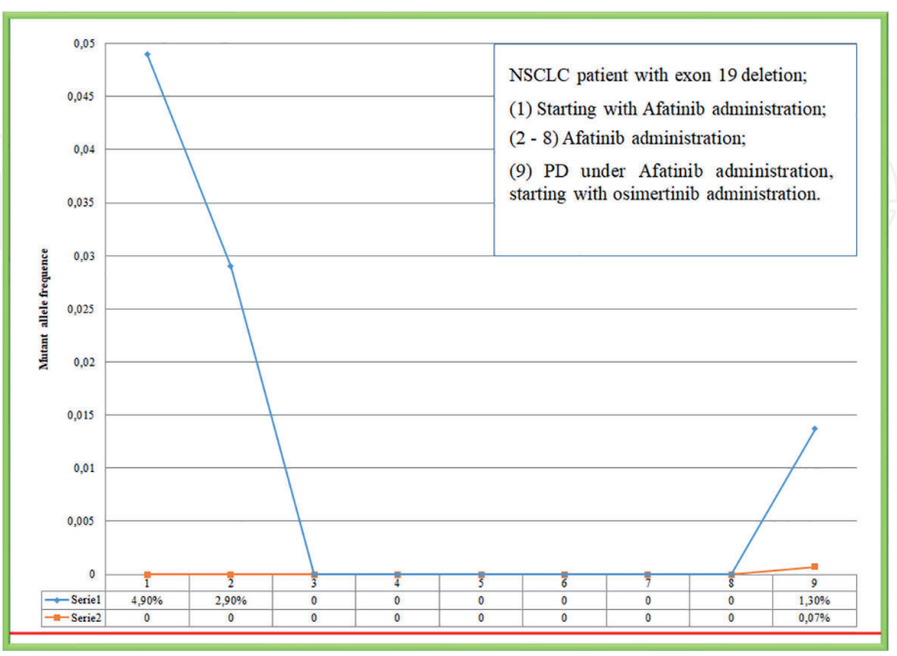

Figure 3.
Molecular follow up of a NSCLC patient. The figure shows the behavior of inspected molecular alterations [exon 19 deletion (blue line) and p.T790M point mutation (red line)] analyzed by serial liquid biopsy specimens in relation to the clinical outcome of a NSCLC patient.

receptor-1 (PD-1) /PD-1 ligand 1 immune checkpoint inhibitors (IC) in clinical trials indicates their efficacy in immune therapy against cancer.

These immune checkpoint inhibitors have been shown to have an effective therapeutic response, particularly in tumors with high tumor mutation burden (TMB) [72]. If the ctDNA can be used to guide and monitor immune therapy is just beginning to be evaluated [73].

In several studies, tumor mutation burden (TMB) can be estimated from ctDNA with good concordance with tissue results [74, 75]. Moreover, the identification of a high TMB in the ctDNA correlates closely with the two inhibitors of programmed cell death (PD)1 and its ligand (PD-L1) [74].

In a recent study it was shown that ctDNA TMB it has a closer correlation with metastatic tissue than primary tumor. Furthermore, this concordance is greater in cases with high ctDNA concentrations [76].

Another possible application of ctDNA in immunotherapy is in response monitoring. Has been proven a good correlation between ctDNA modification and clinical response. Some studies show indeed that the amount of cfDNA correlate with the response to immunotherapy and a decrease of cfDNA was an indicator of good response. Moreover, also chromosomal instability, predictive for immunotherapy response canbe evaluated by NGS in cf-DNA [77, 78].

A new interesting area is represented by the evaluation of tumor microenviroment adopting liquid biopsy specimen in the clinical administration of lung cancer patients. Cai et al. compared IC positive outcome and inflammation markers (INFγ, lymphocites ratio CD4/CD8) in relation to the conventional target biomarkers (PD-L1, CTLA4) for the selection of lung cancer patients to IC administration. They showed that a wide range of inflammatory biomarkers may integrate clinical outcome in NSCLC patients clinical administration [79].

Other applications of cfDNA are likely to emerge in the near future, in the field of immuno oncology such as the detection of minimal residual disease for adjuvant immunotherapy, and the identification of resistance mechanisms linked to the onset of new mutations such as the acquired JAK1/2 or B2M mutations [73].

However, in this context it is not yet possible to think of a complete replacement of the liquid tissue biopsy. The tumor biopsy will have more and more value, both in the evaluation of PD-L1 expression on tumor cells and in the analysis of the tumor microenvironment.

And while it is possible to identify resistance mechanisms as acquired mutations in the blood, however, acquired resistance could be linked to dynamic changes in the microenvironment, which cannot be detected by a simple blood draw.

7.5 Liquid biopsy in the management of other tumors

Several pre clinical studies were performed in order to evaluate the role of cfDNA in the management of other tumor patients.

Wang et al. [80] demonstrated that ctDNA may be considered a diagnostic biomarker in head and neck squamous cell carcinoma (HNSCC) patients. 93 saliva and matching blood specimens were collected from HNSCC patients to identify somatic mutations in genes (TP53, PIK3CA, CDKN2A, HRAS, NRAS) that could play a potential clinical role in the management of this patient cohort. Results showed that ctDNA was successfully isolated from 76% and 87% of saliva and plasma samples, respectively. The authors, highlighting a percent detection rate for hot spot mutations of 100% and 76% for saliva and blood specimens respectively, defined how cfDNA extracted from saliva samples may be considered a reliable tool to identify HNSCC malignant lesions in early stage setting. Similarly, Salvi et al. [81] discussed how cfDNA may be adopted in early stage section for prostate cancer patients by

elucidating an accuracy of 80% to discriminate cancer patients from benign lesions. In addition, Christensen et al. [82] showed how a digital droplet PCR approach is suitable to identify somatic alterations in urine cell free DNA (Ucf-DNA) by demonstrating how a correlation between Ucf-DNA alteration and tumor stage, size and grade, were statistically significant. In relation to this section, stool DNA also represents a promising diagnostic tool to early detect colorectal carcinoma patients in the early stage. According to this point, Imperiale et al. [83] showed that the sensitivity of novel technical approach DNA-based were characterized by higher analytical performance in the analysis of both CRC (92.3%) and advanced precancerous lesions (42.4%) respect the conventional screening test commercially available.

8. Conclusion

Is tissue still the issue?

One of the key questions facing oncologists today is whether ctDNA can replace biopsy or ribiopsy in clinical practice.

Liquid biopsy resulted improvements in the management of patients with NSCLC, offering an alternative to standard procedures in cases where tissue biopsy samples are insufficient or not feasible and providing a quick and dynamic assessment of emerging resistance mechanisms that can be used for guide treatment decisions so has been suggested to be included in clincal practice. However the fields of investigation using liquid biopsy are still restricted in routine practice. To date, FDA has approved the analysis EGFR gene assessment that is currently based on standardized and international practices recommendations and authorizes the administration of TKI. Recently, a document of the IASLC, stated that an EGFR, ALK, ROS1 or BRAF positive result of an NGS liquid biopsy analisys should be considered adequate for initiating first-line therapy in advanced NSCLC.

Although we can firmly state that liquid biopsy is a great help in NSCLC patient managing, however, in our opinion, for now it cannot replace tissue biopsy, which will remain the gold standard. Especially in the context of diagnosis where the definition of the tumor subtype can only be clarified through a cyto-morphological analysis and immunohistochemical criteria. Furthermore, even the exact stage of cancer can only be obtained with tissue sampling. Moreover, in the setting of detect EGFR-sensitizing alterations in peripheral blood a negative plasma test it should be considered non-informative and will always need tissue confirmation.

Moreover, results of clinical studies have highlighted the existence of significant critical issues in the execution of a mutational test on liquid biopsy, either in the pre-analytical phase both in the analytical phase. Standardization of the various stages of the process is fundamental. It is certainly important that the analysis be performed in laboratories highly specialized, who already have experience in using highly sensitive molecular techniques in order to avoid false positives or false negatives results.

As indicated, there are numerous methods of studying circulating nucleic acids. In view of the large number of actionable targets in NSCLC, guidelines support the position that broad-based testing by NGS. The NGS technique requires workflow complex and use of multigene panels also requires the use of sophisticated software and, sometimes, the help of bioinformatics.

So it is therefore evident that the choice between the different technologies must hold account of their sustainability and the clinical use required in the context in which you operate.

In conclusion we think that ctDNA can play complementary roles in the management of patient NSCLC and act as a prognostic or predictive biomarker as a part of a thorough clinical evaluations to assess the disease, that include comparative sequence analyses of plasma DNA, and biopsies in combination with imaging studies and detailed functional studies.

Conflict of interest

The authors declare no conflict of interest.

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