

Clinical Utility and Application of Liquid Biopsy Genotyping in Lung Cancer: A Comprehensive Review

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Abstract: Precision medicine has revolutionized the therapeutic management of cancer patients with a major impact on non-small cell lung cancer (NSCLC), particularly lung adenocarcinoma, where advances have been remarkable. Tissue biopsy, required for tumor molecular testing, has significant limitations due to the difficulty of the biopsy site or the inadequacy of the histological specimen. In this context, liquid biopsy, consisting of the analysis of tumor-released materials circulating in body fluids, such as blood, is increasingly emerging as a valuable and non-invasive biomarker for detecting circulating tumor DNA (ctDNA) carrying molecular tumor signatures. In advanced/metastatic NSCLC, liquid biopsy drives target therapy by monitoring response to treatment and identifying eventual genomic mechanisms of resistance. In addition, recent data have shown a significant ability to detect minimal residual disease in early-stage lung cancer, underlying the potential application of liquid biopsy in the adjuvant setting, in early detection of recurrence, and also in the screening field. In this article, we present a review of the currently available data about the utility and application of liquid biopsy in lung cancer, with a particular focus on the approach to different techniques of analysis for liquid biopsy and a comparison with tissue samples as well as the potential practical uses in early and advanced/metastatic NSCLC.

Keywords: circulating tumor DNA, liquid biopsy, EGFR, minimal residual disease, sequencing technologies, lung cancer, early-stage, non-small cell lung cancer

Introduction/Background

During the last decade, several tumor molecular alterations have been identified as drivers of cancer proliferation and survival, leading to the development of novel specific targeted agents for different tumors, including lung cancer.^{1,2} Concerning non-small cell lung cancer (NSCLC), the advent of targeted therapy with tyrosine kinase inhibitors (TKIs) improved the overall patient outcomes with a better tolerability and safety profile when compared to platinum-based chemotherapy. Predominant targetable alterations in nonsquamous NSCLC are Epidermal growth factor receptor (*EGFR*), occurring in about 10–15% of Caucasian patients and up to 40% in Asian population, and Kirsten rat sarcoma virus (*KRAS*)-mutations, present in 20–30% of patients, followed by Anaplastic lymphoma kinase (*ALK*) translocations (3–7%), v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutations (2–4%), c-mesenchymal-epithelial transition factor (*MET*) amplifications and exon 14 skipping mutations (2–4%), and c-ros oncogene 1 (*ROS1*) (1–2%), rearranged during transfection (ret proto-oncogene or *RET*) (1–2%) and Neurotrophic tyrosine receptor kinase (*NTRK*) fusions (0.5–1%).^{3–11}

However, many patients present a non-oncogene-addicted disease without any of the above-mentioned driver alterations.³ Thus, the proportion of nonsquamous NSCLC patients presenting a non-oncogene addicted disease is

progressively decreasing.¹² Conversely, the rate of actionable driver mutations among squamous NSCLC is very low, and international guidelines do not recommend comprehensive molecular testing.

The expanding access to various molecular diagnostic strategies, such as next-generation sequencing (NGS) technique, immunohistochemistry and polymerase chain reaction method (PCR), allowed the routine use of molecular tumor assessment to determine the status of the most relevant molecular targets in NSCLC patients.^{13,14} In fact, since 2018 and with the recent update of 2021, the International Society for the Study of Lung Cancer (IASLC) and the European Society of Medical Oncology (ESMO) recommended the adoption of NGS for the molecular assessment of patients with advanced NSCLC.¹⁵

Nowadays, tumor tissue still represents the standard biological sample for molecular analysis. However, this approach has significant limitations due to the potentially difficult technical accessibility of particular sites of disease or the inadequacy of the histological sample for molecular testing, sometimes requiring a new biopsy.¹⁶ To overcome these issues, liquid biopsy has gained increasing attention and currently represents an important alternative source to detect tumor genomic alterations.

Liquid biopsy is a minimally invasive procedure that provides the opportunity to detect, analyze and monitor cancer in various body fluids (such as blood, urine, malignant pleural effusion and ascites) and also permits dynamic and real-time monitoring of cancer molecular alterations.¹⁶ In fact, metabolic and homeostatic mechanisms, along with micro-environmental factors and drug selection pressures, may all influence and change the molecular features of cancer cells, an important phenomenon that physicians need to detect.¹⁴

Although this method has a good specificity in detecting mutations in circulating tumor DNA (ctDNA) (90–100%), the sensitivity levels achieved with the current methodologies are low (60–70%).^{17,18} The ctDNA is significantly diluted in the circulation by the abundance of background cell-free DNA (cfDNA) released by non-tumoral cells.¹⁹ Furthermore, circulating cells, extracellular vesicles, messenger RNA (mRNA), micro RNA (miRNA), proteins, and cell-free DNA (cfDNA) can also be detected in the bloodstream²⁰ (Figure 1).

The term liquid biopsy can be referred to different laboratory techniques that analyze tumor-derived circulating nucleic acids (like ctDNA), tumor-derived fractions of cfDNA in the plasma, mRNA, non-coding RNA such as micro-RNA or long non-coding RNA, tumor-educated platelets (TEP), circulating tumor cells (CTCs), exosomes or proteins, and metabolites.¹⁴ Tumor mutations in ctDNA can be detected using NGS techniques. Thus, ctDNA analysis could be employed as a complementary tool that can provide additional information regarding tumor genotyping associated with tumor tissue biopsy for NSCLC.

Given these concepts, liquid biopsy may represent a less invasive and more feasible alternative to tissue biopsy in many cancer settings, from a promising detection method for minimal residual disease (MRD) and recurrence in patients with resected disease, to a dynamic assessment of tumor mutability in advanced disease.²⁰ This review analyzes the clinical utility and application of liquid biopsy genotyping in NSCLC.

Applications of Liquid Biopsy

When dealing with a NSCLC formalin-fixed paraffin-embedded (FFPE) sample, evaluation of PD-L1 expression through immunohistochemistry (IHC) and analysis of molecular alterations in an increasing number of genes are all needed to define appropriate treatment. Tissue genotyping can be performed with FISH or IHC, multiple PCR hot spot single-gene assays, or wide NGS panels, depending on the costs and availability.²¹

Notwithstanding the cases where biopsy is not feasible due to patient conditions or technical difficulties, even when the tumor tissue is available, sometimes the required analyses are not always successful due to the low quality or scant quantity of available tissue.²²

The NILE trial²³ showed that, out of 282 patients with treatment-naïve advanced NSCLC, as few as 18% of patients had complete tissue analysis of all the biomarkers of interest at the time of the trial (*EGFR* mutations, *ALK* fusions, *ROS1* fusions, *BRAF*^{V600} mutations, *RET* fusions, *MET* amplification, *MET* exon 14 skipping variants, *ERBB2* mutations), while 68.1% had a partial analysis. On the other hand, liquid biopsy with NGS analysis of all recommended genes was successfully performed on 95% of patients, with a sensitivity of 80% for detection of recommended biomarkers and a positive predictive value of 100%. Furthermore, turnaround time (TAT) was shorter with liquid biopsy compared with

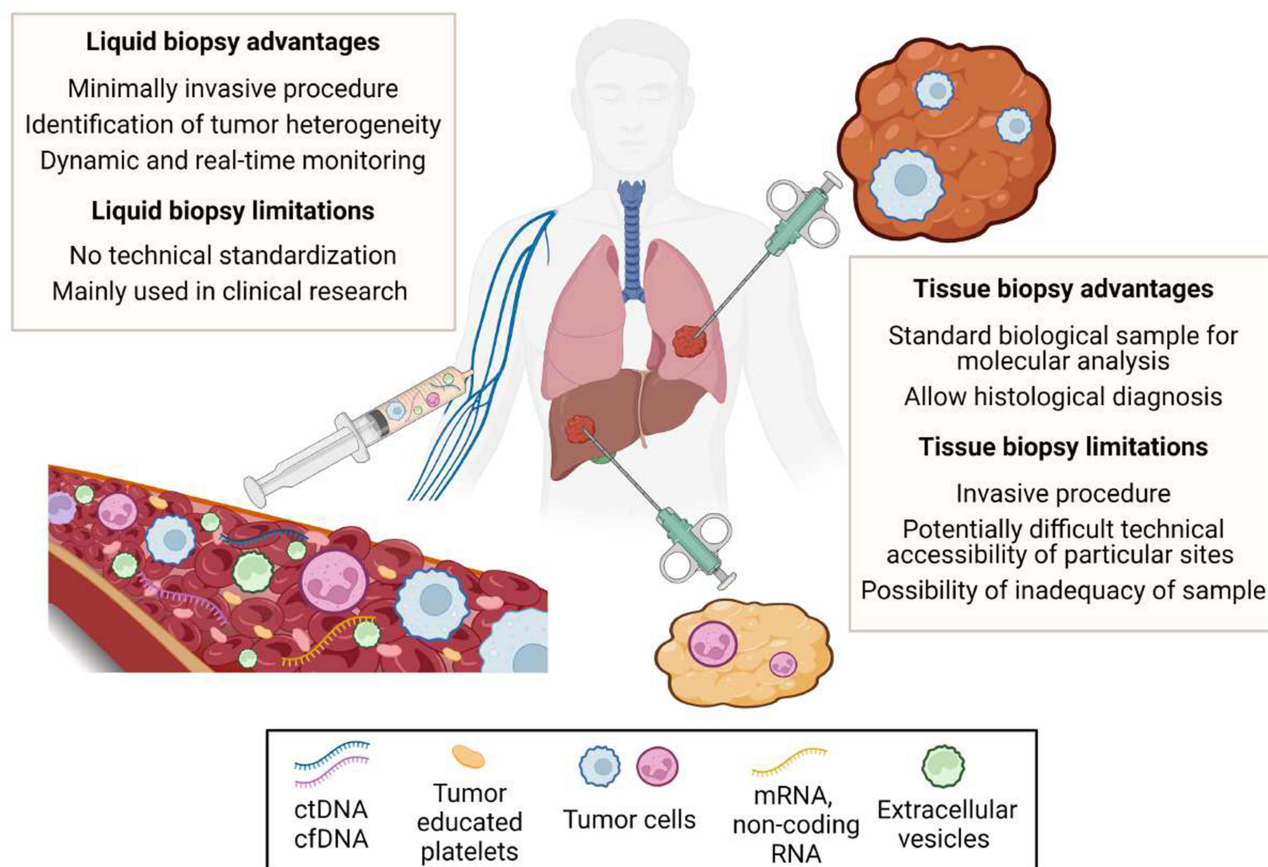


Figure 1 Comparison between tissue and liquid biopsy: advantages and limitations.

Abbreviations: ctDNA, circulating tumor DNA; cfDNA, cell-free DNA; mRNA, messenger RNA; miRNA, micro RNA.

tissue genotyping (9 vs 15 days). The NILE trial is a prime example of how liquid biopsy can bypass the problem of insufficient FFPE tissue and offers quicker turnaround times.

The idea of liquid biopsy actually encompasses several techniques with different analyzed bioproducts. It can be used to analyze circulating tumor cells, circulating free RNA or exosomes, but these techniques are yet too unreliable for clinical use²⁴. Most commonly, liquid biopsy refers to the search for cfDNA and in particular one of its components, ctDNA, defined as the DNA released from cancer cells into biofluids.²⁵

Once the cfDNA has been obtained, real-time PCR-based arrays, like allele-specific polymerase chain reaction (ASPCR) for *EGFR*, digital PCR techniques (such as BEAMing or droplet digital PCR), or multigene NGS-based arrays can be used to analyze the genome.²⁶ The main limitation of PCR-based techniques is that only a finite number of mutations is detectable. For instance, the Cobas[®] *EGFR* mutation test v2 can detect only 42 alterations in exons 18–21, despite a sensitivity of 72.1% and specificity of 97.9%.²⁷ This drawback also applies to digital PCR though with relatively higher sensitivity (70–85%).²⁸

Other than qualitative information, liquid biopsy can also give quantitative data in the form of mutant allele fraction (MAF), meaning the percentage of mutant alleles in a specified locus, which helps track the tumor clonal evolution over time.²⁹ ctDNA can be detected in plasma even if present at low levels: the detection limits of modern kits are as low as an allelic fraction of 0.1% or lower, with a median allelic fraction of 0.5%.^{30,31}

Overall, high specificity and high positive-predicted value are core features of ctDNA, meaning that the detection of targetable genomic alterations, even in low allelic fractions, can be clinically relevant.³² This has been proven with single-gene PCR (specificity of PCR-based *EGFR* tests was close to 98%^{27,33}) and NGS assay (specificity of 97%³⁴) when dealing with targetable variants.

The detection of non-targetable mutations has a more nebulous interpretation, and their origin from tumor DNA is not always certain. Particularly, *JAK2* alterations are usually imputable to white blood cells' DNA, a phenomenon known as clonal hematopoiesis, and this can also apply to *TP53* and *KRAS* mutations.³⁵

On the other hand, false negatives can be more frequent as the sensitivity of ctDNA can be affected by a variable amount of DNA shedding and tumor heterogeneity. Studies report detection rates of around 70–80% for both PCR-based and NGS-based arrays.^{34,36}

A recent study showed that detection rates and concordance percentages between liquid biopsy and tissue samples vary widely, ranging from as low as 14.3% in stage I to as high as 85% in stage IV disease.³⁷ Overall, better results were obtained with more advanced disease stages, higher tumor burden, and in treatment naïve patients or progressing on therapy.³⁷

Overall, several techniques such as real-time PCR, digital PCR or multigene NGS-panel can be applied to liquid biopsy samples, with high specificity results and a wide range of sensitivity rates, depending on the sample, patients' clinical characteristics, and tumor features.

Early-Stage NSCLC

Patients with early-stage (I, II and resectable III) NSCLC are amenable to radical surgery, followed or not by adjuvant therapy on the basis of primary tumor TNM staging and *EGFR* mutational status.^{38–40} Despite the curative intent, 5-years overall survival after radical surgery was about 32%.⁴¹ In fact, 30% to 70% of patients show local or distant disease recurrence,³⁸ which might be attributable to the persistence after surgery of MRD. Therefore, the early identification of individuals at high risk of recurrence is crucial to provide them with appropriate treatment. Likewise, detecting viable cancer cells before they spread to distant sites is a primary goal of cancer research. Various studies analyzed the presence of peripheral blood ctDNA in patients affected by early-stage lung cancer before and after surgical treatment, reporting a rapid decrease in ctDNA levels after surgery.^{42,43} In addition, several analyses showed discrepancies in mutation frequency between tumor-DNA (tDNA), the genetic signature detected on tumor mass analysis, and matched ctDNA detected in patient's blood, depending on tumor stage, with a more significant mismatch in stage I–IIA.^{43,44} This discordance may be correlated with the wide intra-tumor heterogeneity, although it would be expected to be more represented in advanced lung cancer than in early lung cancer due to the persistent interaction between tumor cells and the other cells comprised in the tumor microenvironment.⁴⁵

Detecting ctDNA after surgical resection may guide physicians in identifying patients at higher risk of recurrence and who may benefit from adjuvant therapy. On the contrary, patients with no detection of ctDNA may be initiated to clinical-instrumental monitoring only. A limitation of this approach is that ctDNA is often lacking or present in a meager amount in early-stage disease. To overcome this limitation, more sensitive tests have been developed to detect and quantify extremely low levels of plasma ctDNA.

The CAncer Personalized Profiling by deep Sequencing (CAPP-Seq) is a highly sensitive and specific platform that uses a “selector”, composed of biotinylated DNA oligonucleotides, that is applied first to tDNA to detect specific genetic alterations, then to ctDNA to quantify them.⁴⁴ From the analysis of Chaudhuri et al,⁴⁶ this type of test has proven to be highly effective in identifying MRD after treatment of localized tumors, and it has been able to quantify ctDNA also in stage I lung cancer. According to the paper, about 94% of patients enrolled in this trial who subsequently relapsed had detectable ctDNA in their post-treatment blood specimen, making the test a reliable predictor of relapse. In addition, during analysis, the *EGFR*^{L858R} mutation was identified at the MRD landmark, underlining how ctDNA analysis after radical treatment can help to choose tailored therapies before clinically or radiologically documented disease recurrence.⁴⁶ For example, an 81-year-old patient, enrolled on trial and underwent stereotactic ablative radiotherapy (SABR) for localized lung disease, died for symptomatic brain metastases and concurrently adrenal metastasis 3 months later. In this case, early treatment with an EGFR TKI should have been considered before the development of symptoms in view of detection of *EGFR*^{L858R} mutation at the MRD landmark.⁴⁶

The “Tracking Non-Small-Cell Lung Cancer Evolution Through Therapy (Rx)” (TRACERx) study analyzed the detection of plasma cfDNA of 100 stage IA–IIIA NSCLC patients before and at different time points after surgical treatment to comprehend the clonal evolution of cancer cells from the time of diagnosis to patient's death.^{47,48} The

investigators devised sequencing assays tailored for each patient, starting with multi-region sequencing of the individual tumor exome, targeting both clonal and sub-clonal single nucleotide variants (SNVs). The study found that necrosis, lymphovascular invasion, Ki67 index, and non-adenocarcinoma pattern predict ctDNA shed, in addition to already recognized factors, such as tumor size, stage of disease, and sites of metastasis. cfDNA was identified only in 48% of early-stage patients, and based on a rough estimate, tumors $<10\text{ cm}^3$ of volume have no detectable ctDNA.^{47,49,50} Strengths of the TRACERx trial are the variety of tumor staging included, such as a large cohort of stage I NSCLC characterized by a minimal tumor burden and the analysis interval of ctDNA (that could have been performed sometimes during adjuvant treatment). In some cases, the evidence of MRD allowed for changing the type of adjuvant therapy based on ctDNA changes. On the contrary, a limitation of the TRACERx study⁴⁸ and the CAPP-Seq⁴⁶ was the poor number of certified relapses.

Cancer SEEK is another blood test, based on PCR, able to synchronously evaluate several regions of driver genes typically mutated in different types of cancer (lung, breast, ovary, pancreas, liver, esophagus, stomach and colorectal), detecting early-stage tumors and localizing the primary tissue.⁵¹ The use of multiplex-PCR allowed each original model molecule to be directly and uniquely labeled with a DNA barcode. This approach minimizes the intrinsic biases of massively parallel sequencing and makes optimal use of the small pool of cfDNA. In addition, the total volume of plasmatic DNA was divided into several portions on which independent assays were performed. By doing so, the number of DNA molecules per sample is reduced, but the fraction of each mutant species is increased, making mutations easier to be detected. In this method, PCR assay is combined with eight specific protein biomarkers that can detect various types of cancer early, with a sensitivity $>69\%$ and specificity $>99\%$. The study results showed 90% concordance in mutation detection between plasma and primary tumors, ranging from 100% in ovarian and pancreatic cancers to 82% in gastric cancers.⁵¹ However, although this approach detected about 70% of the total cancers tested faithfully, its sensitivity for stage-I tumors was relatively low (43%). In particular, lung cancer and esophageal cancer showed the lowest detection rates. Furthermore, because there is no specific circulating protein marker for lung cancer, site determination was possible in only 39% of cases, compared with 84% for colorectal cancer, 79% for ovarian cancers, and 81% for pancreatic cancers.⁵² In the screening setting, a next-generation plasma sequencing by using wider panel will be able to help in early tumor diagnosis, considering its good sensitivity, that could be impaired by low tumor spread, and its high specificity, which might be greater in a sample of healthy people such as in a real screening scenario.⁵²

The analysis of ctDNA mediated by the above-mentioned tests may also represent a non-invasive approach for the early diagnosis of NSCLC. Compared with conventional unspecific and insensitive serum tumor markers, occasionally used for early diagnosis of various types of cancer, and similarly unspecific computed tomography (CT) scan,^{53–55} ctDNA was found to be more sensitive and specific to reveal an early-stage tumor. From the study by Chen et al, no patients with benign lesions had detectable ctDNA in their blood, suggesting that, in the case of positive ctDNA, a suspected lesion should be considered likely malignant, requiring a more intensive diagnostic approach.⁴³ Concerning this concept, the TEC-Seq assay, known as “Targeted Error-Corrected Sequencing”, analyzed, in an ultra-sensitive manner, sequence changes in tumor genes commonly mutated in cfDNA, without first knowing the alterations in the tumor, re-reading each DNA code at least 30,000 times.^{56,57} By comparing sequence alterations of genes responsible for cell proliferation in healthy individuals with the cancer-specific alterations in the plasma of patients affected by tumors, the authors highlighted both the potential utilities and limits of large-scale testing of ctDNA modifications for the non-invasive detection of early-stage cancers. The main issue is that the detection rate of circulating tumor cells and ctDNA is typically low ($<30\%$). In addition, any inflammatory disorder may release circulating epithelial cells, twisting the findings.^{58,59}

Ilie et al⁶⁰ selected five chronic obstructive pulmonary disease patients with CTCs identified by an innovative isolation technique by the size of epithelial tumor cells, named ISET, monitoring them with CT scans. All of them subsequently developed lung cancer, diagnosed at an early stage. In contrast, none of the other patients who did not have CTCs at the test developed the tumor. Based on this concept, an earlier analysis of prospective, non-randomized, multicenter AIR study suggested that detection of CTCs in peripheral blood with ISET technique could be used as a screening test for early diagnosis of lung cancer.⁶¹ Later, Marquette et al⁶² revealed that this method is not suitable for cancer screening, showing a CTC detection sensitivity for lung cancer of only 26%.

In summary, although testing ctDNA and CTCs in the blood of high-risk patients can detect lung cancer early, there is no sufficient evidence of its use as a screening method in clinical practice. On the other hand, ctDNA profiling may help identify patients who underwent curative surgery for lung cancer and are less likely to benefit from adjuvant chemotherapy. Vice versa, longitudinal ctDNA analysis may allow the identification of those patients who show increased ctDNA levels during adjuvant chemotherapy and thus may need more careful evaluation. In the future, the use of this new biomarker will depend on novel prospective clinical trials that investigate its clinical benefit as well as its possible integration with diagnostic imaging for earlier detection of disease recurrence.

Advanced/Metastatic Disease – Non-Oncogene Addicted

The liquid biopsy has no well-defined indications in patients with advanced non-oncogene addicted NSCLC. Promising areas of investigation include identifying patients who could benefit the most from immunotherapy, collecting the dynamic genomic heterogeneity and laying a tumor molecular profile during the disease course.⁶³

The advent of immunotherapy has revolutionized the natural history of patients with non-oncogene addicted NSCLC. Identifying predictive biomarkers for anti-PD-1/PD-L1 therapies in NSCLC is crucial for selecting patients who will benefit from immunotherapy while limiting ineffective treatment and their related toxicities.^{63,64} The only validated biomarker is PD-L1 expression by immunohistochemistry on tumor tissue.⁶⁵ However, tumor PD-L1 expression is not completely reliable, as improved survival outcomes have also been assessed in negative or low PD-L1 expression, even if the 50% of tumor proportion score represents the best threshold for upfront single-agent PD-(L)1 inhibitor efficacy as demonstrated by several prospective trials.⁶⁶ Tumor mutational burden (TMB) is defined as the total number of somatic, non-synonymous mutations in the tumor genome. It is garnering a lot of attention, and several studies have reported the evaluation of TMB in blood (bTMB) as this may be estimated from cfDNA NGS.⁶⁷ It has been suggested that highly mutated tumors tend to produce neoantigens, which can be targeted by activated immune cells. In many tumor types, TMB has been shown to correlate with patient response to both CTLA-4 and PD-(L)1 inhibition.^{68,69} It seems that TMB and PD-L1 show only a slight overlap in NSCLC, suggesting that these assays measure various aspects of the immune phenotype.⁷⁰

A meta-analysis performed by Yu et al⁷¹ in 14,395 patients demonstrated that the combined use of PD-L1 expression and TMB are promising biomarkers to assess patient survival and response to immunotherapy. Moreover, the matched evaluation of CD8⁺ tumor-infiltrating lymphocytes, PD-L1, and TMB expression had prognostic relevance, although the predictive value of this assessment needs to be validated in further prospective studies.⁶¹ The study by Gandara et al⁶⁷ optimized a gene panel and an algorithm for bTMB, demonstrating the possibility of using cfDNA to determine TMB and showing its predictive value for NSCLC patients treated with immunotherapy. Likewise, Phase 2 POPLAR and Phase 3 OAK studies revealed that high bTMB was associated with improved response rate and PFS from atezolizumab in single-agent regimen.^{67,72} In the prospective proof-of-principle B-FIRST (NCT02848651) trial, bTMB (cutoff greater than or equal to 16 mut/Mb), assessed with the FoundationOne Liquid CDx assay, was evaluated as a predictive biomarker of response to first-line atezolizumab in PD-L1-unselected patients with advanced NSCLC.⁷³ A bTMB greater than or equal to 16 mut/Mb was correlated with increased ORR. The Phase 3 Blood-First Assay Screening Trial (NCT03178552) is randomly evaluating bTMB greater than or equal to 16 mut/Mb. Instead, Rizvi et al⁷⁴ in the phase 3 MYSTIC study compared durvalumab with or without tremelimumab with platinum-based chemotherapy using the Guardant OMNI 500-gene panel to assess bTMB. They demonstrated that a bTMB greater than or equal to 20 mut/Mb in the double checkpoint inhibition arm is associated with improved overall survival, PFS and ORR compared to chemotherapy. In addition, less clinical benefit was shown with durvalumab alone versus chemotherapy.⁷⁴ In conclusion, the techniques used for the analysis and interpretation of TMB widely vary, and even if high TMB was linked to greater efficacy of immunotherapy, several limitations and conflicting results in different studies have slowed down its translation into clinical practice.⁶³

cfDNA analysis is also emerging as a new plasma biomarker to evaluate disease activity and treatment-related changes, especially as a predictive marker of immunotherapy response.^{75,76} Composed of double-stranded fragments of 150–200 base pairs, cfDNA derives from normal physiological tissue remodeling events,⁷⁷ although it may result from necrosis and apoptosis of cancer cells. cfDNA has a short half-life of about 2 hours, allowing a dynamic assessment of

tumor-related changes and providing real-time monitoring of response or recurrence.⁶³ In a proof-of-concept study, variations in cfDNA concentration were pointed out as a suitable tool to assess tumor response in patients treated with PD-1 inhibitors.⁷⁸ The percentage of ctDNA on total cfDNA in cancer patients varies dramatically, ranging from less than 0.1% to more than 90%.^{77,79,80} ctDNA is isolated uniquely through specific somatic genomic alterations and tends to be more fragmented, with sizes ranging from 90 to 150 base pairs.⁵³ ctDNA changes may appear earlier than radiographic ones, enabling a prompt evaluation of response to treatments.^{1,81} In addition, several studies have shown that early changes in ctDNA levels during immunotherapy correlated with radiological response and could identify patients who will achieve long-lasting benefits.^{78–82} Therefore, cfDNA and ctDNA levels may be early markers of efficacy in patients treated with immune checkpoint inhibitors (ICIs).

CTCs are released into the bloodstream from the primary tumor and are characterized by a very short half-life (1–2.4 h).⁶³ The percentage of CTCs in the blood is very low, about 1 CTC for every 106–107 leukocytes.⁶³ Initially, CTCs have an epithelial phenotype that is unlikely to migrate into the bloodstream. Nevertheless, they can undergo epithelial-to-mesenchymal transition (EMT), enhancing plasticity and ability to migrate, invade, and disseminate.⁶³ Some authors found an association between a high number of baseline CTCs and poor overall survival, with an increased risk of death and progression.⁸³ This concept may also be an indicator of the aggressiveness of these CTCs, which may show features of the mesenchymal phenotype (via EMT).⁸⁴

Moreover, the joint analysis of cfDNA with CTCs helped to discriminate against a low-risk population that might benefit from continuing nivolumab beyond progression. This analysis suggests that the combined evaluation of cfDNA and CTCs could help to select those patients who will benefit most from immunotherapy.⁶³

Advanced/Metastatic Disease – Oncogene Addicted

Nowadays, ctDNA analysis in advanced or metastatic NSCLC has been extensively validated for the assessment of *EGFR* mutational status when tissue is insufficient for tumor genotyping of treatment-naïve patients, for the detection of T790M resistance mutation, and the monitoring of *EGFR* sensitive mutations during TKI treatment.^{85,86}

Despite the limitations (non-DNA biomarkers not evaluable, increased costs if used concurrently with tissue testing and false negatives), there is solid evidence to support the use of plasma ctDNA in clinical practice in genotyping multiple other targetable oncogenic drivers through a broad-based platform, such as NGS.^{23,87,88} Liquid biopsy could be used in newly diagnosed tumors with tumor tissue available for initial genotyping when the tissue sample results are inadequate, or it could be performed simultaneously with tissue analysis to increase the identification rates of relevant targeted oncogenic drivers.²³ In fact, the study by Leigh et al²³ suggests that initial biomarker evaluation using cfDNA improves biomarker discovery rate and number of newly diagnosed mNSCLC patients receiving guideline-complete biomarker testing. Although this approach represents the more expensive alternative, the simultaneous modality appears to be the most effective option in the case of small tumor biopsies with uncertain adequacy.³² Furthermore, liquid biopsy performed prior to tissue sampling, according to the “plasma first” modality, should represent the standard of care for patients with oncogene-addicted NSCLC progressing after targeted therapy.¹

EGFR Mutations

NGS analysis of ctDNA can detect both on-target (secondary mutations or amplification of the target gene) and off-target (“bypass trace”) acquired molecular resistance mechanisms, suggesting tailored treatment at the time of disease progression to targeted agents.

In the FLAURA study,⁸⁹ ctDNA analysis was performed at the time of disease progression in both study arms (osimertinib vs investigator choice of gefitinib or erlotinib). Several studies have analyzed the agreement between the assessed mutational status in plasma and tumor tissue samples, showing a robust correlation^{28,90–97} (Table 1). Recently published meta-analyses (Table 2) have a sensitivity for the detection of EGFR mutations ranging from 60% to 70% and a specificity of 80–98%.^{98–103} However, given the different technologies included, the sensitivity values are very variable. These data suggest the need to standardize procedures through validation of techniques for large-scale implementation. Recent sequencing technologies, such as digital PCR or plasma digital droplet PCR (ddPCR), use probes that allow the detection of del19 and L858R with very high sensitivity rates (greater than 80%) and 100%

Table 1 ctDNA plasma detection of *EGFR* mutation

Reference	Method of Detection	Study Design/Sample Size	Sensitivity	Specificity	Concordance/OPA Between Tissue and Liquid Biopsy
Douillard J et al ⁹¹	QUIAGEN theascreen EGFR RGQ PCR Kit	Prospective, single-arm Phase IV study (IFUM study); N = 652	65.7%	99.8%	94.3%
Reck M et al ⁹²	QUIAGEN theascreen EGFR RGQ PCR Kit; Cobas EGFR mutations test version 2; Cycleave; PNA-LNA PCR Clamp; other	Multicenter (ASSESS study); N = 1162	46%	97%	89%
Wu YL et al ⁹³	QUIAGEN theascreen EGFR RGQ PCR Kit	Phase III, Prospective (Lux-Lung 3 and 6 studies); N = 334 (plasma); N = 287 (serum)	60.5% (plasma) 28.6% (serum)	n.r.	n.r.
Han B et al ⁹⁴	Cobas EGFR mutations test version 2	Multicenter (IGNITE study); N = 2561	46.9%	95.6%	80.5%
Karlovich et al ⁹⁶	Cobas EGFR mutations test version 2; BEAMing (Symex Inostics GmbH)	Prospective, multi-centre, observational and phase-I TIGER-X; N=153	(Cobas/BEAMING) Activating mutations: 73%/82% T790M: 64%/73%	(Cobas/BEAMING) Activating mutations: 100% T790M: 98%/50%	(Cobas/BEAMING) Activating mutations: 80% T790M: 86%/67%
Oxnard et al ²⁸	BEAMing (Symex Inostics GmbH)	Retrospective (AURA Phase I) N = 216	T790M: 70% L858R: 86% Del 19: 82%	T790M: 69% L858R: 97% Del 19: 98%	n.r.
Sacher et al ⁹⁷	Droplet digital PCR (ddPCR)	Prospective; N = 180 (120 newly diagnosed + 60 acquired resistance)	L858R: 74% Del 19: 82% T790M: 77%	L858R: 100% Del 19: 100% T790M: 63%	L858R: 80% Del 19: 91% T790M: 40%
Zheng D et al ⁹⁸	Droplet digital PCR (ddPCR)	N=117	T790M: 81%	T790M: 100%	88%

Abbreviations: N.R., not reported; NSCLC, Non-small cell lung cancer; EGFR, Epidermal growth factor receptor; ddPCR, Droplet digital polymerase chain reaction; BEAMing, Beads, emulsion, amplification, and magnetics; OPA, overall percent agreement.

Table 2 Meta-analysis on the diagnostic accuracy of liquid biopsy for detecting *EGFR* mutations

Reference	Study Design (N of Patients)	Sensitivity	Specificity	Concordance/OPA Between Tissue and Liquid Biopsy
Luo et al ¹⁰⁰	20 studies (N=2012)	67.4%	93.5%	n.r.
Qiu M et al ¹⁰¹	27 studies (N=3110)	62%	95.9%	n.r.
Mao et al ¹⁰²	25 studies (N=2605)	61%	90%	79%
Zhou et al ¹⁰³	32 studies (N=4527)	70%	98%	n.r.
Passiglia et al ¹⁰⁴	21 studies (N=1639)	67%	80%	n.r.

Abbreviations: OPA, overall percent agreement; n.r., not reported.

specificity.^{96,97} The most common resistance mutation in patients who did not receive osimertinib upfront was the occurrence of *EGFR* exon 20 p.T790M mutation (47%). Instead, no cases of acquired *EGFR* exon 20 p.T790M mutation were documented at the time of disease progression among patients who received osimertinib as first-line therapy. The most commonly detected molecular resistance mechanisms in this arm were secondary *EGFR* mutations, such as exon 20 p.C797X and *MET* amplification.⁸⁹ Recent studies suggested that acquired resistance through *MET* amplification during the treatment with osimertinib could be overcome by combining a *MET* inhibitor with the third-generation EGFR-TKI.^{104,105}

The FLAURA trial also evaluated the prognostic impact of detectable *EGFR* mutations on ctDNA at baseline, and the persistence versus the clearance of mutant *EGFR* ctDNA levels during the targeted therapy. Progression-free survival (PFS) was significantly longer in patients without detectable *EGFR* mutations at baseline in both treatment arms than in patients with detectable baseline *EGFR* mutations on ctDNA.⁸⁹ Moreover, the reduction of detectable *EGFR* mutant ctDNA following TKI administration correlated with longer median PFS and OS.^{105,106}

ALK Rearrangements

Detection of an ALK rearrangement can be achieved in a tissue sample by fluorescent in situ hybridization (FISH), immunohistochemistry (IHC), reverse transcription polymerase chain reaction (RT-PCR) or integrated into a multiplex assay using NGS.¹⁰⁷ Regarding the use of liquid biopsy for ALK fusion detection, the results are promising, although the sensitivity is not as high as for EGFR. A PCR-based target sequencing showed low sensitivity, 50% with 100% specificity.¹⁰⁸ Other several recent investigations suggest a potential use of liquid biopsy in the management of *ALK*-positive NSCLC.^{109,110} Two studies demonstrated that the Guardant360 test reliably detects *ALK* fusions and kinase domain mutations in patients with TKI-resistant *ALK* disease.^{110,111} On the other hand, many studies evaluated the evolution of resistance to *ALK*-directed TKIs during treatments. Dagogo-Jack et al¹¹² demonstrated that *ALK* resistance mutations increase with each successive generation of *ALK*-directed TKI and can be effectively detected by liquid biopsy. Considering the increasing number of available *ALK*-directed therapies and the possibility of sequentially administering them, defining the role of liquid biopsy in the dynamic management of these patients is gaining importance.

ROSI Rearrangements

ROSI rearrangements are commonly detected by fluorescent in situ hybridization (FISH) or NGS.¹¹³ Due to incomplete *ROSI* intron coverage, DNA-based NGS panels are more prone to false negatives than RNA-based ones.¹¹⁴ Detecting rearrangements in plasma via liquid biopsy is more complex than identifying short variants such as point mutations and insertions/deletions.⁴⁴ Few studies evaluated the feasibility of plasma genotyping in *ROSI*-positive NSCLC.^{44,115} In a study of Dagogo-Jack et al,¹¹⁶ a plasma molecular analysis of patients with *ROSI*-positive NSCLC was performed. The results obtained from the plasma analysis were compared with those obtained from the investigation conducted on the tissue, to evaluate the potential role of fluid biopsies in managing this subgroup of NSCLC. The results showed that NGS-based plasma genotyping is reliable for identifying *ROSI* fusions and detecting resistance molecular alterations. However, in those cases where no genetic alteration is detected, it is necessary to integrate the analysis with the histological study of the tissues to identify the possible molecular drivers of resistance.

BRAF Mutation

Several studies showed that detectable *BRAF* mutations on plasma of patients with *BRAF* mutated NSCLC correlate with tumor burden and disease dissemination in specific metastatic sites.^{37,117} A study by Sandra Ortiz-Cuaran et al¹¹⁷ analyzed a prospective cohort of 78 *BRAF*-positive NSCLC patients. Detectable *BRAF* mutations were found in 34 of 47 (72%) blood samples of patients naïve to *BRAF*-targeted therapies and correlated with systemic disease. From the results, it would also seem that the study of ctDNA in the early stages of treatment can be used as a predictor of therapy response.^{118,119} With regard to this aspect, ctDNA clearance at the first radiological evaluation during targeted therapy was observed in 63% (12/19) of cases and was associated with longer PFS and OS, while ctDNA positivity resulted to be correlated with worse survival outcomes.¹¹⁷ Serial monitoring of *BRAF*-ctDNA levels during treatment could be a potential marker of tumor response, as well as a surrogate for survival, like what has already been seen in patients with *EGFR* mutant NSCLC.²⁸ However, further studies are needed to evaluate the reliability of the method, especially in the ability to individuate concomitant oncogenic alterations capable of generating primary resistance to anti-*BRAF* therapy.¹⁰⁵

Other Molecular Targets

Since the first IASLC liquid biopsy statement,¹⁰⁶ many studies have been designed to support the extension of ctDNA analysis to other recommended and treatable oncogenic drivers according to international guidelines, including *MET* amplification and exon

14 skipping mutations,¹²⁰ *RET* rearrangements,¹²¹ *HER2* mutations,¹²² and, lastly, exon 2 *KRAS* p.G12C mutation.¹²³ Currently, all these studies are ongoing. Enrollment of patients in clinical trials should be encouraged to further validate the methods.

Conclusion

Considering the wide range of milestones achieved by personalized therapy in many solid tumors, exploiting pioneering molecular-based methods on more accessible tissue such as blood for managing cancer patients represents a practice-changing opportunity in oncology.

As for lung cancer, liquid biopsy has demonstrated to play a key role in both localized and metastatic disease (Figure 2). For early-stage disease, detecting ctDNA and MRD is effective in the early finding of recurrence after loco-regional treatment or as a screening tool. In the metastatic setting, oncogene-addicted NSCLC patients seem to benefit the most. In this subgroup, liquid biopsy is emerging as an integrative analysis of molecular tissue characterization and as a “plasma first” approach for assessing predictive and prognostic biomarkers at the time of diagnosis and during the treatment.

In conclusion, liquid biopsy is an up-and-coming tool in NSCLC management, but further studies are needed before it enters routine evaluation practices.

Author Contributions

All authors made significant contributions to the reported work, whether in conception, study design, execution, data acquisition, analysis and interpretation, or all of these areas; participated in the drafting, review or critical review of the

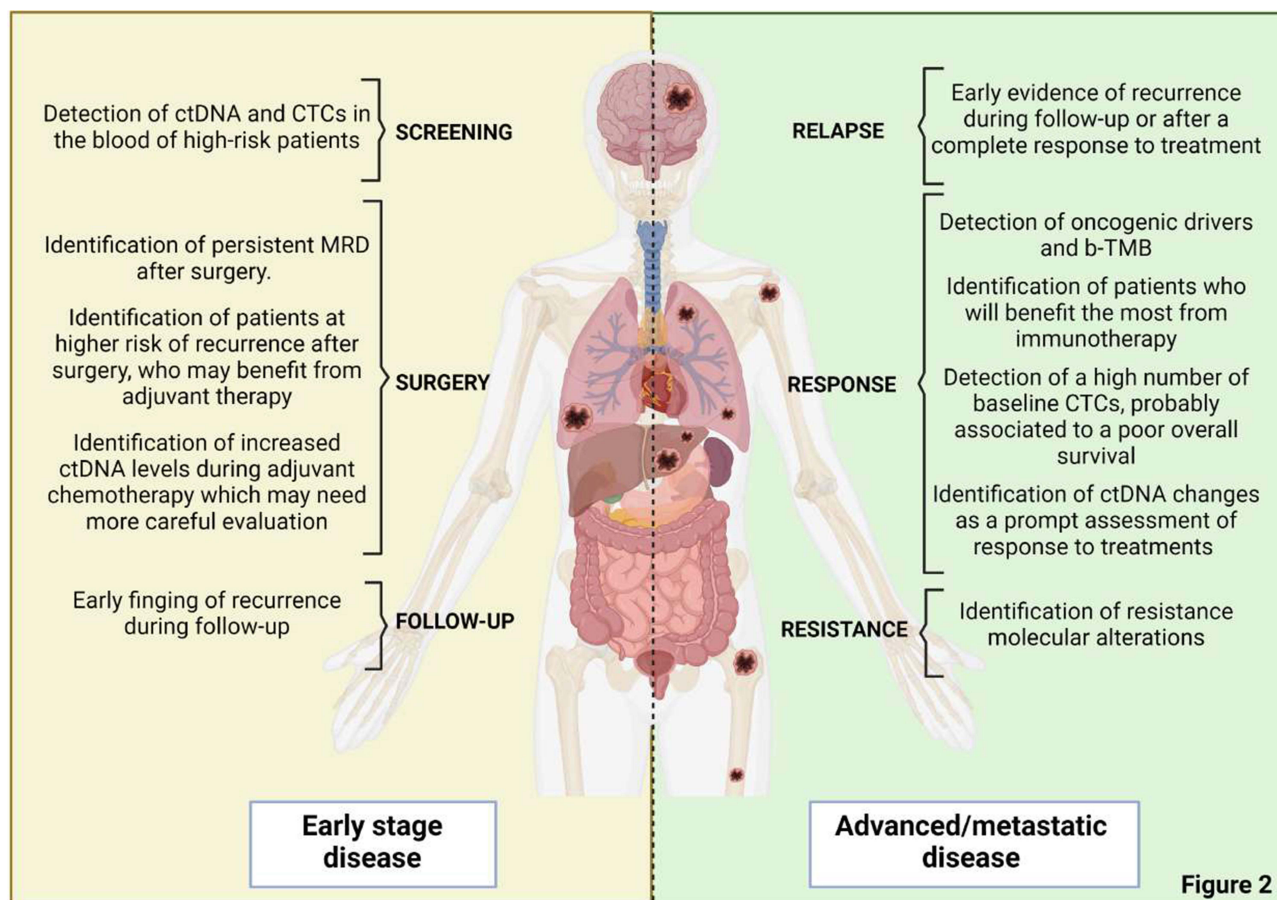


Figure 2

Figure 2 Potential applications of liquid biopsy in both localized and metastatic non-small cell lung cancer.

Abbreviations: b-TMB, blood tumor mutational burden; ctDNA, circulating tumor DNA; MRD, minimal residual disease.

article; gave final approval of the version to be published; agreed on which journal the article was submitted to; and agrees to be responsible for all aspects of the job.

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