REVIEW ARTICLE

Liquid Biopsy for Advanced Non-Small Cell Lung Cancer (NSCLC): A Statement Paper from the IASLC

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Christian Rolfo, MD, PhD, MBA,[a] Philip C. Mack, PhD,[b]
Giorgio V. Scagliotti, MD, PhD,[c] Paul Baas, MD, PhD,[d] Fabrice Barlesi, MD, PhD,[e]
Trever G. Bivona, MD, PhD,[f] Roy S. Herbst, MD, PhD,[g] Tony S. Mok, MD,[h]
Nir Peled, MD, PhD,[i] Robert Pirker, MD,[j] Luis E. Raez, MD,[k] Martin Reck, MD, PhD,[l]
Jonathan W. Riess, MD,[b] Lecia V. Sequist, MD, MPH,[m] Frances A. Shepherd, MD,[n]
Lynette M. Sholl, MD,[o] Daniel S. W. Tan, MBBS, PhD,[p] Heather A. Wakelee, MD,[q]
Ignacio I. Wistuba, MD,[r] Murry W. Wynes, PhD,[s] David P. Carbone, MD, PhD,[t]
Fred R. Hirsch, MD, PhD,[u,*] David R. Gandara, MD[b]

[a]Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland
[b]University of California Davis Comprehensive Cancer Center, Sacramento, California
[c]University of Turin, Department of Oncology at San Luigi Hospital, Orbassano, Italy
[d]Department of Thoracic Oncology, The Netherlands Cancer Institute and Department of Pulmonary Disease, Academic Medical Center, Amsterdam, The Netherlands
[e]Multidisciplinary Oncology and Therapeutic Innovations Department, Assistance Publique Hôpitaux de Marseille, Aix Marseille University, Marseille, France
[f]Department of Medicine, Division of Hematology/Oncology, University of California San Francisco, San Francisco, California
[g]Yale Cancer Center, New Haven, Connecticut

[h]State Key Laboratory of South China, Hong Kong Cancer Institute, The Chinese University of Hong Kong, Hong Kong, China
[i]Institute of Oncology, Soroka Medical Center and Ben Gurion University, Beer Sheva, Israel
[j]Department of Medicine I, Medical University of Vienna, Vienna, Austria
[k]Memorial Cancer Institute, Memorial Healthcare System/Florida International University (FIU) Miami, Florida
[l]Department of Thoracic Oncology, Lung Clinic Grosshansdorf, Airway Research Center North (ARCN), German Center for Lung Research (DZL), Grosshansdorf, Germany
[m]Massachusetts General Hospital Cancer Center and Harvard Medical School, Boston, Massachusetts
[n]University Health Network and Princess Margaret Cancer Centre, Toronto, Ontario, Canada
[o]Brigham and Women's Hospital and Department of Pathology, Harvard Medical School, Boston, Massachusetts
[p]National Cancer Centre Singapore and Genome Institute of Singapore, Singapore
[q]Department of Medicine, Division of Oncology, Stanford University School of Medicine, Stanford, California
[r]Department of Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas
[s]International Association for the Study of Lung Cancer, Aurora, Colorado
[t]Division of Medical Oncology, Department of Internal Medicine, The Ohio State University, Columbus, Ohio
[u]Division of Medical Oncology, Department of Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado, and the International Association for the Study of Lung Cancer, Aurora, Colorado

ABSTRACT

The isolation and analysis of circulating cell-free tumor DNA in plasma is a powerful tool with considerable potential to improve clinical outcomes across multiple cancer types, including NSCLC. Assays of this nature that use blood as opposed to tumor samples are frequently referred to as liquid biopsies. An increasing number of innovative platforms have been recently developed that improve not only the fidelity of the molecular analysis but also the number of tests performed on a single specimen. Circulating tumor DNA assays for detection of both EGFR sensitizing and resistance mutations have already entered clinical practice and many other molecular tests — such as detection of resistance mutations for Anaplastic Lymphoma Kinase (ALK) receptor tyrosine kinase rearrangements — are likely to do so in the near future. Due to an abundance of new evidence, an appraisal was warranted to review strengths and weaknesses, to describe what is already in clinical practice and what has yet to be implemented, and to highlight areas in need of further investigation. A multidisciplinary panel of experts in the field of thoracic oncology with interest and expertise in liquid biopsy and molecular pathology was convened by the International Association for the Study of Lung Cancer to evaluate current available evidence with the aim of producing a set of recommendations for the use of liquid biopsy for molecular analysis in guiding the clinical management of advanced NSCLC patients as well as identifying unmet needs. In summary, the panel concluded that liquid biopsy approaches have significant potential to improve patient care, and immediate implementation in the clinic is justified in a number of therapeutic settings relevant to NSCLC.

Introduction

One of the hallmarks of NSCLC is represented by the expanding array of effective targeted therapies with activity in specific molecular subsets of this disease. Because acquired resistance to targeted inhibitors is nearly universal, development of next-generation agents able to overcome common resistance mechanisms has been a vital key of experimental and therapeutic research. As a primary example, the approval of first-generation EGFR tyrosine kinase inhibitors (EGFR TKIs) in 2009 was rapidly followed by the development of second- and third-generation TKIs, with a fourth-generation inhibitor currently being studied.[1-7] In particular, third-generation inhibitors, such osimertinib, were designed to selectively target specific mutant forms of EGFR. This new class of agents provide several advantages: high potency against common EGFR activating mutations, the ability to inhibit the EGFR protein harboring the T790M mutation that confers resistance to first- and second-generation EGFR TKIs, and its relatively lower affinity for wild-type (WT) EGFR, which substantially reduces class toxicities. Similarly, an expanding repertoire of agents that target anaplastic lymphoma kinase (ALK) fusion kinase provides significant therapeutic options for patients with acquired resistance to the first-generation ALK TKI crizotinib.[8] Approximately one-third of patients acquire resistance to crizotinib through emergence of any one of the growing list of ALK-specific point mutations that interfere with drug binding. Next-generation ALK TKIs such as alectinib, ceritinib, brigatinib, ensartinib, and lorlatinib are capable of binding to and inhibiting mutant forms of ALK. However, these drugs have different binding affinities in the context of different resistance mutations and optimal patient treatment may benefit from identification of the specific resistance mutation to deliver to the patients the most appropriate agent to restore activity.[8]

As a consequence, accurate identification of predictive genetic alterations — at both baseline and progression — is crucial not just for patient management but also for advancing our understanding of treatment-induced tumor evolution. Although repeated tissue biopsies have been advocated as a tool for monitoring the evolution of the tumor, the feasibility of the approach in a real-world experience may be too burdensome to be applied to every patient. To this end, a variety of liquid biopsy platforms have been developed that can serve as a complement to routine tissue-based diagnostics and, pivotally, as a feasible means of identifying acquired resistance mechanisms. Liquid biopsy is recommended in 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.[9] Although not recommended as a replacement for a diagnostic tissue biopsy, liquid biopsy is recommended in cases with insufficient tumor tissue specimens or in cases where tissue specimens are not obtainable. Firstly, a significant subgroup of patients cannot undergo a biopsy or rebiopsy due to their suboptimal clinical condition or an unfavorable tumor site such as bone or central nervous system or multiple small pulmonary nodules that are not safely amenable to biopsy or where adequate tissue for molecular testing is unlikely to be achieved.[10] Secondly, liquid biopsy may spare the patient an invasive procedure with the incumbent risks of major complications, observed in 5% of computed tomography–guided transthoracic lung biopsies.[11] Thirdly, scarcity of tumor tissue in the tumor biopsy can prevent the pathologist from performing all the required analyses, thus making it necessary to obtain new tissue for further tests. Fourthly, performing a tissue biopsy is considerably more expensive than a blood draw, suggesting liquid biopsy as a cost-effective alternative for patients under follow-up or progressing on a targeted therapy. Fifthly, turnaround time may be shorter for liquid biopsy than for tissue biopsy, when one considers the scheduling time involved.[12] Finally, circulating markers are theoretically more likely to reflect systemic tumor burden and are, as such, more effective in depicting the intratumoral heterogeneity and emergent biology in actively growing metastatic lesions which may be missed by single-site tissue biopsies.[13,14] Notwithstanding these advantages, there are a number of questions regarding sensitivity and clinical utility of liquid biopsy and its role in clinical practice that require further clarification.

To address this topic, the IASLC convened a multidisciplinary panel composed of experts in the field of thoracic oncology. The panel held several meetings over the course of 2 days following the International Lung Cancer Congress and subsequent written correspondence until January 2018 during which time the available scientific evidence concerning the role of liquid biopsy in clinical practice was discussed. The panel presents this consensus-statement paper that describes its majority-shared opinions regarding: sample acquisition and handling, with subsequent extraction of circulating cell-free tumor DNA (ctDNA); platforms and requirements for molecular analysis; role of liquid biopsy in treatment-naive, treatment response, and progressive disease settings; results reporting; ethical considerations; and future perspectives with regard to immune oncology, circulating tumor RNA (ctRNA), circulating tumor cells (CTCs), and liquid specimens beyond peripheral blood.

There are a variety of biologic elements that can be isolated from peripheral blood beyond proteins and ctDNA.[15] The definition of liquid biopsy includes also CTCs, circulating exosomes, platelet RNA and ctRNA.[16,17] Whereas each of these modalities has potential to provide novel diagnostic information and their exploration is highly encouraged, ctDNA certainly represents the most mature example of the investigation of the liquid biopsy in clinical practice for lung cancer patients. Therefore, this consensus report will focus mainly on the clinical value of the ctDNA. Additionally, members of the panel are mindful of the diversity of the available diagnostic facilities worldwide. The accessibility to different techniques, platforms, test reimbursement, and drugs is still far from being equally distributed. Nevertheless, the panel believes that this consensus should report the best possible standard-of-care, an educative paradigm that has to be pursued by physicians, regulatory institutions, and governments in general. The panel advises that the anticipated clinical benefit for the patient should be taken into account when considering the views expressed in this consensus — for example, druggable mutations should not be investigated if there is no local availability for that drug. These recommendations are for general oncologists and clinicians that are seeking practical ways to implement all the new information that is emerging with liquid biopsies.

ctDNA Extraction and Analysis Requirements

Accurate blood sample collection, handling, and storage procedures are essential for reliable ctDNA extraction and subsequent molecular analysis. Several blood collection and preservation strategies have been investigated for ctDNA isolation, each with their particular advantages and cost implications. The conditions in which the blood samples are stored and shipped, as well as the amount of time that passes between blood

withdrawal and plasma extraction are just some of the factors that influence the accuracy of ctDNA analysis. Herein, we give a brief overview of the different possibilities and recommended procedures.

What are the Requirements for Blood Sample Handling and Subsequent ctDNA Extraction?

The two most prominent methods of blood collection for ctDNA isolation involve the use of ethylenediaminetetra-acetic acid (EDTA) tubes for plasma extraction or the use of preservative tubes designed specifically for cell-free DNA isolation; several institutions acquire 20 mL of blood but still there is no standard collection volume.[18,19] The major advantage of EDTA tubes is their widespread availability and low cost compared to expensive preservative tubes. The drawback to using EDTA tubes is the requirement to process the sample within a short timeframe, which will often entail processing at the collection site.[20] Conversely, preservative tubes, such as Streck (La Vista, Nevada) Cell-Free DNA BCT, are designed to maintain the quality of small DNA fragments for multiple days at room temperature without any significant on-site processing requirements.[21] According to the manufacturer, Streck tubes maintain cfDNA stability for up to 14 days if properly stored; whereas CTCs are reported as stable for up to 7 days.[22] This technology allows convenient shipping of the tubes to analysis labs, decreasing interinstitution variability in processing and handling. Cell-free DNA (cfDNA) by definition represents the entirety of all circulating DNA, regardless of origin; in contrast to ctDNA which defines the subset of DNA that is specifically tumor in origin. The principle behind cfDNA collection tubes is the inclusion of preservative reagents that stabilize nucleated blood cells, preventing their degradation and subsequent release of genomic DNA into the tube. Additionally, preservative tubes may also inhibit exonuclease activity that would result in cfDNA degradation. These processes may be disrupted if the tubes are not maintained at or near room temperature. Strict adherence to product guidelines is recommended for storage and transport.[23,24]

If EDTA tubes are to be used in lieu of preservative tubes, then rapid processing is required to prevent cellular and nucleotide degradation; it is recommended to process the sample within 1-2 hours from the collection.[25] Perhaps the most significant threat to ctDNA analysis is contamination from genomic DNA released from lysed white blood cells if samples are not promptly processed. Large systematic analyses of ctDNA have shown that even under ideal conditions, the proportion of tumor-derived mutant forms of an allele is often less than 1% of the total DNA (mutant plus WT) for that sequence.[26-28] Thus, any significant release of

normal genomic DNA due to suboptimal handling will further dilute the mutant species, possibly to below the level of detection. Furthermore, cfDNA fragments will degrade over time if left unattended in the collection tube at room temperature. Studies have indicated that the amount of total circulating cfDNA extracted from EDTA-collected blood samples is stable up to 6 hours after collection.[29] Conservation at room temperature or on ice (+4°C) did not alter the amount of cfDNA or the sensitivity in detecting mutations using droplet digital polymerase chain reaction (ddPCR) over short periods.[23] To further prevent genomic DNA contamination, it is also suggested that the blood tube be centrifuged twice, once in the original collection tube and then in a second tube after transferring the plasma, to ensure a more efficient purification.[25]

Comparison of Serum and Plasma

Different studies compared serum and plasma for variant detection in ctDNA.[30,31] The phase III randomized clinical trials for gefitinib (IPASS) and afatinib (LUX-Lung 3) examined *EGFR* mutational status in tumor DNA extracted from standard tissue biopsy specimens as well ctDNA extracted from serum liquid biopsy specimens.[1,32] Overall, the sensitivity level reached in these studies using a real-time PCR (qPCR)-based assay was 43.1%, considering the tissue-based analysis as reference. Based on these results, the phase IV clinical trial for gefitinib (IFUM) and the phase III clinical trials for afatinib (LUX-Lung 6 & 3) included the plasma sample analysis of *EGFR* mutation status.[32-34] The plasma test sensitivity was 65.7% and 60.5%, respectively (the mutation detection rate using serum samples was dramatically inferior, 28.6% in the Lux-Lung 6 & 3 trials), by using a qPCR-based assay, hereto considering the tissue-based analysis for *EGFR* mutations as the reference standard.[35] In the real-world data coming from the ASSESS trial —including 1288 patients form Japan and Europe — the concordance among plasma samples and tissue for EGFR mutation was 89%, with a high specificity (97%) but a low sensitivity (46%). The positive predictive value was 78% and negative predictive value 90%. This study showed the necessity to perform tests in labs with high expertise.[36] In the phase III study of erlotinib (EURTAC), a secondary objective was to assess the feasibility of using ctDNA from blood samples (serum and plasma for each patient) as a surrogate for tumor biopsy for determining *EGFR* mutation status and to correlate *EGFR* mutations in ctDNA with outcome.[37] In particular, *EGFR* mutations were examined in ctDNA isolated from 97 baseline blood samples by a peptide nucleic acid–mediated 5′ nuclease qPCR (TaqMan; Applied Biosystems, Foster City, California) assay and *EGFR* mutations in ctDNA were detected in

78% of patients (n = 76) with a specificity of 100%.[38] In this study, serum or plasma samples were allowed for the analysis; unfortunately, there is no information about the detection rate differences between sample types.

Recommendations

In the absence of conclusive evidence, the panel reached an agreement in favor of the following claims based on this existing knowledge. Plasma is preferred over serum for ctDNA extraction. The suggested maximum time from blood withdrawal to plasma extraction is 2 hours for EDTA tubes and 3 days for preservative tubes. Operators dealing with blood collection, handling, and eventual shipping should be cognizant of these time constraints. A "double spin" plasma isolation procedure is highly recommended. Blood should never be frozen before plasma extraction regardless of tube type. EDTA tubes and cfDNA preservative tubes are both feasible for ctDNA extraction. EDTA tubes may require on-site processing and increase risk of cfDNA degradation and genomic DNA contamination if not handled promptly and carefully. In contrast, preservative tubes such as the Streck Cell-Free DNA BCT allow for greater flexibility with regard to processing time and reduce the risk of degradation and contamination. Draw of two tubes is recommended to ensure adequate analysis. Volumes are dependent upon institutional standard operating procedures. Many labs request two standard 10-mL tubes. DNA extraction should be performed using protocols or kits designed for small fragmented DNA.

What are the Methods for ctDNA Analysis?

A wide variety of analytical methods have been developed for the identification of molecular alterations using ctDNA. An important requirement is the sensitivity of these tools, as the amount of ctDNA can vary substantially from patient to patient and in many cases will be limited.[24] A comparison has to be made between targeted (narrow) approaches and untargeted (broad) approaches. The former can detect alterations in relatively small regions of DNA and nearly all PCR-based methods belong to this group. Next-generation sequencing (NGS)–based methods are part of the second group, designed to interrogate larger regions of multiple genes in a single run, and are often focused on a panel of genes relevant to cancer therapeutics. Several features — such as the diagnostic accuracy, the turn-around time and the costs — differ from test to test and all of these should be considered when assessing which platform fits best with the clinical practice. In addition, the amount of total cfDNA extracted can have a critical impact on the sequence accuracy, as well as the technology used.

qPCR

The commercially available cobas EGFR mutation test, originally v1 and now v2, (Roche Molecular Diagnostics, Branchburg, New Jersey) is perhaps the most investigated test of a PCR-based platform used in patients with NSCLC.[35] Real-time or quantitative PCR (qPCR) differs from classic PCR because the intensity of a fluorescent light emitted by the probes is read every cycle, which allows for an estimate of the quantity of the loaded sample based on the number of cycles needed to obtain a threshold fluorescent signal.[39] Currently, cobas is the only technology approved by the U.S. Food and Drug Administration (FDA) for the molecular analysis of liquid biopsy specimens in NSCLC, although other countries may have alternative approvals.[40] Particularly, its indications cover only some of the EGFR gene alterations: the cobas test was approved by the FDA for the detection of exon 19 deletions and L858R and T790M mutations from plasma DNA.[41] Despite this, its sensitivity has already been significantly outperformed by other platforms, raising the issue of false negatives.

ddPCR

ddPCR is a method based on sample emulsion and fractionation in droplets, which outperforms the precision of classic PCR. Specifically, ddPCR works through a microfluidic technology that partitions the sample in a way that each droplet has either 1 or 0 molecules of DNA to be amplified. Reading the signal from each droplet reveals a ratio of positive and negative droplets, and by using the Poisson distribution, allows an estimation of the initial ratio of mutant-to-WT DNA. This quantification, as stated before, is considered more precise than that performed with qPCR.[42]

BEAMing

BEAMing (beads, emulsions, amplification and magnetics) is based on the same principles of ddPCR but with DNA templates bound to magnetic microbeads before emulsion into droplets. After amplification, thousands of copies of DNA coat each bead; beads can be isolated afterwards through centrifugation or through a magnet. The amount of amplified DNA that retains a specific mutation of interest is sufficient to allow precise quantification. Optical scanning or flow cytometry are used to quantify the DNA on the beads; such DNA can be also used for further sequencing with high throughput methods.[43] Although it is more sensitive than qPCR, use of BEAMing incurs high costs that might not be feasible in routine practice. Also, the learning curve for this technique is steep.

NGS

The term next-generation sequencing (NGS) — also referred to as high throughput sequencing — comprises a group of platforms that perform the sequencing of nucleic acids through a large number of parallel reads and their subsequent alignment to a genomic reference standard.[44] As an example, deep sequencing performs tens of thousands of reads of the same strand and can use statistical approaches to call a mutation and to define its frequency.[45] Single base mutations, small insertions and deletions, large genomic deletions or amplifications, and rearrangements, such as inversions and translocations, can all potentially be detected with NGS. This methodology can be used to sequence whole genomes, whole exomes, or panels of a few to hundreds of targeted regions of exons or introns. Targeted panels have the advantages of higher throughput, better sensitivity, efficient use of limited DNA, lower costs per analyses, and a wider range of mutation detection. For targeted panels there are two types of target enrichment: 1) hybrid capture, which interrogates the entire coding sequence of oncogenes and tumor suppressor genes and the introns of selected genes and provides information on the entire spectrum of DNA changes; and 2) PCR capture, which is designed as a "hot-spot" test, sequencing predefined areas of genes — these have high sensitivity for mutations but lower for indels and routinely do not detect copy numbers (amplifications and deletions) and gene fusions, in contrast to other NGS approaches.[46]

Different NGS-based methods have been developed and subsequently validated for NSCLC ctDNA mutation detection.[47] Two of the more commonly used sequencing platforms are Illumina (San Diego, California), which uses sequencing-by-synthesis chemistry that simultaneously identifies DNA bases, while incorporating them into a nucleic acid strand, by using four-color optical imaging of fluorescently labeled nucleotides, and Ion-Torrent (ThermoFisher Scientific, Waltham, Massachusetts), which also uses sequencing-by-synthesis but instead of using optical signals it uses a semiconductor to measure a change in pH due to the release of an H+ ion following the addition of a nucleotide. There are distinctions between the two platforms that the user should be aware of, such as reports of pH read mistakes and a higher rate of sequencing errors with the Ion Torrent relative to the Illumina platform.[48,49] In addition, library preparation is critical for accuracy and the ability to detect fusions and small indels; therefore, each lab should perform technical validation studies to determine the lower limit of detection and consider the advantage/disadvantage of each technology.

Notably, the Centers for Medicare and Medicaid Services recently published a note supporting the use of NGS in the daily clinical practice.[50]

Pros and Cons

Historically, PCR-based methods were characterized by shorter turnaround times — approximately 2 to 3 days in optimal situations — and an affordable price when compared to NGS technology.[51,52] While this is still generally the case, with the advent of new technologies, the turnaround time for NGS is now acceptable for clinical management — approximately 13 days — and costs have been significantly reduced[53]; this is a trend that is expected to continue in the near future. Furthermore, many NGS platforms are capable of simultaneous detection of mutations, indels, copy number variations, and genomic rearrangements.

In contrast to PCR-based methods, NGS can detect rare and previously uncharacterized alterations in the sequenced gene. Depending on the size of the panel analyzed, a single NGS report can provide information on dozens of targetable genetic abnormalities simultaneously, thus giving added value by obtaining further useful information from the same specimen.[54] This is particularly relevant considering the expected rise in the enrollment of basket trials or in expanded access programs requiring molecular assignment.[55] NGS-based platforms, in contrast to PCR, also have the capacity to evaluate tumor suppressor genes, where deleterious mutations can occur at myriad locations throughout the gene, as opposed to oncogenes where gain-of-function mutations typically occur at highly specific, previously recognized locations. Additional advantages of an NGS approach with specific regard to ctDNA are the ability to quantitate gene copy number variations, including gene amplification, and identify chromosomal rearrangements such as oncogenic fusions. Copy number variations are typically assessed as read depth in one specific sequence area relative to all other measured sequences and its precision is based on the proportion of tumor to non-tumor cfDNA and the total amount of cfDNA detected. A variety of publically available and proprietary bioinformatics tools have been developed to assist in these calculations. Likewise, the ability to detect gene fusions in DNA, which are significantly more diverse and complex than post-processed RNA, is highly dependent on analytical algorithms. NGS is likely to under-call the presence of gene fusions; however, it remains the best tool available for their identification in fragmented DNA and a positive finding can provide far more detailed information on fusion partners and breakpoint locations than traditional fluorescent in situ hybridization assays. Another feature that distinguishes NGS and ddPCR from qPCR is that the former methods can also quantify the amount of DNA that carries a particular alteration, while the latter is merely a semiquantitative method. The clinical significance of tracking the variant allele frequency (VAF) — the relative

proportion of mutant forms compared to WT forms at a given locus — over the course of a patient's treatment is actively under investigation, and is a promising opportunity for long-term disease management.[56]

Concerns regarding error rates innate to NGS platforms are particularly pertinent to the analysis of ctDNA from plasma, where VAFs can be at such low frequencies that a single false-positive read may impact the interpretation of the data. To address this, leading commercial and academic laboratories have adopted sophisticated error-proofing techniques and algorithms to dramatically improve the specificity of the sequencing, in some cases greatly diminishing the possibility of false positive calls.[57,58] This, in turn, leads to greater sensitivity (in this context: detection of low frequency variants) by increasing confidence in calls made from extremely limited counts. "Duplex" and "digital" NGS are leading examples of error-proofing, which often use molecular barcoding of individual DNA fragments and confirmation of mutations via matching base substitutions in the complementary DNA strand.[57-59] While these techniques have significant effects on the workflow process, they are a necessity for sequencing of ctDNA and enable more advanced analyses such as longitudinal VAF monitoring and subclone detection.

Feasibility of platforms in one's own region is a prime concern and each clinical setting will have to evaluate and balance between availability, cost, and assay sensitivity.

Recommendations

Validated qPCR-based methods are acceptable for targeting specific mutations such as the *EGFR* mutations. This technology has limited capabilities, such as only interrogating a discrete set of previously defined mutations in a constrained number of oncogenes; therefore, missing rare but nonetheless clinically relevant mutations. NGS multiplex panels focused on clinically relevant (actionable) genes that: 1) use validated error proofing technologies with sufficient technical sensitivity and specificity for ctDNA applications; and 2) comprehensively evaluate single-base variants, indels, copy number variations, and chromosomal rearrangements are preferred. However, they are more costly at the present time and not as widely available. An expected turnaround time of more than 2 weeks for the tissue biopsy analysis can be considered a reasonable time limit over which a liquid biopsy should be considered despite the tissue biopsy analysis.

ctDNA in Clinical Practice

Currently, there are two important scenarios in which the liquid biopsy might confer an advantage to patients with advanced NSCLC: initial molecular diagnosis and progression during targeted therapy. However, a treatment strategy that considers the patient's clinical status, clinical relevance of test results, and local feasibility of the different testing methods must be considered when planning diagnostic procedures to avoid potential delays in identifying therapeutically actionable resistance mechanisms.

What is the Role of Liquid Biopsy in Treatment-Naive Patients?

Currently there are several therapeutic options for the first-line treatment of patients with NSCLC. The liquid biopsy has the potential to provide much of the information needed for clinical application of targeted therapy. All patients whose molecular status should be investigated according to the existing guidelines are eligible for molecular determination in ctDNA.[60] The liquid biopsy provides a clinical advantage to the treatment-naive patient by sparing diagnostic tissue for additional analyses, including immunohistochemistry relevant for immune oncology. However, awareness of the advantages/disadvantages of current technologies is required, as ctDNA platforms vary in their breadth and sensitivity. Critically, not all tumors shed sufficient amounts of DNA into peripheral circulation for detection, and even the most sensitive of assays appear to achieve approximately 85% sensitivity in advanced stage disease. Treatment-naive patients with indolent, slow-growing tumors may be at more risk of a false-negative finding in plasma compared to patients with a more disseminated cancer. Therefore, it is imperative that caregivers are aware of the possibility of a false-negative result from the liquid biopsy. Additionally, it is important to remember that therapeutic intervention can drastically change the VAF in circulation. Patients should be drawn before any therapy, as even one or two weeks of effective treatment can render a previously plasma-positive patient undetectable.[61] Figure 1 depicts a flowchart which summarizes panel believes for the use of the liquid biopsy in tretament naive patients.

Recommendations

The criteria used to select treatment-naive patients for molecular testing of ctDNA is the same used for molecular testing using DNA isolated from tissue: 1) all patients with advanced/metastatic nonsquamous NSCLC; 2) patients with squamous NSCLC if they have clinical features, for example, a never smoker and/or younger age, that may suggest they have a molecular driver; and 3) there is a nonsquamous component in the diagnostic histology. A clinical trial with molecular eligibility would represent another example, although
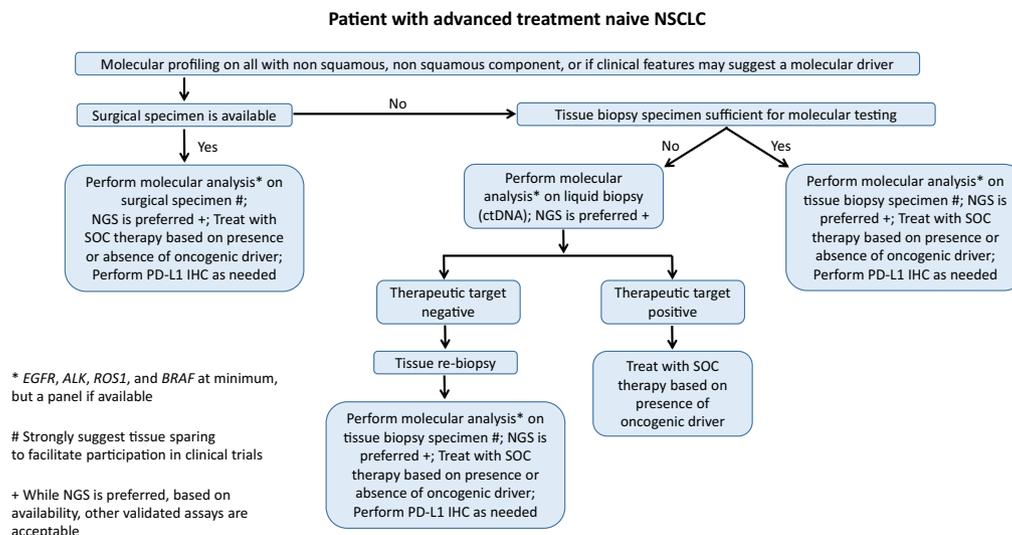
Patient with advanced treatment naive NSCLC

Molecular profiling on all with non squamous, non squamous component, or if clinical features may suggest a molecular driver

Surgical specimen is available — No → Tissue biopsy specimen sufficient for molecular testing

Yes ↓

Perform molecular analysis* on surgical specimen #; NGS is preferred +; Treat with SOC therapy based on presence or absence of oncogenic driver; Perform PD-L1 IHC as needed

No ↓   ↓ Yes

Perform molecular analysis* on liquid biopsy (ctDNA); NGS is preferred +

Perform molecular analysis* on tissue biopsy specimen #; NGS is preferred +; Treat with SOC therapy based on presence or absence of oncogenic driver; Perform PD-L1 IHC as needed

Therapeutic target negative   Therapeutic target positive

Tissue re-biopsy   Treat with SOC therapy based on presence of oncogenic driver

Perform molecular analysis* on tissue biopsy specimen #; NGS is preferred +; Treat with SOC therapy based on presence or absence of oncogenic driver; Perform PD-L1 IHC as needed

* *EGFR*, *ALK*, *ROS1*, and *BRAF* at minimum, but a panel if available

Strongly suggest tissue sparing to facilitate participation in clinical trials

+ While NGS is preferred, based on availability, other validated assays are acceptable

Figure 1. Patient with advanced treatment-naive NSCLC. NGS, next-generation sequencing; SOC, standard of care; PD-L1, programmed death ligand 1; IHC, immunohistochemistry; *ALK*, ALK receptor tyrosine kinase; ctDNA, circulating tumor DNA.

physicians should confirm that plasma-based testing is acceptable.

Liquid biopsy can be considered at the time of initial diagnosis in all patients who need tumor molecular profiling, but it is particularly recommended when tumor tissue is scarce, unavailable, or a significant delay potentially greater than 2 weeks is expected in obtaining tumor tissue. This scenario is most frequent in the case of very scant tissue, in patients for whom invasive procedures may be risky or contraindicated, or with bone biopsies, because although bone biopsies can be sufficient for a histologic diagnosis, they may not be compatible with molecular testing as some decalcification solutions can damage tumor DNA.[61]

A positive finding of an actionable mutation in ctDNA, if using a validated assay, represents sufficient evidence to initiate targeted treatment. However, a negative result should be considered inconclusive and followed up with a secondary test.

Sensitivity, specificity, and concordance rates of different platforms are depicted in Table 1. A good molecular ctDNA test should retain an acceptable concordance to molecular testing in the tumor tissue. As previously mentioned, the cobas test is the only FDA-approved method for the determination of some *EGFR* sensitizing alterations in ctDNA but investigations have shown that while cobas has an optimal specificity its sensitivity is modest. Methods such as ddPCR, BEAMing, and NGS can reach higher levels of sensitivity for the detection *EGFR* sensitizing alterations without diminishing the specificity. These methods are currently being used widely and it is predicted that their application will continue to expand in the near future. However, even with the increased sensitivity, a negative result from one

of these tests is not sufficient to exclude the potential existence of an *EGFR* driver alteration; therefore, in these circumstances tissue analysis should be performed.

In contrast to *EGFR*, where substantial data exists (Table 1), *ALK* rearrangement assessment using ctDNA in treatment-naive patients is more limited and no prospective cohorts have been evaluated to date. Retrospective data suggests that qPCR probably is not effective enough for the detection of *ALK* rearrangements in ctDNA — efficacy in platelet-derived or CTCs-derived RNA may be more promising but on a research level with a prospective cohort validation is still missing.[62] While ddPCR has been shown to be far more effective at detecting *ALK* rearrangements in ctDNA compared to reverse transcriptase PCR, a prospective validation study is still missing. No studies using the BEAMing platform have been reported for the detection of *ALK* rearrangements or mutations. Conversely, NGS reached acceptable levels of sensitivity combined with optimal specificity in prospective cohorts — even though *ALK* rearrangement-specific data are not available.[57,63,64]

The recent evidence for *BRAF* TKIs in combination with an MEK inhibitor in patients with *BRAF*-mutated NSCLC has introduced a new treatment option for the first-line treatment of these patients, making it necessary to include *BRAF* mutation detection — particularly V600E mutation — in the workup of patients with nonsquamous NSCLC.[60,65] The list of druggable molecular alterations in NSCLC also includes *ROS1* rearrangement and MNNG HOS Transforming gene (*MET*) exon 14 skipping mutation; nevertheless, limited experience exists for the detection of these genetic aberrations in peripheral blood samples.[26] Moreover, as the number of druggable alterations is likely to increase over time, the

Table 1. Sensitizing Mutations for Treatment-Naive Patients

Platform	Patients	Alteration	Specificity (%)	Sensitivity (%)	Concordance (%)	Ref
cobas	238 patients from the FASTACT-2 study	L858R and exon 19 deletion	96	75	88	77
cobas	196 patients with LUAD	L858R and exon 19 deletion	96	60	91	128
qPCR	96 patients from EURTAC study with either L858R or exon 19 deletion	L858R and exon 19 deletion	—	78	—	38
ddPCR	120 newly diagnosed patients with nonsquamous NSCLC with either L858R or exon 19 deletion	*EGFR* exon 19 deletion	100	86	91	12
		EGFR L858R mutation *KRAS* G12X mutation	100	69	80	
			100	64	72	
ddPCR	58 patients from South Korea	L858R exon 19 deletion	—	70.8	87.9	75
				76.5	86.2	
BEAMing	Retrospective cohort of 216 patients from the AURA-1 study	L858R exon 19 deletion	97	86	—	129
			98	82	—	
BEAMing	38 patients from the AURA 1 trial	L858R exon 19 deletion	100	93	95	130
			93	100	95	
cobas	38 patients from the AURA 1 trial	L858R exon 19 deletion	100	90	97	130
			100	86	89	
ddPCR	38 patients from the AURA 1 trial	L858R	100	90	97	130
BEAMing	72 patients from the AURA 1 trial	L858R exon 19 deletion	97	87	—	130
			97	82	—	
Cobas	72 patients from the AURA 1 trial	L858R exon 19 deletion	97	82	—	130
			97	82	—	
Cobas	226 patients from the AURA 3 trial	L858R exon 19 deletion	100	59	—	131
			99	85	—	
ddPCR	208 patients from the AURA 3 trial	L858R exon 19 deletion	98	69	—	131
			100	72	—	
NGS	227 patients from the AURA 3 trial	L858R exon 19 deletion	99	62	—	131
			99	81	—	
BEAMing	Retrospective cohort of 44 patients with confirmed *EGFR* mutated status	*EGFR* activating alterations	—	72.7	—	132
NGS	Prospectively enrolled cohort of 165 patients with stage III-IV solid tumors	54 genes pane	99	85	—	57
NGS	50 prospectively enrolled patients with NSCLC	70 genes panel	—	—	79	133
NGS	68 nonsmoker patients with NSCLC	*EGFR*, *KRAS*, *BRAF*, ERBB2, and PI3KCA genes	87	58	68	63
qPCR	Cohort of 32 patients with NSCLC – with 14 patients having an *ALK* rearrangement confirmed by primary tumor real– time PCR and/ or FISH	*ALK* rearrangement	100	21	—	62
ddPCR	Retrospective cohort of 102 consecutive patients with lung adenocarcinoma	*ALK* rearrangement	100	93	—	134
NGS	88 retrospectively enrolled consecutive patients with LUAD	*EGFR* alterations detection	—	—	80.8	135
NGS	100 patients with NSCLC	*EGFR* alterations detection	—	—	79	133
qPCR	Retrospective cohort of 107 patients with lung adenocarcinoma	*BRAF* mutation	93	28.6	—	101
NGS	48 patients with advanced, progressive NSCLC	Different alterations	100	77	—	136

qPCR, qualitative PCR; ddPCR, digital droplet PCR; NGS, next-generation sequencing; *ALK*, ALK receptor tyrosine kinase; LUAD, lung adenocarcinoma; BEAMing, beads, emulsions, amplification and magnetics; PCR, polymerase chain reaction; FISH, fluorescent in situ hybridization; Ref, reference number.

panel proposes to update this document periodically to include new data concerning indications for the detection of these alterations in ctDNA.

Finally, different studies showed that the presence of *EGFR*, *ALK,* or *METex14splice* alterations was a negative predictor of response to therapy with immune checkpoint inhibitors: to this point, it seems that a comprehensive analysis of the molecular profile is likely to give useful clinical information to all the patients and these biomarker should be included in panels developed either for tissue or plasma testing.[66-69]

Recommendations

cobas *EGFR* Mutation Test v2 is an acceptable test for use with ctDNA to detect the presence of *EGFR* sensitizing mutations and a positive result is sufficient to start a first-line treatment with an EGFR-TKI; however, negative results must be interrogated further with either DNA from a tumor biopsy or a more sensitive test (e.g., ddPCR or NGS) using ctDNA.

ddPCR could be considered for the detection of sensitizing *EGFR* mutations and a positive result should be sufficient for initiating therapy targeting these alterations; however, a negative result should prompt further evaluation with either a NGS-based test using ctDNA or using DNA from a tumor biopsy.

PCR-based methods should not be routinely used for *ALK* or *ROS1* rearrangement detection from ctDNA.

Multiplex panels using NGS platforms are reliable and preferred as they detect beyond the common mutations and can be designed for accurate assessment of indels, copy number changes, and translocations.[61] NGS can reach acceptable levels of sensitivity and optimal levels of specificity.[70] A positive result for *EGFR*, *ALK*, *ROS1*, or *BRAF* should be considered reliable and adequate to initiate first-line therapy. Detection of an oncogenic driver mutation other than *EGFR*, *ALK*, *ROS1*, or *BRAF* may be adequate for enrollment on a clinical trial depending on the trial criteria. A negative result from NGS for oncogenic driver alterations is not sufficient to exclude therapy and requires a confirmation from tumor biopsy.

Clinical Value in Patients With Progressive Disease on a Targeted Therapy

Patients with both *EGFR*-mutated and *ALK*-rearranged NSCLCs can benefit from successive lines of TKIs that can specifically target acquired resistance mutations. Patients experiencing progressive disease during TKIs must have repeat tumor sampling to assess which mechanism of resistance is involved, currently done through a biopsy at the progressing site. In this context, liquid biopsy has the potential of sparing an invasive procedure to the patient, with the added advantage that it monitors the patient systemically without the bias of a single, localized cell population represented by a needle biopsy.

Patients Progressing During *EGFR* TKIs

The most common mechanism of resistance to first- and second-generation *EGFR* TKIs in the first-line setting is the *EGFR* T790M mutation, with the less common resistance mechanisms being *MET* and erb-b2 receptor tyrosine kinase 2 (*HER2*) amplification, and SCLC transformation.[71,72] Table 2 lists several of the studies that investigated detection of T790M from ctDNA in the context of *EGFR* TKI resistance. Prompt detection of T790M in these patients allows starting treatment with the third-generation *EGFR* TKI osimertinib.[6] Similarly, the profile of resistance alterations in patients progressing on osimertinib treatment is continually emerging; therefore, their evaluation is expected to become clinically relevant, particularly with the advent of fourth-generation *EGFR* TKIs that can inhibit T790M/C797S mutant *EGFR,* and with strategies to curtail parallel pathway activation under study in clinical trials.[7,73,74] As first-line use of osimertinib gains popularity with the results of the FLAURA trial, the frequency of T790M will decline, but understanding mechanisms of resistance to osimertinib will likely be of clinical utility to patients in the near future.

The cobas test, as previously mentioned, can reach an acceptable grade of specificity but lesser sensitivity in detection of *EGFR* mutations in ctDNA. In 14 patients from South Korea who developed a T790M mutation, the mutation was detectable 2 to 12 months before radiologic progression in 8 patients, whereas 6 of them had the mutation detectable only at the time of progression.[75] Whether this is clinically relevant is still under investigation.

NGS can reach higher values of sensitivity compared to PCR-based methods.[40] As previously mentioned, the NGS concordance rate with tumor tissue for *EGFR* alterations is very high. The considerable advantage of NGS methods over PCR-based methods, in this setting, stems from the possibility of revealing resistance alterations other than T790M in the same analysis, which could potentially be useful for trials enrollments or expanded access programs. Tumor genomic complexity increases with *EGFR*-TKI treatment based on ctDNA analysis by NGS.[19] Notably, so far, detection of T790M remains the primary goal in patients progressing after first- or second-generation *EGFR* TKI as it is the alteration that can be treated most effectively. T790M could also precede radiological progressive disease and could be potentially used to monitor response

to first- and second-generation *EGFR* TKIs: 45% of patients harboring a T790M mutation could have this alteration detected before progressive disease through ddPCR assay of ctDNA[76]; similarly, the persistence of *EGFR* mutations in ctDNA from patients of the FASTACT-2 study in treatment with erlotinib could predict a poorer progression-free survival (PFS) rate[77]; finally, in 143 patients of the AURA 1 study with detectable T790M mutation in the ctDNA, persistence of T790M 6 weeks after initiation of treatment with osimertinib could predict shorter median PFS (5.5 months versus 10.9 months) and decreased objective response rate (35% versus 70%).[78] In addition, it must be remembered that at the time of progression a tumor biopsy may not always be available, as in the case of brain-only progression. The time needed to set up and perform the biopsy must be balanced with the eventual need of promptly starting an active treatment, particularly in symptomatic and rapidly progressing patients. Notably, in the near future, T790M detection might become of secondary importance when third-line *EGFR* TKIs are approved for first-line treatment of patients with activating *EGFR* mutations.[79] Figure 2 depicts a flowchart which summarizes panel believes for the use of the liquid biopsy in patients progressing during TKI treatment.

Recommendations

Testing of *EGFR* alterations with an EGFR assay of sufficient sensitivity is recommended for patients progressing, either clinically or radiologically, during treatment with first- or second-generation *EGFR* TKIs. Negative results, such as the absence of T790M, should be considered inconclusive and be assessed further with a more sensitive and/or more comprehensive test using ctDNA or using DNA from a tumor biopsy.

An NGS multiplex panel is preferred and recommended over PCR-based methods as it detects not only the common resistance mechanism T790M but is capable of detecting a spectrum of alterations. A positive result for *EGFR* T790M should be considered adequate to initiate osimertinib in the second-line setting after progression on therapy with a first- or second-generation *EGFR* TKI; however, a negative result requires confirmation with molecular analysis on a tissue biopsy, if possible. If the tissue biopsy is also negative for T790M, then the results

Table 2. Detection of T790M in ctDNA

Platform	Patients	Alteration	Specificity (%)	Sensitivity (%)	Concordance (%)	Ref
Cobas	24 patients with NSCLC treated with Nivolumab	T790M			61.4% positive agreement	101
					78.6% negative agreement	
ddPCR	60 patients progressing after *EGFR* TKI treatment	T790M	63	77		12
BEAMing	Retrospective cohort of 216 patients from the AURA-1 study	T790M	69.0	70	—	129
BEAMing	23 patients progressing after a *EGFR* TKI	T790M	—	10 mutations detected	—	132
cobas	38 patients from the AURA 1 trial	T790M	100	41	57	130
ddPCR	38 patients from the AURA 1 trial	T790M	83	71	74	130
BEAMing	38 patients from the AURA 1 trial	T790M	67	71	70	130
cobas	72 patients from the AURA 1 trial	T790M	67	73	—	130
BEAMing	72 patients from the AURA 1 trial	T790M	58	81	—	130
cobas	226 patients from the AURA 3 trial	T790M	—	51		131
ddPCR	208 patients from the AURA 3 trial	T790M	—	57		131
NGS	227 patients from the AURA 3 trial	T790M	—	65%		131
NGS	100 patients with NSCLC	T790M	T790M was identified in 4 tumor DNA samples and 8 ctDNA samples			133
NGS	63 patients with cancer (51 were NSCLC and 48 were stage IIIB-IV)	T790M	100	90.5	97	52
qPCR	306 patients with NSCLC from AURA 17	T790M	—	49		137
cobas	306 patients with NSCLC from AURA 17	T790M	—	42		137
ddPCR	306 patients with NSCLC from AURA 17	T790M	—	56		137
NGS	48 patients with NSCLC	T790M	A comparison was not present; this mutation was detected in 50% of the patients			138

qPCR, qualitative PCR; ddPCR, digital droplet PCR; NGS, next-generation sequencing; TKI, tyrosine kinase inhibitor; ctDNA, circulating tumor DNA; BEAMing, beads, emulsions, amplification and magnetics; PCR, polymerase chain reaction; Ref, reference number.
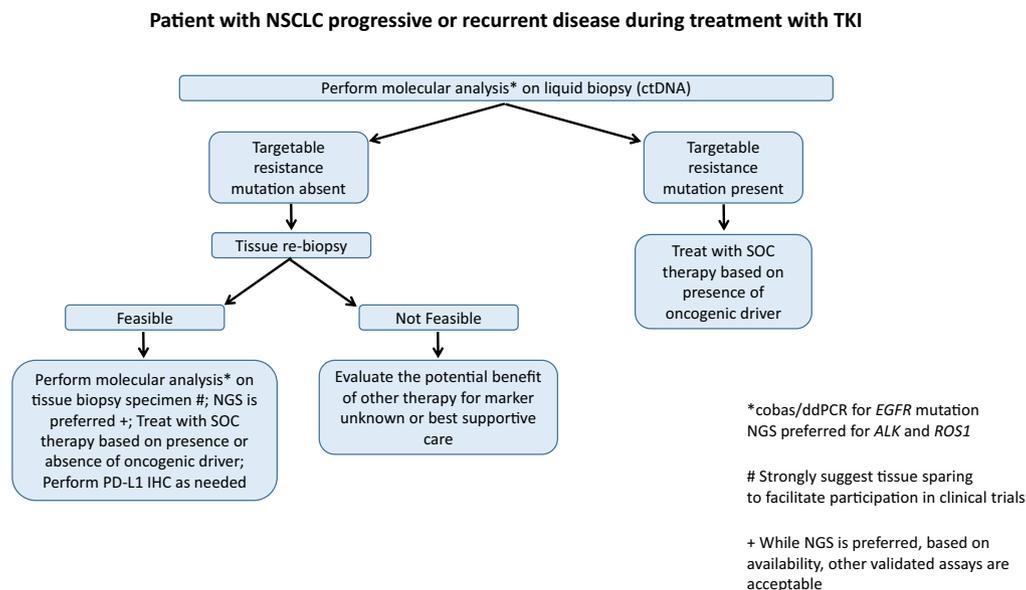
Patient with NSCLC progressive or recurrent disease during treatment with TKI

Figure 2. Patient with NSCLC progressive or recurrent disease during treatment with TKI. TKI, tyrosine kinase inhibitor; ctDNA, circulating tumor DNA; NGS, next-generation sequencing; SOC, standard of care; PD-L1, programmed death ligand 1; ddPCR, digital droplet polymerase chain reaction; ALK, ALK receptor tyrosine kinase.

from the NGS panel may reveal other mechanisms of resistance to guide clinical management via a clinical trial or expanded access. A negative T790M result may have more credence if a simultaneous assessment for the primary EGFR mutation is positive; however, because the T790M will often be at a lower mutant allele frequency MAF than the primary mutation, it may still be missed in the plasma, and further investigation is always warranted. In the setting where both are negative, it is more likely the tumor is not shedding adequate levels of DNA for detection.

Patients Progressing During ALK TKIs

Detection of ALK TKI resistance variants in the setting of disease progression after first-line therapy is expected in the near future to have clinical value for the management of patients. Table 3 lists a number of the studies that

investigated detection of ALK acquired resistance mutations from ctDNA. Similar to patients progressing during EGFR TKIs treatment, patients progressing during ALK TKIs may benefit from further molecular analysis of the progression lesion as later ALK TKIs have mutation-specific inhibitory characteristics. To date, no studies measured sensitivity and specificity of PCR-based or BEAMing platform methods for these alterations in patients with NSCLC. Currently, the majority of reports for detection of ALK mutation in ctDNA have been made with NGS. Moreover, the wide range of mutations that have to be covered and the number of upcoming new drugs suggest that NGS will be the optimal method for determination of ALK mutations from ctDNA. Among others, early preclinical evidence suggests that the L1196M or S1206Y mutations confer resistance to crizotinib but not to ceritinib[8]; the I1171T and the V1180L mutations confer resistance to alectinib and crizotinib but not to ceritinib[80];

Platform	Patients	Alteration	Specificity	Sensitivity	Ref
ddPCR	Cohort of 101 patients with neuroblastoma	Different ALK point mutations	100%	From 85.7% to 92.4% according to the mutation	139
ddPCR	20 patients progressing during therapy with Crizotinib	ALK mutations		10 mutations	140
		KRAS mutations		5 mutations	
NGS	22 ALK-positive patients with acquired resistance to ALK TKIs	ALK mutations ALK fusions		Concordance for ALK mutations calls from liquid biopsy and tissue biopsy was 100%	31
NGS	Reports providing estimations for the sensitivity of ALK fusion detection with NGS are expected in the near future.				

Table 3. Detection of ALK Acquired Resistance Mutations From ctDNA

Ref, reference number; ALK, ALK receptor tyrosine kinase; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; PCR, polymerase chain reaction; NGS, next-generation sequencing; TKI, tyrosine kinase inhibitor.

G1202R, G1123S, or F1174 mutations confer resistance to crizotinib. Finally, a new platform (cSMART PCR), already tested for *EGFR* mutation detection, is soon going to be tested for the detection of *ALK* mutations.[81,82]

Recommendations

Detection of *ALK* acquired resistance mutations in patients progressing during *ALK* TKIs is not required in clinical practice to switch them to a different *ALK* TKI. However, such information may be valuable in determining the optimum choice of next-generation TKIs, which have differing activity against distinct mutations. When re-biopsy of the progressing site is not feasible, comprehensive testing such as a NGS panel using ctDNA is preferred because this method can provide information not only on *ALK* resistance mutations but also on other molecular mechanisms of resistance for which the patient may receive treatment either through a clinical trial or expanded access.

Other Oncogenic Drivers in NSCLC

As previously mentioned, a wide range of potentially actionable alterations are detectable in patients with NSCLC, including *ROS1*, fibroblast growth factor receptor 3 (*FGFR3*), and neurotrophic receptor tyrosine kinase 1 (*NTRK1*) rearrangements and *MEK, AKT, BRAF, HER2, MEK1/2, NRAS,* and *KRAS* mutations. Abnormalities in the *MET* receptor tyrosine kinase, including mutations, amplification, and structural changes (e.g., Exon14 skipping events), are of particular interest clinically. All of these abnormalities have been detected in cfDNA isolated from plasma, and are occasionally included on NGS panels. Some of them are potentially druggable and thus their detection can bring a clinical advantage through a clinical trial or extended access program for a patient with no other treatment alternatives. While not currently targetable by approved agents, *KRAS* mutations nevertheless provide predictive information in several ways. The presence of a *KRAS* mutation can be used for assignment to clinical trials widely available at many academic and community institutions. Furthermore, detection of a *KRAS* mutation essentially rules out the possibility of other actionable driver alterations in treatment-naive patients, indicating that further molecular profiling may be unwarranted. Given this scenario, NGS and NGS-commercial panels may prove more expedient than PCR-based methods as they can test alterations in multiple genes simultaneously.

How Should the Results of Liquid Biopsy be Reported and Discussed?

An accurate, concise, and clear report of the molecular alterations investigated in ctDNA is important for optimizing therapy. The liquid biopsy report should be thorough and complete yet easy to interpret. Standards of molecular diagnostics reporting have been published by laboratory accrediting organizations including the CAP and the European Society of Pathology Task Force on Quality Assurance in Molecular Pathology and the Royal College of Pathologists.[29] Certain minimum elements are required in all reports for CAP-accredited laboratories. These elements include (in addition to patient identifiers and specimen type) assay methodology, basic clinical performance characteristics including clinical and analytic sensitivity (limit of detection) and specificity, assay results, and interpretation.[83] Evidence suggests that tumoral heterogeneity, in particular the proportion of tumor comprised of treatment-sensitive subclones, contributes to response to targeted therapies.[84,85] ddPCR, BEAMing, and NGS are among the assays that can provide a quantification of the fraction of DNA that harbors a specific alteration. The closest equivalent to percent positivity is the variant (or mutant) allele frequency (VAF), which should not be misconstrued as representing the fraction of tumor cells positive for that variant. The definition of VAF is simply the ratio of variant alleles to WT alleles, such that a 1% VAF for an *EGFR* L858R mutation means that out of every 100 captured DNA fragments that contain the relevant *EGFR* sequence, 1 is mutant and 99 are WT at the L858R locus. Many biological factors contribute to the amount of tumor-DNA shed specifically from cancer cells, as well as to the amount of nontumor DNA in peripheral circulation — including a significant fraction of cfDNA coming from cells of the immune system.[86] The amount of tumor DNA shed by cancers is thought to be dependent on total tumor burden, location and extent of metastases, proliferation rate, apoptotic potential, and genome instability among other processes.[56] Furthermore, gene amplification and loss will affect the proportion of mutant alleles detected. Because of this interpatient variability in both the amount of shed tumor DNA and background WT DNA, the biologic significance of the VAF should be interpreted with caution. Nevertheless, there are many potential advantages to an accurately determined VAF. For instance, if multiple abnormalities are detected in the same draw from the same patient, differences between their VAFs may indicate that a particular mutation is subclonal compared to others. In serial analyses, changes in VAFs over time may be indicators of increase disease burden, tumor evolution, or treatment activity. Increasing VAFs over time would portend a poor prognosis; whereas decreasing VAFs over time would likely be indicative of a successful therapeutic intervention.

To address these points, the recent tier classification of molecular alterations designed by the AMP, the American Society of Clinical Oncology, and the CAP seems suitable for these needs as they provide a complete list of the alterations discovered in a single test prioritizing explicitly those that are likely to be clinical meaningful.[87] Finally, we emphasize that internal validation and ongoing proficiency testing of in-house methods (laboratory developed tests) is an essential prerequisite to avoid disparities in the reliability of every kind of platforms.[49]

Recommendations

The liquid biopsy report should include the platform used and all the findings of the molecular analysis. It should be specified whether a detected alteration is clinically relevant according to present evidence. Established tier classification systems can provide guidance for reporting of clinical significance of genetic alterations. The VAF of a given mutation should be reported, and will likely be informative in longitudinal analyses.

Development of the Molecular Tumor Boards

With the advent of liquid biopsy, the availability of new molecular testing for a growing number of patients will be expanding over the next few years and, possibly, a solo practitioner might not be able to decide the optimal treatment for every patient. Moreover, it is to be expected that molecular test results from liquid biopsies will have to be considered in the context of all treatment modalities including radiation oncology and surgical oncology. In this scenario, we propose that, to improve the standard of care of patients, a Molecular Tumor Board (MTB) should be established in every hospital. Tasks of the MTB would be to discuss the best treatment option for the patient taking into consideration results of molecular testing, including those coming from liquid biopsies. Some experience in all solid tumors has already been made in this direction.[21] Rolfo et al.[88] instituted an MTB that retrospectively evaluated 141 patients, recommending a treatment in 78 (55%) of the patients. The group of Harada et al. also used the MTB for the selection of patients with cancer who should be advised a genetic testing; this resulted in approval of 132 cases of 191 for NGS analysis.[89]

Ethical Considerations and Informed Consent for Germline Variants

In a typical output of a sequencing analysis, germline mutations appear as peaks of allelic fraction that stand around 50% and 100% for heterozygous and homozygous mutations, respectively.[57] In contrast, tumor-specific DNA mutations rarely exceed 10% of the allelic fraction detected. In a large study with 31,414 patients who underwent NGS, the T790M mutation was found in 48 patients (of which 43 were patients with nonsquamous NSCLC).[90] In a series of 1000 consecutive patients who underwent tissue NGS, 2.3% of patients were discovered to be carriers of a previously unrecognized germline mutation.[91] cfDNA testing may identify high-risk germline (hereditary) DNA variants. Although somatic and germline variants should be readily distinguished based on VAF, in a small subset of patients with high ctDNA burden this may not be possible and patients should be informed of the possibility that high-risk germline variants may be incidentally detected in a liquid biopsy. The informed consent should clarify whether the patient wants to be informed about these incidental findings. Patients identified with a previously unrecognized germline mutation, and who asked to be informed of such finding, should be promptly referred for genetic counselling. Besides the incidental germline variants, there are other risks that should be considered in the informed consent. There is a certain probability that either the sequencing does not provide any result or it only finds mutations that are not druggable. This can be frustrating for the patient, particularly if they paid out-of-pocket for some nonroutine analysis, which can be the case for some commercial NGS-platforms.

Future Directions

Liquid biopsy is a rapidly growing field in oncology, particularly in lung cancer. There are different scenarios in which the liquid biopsy is likely to become part of the clinical practice, such as with immuno-oncology (I-O) and the monitoring of residual disease. The value of ctDNA in NSCLC is going to be further investigated in different studies. Currently, some trials are ongoing in this field. In addition, the use of ctRNA seems a very promising approach in NSCLC, although still remains to be validated. Here we summarize the most important concepts to be developed in the near future.

Liquid Biopsy and Immuno-Oncology

I-O is an expanding field, particularly in NSCLC. Although prediction and monitoring of the response to immune checkpoint inhibitors is still incompletely understood, some experiences with the implementation of liquid biopsy have already been made. As therapy with immune checkpoint inhibitors is reflected in a change of the profile of immune system's cells, identification of such changes are being used to predict the response or to monitor it.[92-94] In patients with NSCLC, T cell receptor expansion in peripheral blood lymphocytes has been

proposed as a marker for immune checkpoint inhibitor monitoring.[95]

In a study from Gandara et al.,[96] ctDNA was used to assess tumor mutational burden using a 394 gene panel. Cut points were determined in the POPLAR study and validated in the OAK study.[97] Patients with a higher number of mutations had improved PFS from the immune checkpoint inhibitor atezolizumab. Similarly, among 69 patients with NSCLC who underwent immune checkpoint blocker therapy, a significantly higher PFS (not reached versus 10.72 months) was observed in patients harboring more than three variants of unknown significance among the 73 genes analyzed in ctDNA; overall survival (OS) trended to be higher in patients with a higher mutational burden but this difference did not reach significance.[98] However, a report presented at the 2017 IASLC World Conference on Lung Cancer showed that a higher mutant allele frequency was correlated with shorter OS and PFS.[99] Finally, in patients with NSCLC treated with durvalumab, a decrease in VAF was seen in patients with partial responses; whereas, increases were observed in those with progressive disease.[100]

Another approach consists in monitoring CTCs. Some exploratory data showed that patients who experienced a decrease of the number of CTC expressing programmed cell death ligand 1— comparing numbers between baseline and 6 months — were more likely to receive a clinical benefit from therapy with nivolumab.[101] Development of proteins microarray from peripheral blood could be also a future implementation in I-O.[102]

Liquid biopsy in I-O needs further prospective validations. Particularly, tumor mutation burden prediction and detection of circulating markers of resistance seem to be the most promising fields for liquid biopsy in I-O.

RNA in the Liquid Biopsy

As previously mentioned, the circulating DNA it is not the only molecule that can be measured in liquid biopsy specimens. RNA-sequencing performed on platelets from 60 patients with NSCLC (56 were metastatic) and on 55 healthy donors could identify a signature able to precisely distinguish patients with and without cancer — area under the curve of the validation set was 0.977.[103] In a meta-analysis of 29 studies, high levels mir-21 and -155 resulted in a poorer OS and PFS —only approximately one-quarter of the patients had their micro-RNA (miRNAs) evaluated in the serum or the plasma whereas the others had it measured from the primary tumor.[104] Dosing of two miRNAs extracted from peripheral blood mononucleated cells could reach 71.43% sensitivity and 82.61% specificity for the diagnosis of NSCLC in a validation cohort of 56 patients with lung cancer and 46 smokers as controls.[105] Also, circulating free RNA

(cfRNA) can be used for the measurement of RNA transcripts of fusion genes (SLIT and NTRK like family member 1 [NTRK], ALK, ROS1, and ret proto-oncogene [RET]) and MET-14 splicing variant by rt-PCR.[106-108] Programmed cell death ligand 1in blood in NSCLC is being explored as tool to predict response to immunotherapy. Raez et al. have found a strong correlation between clinical responses assessed by computed tomographic scans with changes in plasma levels of cfRNA in patients with NSCLC, some of these were documented weeks before imaging was performed.[109,110] Moreover, the EML4-ALK fusion transcripts have been identified in the exosomal RNA of NSCLC patients and miRNA analysis of exosomes might be useful for the diagnosis and prognosis of lung adenocarcinoma and these particular miRNAs can offer prognostic information in advanced NSCLC for response to osimertinib.[111-117] In addition, miRNAs extraction from circulating exosomes has also been investigated.[118] A panel of four exosomal miRNAs could diagnose lung cancer with 96% sensitivity and 60% specificity in a retrospective cohort of 30 people of whom 10 were patients with lung cancer.[113] These promising preliminary results are still far from the clinical application.

Monitoring Residual Disease

Measurement of residual disease after primary tumor treatment is an area of active investigation. The first valuable experience came from a cohort of 18 patients with colorectal cancer who underwent multimodal therapy for primary tumor, in which the presence of ctDNA could be used as a reliable method for disease recurrence monitoring.[119] In a small cohort of lung cancer patients with stage I-III lung cancer (37 with NSCLC and 3 with SCLC) undergoing local treatment with radiation, surgery, or both, the presence of ctDNA after local treatment was highly predictive of disease recurrence.[120] ctDNA sequencing was performed using targeted panel NGS optimized for ctDNA analysis and, intriguingly, the number of mutations identified was an optimal predictor of the mutational burden, thus suggesting that this approach could also be useful for the above-mentioned needs in I-O.

The Liquid Biopsy in Other Fluids

Potentially, the blood is not the only source of useful clinical information in patients with NSCLC as many other fluids can be used to perform different molecular analysis. ddPCR and Illumina MiSeq were used to quantify the presence and the kinetics of EGFR alterations from serially collected samples of urine of nine patients with NSCLC undergoing therapy with erlotinib or afatinib and then, at the moment of progression, with

osimertinib. *EGFR* activating alterations and T790M mutation were confirmed in tumor tissue.[121] In patients under treatment with rociletinib, different investigators found a sensitivity for the detection of different *EGFR* alterations in urine free DNA comparable to the one of free DNA from plasma; moreover, plasma and urine together could identify patients with undetected T790M mutation in the tissue.[122,123] Spikes in the amount of T790M mutated DNA, days after osimertinib therapy initiation, was likely to reflect massive apoptosis of lung cancer cells, thus reflecting the value of this methods and the potential of exact mutation quantification. Detection with CellSearch Assay (Menarini Silicon Biosystems Inc, Huntington Valley, PA) of CTCs from the cerebrospinal fluid from 21 patients with NSCLC suspected for central nervous system metastasis was found to be more sensitive than magnetic resonance imaging for the detection of leptomeningeal involvement at 95.5% versus 47.6%, respectively. Moreover, mutations in CTCs were highly concordant to those identified in primary tumor (17 of 19 [89.5%]).[124] In addition, other fluids such as pleural effusion and saliva have been studied as noninvasive tools for EGFR mutation and other aberration detection.[125-127]

Conclusions

Liquid biopsy has shown considerable promise toward improvements in the management of NSCLC patients, offering an alternative to standard procedures when tissue biopsy specimens are insufficient or unfeasible, and providing a rapid and dynamic assessment of emergent resistance mechanisms that can be used to guide treatment decisions.

We can firmly state that technologies that detect *EGFR*-sensitizing alterations in peripheral blood in patients with treatment-naive NSCLC have reached such high reliability that their use in clinic is highly recommended. However, a negative cfDNA result should be considered uninformative, and should be followed by conventional tumor testing. The clinical benefit of combining, in this setting, the liquid biopsy with the tumor biopsy is significant for the above-discussed reasons. The liquid biopsy is reliable enough to be considered an acceptable surrogate of the tumor tissue biopsy whenever the latter, for whatever reason, cannot be obtained, particularly for the detection of the T790M mutation. To this point, the liquid biopsy is still far from replacing the tumor biopsy, which remains a cornerstone of the patient's diagnosis — including, of course, the histology of NSCLC and the molecular characterization whenever liquid biopsy–based approaches fail to give a positive result. Nonetheless, we strongly believe that with the growing relevance of molecular testing in every field of cancer, physicians dealing with cancer have developed confidence in the liquid biopsy technologies. In our opinion this is aided through the conformity and the accuracy of the pathologists' reports and through the creation of a specific board that takes care of considering the molecular identity of the patient's tumor in a multidisciplinary team.

Finally, topics discussed in the future perspectives section should be considered as promising fields of investigation for the near future, but not as validated tools appropriate for routine clinical practice.

It is the commitment of IASLC to update this consensus yearly to include all the new developments in the exciting topic of liquid biopsy.

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