# Liquid Biopsy in Non-Small Cell Lung Cancer (NSCLC)

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## Current Status of Lung Cancer

Lung cancer is the leading cause of cancer deaths worldwide [1], being 85% of those non-small cell lung cancer (NSCLC). The last data published by Cancer Research UK reported the 1-year overall survival rate of 32% for lung cancer patients, while the 5-year survival rate is around 10%. Besides the development of new effective therapies, lung cancer is still today a disease difficult to control.

The advent of targeted agents represents the most important innovation in the treatment of lung cancer over the last years. The discovery of epidermal growth factor receptor (EGFR)-activating mutations in 2004 as oncogene driver in a subgroup of patients with NSCLC led to the development of a new family of biological agents, called EGFR-TKIs, which were able to selectively bind and inhibit the EGFR molecular pathway. About eight phase III randomized clinical trials compared EGFR-TKI gefitinib, erlotinib, or afatinib vs platinum-based chemotherapy as first-line treatment for EGFR-mutated NSCLC patients, all showing a significant survival benefit in favor of EGFR-TKIs. These drugs have revolutionized the clinical management of about 40% Asian and 12% Caucasian NSCLC patients harboring EGFR mutations, whose survival outcomes nearly doubled compared to standard chemotherapy. Later the discovery of the EML4-ALK fusion gene in about 3–8% of patients with NSCLC and the subsequent clinical development of crizotinib represented an amazing success story leading to the recent approval of this compound as new standard first-line treatment in this

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subgroup of patients [2]. Nevertheless in both cases, despite an initial impressive benefit, patients inevitably experience tumor progression, because the tumor can generate resistance to these treatments through genetic modifications like mutations or amplifications. To avoid this problem, pharmaceutical industries are developing new drugs that are able to overcome resistance mechanisms. New generations of EGFR and ALK inhibitors have been recently investigated in randomized clinical studies, showing a great efficacy and tolerability in patients who failed prior TKIs. Particularly osimertinib is the third-generation EGFR-TKI in most advanced stage of clinical development which is active against both EGFR-sensitizing and EGFR-resistant T790M mutation. The phase III AURA 3 study has recently shown a significant survival benefit in favor of osimertinib over platinum chemotherapy in NSCLC patients who progressed to prior EGFR-TKI and were T790M positive [3]. Similarly the new-generation ALK inhibitors alectinib and ceritinib also demonstrated a significant superiority over platinum chemotherapy in ALK-rearranged patients who failed prior therapy with crizotinib [4]. However, there are already some data showing that resistance mechanisms can occur also for these new-generation drugs [5, 6]. In this scenario biomarker investigations have become one of the most interesting and studied fields of translational lung cancer research with the aim to estimate patients' prognosis, to monitor treatment response and to eventually predict both treatment efficacy and tumor recurrence [7, 8].

The genetic analysis of both EGFR mutations and EML4-ALK translocation is a crucial step at the time of diagnosis, in order to plan the optimal treatment strategy for each patient. Furthermore, the analysis of EGFR mutations has acquired a growing importance also in the follow-up of TKI-treated patients. In fact, almost in nearly 60% of TKI-treated patients, the treatment efficacy fails due to resistance mechanisms. The most common cause of TKI failure depends on the onset of secondary mutations; the exon 20 T790M is the most characterized resistance mutation in EGFR [9].

Therefore EGFR mutational status should be monitored during treatment and mostly at relapse to choose the proper subsequent therapy. To date, the gold standard for the molecular analysis of a patient affected by NSCLC is the tissue biopsy.

Even if there is a big consensus about the use of tissue biopsy as a primary source of genetic information, we still have to face the situation when "the tissue becomes the issue". This may happen when a strict "molecular follow-up" is mandatory to evaluate patient's disease evolution. To solve this problem, liquid biopsy has raised as the "new ambrosia of researchers" as it could help clinicians to identify both prognostic and predictive biomarkers in a more accessible way [10].

## The Importance of Liquid Biopsy in NSCLC

One of the new hallmarks of cancer is the "genome instability and mutation" [11]. In lung cancer, it becomes a very relevant issue because of the high heterogeneity of this tumor. Lung cancer is characterized by different driver molecular alterations, with EGFR mutations, ALK-EML4 translocations, and RAS mutation being the most common among others [12, 13]. The new targeted therapies against these driver mutations have nearly doubled patients' survival [14, 15]. However, due to the genomic instability of cancer and its peculiar ability to adapt to the tumor microenvironment, cancer cells usually develop resistance mechanisms such as the EGFR-T790M mutation or the L1196M mutation during first-generation EGFR-TKIs and crizotinib treatment, respectively [16, 17]. Recent evidences showed that the tumor molecular alterations may not be homogeneously distributed within the same lesion and, what is most relevant, the metastasis can present a completely different molecular profile as compared to the primary tumor [18, 19].

Therefore, the molecular analysis of the tumor and/or of the metastatic lesions is becoming more and more requested at the time of PD. Unfortunately, tissue biopsy is a procedure

often limited by several features, including its invasiveness, the not easy access to different tumor sites, the high intra-tumor heterogeneity, and not ultimately the low patients' compliance [20]. Thus, in the last decade, many new noninvasive approaches have been studied to overcome the aforementioned issues. Among these, liquid biopsy represents a valuable alternative for the detection of EGFR mutational status once it cannot be performed on tissue samples according to international guidelines. Furthermore a liquid biopsy can be easily repeated at different time points allowing to follow the tumor molecular status during the treatment course [21]. This could help clinicians to predict disease progression over time, to identify new acquired molecular alterations, and to observe how all these characteristics correspond to patient's status.

## Liquid Biopsy in Non-small Cell Lung Cancer

As we are describing in this book, there are many definitions of "liquid biopsy". The definition is complex since different body fluids as urine, ascites, saliva, cerebrospinal liquid or plasma can be considered as valuable sources of tumor components.

In this chapter, we are going to focus our attention on the main published studies investigating circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and exosomes and other extracellular vesicles (EVs) in lung cancer. The last paragraph will be destined to describe the uncommon components of the liquid biopsy such as platelets.

## Circulating Tumor Cells (CTCs)

The circulating tumor cells are shed from both primary and metastatic tumor; thus they are representative of the tumor from which they detached. It is known that lung cancer releases a limited number of CTCs, and therefore they were not so far considered a good field of study. Nevertheless, limitations of CTCs detection in lung cancer were mainly due to the limited available isolation methods. Thanks to the increase of knowledge about CTCs' biological and physical characteristics, detection and isolation methods have been consequently improved. Nowadays, CTCs may become a promising field of study also in lung cancer [22].

CTCs can be used for two different aims: to evaluate the risk of metastasis and as a source of nucleic acid for molecular characterization. Indeed, CTCs are shed to the bloodstream and can play an important role in the metastatic process. Moreover, since CTCs spread directly from the tumor, they might harbor the same mutational landscape that can be investigated through molecular analysis.

The studies on CTCs in lung cancer have shown heterogeneous results, mainly due to the different techniques and criteria used for the experiments. Tanaka et al. demonstrated that the number of CTCs is higher in patients with lung cancer than in those with benign disease, and the number of CTCs is significantly increased in patients with distant metastasis than in the primary ones. In the same study the authors demonstrated a significant correlation between the number of CTCs in the bloodstream and the stage of the disease [23], but other studies have not showed the same results [24, 25]. The number of CTCs can be also a good marker of tumor growth and prognosis. Krebs et al. demonstrated that patients with five or more CTCs in 7.5 mL of total blood, after one cycle of chemotherapy, have a worse prognosis as compared to those with a lower number [26].

The molecular characterization of CTCs is technically challenging mainly because of the limited performance of isolation and detection methods. Moreover, the amount of extracted nucleic acids is always very poor, limiting the downstream applications. Indeed, new highly sensitive techniques, such as next-generation sequencing (NGS), are now available and offer the possibility to analyze the molecular alteration of CTCs in a relatively simple way.

The detection of EGFR-activating mutations in CTCs has revealed contradictory results. Maheswaran et al. first published in 2008 an

article describing the identification of EGFR mutations in CTCs, providing exciting results. They analyzed EGFR mutations in both CTCs and ctDNA using a SARMS assay in patients already tested positive in tissue samples. Mutations in CTCs were detected in 19 out of 20 patients with 95% sensitivity; they also detected T790M mutation in 2 out of 6 responding patients and in 9 out of 14 progressive patients. Moreover, in four patients they reported that levels of activating and resistance mutation (exon 19 deletion and exon 20 T790M, respectively) floated according to disease status [27]. However, a study carried by Punnoose et al. showed disparate results. In this paper the authors analyzed the EGFR expression through FISH showing very heterogeneous results. Indeed, they revealed CTCs with very strong signal (3+), others with very low (0), and other with intra-heterogenic results ranging from 3+ to 0, and this expression was not correlated with the EGFR status on tissue. Moreover, when the DNA from CTCs was analyzed to detect EGFR mutations, only one out of eight EGFR-mutated patients was detected [28].

Besides EGFR mutations, it has been proposed that ALK-EML4 translocations are detectable in CTCs using immunohistochemistry and FISH. The results reported in literature showed a high correlation between ALK-EML4 detection in tissue and in CTCs even if the cutoff value was different among the studies due the various techniques used for CTCs isolation [29–31]. Moreover, a study performed by He et al. investigated a new technique for CTC isolation comparing the results with the FDA-approved methods, the CellSearch system. They demonstrated a correlation between the ratio ALK-EML4 rearrangement signal/CTCs and TNM stage, and similarly to other studies, the count of CTCs was related to the disease status [32]. Therefore, CTCs can be useful for disease follow-up as they offer the opportunity to evaluate both EGFR and ALK-EML4 alterations, as a surrogate biomarker for treatment response and to promptly identify resistance mutations responsible for treatment failure.

The CTC study has risen with the implementation of new isolation techniques that allow more reliability and the improvement of the molecular analysis techniques such as one-cell genotyping. However, a standardization of the techniques is needed and a big consensus on how the samples must be analyzed is fully required to make the CTC analysis truth.

## Circulating Tumor DNA (ctDNA)

The investigation of ctDNA can hypothetically reveal a wider genomic landscape of a tumor [33]. For this purpose, new sensitive technical approaches are available to analyze EGFR mutational status from plasma-derived ctDNA. In particular, digital PCR (dPCR) and next-generation sequencing (NGS) platforms represent to date the most studied approaches for application in clinical practice. Through the dPCR approach, the DNA sample is partitioned into thousands of single PCR reactions. As in the qPCR approach, analysis software allows to identify a positive or a negative signal indicating the presence or absence of a target sequence. Therefore, a mutated ctDNA can be detected in a wide background of wild-type sequences [34]. The introduction of NGS technologies in clinical practice is the most important revolution that we have experienced since the discovery of polymerase chain reaction (PCR) and Sanger sequencing. Until now we have been working analyzing *one gene at a time* and *one patient at a time*, with NGS techniques this assertion has been revolutionized and we can analyze *multiple genes and multiple patients at a time* with a consistent reduction in time and costs [35]. NGS is a high-throughput technique, based on massive parallel sequencing of thousands of DNA molecules [36]. There are several NGS platforms that differ mainly in the detection chemistry, but they all share some important steps: library preparation, library amplification, sequencing, and data analysis. At the end of the analysis they all provide a plethora of information about the mutational landscape of the analyzed samples that can be used in clinical practice. Another great advantage of NGS compared to Sanger sequencing is the higher sensitivity, which is important when we

have to look for somatic and rare mutations. This is the case of liquid biopsy and specifically of circulating tumor DNA (ctDNA) analysis. The information arising from ctDNA analysis will broad from early diagnosis to prognosis as well as response to drug administration and real-time monitoring of the disease.

## Diagnostic Role of ctDNA

To date, several studies and meta-analysis deeply highlighted the diagnostic value of plasma-based EGFR testing in NSCLC patients, showing an interesting accuracy of ctDNA in terms of sensitivity and specificity if compared with the gold standard tissue genotyping [37–40]. Therefore, the isolation of ctDNA from plasma or serum would be helpful for EGFR testing in all those patients whose tissue is not available at diagnosis or tissue analysis results are inconclusive. Sacher et al. have recently evaluated the reliability of plasma analysis. This study demonstrated a high specificity (100%) and sensitivity (74–82%) in 80 patients with advanced NSCLC harboring activating EGFR del19/L858R mutations [41] using droplet digital PCR (ddPCR). The same promising results at diagnosis have been also showed within the multicenter ASSESS study in which a similar concordance rate of 89% (sensitivity 46%, specificity 97%) has been found in a cohort of 1162 patients with advanced NSCLC [42]. Furthermore, despite real-time PCR and ddPCR techniques are definitely the most used for ctDNA analysis, NGS is emerging as an important tool that can complement or substitute tissue NGS analysis. Indeed, there are several commercially available NGS panels specifically designed for ctDNA testing in lung cancer. Recently Villaflor et al. assessed the utility of two ctDNA panels in a clinical series of 68 NSCLC patients; the 54-gene panel includes only mutations, whereas the 68-gene panel includes also *ALK, RET*, or *ROS1* fusions [43]. In this paper, it was also investigated the concordance between paired tissue and blood samples whenever possible. The results reported that 80% of patients have detectable ctDNA, with 83% presenting at least one non-synonymous ctDNA alteration. As expected the most frequent mutations were reported in TP53, KRAS, and EGFR genes [43]. Another recent paper published on December 2016 supports these evidences. NGS was used to characterize 112 plasma samples from 102 patients with advanced NSCLC, detecting 275 alterations in 45 genes in 84% of patients (86 of 102). As well as reported in the paper from Villaflor [43], NGS was able to detect mutations in additional genes for which experimental therapies, including clinical trials, were available. The concordance between tissue and plasma was 79%, and interestingly the concordance increases when a shorter time interval between tissue and blood collection was reported [44]. Moreover, ctDNA sequencing enabled the detection of resistance mutation in eight patients who experienced progressive disease during targeted therapy and for whom tissue analysis was not possible. Finally, Chen et al. prospectively evaluated the detection of ctDNA mutations in early-stage NSCLC patients (IA, IB, and IIA) by targeted sequencing in plasma and paired tissue samples. They found a considerable ctDNA concentration in 52 out of 58 patients, suggesting that ctDNA might be related to tumor cancer spread. Furthermore, plasma ctDNA mutations were identified in 35 out of 58 patient samples, with 50% concordance between plasma and tissue [45]. These results suggest that ctDNA analysis may also be applied in early-stage disease.

## Prognostic Role of ctDNA

The prognostic role of ctDNA has been deeply investigated. In 2014 the group of Wang et al. tested the ability of dPCR to identify T790M in plasma ctDNA compared to a non-digital approach (ARMS). They showed a statistical correlation between survival and allele fraction of circulating T790M before and after EGFR-TKI administration. Patients with increasing levels of circulating T790M during EGFR-TKI treatment showed better progression-free survival (PFS) and overall survival (OS) if compared with patients who do not display any significant

T790M variation [46]. Furthermore, in 2016 the same research group confirmed that patients with circulating T790M had a better clinical outcome compared to plasma T790M-negative patients [47]. Recently, Thompson et al. correlated survival with ctDNA levels and number of variants using NGS in plasma specimens of metastatic NSCLC patients. The high levels of ctDNA (>3 ng/mL), irrespective of mutational profile, were associated with decreased survival. Conversely, patients with ctDNA levels lower than 3 ng/mL showed a better median survival (24 months vs 46 months, respectively). Furthermore, OS seems to be strictly correlated with number of variants detected in plasma. Indeed, a number of variants greater than 3 determined an OS reduction from 62 to 46 months, giving thus a poorer prognosis [44]. Therefore, it seems that mutational load itself may be a good prognostic marker.

## ctDNA Value in Real-Time Monitoring of the Disease

The translation in clinical practice of liquid biopsies is strictly requested in all those cases in which a disease progression monitoring is needed (Fig. 12.1). Indeed, on November 2015 the FDA-approved osimertinib as new treatment option for patients with metastatic EGFR T790M-positive NSCLC patients who failed prior EGFR-TKIs [48, 49]. Patients' selection is strictly based on the identification of T790M mutation, and for the first time the molecular analysis can be performed either through tissue re-biopsy or in plasma samples [50, 51]. The noninvasive potential of ctDNA has been deeply studied by Oxnard in many studies specifically focused on the molecular biology of NSCLC. In 2014, one of the first studies performed by its group highlighted the possibility to anticipate clinical evidence of progression through early molecular evidences. Indeed, the analysis of ctDNA through ddPCR, in serial plasma sampling, allowed the detection of resistance mutations (T790M) weeks and sometimes months prior to radiological progression [52].

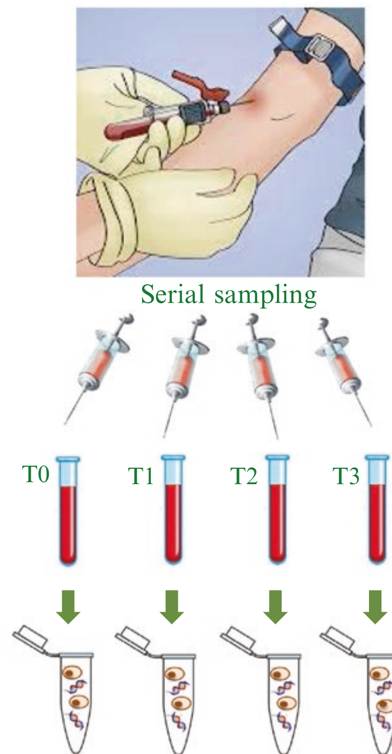


**Fig. 12.1** Serial monitoring of NSCLC patients during treatment. Serial blood withdrawal can be obtained at different time points (T0, T1, T2 and T3) to detect CTCs, ctDNA, and exosomes; the dynamic changes of these different components of liquid biopsy may be useful for clinical management of lung cancer patients

Recently his group prospectively evaluated the sensitivity and specificity of plasma genotyping by ddPCR in 180 patients with advanced NSCLC, including 60 patients with acquired resistance to EGFR-TKI. Plasma genotyping by ddPCR exhibited 79% specificity and 77% sensitivity in the detection of T790M mutation, which are lower than those observed with EGFR-activating mutations at baseline. In addition Oxnard et al. showed that outcomes of T790M-positive patients included in the phase I AURA study were similar if T790M was detected in plasma or tumor tissue. Conversely both RR and PFS of T790M-negative patients on plasma were significantly higher than T790M-negative on tissue, and further tumor genotyping of plasma T790M-negative patients allowed to identify a subgroup of T790M-positive patients on tumor

tissues who had better outcomes. According to these data, the authors suggest that plasma genotyping could represent the first step for the detection of T790M status at the time of PD. However, because of the low sensitivity (70%) of the current available technologies which are associated with a 30% false negative rate, patients with T790M-negative on plasma should repeat tumor tissue biopsy to further investigate the presence of such molecular alteration [53]. Clinical utility of ctDNA testing through NGS could be also proven in treatment monitoring, for the evaluation of tumor clone response to target treatment administration. Indeed, NGS analysis, as well as dPCR, provides also data concerning the mutations allele fraction. Therefore, it is possible to trace allele fraction modifications over time during a given targeted treatment and correlate these data with treatment response but also to predict relapse and disease progression [54]. In support of the high tumor heterogeneity, CAPP-Seq ctDNA studies performed by Chabon et al. on 41 patients harboring both EGFR-activating and EGFR-resistant T790M mutations on tumor tissue after progression to prior EGFR-TKI therapy revealed additional molecular alterations, including MET alteration or HER2 increased gene copy number (GCN) and/or single nucleotide variations [55]. Since the simultaneous presence of such a plethora of different molecular alterations has been associated with poorer outcomes to TKI therapies, ctDNA analysis could represent a valuable option in guiding clinicians in the choice of the proper treatment strategy. Notably it has been recently developed a novel targeted NGS approach for the detection of both driver mutations and rearrangements in ctDNA from advanced NSCLC patients [56]. This approach relies on the use of specific intronic probes that enable the detection of genome-level rearrangements that create chimeric gene fusions in ALK, ROS1, and RET. The assay and analysis software was able to identify mutation present at 0.1% even if the diagnostic performance was better, reaching 100% sensitivity and specificity, when mutations were present at an allelic frequency 0.4% or greater [56]. In addition to plasma, urine genotyping has also shown a high sensitivity in detecting T790M mutation status, ranging from 72% to 93%, in preliminary studies including few patients and is currently under investigation in trials including larger cohorts of patients [57].

## Exosomes

The interest of the scientific community on the role of exosomes in NSCLC is growing, and as it happens with CTCs, exosomes are nowadays a pending subject to understand. Despite the misunderstanding of the exact exosome composition and function, this is becoming one of the most interesting fields of study in liquid biopsy. As aforementioned, exosomes contain a wide variety of material like miRNAs, proteins, and finally, messenger RNA that are surrounded by a lipid bilayer that confers stability. The exosomes differ from the other components of the liquid biopsy because they are actively released by the cells, earning a potential role in tumor progression.

The implementation of exosomes in clinical practice is several steps back as compared to ctDNA in NSCLC. This is mainly due to the lack of consensus in the best way of isolating exosomes from body fluids, but also to the high quantity of material needed to their study. For this reason, in this chapter we will talk about the principal advances in the study of exosomes in NSCLC that could lead to an implementation in the clinical practice in the following years.

The study of exosomal miRNAs is very promising, and new techniques have improved miRNA detection in NSCLC [58]. The new high-throughput technologies have allowed to identify differential miRNA expression between tumor-derived exosomes and exosomes derived from healthy volunteers. This has permitted the description of different miRNA profiles that can help for both tumor diagnosis and/or disease monitoring [59]. For example miRNA-373 and miRNA-512 seem to restrict both the growth and invasiveness of the tumor in normal conditions. However, in cancer patients these ncRNAs are

epigenetically silenced, meaning a poor prognosis for the patients, while if the silencing disappears, the re-expression of the miRNAs inhibits the cell migration [60]. Regarding the treatment follow-up, the overexpression of miR-208a and miR-1246 seems to promote the resistance to chemotherapy in NSCLC patients. Similarly our group described that the overexpression of miR-221-3p and -222-3p in patients treated with third-generation TKI (osimertinib) is associated with better prognosis [58, 61, 62].

The sequencing of the exosomes transcriptome is a novel field of study, and thus, the information available are still limited. Through RNA sequencing it was also possible to detect EGFR mutations inside exosomes [63]. Accordingly we have recently detected the EML4-ALK translocation within exosomes derived from plasma of NSCLC patients [64].

The high-throughput technologies for exosomes proteomic analysis may allow to identify the primary tumor and analyze its molecular profile to better understand it. Regarding this, Yamashita et al. demonstrated that the presence of EGFR protein was significantly higher in the membrane of exosomes isolated from NSCLC patients compared to healthy donors [65]. Some other proteins have been described to be important prognostic biomarkers; for example, Sandfeld-Paulsen described CD171 on the membrane of the exosomes as a marker for positive overall survival in NSCLC [66], and also FAM3C have been described to be a good prognostic factor in squamous cell carcinoma patients [67]. The exosomes may be also helpful to the tumor diagnosis. Indeed, the same group has described different markers to discriminate the subtype of tumor, including also multi-marker models with a better discrimination curve [68].

A peculiar feature of the exosomes is their ability to be specifically phagocytized by cancer cells. This could lead to the use of exosomes as drug delivery components. It has been already demonstrated that in lung cancer mice models, Paclitaxel encapsulated in the exosomes could be an effective treatment option [69]. Moreover, two clinical trials using this innovative approach of drug delivery have been performed. The first approach is a Phase I trial using dendritic cell-derived exosomes (DEX) immunotherapy; the second one is a Phase II trial where a vaccination with DEX carrying IL-15Ra and NKG2D in association with cyclophosphamide after platinum-based chemotherapy. The main objective of both studies was to measure the toxicity and the feasibility to produce autologous DEX. The results from both studies are eagerly awaited also because it has been shown that DEXs were also able to activate both the adaptive and the innate immune system [70, 71].

## How the Future of Liquid Biopsy Looks Like: Platelets as a Source of Tumor-RNA

Rearrangements of ALK, ROS1, and RET genes are now important as much as EGFR-activating mutations because in this subgroup of patients very effective targeted treatment can be used. Nevertheless, it is important to point out that for this kind of analysis RNA is requested instead of DNA. This may represent a problem because circulating RNA undergoes degradation very quickly unless plasma samples are rapidly processed after withdrawal. Several research groups are trying to overcome this inconvenient. Indeed recent studies have shown that platelets can engulf tumor-related RNA preserving it from degradation, and thus permitting to identify primary tumor profiles with very high accuracy, and, in many cases, discriminate if the patients are metastatic or not [72, 73]. In 2016 Nilsson et al. have first shown that EML4-ALK translocation can be detected through RT-PCR from platelet-derived RNA with 65% sensitivity and 100% specificity [74] (Table 12.1).

**Table 12.1** Summary of the useful components described along the chapter and their clinical utility

| Liquid Biopsy Component | Detection | Utility | References |
|---|---|---|---|
| Circulating Tumor Cells | CTCs enumeration | ↓Prognosis | [23, 26] |
| | EGFR | Diagnosis/Recurrence | [27] |
| | ALK-EML4 | Diagnosis | [29–31] |
| Circulating Tumor DNA | EGFR | Diagnosis/Recurrence | [37–42] |
| | TP53 | Diagnosis | [43] |
| | KRAS | Diagnosis | [43] |
| | T790M | ↑Prognosis | [46, 47] |
| | [ctDNA] ng/mL | ↓Prognosis | [44] |
| | T790M | Follow-up | [52] |
| Exosomes | miR -373, -512 | ↑Prognosis | [60] |
| | miR -208a, -2223p | ↓Prognosis | [61, 62] |
| | miR -221-3p, -223-3p | ↑Prognosis | [58] |
| | EGFR | Diagnosis | [63] |
| | EML4-ALK | Diagnosis | [64] |
| | CD171 | ↑Prognosis | [66] |
| | FAM3C | ↑Prognosis | [67] |
| Platelets | EML4-ALK | Diagnosis | [74] |

## Conclusions

In conclusion, the incorporation of ctDNA analysis can definitely improve lung cancer patients' management because it can provide a better molecular stratification even when tissue cannot be obtained due to ethical and safety reasons. Although the implementation of both exosomes and CTCs in clinical practice is several steps back, the new advances and discoveries makes them, together with the ctDNA, a very promising tool. Liquid biopsy analysis can be used in different moments starting from diagnosis to relapse, earning multiple clinical meanings. In fact, at diagnosis, it can help in obtaining a better patients' stratification with both prognostic and predictive value, rather than during treatment, and it can be a valuable and simple test to follow tumor response and moreover to identify resistance mechanisms. Therefore it is clear that liquid biopsy has already improved NSCLC patients' management as it offers a noninvasive but valid method to detect actionable mutations.

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