Review Article

Circulating free tumor DNA in non-small cell lung cancer (NSCLC): clinical application and future perspectives

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Abstract: Major advances in the treatment of non-small cell lung cancer (NSCLC) patients have been obtained during the last decade. Molecular testing of tumor samples is therefore mandatory in routine clinical practice. Tumor DNA is also present as cell-free molecules in blood, which is therefore a very useful and convenient source of tumor DNA. In this review, we discuss pre-analytical and analytical aspects of circulating tumor DNA (ctDNA) analysis. We also describe the use of ctDNA analysis in routine clinical practice, and discuss the potential use of ctDNA monitoring both to identify minimal residual disease and as a potential tool to early identify patients' response to treatment.

Keywords: Circulating tumor DNA (ctDNA); non-small cell lung cancer (NSCLC); *EGFR* mutation; ALK translocation; tumor mutation burden (TMB); minimal residual disease (MRD)

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Introduction

Over the past decade, molecular characterization of non-small cell lung cancer (NSCLC) has uncovered molecularly defined subsets of tumors (1,2). Somatic molecular alterations in NSCLC can lead to oncogene activation through multiple mechanisms, including point mutations, insertions, deletions and gene rearrangements. For a subset of patient, the treatment of cancer has thus evolved from broad chemotherapeutic approaches to therapies targeted towards some of these specific molecular abnormalities that drive tumor growth. To date, there are a few number of drugs approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for NSCLC presenting specific molecular alterations (*Table 1*). However, an increasing number of agents targeting genetic alterations are being evaluated in clinical trials.

Therefore, in routine clinical practice, robust and accurate assessment of molecular alterations within tumors is mandatory to determine which patients are suitable for these targeted therapeutics. Molecular testing is performed using formalin fixed paraffin embedded (FFPE) tumor tissue obtained by biopsy or surgery. Adequate tumor samples (tissue or cytology) taken in a suitable form are clinically important for a complete pathological diagnosis including tumor typing and sub-typing, and analysis of predictive markers. Molecular testing guidelines and recommendations have been published recently (3,4).

However, it can be challenging to obtain sufficient tumor tissue for molecular testing (particularly when biopsy

Table 1 Specific molecular alterations and approved drugs in Europe (at time of writing)

Gene	Molecular alteration	Approved drugs
EGFR	Activating mutations	Erlotinib, gefitinib, afatinib, osimertinib
	Resistance T790M mutation	Osimertinib
BRAF	V600 mutation	Dabrafenib + trametinib
ALK	Translocation	Crizotinib, alectinib, ceritinib, brigatinib
ROS1	Translocation	Crizotinib

samples are small and/or a very few percentage of tumor cells are present or are prioritized for disease diagnosis). In addition, some invasive biopsy procedures may present with a health risk for some patients. In this context, up to 20–30% of NSCLC patients may be unable to provide a tumor sample suitable for molecular testing at diagnosis (5-7). Similarly, for patients progressing on treatment, a tumor rebiopsy is not always available or may not be of sufficient quality to allow molecular testing (8). Thus, analysis of circulating free tumor-derived DNA (ctDNA) has been proposed as an alternative or a complementary minimally invasive method for the detection of molecular alterations in NSCLC patients.

Cell-free nucleic acids and circulating tumor DNA

The presence of nucleic acids in the circulation was first reported by Mandel and Metais in 1948 (9). Circulating cell-free DNA (cfDNA) is a common constituent of blood samples, present at a very low concentration (5–10 ng/mL) in healthy individual (10,11). This basal level can be increased in inflammation (12), during pregnancy (13), and the concentration of cfDNA was first demonstrated to be increased in cancer patients in 1977 (14).

In NSCLC patients, the baseline concentration of ctDNA is correlated with tumor burden measured by CT scan (15-18), with tumor metabolism as assessed by PET scan (18-21) and to TNM stages (21,22). High concentrations of baseline ctDNA constitute a poor prognostic factor in progression-free survival (PFS) and overall survival (OS), independently of age, stage, nature of the treatment, histological subtype or smoking status (17,19, 22,23). Thus, Yang *et al.* recently proposed to incorporate ctDNA analysis (blood-based liquid biopsy) in a modified TNMB staging system (24).

Circulating tumor DNA is a part of cfDNA coming from tumor cells. The process by which tumor DNA enters the bloodstream is not fully understood (25-27). The length of ctDNA is in the range 180–200 base pairs, suggesting that ctDNA is mainly released by apoptotic cells (28). Circulating tumor cells observed in NSCLC patients are usually in a quite low number, suggesting that these cells are probably not a major source of ctDNA. Moreover, it has been suggested that tumor cells may actively secrete DNA fragments via extracellular vesicles including exosomes (29-31).

CfDNA and ctDNA are also present in other biological fluids allowing, for instance, the detection of *EGFR* mutations in urine (32,33) and in spinal fluid (34-36), but this will not be detailed further in this review which will be focused on plasma-derived ctDNA.

Preanalytical steps

Blood collection and handling are key steps in order to optimize the chance to detect a molecular alteration. Plasma (not serum) should be used for cfDNA mutation analysis, preventing contamination of plasma samples by wild-type DNA released from circulating leukocytes during clotting (11,37). Common anticoagulants such as EDTA and citrate are both suitable for processing of blood samples for cfDNA analysis (38), but EDTA is by far the most used to date. Again, in order to prevent release of normal DNA from blood cells, it is recommended to process blood to plasma within 4 hours of draw (39). Alternatively, use of stabilization collection tubes containing fixatives, such as the Cell-Free DNA BCT tubes (Streck) (40,41) or the cell-free DNA collection tubes (Roche Diagnostics) (42) allow blood processing at a later time, up to 10 days after collection (43).

Plasma is obtained via centrifugation of the blood sample (1,200–2,000 g, 10 min, 25 ℃). A second, high-speed spin must be performed before or after freeze/thaw (3,000–16,000 g, 3 min) in a microcentrifuge to generate clean samples for mutation analysis.

Table 2 Comparison of the most widely used ctDNA testing methodologies

Test	Detection limit	Advantages	Disadvantages
Allele-specific PCR	0.1–1%	Easy to set-up	Detection of small number of alterations per sample
		Short time to result	Lower sensitivity
		FDA approved/CE-IVD kits available	
		Specific equipment not always required	
		Low cost	
Digital PCR	0.01–0.1% (49,50)	High sensitivity	Detection of small number of alterations per sample
		Short time to result	Dedicated equipment necessary
		Low cost of reagents	
		Absolute quantification (copies/mL)	
NGS	0.01–2%	Large coverage	Reagents more expensive
		High sensitivity (if deep sequencing methods are used)	Dedicated equipment necessary
			Longer turn-around time
			Relative quantification (allelic fraction)

NGS, next generation sequencing.

DNA extraction can then be performed using one of the numerous commercially available kits specifically designed to extract cfDNA from plasma.

Technical issues

The improvement in detection techniques has allowed to detect molecular alterations in ctDNA. In theory, all the molecular techniques allowing to detect a mutation can be used. But the fraction of ctDNA can be very low, therefore requiring highly sensitive techniques. Three main approaches are commonly used: allele-specific PCR (e.g., COBAS, Roche Diagnostics; Therascreen, Qiagen), digital PCR (dPCR) [including droplet digital PCR (ddPCR) and Beads, Emulsion, Amplification, and Magnetics (BEAMing)] and next generation sequencing (NGS). Several head-to-head comparisons have been performed (44-46), and detailed reviews have now been published (39,47,48). The main advantages and disadvantages of each technique are summarized in *Table 2*.

The first two approaches have in common that they are designed to detect specific alterations. This is convenient when the number of alterations which could be detected is limited (typically the *EGFR* T790M resistance mutation). But this is a limitation when a significant number of genes/alterations have to be analyzed at once. In such circumstances [*ALK* resistance mutations, Tumor Mutation Burden (TMB), …], NGS approaches are clearly required.

Clinical use of ctDNA testing

The clinical use of ctDNA analysis can be split in two categories:

- Detection of targetable molecular alteration (at diagnosis and/or at progression) is nowadays performed in routine practice. We will address the main issues related to these applications;
- Monitoring ctDNA over time could be useful for monitoring treatment efficiency and relapse in a relatively non-invasive way, but this is not yet used in routine practice. These potential future application of ctDNA testing in clinical practice will be discussed in the last part of this review.

EGFR: from activating mutations to resistance mutations

Many studies reported the detection of *EGFR* activating

Table 3 Advantages and disadvantages of ctDNA testing

Advantages
Comfort of patient
Serial monitoring possible
Lower sensitivity to tumor heterogeneity (66-68)
Reduced time to result (58)
Disadvantages
No access to histology (SCLC transformation)
ctDNA not present in all plasma samples

ctDNA, circulating tumor DNA; SCLC, small cell lung cancer.

mutation in ctDNA of patients with NSCLC. Some of these studies have been included in meta-analyses (51-53). Altogether, these studies indicate that it is feasible to detect *EGFR* mutation in ctDNA, with in most cases a reasonable sensitivity (pooled sensitivity 62–75%) and a good specificity (pooled sensitivity 79–96%).

Prospective clinical trials have allowed to validate these findings with large series of patients (6,54-56). For instance, in the open-label IFUM study of Caucasian patients with *EGFR* mutation-positive NSCLC, a mutation status concordance of 94.3% [sensitivity 65.7%, specificity 99.8%, positive predictive value (PPV) 98.6%, negative predictive value (NPV) 93.8%] was observed between 652 matched tissue/cytology and plasma samples (6).

Finally, the multicentre, non-interventional, diagnostic ASSESS study investigated ctDNA for *EGFR* mutation testing in patients with advanced NSCLC in the real-world setting (57). Overall, the data obtained confirmed that ctDNA is a feasible sample type for real-world *EGFR* mutation testing, if robust and sensitive DNA extraction and mutation analysis methodologies are employed (57).

More sensitive techniques such as dPCR have shown higher sensitivity to detect *EGFR* mutations in NSCLC patients (58). In few cases, *EGFR* mutations detected at low levels in ctDNA were found to be subclonal in the tumor tissue (59). However, these are rare cases because *EGFR* mutations are almost always clonal in NSCLC patients (60).

Altogether, these data confirmed ctDNA as a powerful alternative sample for *EGFR* mutation analysis in patients with advanced NSCLC, particularly when no tissue sample is evaluable or available. In agreement with this conclusion, the EMA approved the use of ctDNA obtained from a blood (plasma) sample for *EGFR* mutation assessment before treatment with gefitinib in 2015. In June 2016, the FDA approved cobas *EGFR* Mutation Test v2 (Roche Molecular Systems, Inc.) using plasma specimens as a companion diagnostic test for the detection of exon 19 deletions or exon 21 mutations in the *EGFR* gene to identify patients with metastatic NSCLC eligible for treatment with erlotinib.

Most NSCLCs with activating *EGFR* alterations respond dramatically to tyrosine kinase inhibitors (TKIs). However, all these patients will ultimately relapse. The most frequent alteration associated with TKI resistance is the *EGFR*-T790M gatekeeper mutation (61-64). Third generation EGFR TKIs have been developed to irreversibly inhibit mutant *EGFR*, including the *EGFR* T790M variant. Osimertinib has shown to be very efficient in T790M-positive patients (65), and was thus first approved in this context. In the clinical setting, it is therefore mandatory to test *EGFR*-mutated patients treated with EGFR inhibitors for the presence of the T790M mutation at relapse. The European approval of osimertinib indicates that this test can be performed using either tumour DNA derived from a tissue sample or ctDNA obtained from a plasma sample. In this context, liquid biopsies using ctDNA have distinct advantages over traditional biopsy methods (listed in *Table 3*). If this mutation is found, the patient can be treated with osimertinib. Indeed *EGFR*-mutant NSCLC patients with T790M mutation detected by ctDNA benefit treatment with osimertinib (69). However, if the ctDNA test is negative, it is advisable to follow-up with a tissue test wherever possible due to the potential for false negative results using a plasma-based test (39,70).

At the molecular level, the T790M mutation is a single nucleotide change (c.2369C>T). In this context, techniques that focus on specific alterations in order to be as sensitive as possible might be adequate for this analysis. dPCR approaches are the most sensitive techniques in this context (44), even if the COBAS assay turned out to be the most robust approach in the first round of external quality assessments performed in France (71).

T790M-positive patients treated with osimertinib finally relapse and acquire new molecular alterations. Novel mutations of the *EGFR* gene associated with resistance to osimertinib including the C797S mutation (72-75) and others (76) have been described. A variety of additional alterations including *KRAS* mutation (74), *BRAF* V600 mutation, *HER2* amplification and *MET* amplification have also detected in tumors and/or ctDNA collected at progression after osimertinib (77-80). When novel treatments targeting these alterations will be available/approved, it will be most convenient to look for all these alterations in ctDNA by using NGS approaches.

Osimertinib has recently been shown to be also very efficient in first line treatment of *EGFR* mutated patients (81,82). At progression, patients do not develop the T790M mutation (80). Therefore, if the use of osimertinib in frontline becomes a standard of care, the T790M testing in ctDNA will not be required anymore.

On the other hand, patients progressing on first line osimertinib have been shown to develop molecular mechanisms of resistance that are similar to those described in second line of treatment. Some treatment naive patients have been enrolled in the AURA trial (80). Plasma samples were collected at progression on osimertinib and several potential resistance mechanisms were reported (80). The *EGFR* C797S mutation was detected in 2 patients. Interestingly, cells lines presenting the C797S mutation (but not the T790M) in addition to an activating mutation were sensitive to first generation (gefitinib) and second generation (afatinib) EGFR TKI inhibitors (83), suggesting that these inhibitors could be efficient as second line treatment in this context. Other alterations including amplification of *MET*, *EGFR* and *KRAS*, mutation of *MEK1*, *KRAS*, *PIK3CA*, *HER2* and *JAK2* have been described in patients progressing on osimertinib treatment (80). Gene fusion have also been reported as mechanisms of resistance to osimertinib (84,85). Recent reports indicated that some of these alterations can be successfully targeted (84,86-88). Therefore, the analysis of ctDNA collected at progression in patients treated with osimertinib front-line will be most useful to guide effective second-line therapy.

ALK: from gene rearrangements to resistance mutation

In routine practice, the detection of *ALK* rearrangements is usually performed by immunohistochemistry and fluorescent in-situ hybridization (FISH) on tissue specimens (89-91). NGS-based gene capture approaches using DNA from FFPE samples have been described (92). Using a similar approach, McCoach *et al.* were able to detect *ALK* fusions in ctDNA (93).

RNA-based strategies (RT-PCR and RNA sequencing) have also been demonstrated to detect ALK rearrangements in tissue samples with a high sensitivity and specificity, and allowing to identify *ALK* fusion variants (94-97). RT-PCR has also been used to detect fusion transcripts in circulating RNA (98,99), but these approaches are not yet used in routine clinical practice.

Secondary *ALK* mutations that confer acquired resistance to crizotinib have been described (100,101). These alterations do not appear to prevent efficacy of second generation ALK inhibitors such as ceritinib (102). Other acquired mutations have been described upon treatment with second line ALK inhibitors (103). These mutations can be detected both in tumor tissues and in ctDNA (101). Since many different mutations have been described, NGS approaches are the most appropriate techniques in this context. However, there is currently insufficient evidence to support the use of testing *ALK* mutational status for lung adenocarcinoma patients with sensitizing *ALK* translocation who have progressed after treatment with an ALK-targeted TKI (3).

BRAF gene

The *BRAF* V600E mutation is frequent in metastatic melanoma, and a number of studies reported the clinical use of ctDNA testing in this context (10,104-106). This mutation is much less frequent in NSCLC (approximately 1% of cases). Recent clinical trials have demonstrated a significant effect of the combination of a BRAF inhibitor combined to a MEK inhibitor (107). Several reports demonstrated a clinical use of ctDNA equivalent to that described for *EGFR* (108,109).

Mutation load and other genetic/epigenetic alterations

Several clinical trials recently demonstrated that a high TMB is associated with improved efficacy of PD-1/PD-L1 inhibitors (110,111). Interestingly, Gandara and colleagues have recently demonstrated that it is possible to use ctDNA to determine TMB (112). They used plasma samples collected in two large randomized trials (OAK and POLAR), evaluating atezolizumab (anti-PD-L1) in second-line and higher. The TMB score was determined by identifying all base substitutions present at an allele frequency of ≥0.5% across the coding region of 394 genes (1.1 Mb). They demonstrated a relationship between clinical outcomes and the ctDNA TMB (112). Further studies are necessary to establish the conditions of using this test in routine clinical practice, in particular the cut off value that should be used.

Many different genetic or epigenetic alterations, such as of gene methylation (113-115) or detection of microsatellite alterations (116,117) can be detected using ctDNA. But detection of these modifications has no clinical application at present.

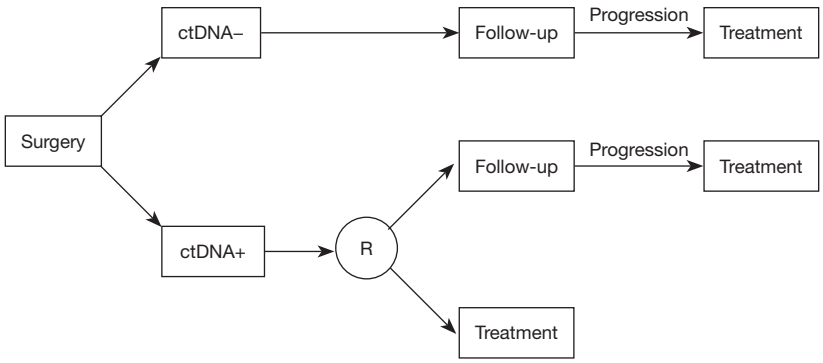

Figure 1 Design of a clinical trial investigating the benefit of ctDNA testing in the adjuvant setting. Following surgery, patients will be tested on plasma. If the test is negative, the patients will be followed until progression, and then treated with adjuvant therapy. If the initial plasma test is positive, patients will be randomized. In the control arm, the patients will be followed until progression, and then treated. In the experimental arm, the patients will be treated with adjuvant therapy directly. ctDNA, circulating tumor DNA.

ctDNA and minimal residual disease (MRD)

At early and locally advanced stages, the reference treatment of NSCLC remains surgical resection, combined with adjuvant chemotherapy or chemoradiotherapy. Despite therapeutic developments in this area, cancer recurrence remains the leading cause of postoperative NSCLC mortality, with significant relapse rates, while the OS benefit of adjuvant chemotherapy remains low (118). In a meta-analysis of five clinical trials of 4,584 patients, Pignon *et al.* showed that adjuvant chemotherapy had a deleterious effect on OS in patients with stage IA, and possibly stage IB NSCLC, while the recurrence rate was up to 36% in these patients (119). This issue has generated a great interest in the development of markers predictive of postoperative recurrence, to strengthen adjuvant therapies and postoperative follow-up in patients at risk of recurrence, while limiting exposure to cytotoxic agents and ionizing radiations in low-risk patients.

As the detection of ctDNA in plasma indicates the presence of residual tumor tissue, it has been proposed to use this biomarker to assess the MRD after surgical resection in locally operable advanced NSCLCs. Chaudhuri *et al.* showed that post-surgical detection of ctDNA was associated in 100% of cases (20/20 patients) with recurrence of the disease (21). In addition, detection of ctDNA preceded radiological detection of progression in 72% of patients, with a median of 5.2 months. The detectability of ctDNA in this study was an independent prognostic factor: patients with detectable ctDNA on a sample collected less than 4 months after surgery had a very significantly lower PFS and OS than those for whom ctDNA was undetectable (36-month PFS =0% *vs.* 93%, respectively). Nevertheless, at the end of the study, 6% of patients with undetectable ctDNA also had a recurrence. Similar results have been observed in other studies with NSCLC patients (6,14,15,18,120,121).

While the use of ctDNA for post-surgical MRD evaluation of NSCLC appears promising, the interpretation of an undetectable ctDNA remains difficult because of the limited detection sensitivity of molecular biology tests, particularly after the surgical reduction of the tumor burden. Prospective studies are required to determine whether the use of adjuvant therapy based on the detection of ctDNA would improve clinical outcomes. Following surgery, patients will be tested on plasma. If the test is negative, the patients will be followed until progression, and then treated with adjuvant therapy. If the initial plasma test is positive, patients will be randomized. In the control arm, the patients will be followed until progression, and then treated, as patients with a negative plasma test. In the experimental arm, the patients will be directly treated with adjuvant therapy (*Figure 1*).

ctDNA monitoring and early assessment of response

ctDNA testing is also an appealing way of monitoring the activity of systemic treatments in the metastatic stage. Numerous studies evaluated the correlation between therapeutic response and longitudinal quantitative changes in plasma ctDNA. The first ctDNA monitoring applications for therapeutic follow-up implicated cohorts

of patients treated with EGFR TKI for advanced NSCLC. The existence of a mutation of the *EGFR* gene, common to all these patients, allowed quantification of ctDNA by quantitative targeted techniques, such as dPCR.

For instance, Mok *et al.* showed that in 66 patients treated with erlotinib plus gemcitabine for an *EGFR*-mutated NSCLC and whose activating mutation was detectable at baseline, the response rate of patients whose ctDNA had become undetectable at 12 weeks was greater to that of patients whose ctDNA remained detectable at 12 weeks (83% *vs.* 67%) (122). The undetectability of ctDNA at 12 weeks was also associated with a significant benefit in PFS and OS [hazard ratio (HR) =0.38, P=0.0083 and HR =0.38, P=0.0831, respectively] (122).

More recently, Taus *et al.* observed changes in ctDNA in patients treated with TKI or chemotherapy for advanced *EGFR*-mutated NSCLC, whose EGFR-activating mutation was detectable in dPCR at baseline (123). A decrease in ctDNA was observed in 13 of 14 evaluable cases (93%), 38 days before the radiological response in median (20 to 69 days). Conversely, in 17 of 19 evaluable cases (89%), an increase in ctDNA was found 80 days in median (12 to 292 days) before radiological progression assessed by CT scan. Patients whose circulating *EGFR* mutation became undetectable during follow-up had a significantly better PFS than those for whom ctDNA was still detectable (median: 295 *vs.* 55 days, respectively).

The recent development of immune checkpoint immunotherapies has generated a particular interest in early identification of therapeutic response by longitudinal analysis of ctDNA concentrations. Indeed, the response to immunotherapies may be difficult to assess on imaging, because it can be characterized by an increase in the apparent tumor volume or the appearance of new lesions, due to the leukocyte infiltration of the tumor that it induces. Despite the development of radiological criteria adapted to immunotherapies, the identification of non-responders remains late (124). One of the main difficulties associated with ctDNA monitoring in these patients is that they do not carry consensual genetic alteration: for this reason, most of the studies evaluating the ctDNA interest in this application have quantified ctDNA in NGS, by measuring the variant allelic frequency (VAF) of mutations identified by screening of a large panel of genes.

Giroux Leprieur *et al.* recently demonstrated, on a cohort of 15 patients treated with nivolumab and with detectable somatic alteration at baseline in NGS, that the absence of a significant increase in VAF at 2 months (defined as an increase of more than 9 % relative to baseline) predicted a disease control of at least 6 months with sensitivity of 71% and specificity of 100% (125). Moreover, the absence of ctDNA increase was associated with a significantly higher PFS and OS (median: 0.7 *vs.* 12.0 and 2.1 months *vs.* not-reached, respectively) (125).

In another study, Goldberg *et al.* defined a "ctDNA response" as a decrease in VAF greater than 50% of the baseline, and confirmed on a second sample (126). This study, carried out on 28 patients treated by anti-PD-1 or anti-PD-L1 immunotherapy and carrying a somatic detectable alteration at baseline in NGS, showed that patients presenting a "ctDNA response" during follow-up presented a longer duration of treatment, PFS and OS than those who did not (median: 206 *vs.* 69 days; HR =0.17, 95% CI: 0.05–1.02 and HR =0.13, 95% CI: 0.03–0.51, respectively). On this cohort, the "ctDNA response" was obtained 42.5 days before the radiological confirmation of the response on CT scan, in median (126).

Finally, Raja *et al.* conducted an NGS analysis of 28 durvalumab-treated patients with somatic detectable alteration at baseline (127). The changes in the VAF of this mutation were correlated with the therapeutic response, with a mean decrease of 2.7% in responder patients compared to a mean increase of 1.7% in non-responder patients. The authors also showed that a decrease in VAF at 6 weeks of treatment was associated with a benefit in PFS and OS (median: 1.45 *vs.* 13.7 months and 9.07 *vs.* 28.13 months). The decrease in ctDNA preceded from 1 to 12 months the radiological confirmation of the therapeutic response in 70% of patients (127).

Some studies have also demonstrated the existence of a peak concentration of ctDNA very early after the start of treatment, probably related to the massive release of tumor DNA by treatment-induced cell lysis (128,129). Whether this very early increase of ctDNA is associated with a better outcome of patients remains to be determine in a large prospective study.

Overall, all the studies evaluating the relevance of kinetic analysis of ctDNA for predicting response of NSCLC to systemic therapies tend to show that the reduction of ctDNA at the beginning of treatment makes it possible to predict the therapeutic response earlier than imaging and is associated with a more favorable prognosis in PFS and OS. A significant increase in ctDNA concentration during follow-up also seems to predict disease progression earlier than radiological monitoring. These data have not yet been applied in the management of patients in clinical practice.

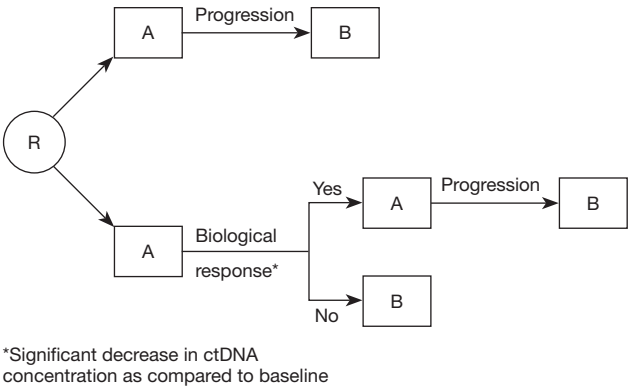

Figure 2 Design of a clinical trial investigating the potential of ctDNA monitoring to guide therapy in the metastatic setting. In the reference arm, patients will be treated with A, then with B following radiological or clinical progression. In the experimental arm, early ctDNA analysis (after for instance 2 or 3 weeks of treatment), will allow to identify a change in ctDNA concentration as compared to the pre-treatment assay performed. In case of significant decrease, suggesting that the patient is responding, treatment A will be continued until radiological or clinical progression. If there is no decrease in ctDNA concentration, suggesting that the patient is not responding to treatment A, the treatment will be changed to B. ctDNA, circulating tumor DNA.

Thus, clinical trials are required. A design for such trials is presented on *Figure 2*. In the reference arm, patients will be treated with A, then with B following radiological or clinical progression. In the experimental arm, early ctDNA analysis (after for instance 2 or 3 weeks of treatment), will allow to identify a change in ctDNA concentration as compared to the pre-treatment assay performed. In case of significant decrease, suggesting that the patient is responding, treatment A will be continued until radiological or clinical progression. If there is no decrease in ctDNA concentration, suggesting that the patient is not responding to treatment A, the treatment will be changed to B.

Such use of ctDNA kinetics will be limited by the need to identify a molecular alteration in the patients' tumor, which is not always the case, even using broad NGS approaches. Furthermore, some patients have no ctDNA detectable at baseline.

It might be also necessary to use techniques allowing an absolute quantification of ctDNA. NGS allows only a relative quantification of the mutated copies, as compared to the wild-type alleles. But multiple physiopathological or preanalytical factors are likely to induce an increased release of non-tumor DNA into the plasma, including inflammation, stimulation of antitumor immunity, lysis of the healthy parenchyma during tumor progression, and *in vitro* leukocyte lysis related to a delay in sample processing or a traumatic puncture (130). dPCR allows an absolute quantification of the mutated copies concentration in the sample, independently of the "background noise" induced by the presence of non-tumor DNA. However, it remains a targeted technique since each test allows the quantification of only a limited number of previously determined mutations on the tumor tissue. Demuth *et al.* recently proposed a test protocol combining a broad screening of ctDNA at baseline by NGS to identify one or more somatic mutations, and an absolute quantification of these mutations on circulating DNA during follow-up, by custom dPCR analyzes for each patient according to the alterations found in NGS (131).

Finally, the lack of consensual evaluation criteria for longitudinal variations of ctDNA is one of the major limitations to the use of this biomarker. Indeed, many studies base their results on the notion of detectability of somatic mutations on circulating DNA. However, this notion is particularly dependent on the pre-analytical and analytical processes used: in the absence of a common methodology, the results of these different studies can therefore hardly be compared and reproduced. For the analysis of quantitative variations of ctDNA during follow-up the accuracy of the method used should be considered, and it depends on the number of mutated copies in the sample (for dPCR) (132) or on the allelic frequency (for NGS) (133). In a recent study on the monitoring of metastatic cutaneous melanoma treated with anti-PD-1 immunotherapy, we recently proposed interpretation criteria based on the point-to-point statistical comparison of ctDNA concentrations measured during follow-up. We defined the biological response (bR) as a significant decrease in ctDNA compared to the baseline measurement, given the inaccuracy of the two measurements. Biological progression (bP) was defined as a significant increase in ctDNA compared to the nadir measurement. In an evaluation of 22 patients, bP was predictive of progression on average 79 days before radiological progression, with 100% sensitivity and 100% specificity, while bR predicted the therapeutic response to average 115 days before the objective radiological response with a sensitivity of 100% and a specificity of 50%. We also showed that the absence of bR at the 2nd week of treatment, in 10/22 patients, was associated with a lack of clinical benefit, with a 0% PFS

rate at 4 months (105). The development and evaluation of similar criteria incorporating the inaccuracy of the ctDNA measurement for the interpretation of its variations could improve the predictive value of this biomarker for the therapeutic monitoring of NSCLC.

Conclusions

ctDNA is already used in routine practice for the detection of molecular alterations, allowing to guide therapy. When new drugs targeting other genes will be approved, it will be easy to use this source of tumor DNA to set up these new assays.

More work is required to determine whether a therapeutic strategy guided by ctDNA analysis can improve PFS, OS and patients' quality of life as compared to radiological/clinical monitoring.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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