Review Article

Liquid biopsy based biomarkers in non-small cell lung cancer for diagnosis and treatment monitoring

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Abstract: Advances in the knowledge of the biology of non-small cell lung cancer (NSCLC) have revealed molecular information used for systemic cancer therapy targeting metastatic disease, with an important impact on patients overall survival (OS) and quality of life. However, a biopsy of overt metastases is an invasive procedure limited to certain locations and not easily acceptable in the clinic. Moreover, a single biopsy cannot reflect the clonal heterogeneity of the tumor. The analysis of peripheral blood samples of cancer patients represents a new source of cancer-derived material, known as liquid biopsy, and its components can be obtained from almost all body fluids. These components have shown to reflect characteristics of the status of both the primary and metastatic diseases, helping the clinicians to move towards a personalized medicine. The present review focuses on the liquid biopsy components: circulating tumor cells (CTCS), circulating free DNA (cfDNA), exosomes and tumor-educated platelets (TEP); the isolation technologies used and their potential use for non-invasive screening, early diagnosis, prognosis, response to treatment and real time monitoring of the disease, in NSCLC patients.

Keywords: Liquid biopsy; non-small cell lung cancer (NSCLC); circulating tumor DNA (ctDNA); circulating tumor cells (CTCs); exosomes; tumor-educated platelets (TEP)

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Introduction

Lung cancer is the leading cause of cancer death worldwide, with non-small cell lung cancer (NSCLC) accounting for the majority of cases. It is the most frequent cancer both in terms of incidence (1.2 million new cases, 12.3% of the global total) as well as with respect to mortality, approximately 1.2 million deaths per year, which is equivalent to 17.8% of the world total (1).

Patients with NSCLC experience poor overall survival (OS) (2) due to the presence of metastatic diseases that are not adequately detected and therefore many patients are subjected to aggressive localized treatments, such as surgery or radiotherapy (RT), when in reality they present advanced distant disease.

Tissue biopsy has always been the standard diagnostic procedure for tumors and also to provide enough material for genotyping, which can assist in the targeted therapies of cancers. However, tissue biopsy-based cancer diagnostic procedures have limitations in their assessment of cancer development, prognosis and genotyping, due to tumor heterogeneity and evolution (3). As a matter of fact, there is growing evidence for intratumor heterogeneity indicating that single-site biopsies fall short of revealing the complete genomic landscape of a tumor (4).

A liquid biopsy, or blood sample, can provide the genetic landscape of all cancerous lesions (primary and metastases). In addition, it offers the opportunity to systematically monitor cancer disease as the quantification of circulating

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tumor DNA (ctDNA) has been shown to correlate with tumor burden (5). Circulating free DNA (cfDNA), exosomal RNA as well as circulating tumor cells (CTCs) or tumor-educated platelets (TEP) offer the potential for non-invasive screening, diagnosis, prognosis, and measuring response to treatment (6-8).

The practice of liquid biopsy as a diagnostic and prognostic tool in lung cancer patients is gradually becoming an appealing approach in the clinical routine practice, since it is noninvasive and can be easily repeated (3). In particular, this approach allows real-time monitoring of the patient during treatment, as well as the detection of different genomic alterations potentially sensitive to targeted therapy or associated with treatment resistance. It also allows rapid access to biomarker assessment in vulnerable lung cancer patients for whom solid biopsies are inaccessible or difficult to repeat. This attractive approach could promote a change in therapy, even before detection of tumor progression or relapse (5).

This Review will explore how tumor-associated genetic alterations detectable in the blood can be used in the clinic for diagnosis, assessment of prognosis, early detection of disease recurrence, and as surrogates for traditional biopsies with the purpose of predicting response to treatments and the emergence of acquired resistance.

Liquid biopsy

The term "liquid biopsy" qualifies different potential approaches for the detection of biomarkers found in circulating blood in cancer patients. Liquid biopsy analysis is a rapidly expanding field in translational cancer research and might be useful at different points of the diagnostic/therapeutic course of cancer patients, such as early diagnosis, estimation of the risk for metastatic relapse or metastatic progression (prognostic information), stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms (predictive information), and understanding metastasis development in cancer patients. Different liquid-derived materials can be employed, such as CTCS, ctDNA, exosomes, circulating RNA or microRNAs (9). They may be obtained from almost all body fluids (blood, serum, plasma, urine, pleural effusion, ascites, etc.) (10) and are likely to contain a wider presentation of genomic data from multiple metastatic sites, whereas mutations present in a single biopsy or minor sub-clone may be missed. In addition, the information obtained from liquid biopsies is allowing an increase into the knowledge of the pathophysiology of lung cancer and into the process of metastatic dissemination. .

Molecular profiling studies performed on tumor biopsies of NSCLC have identified genetic alterations that predict tumor responses to targeted therapies such as protein tyrosine kinase inhibitors (TKI). With an even larger repertoire of TKI entering the clinic, screening tumors for genomic aberrations is increasingly important (11). Detection of these driver mutations, such as EGFR mutations, in cfDNA or CTCs, has allowed to use targeted therapies avoiding invasive procedures and to identify resistance mechanisms for TKIs, while quantification of these mutations has helped to monitor tumor burden and dynamics (12,13). However, the mutational analysis of cfDNA demonstrated a significantly better sensitivity when compared with CTCs, establishing cfDNA as the best source of material for mutation detection (14).

In addition, the biological properties of mRNA content of exosomes and platelets have also contributed to provide a comprehensive real-time picture of the tumor burden, improving the application of genetic profiling of tumor associated RNA and DNA derived from biofluids and the understanding of cancer as a dynamic disease.

CTCs

CTCs are formed by cell detachment from the primary tumor mass, determining the migration of tumor cells to secondary sites via the lymphatic and blood system. The presence of CTCs has been demonstrated in the blood of patients with various solid tumors and it has been associated with poor outcome in metastatic NSCLC patients as well as in other tumors (10,15). Although CTCs were first described in 1869, their possible role the in the clinical setting since has progressively gained attention (16).

Detection of CTCs is technically challenging since it is estimated that as few as one CTCs can be found per 10^6–10^7 peripheral blood mononuclear cells in patients with advanced solid tumors, with less quantity of cells in patients with early-stage diseases. Due to this low concentration of CTCs, its detection and characterization require highly sensitive and specific methods, which consist in a combination of different strategies (17).

CTCs enrichment is either based on biological or physical properties (18). Biological properties include those approaches targeting epithelial specific surface markers like epithelial cell adhesion molecule (EpCAM) for positive selection (e.g., CellSearch™, microfluidic devices or the

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nanodetector) or CD45 for negative depletion (19,20).

The CellSearch^{TM} (Veridex LLC) is the only approved methodology by the U.S. Food and Drug Administration (FDA). It utilizes EpCAM-coated magnetic beads to isolate CTCs but has shown a limited detection efficiency in several types of carcinomas. In metastatic NSCLC disease, Krebs *et al*. reported only 32% of patients having ≥2 CTCs before chemotherapy treatment (21).

The development of a unique microfluidic platform, the "CTCs-chip", has been demonstrated to be capable of efficient and selective separation of viable CTCs from peripheral whole blood samples. The chip is based on the interaction of target CTCs with EpCAM-coated microposts under precisely controlled laminar flow conditions, without requisite pre-labelling or processing of sample. This technology successfully identified CTCs in almost 100% samples analyzed of patients with metastatic lung, prostate, pancreatic, breast and colon cancer patient, with an average purity of 52% in NSCLC patients (22).

Despite all the advantages mentioned above, some tumor cell subpopulations can escape EpCAM-based isolation technologies, as some cells may undergo epithelial-mesenchymal transition (23). In consequence, other technologies based on non-immunological properties have been used for CTCs isolation. Physical properties include size (e.g., ISET, isolation by size of epithelial tumor cells) or differential density (e.g., FICOLL, isolation by centrifugation steps) (21,24). Using ISET, Krebs *et al*. reported an 80% detection rate of CTCs in peripheral blood samples collected from 40 chemonaïve, stages IIIA to IV, NSCLC patients, using ISET, compared with 23% using CellSearch^{TM}. A subpopulation of CTCs isolated by ISET did not express epithelial markers and circulating tumor microemboli (clusters of ≥3 CTCs) were observed in 43% patients using ISET but were undetectable by CellSearch^{TM}. They concluded that both techniques should be performed together as this dual analysis allows more complete exploration of CTCs (25).

As CTCs technology has evolved rapidly during last years, several attempts have been performed for this technology to have the potential to be used in tracking the genomic evolution of tumors over time, and may have therapeutic implications in terms of its ability to detect functional events or resistant subclones while avoiding the need to conduct repeated biopsies. Several studies have attempted to establish CTCs counts as a diagnostic, prognostic and predictive tool in NSCLC patients.

A study published in 2013 (26), aimed to determine CTCs detection as a diagnosis marker of NSCLC. CTCs were labeled by a folate receptor (FR) conjugate, showing a sensitivity of 73.2% and a specificity of 84.1% for the diagnosis of NSCLC (67.2% sensitivity in stage I). Of note, FRs are highly expressed in a variety of tumors, particularly in ovarian and lung cancers, whereas most normal tissues express low to negligible levels. A novel FR-based CTCs analysis method had been developed based on these findings (27).

CTCs have long been considered to reflect tumor aggressiveness and have been investigated as a surrogate marker for tumor growth. At this respect, the decrease in the number of CTCs during the treatment has been associated with radiographic tumor response and an increase in the cell counts with tumor progression (9). In particular, the decrease or disappearance of CTCs following surgery has been correlated with improved clinical outcomes (28,29). A recent study (30) has prospectively analysed the kinetics of CTCs in blood samples of 56 patients obtained before surgery and one month after. The results showed that the presence of CTCs after surgery was significantly associated with early recurrence (P=0.018) and a shorter disease-free survival (DFS) (P=0.008).

Changes in CTCs count after RT have also been studied. Dorsey *et al*. (31) analyzed 30 patients with localized NSCLC undergoing radiation treatment. Using a telomerase-based detection assay, 65% of them were CTCs positive prior to treatment, but CTCs numbers significantly dropped after irradiation (9.1 *vs*. 0.6 CTCs/mL; P<0.001).

In metastatic NSCLC patients, a number of studies have reviewed the prognostic significance of baseline CTCs count, concluding that it is an independent negative prognostic factor that correlates with advanced stage a shorter progression-free survival (PFS) and OS (21). Using the CellSearch system, Krebs *et al*. (21) reported median OS of 8.1 *vs*. 4.3 months (P<0.001) and PFS of 6.8 *vs*. 2.4 months (P<0.001) for stage III–IV NSCLC patients when CTCs cut-offs of <5 *vs*. ≥5 were applied. In the multivariate analysis, the number of CTCs was the strongest predictor of OS (HR 7.92; 95% CI, 2.85–22; P<0.001) exceeding the traditional risk factors of stage and performance status. In another study including 46 patients with newly diagnosed or recurrent NSCLC, CTCs were measured at baseline and before every chemotherapy cycle in a subset of patients (n=23). A baseline CTCs count of more than eight prior to chemotherapy was a strong predictor of reduced PFS (P=0.018) and OS (P=0.026). However, no correlation was observed between CTCs count and tumor size after two chemotherapy cycles (32).

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Finally, several studies have provided evidence of an association between a decrease in CTCs counts and radiographic response by FDG-PET/CT or RECIST. In a phase II clinical trial of erlotinib and pertuzumab with 41 patients enrolled, higher baseline CTCs counts were associated with better response to treatment by RECIST (P=0.009), and decreased CTCs counts after treatment correlated with FDG-PET and RECIST responses (P=0.014 and P=0.019) and a longer PFS (P=0.05) (14). However, a more recent study failed to confirm this finding, probably because it included a heterogeneous cohort of patients with respect to stage and histology, which can confound interpretation of FDG uptake and CTCs analysis (33).

In all, these studies indicate that CTCs count might be useful as a prognostic marker in lung cancer patients and can be of helpful in therapy decision making, especially for the selection of those surgically rejected patients at a higher risk of relapse who might benefit from adjuvant therapies (5).

Biomarker detection in CTCs

The development of personalized medicine and targeted therapies has led to an increased interest in CTCs as a source of material for genetic analyses. The detection and characterization of CTCs represent a major opportunity as they reflect both the phenotypic and the genetic information of the primary tumors. Also, the analysis of CTCs allows real-time evaluation of metastasis and can show the molecular condition of the disease (10).

EGFR status has been examined in CTCs from advanced NSCLC patients, allowing the identification of sensitizing mutations and the T790M resistant mutation. Using the CellSearch System coupled with next-generation sequencing (NGS), Marchetti *et al*. (34) identified for the first time *EGFR* mutations in the CTCs of 84% of patients EGFR positive in matching tumor tissue. In another study (35), a novel assay based on real-time PCR and melting curve analysis was developed to detect activating *EGFR* mutations in blood cell fractions enriched in CTCs. Using this assay, EGFR-positive CTCs were detected in pretreatment blood samples from all eight *EGFR*-mutant lung cancer patients studied (35).

Despite good initial responses, all NSCLC patients carrying sensitive *EGFR* mutations eventually develop resistance to *EGFR*-TKIs. The T790M gatekeeper mutation is present in 40–60% of those patients at disease progression. The development of third-generation *EGFR* inhibitors capable of overcoming T790M-associated resistance has led to a need for noninvasive testing of the T790M mutation (36). In a recent study comparing tumor biopsies with simultaneously collected CTCs and ctDNA, the resistance-associated mutation was detected in 47% to 50% of patients respectively, with a concordance ranging from 57% to 74%. (37). In those patients where the paired biopsy was negative or inconclusive, CTCs and ctDNA-based assays together enabled genotyping in 35% of cases.

ALK rearrangements, which are tested in NSCLC for crizotinib treatment, have also been evaluated in CTCs using a filtration enrichment technique and filter-adapted fluorescent in situ hybridization (FA-FISH). All ALK-positive patients were found to have four or more ALK-rearranged CTCs per mL of blood (median, 9 CTCs/mL; range, 4–34 CTCs/mL). In contrast, 0 to 1 ALK-rearranged CTCs/mL were detected in ALK-negative patients (median, 1; range, 0–1). The assay not only enabled diagnostic testing but also monitoring of crizotinib treatment (38). Similarly, ROS1 rearrangements, another target for crizotinib-based therapy, have been detected using ISET and FA-FISH in the CTCs of 4 patients with positive paired tumors for ROS1-gene (39).

In spite of all these technological advances, CTCs are still not used in the routine clinical practice or as a source of material for biomarker analysis in advanced NSCLC Although FDA approved CellSearch as CTCs detection method in metastatic breast, prostate and colon cancer, it has not yet been approved in metastatic lung cancer patients. Several limitations such as the lack of epithelial biomarkers which hamper CTCs detection, the lack of validation in large multicenter studies to evaluate reproducibility, specificity and sensibility and the lack of clinical validation in different cohorts of lung cancer patients highlight the need of additional research in advanced NSCLC in order to implement its application in the clinical setting (5).

Circulating free tumor DNA

ctDNA has the potential to enable noninvasive diagnostic testing for personalized medicine since it can provide similar molecular information as invasive tumor biopsies. Currently, the evaluation of specific predictive biomarkers is mandatory for a proper treatment of advanced-stage NSCLC patients according to the molecular characterization of the disease. In some cases, this molecular profiling can be difficult due to a reduced availability of tumor tissue, and therefore ctDNA testing might be used as a surrogate.

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cfDNA is single- or double-stranded DNA (dsDNA) that exists in plasma or serum. Early studies showed that many cancer-associated molecular characteristics, such as single-nucleotide mutations, methylation changes and cancer-derived viral sequences can be found in the cfDNA. These findings were significant for the development of ctDNA detection technology. Nevertheless, ctDNA-based assays face several challenges. Body cells release cfDNA into the bloodstream, but the majority of cfDNA is often not of cancerous origin, making it sometimes difficult to detect tumor-derived alterations (9). Also, prior knowledge about particular mutations can be of help, but may be difficult to obtain. However, recent technological advances have overcome these limitations, making it possible to identify both genetic and epigenetic alterations in cfDNA of cancer patients (3).

The fraction of cfDNA that is tumor derived in cancer patients ranges from <0.1% to >30% of the total DNA, and depends on tumor burden, stage, cellular turnover, accessibility to circulation and factors affecting blood volume. The ctDNA released by tumor cells will carry the same genetic (somatic) alterations as the tumor itself and this genetic load can be detected and quantified. At this respect, the relative levels of ctDNA have been shown to correlate with tumor burden and response to therapy (40).

Technical developments have allowed the identification of low frequency alterations in cfDNA. These platforms include real-time quantitative PCR (qPCR); digital PCR (dPCR); beads, emulsion, amplification and magnetics (BEAMing); and NGS. The sensitivity of these techniques ranges from 15% to 0.01%, and one of the major obstacles for their clinical applicability is the lack of standardization (41).

Real time qPCR: real time qPCR has been widely used for the identification of genetic alterations and the quantification of nucleic acids. While qPCR is a standardized, relatively inexpensive technique, its precision for quantifying rare allele or genetic alterations present at very low levels is significantly lower compared to other techniques such as digital (dPCR) (42). However, most of the published studies adopted variants of this technology for the analysis of ctDNA in lung cancer.

Regarding detection of *EGFR* mutations in cfDNA using qPCR, Kimura *et al.* showed for the first time that plasma-derived *EGFR* genotype is predictive of subsequent clinical response to an *EGFR*-TKI. A 72.7% concordance was found in the cases analyzed by sequencing of the primary tumor and Scorpion ARMS assay of plasma, (43). Similarly, Mok *et al.* reported a concordance between tissue and cfDNA of 88%, with blood test sensitivity of 75% and a specificity of 96% using the cobas blood test, based on qPCR (44).

EGFR oncogenic mutations have also been analyzed in plasma cfDNA of patients included into different trials, such as a trial evaluating the combination of pertuzumab and erlotinib (14) or the IPASS trial (45), which compared gefitinib with carboplatin/paclitaxel. The sensitivity of ctDNA testing in identifying *EFGR* mutations compared with tumor tissue ranged from 43% to 100%, being able to identify *EGFR* mutations in patients with insufficient or unevaluable tissue.

dPCR: the unparalleled precision of this technology enables accurate measurements for the quantification of low frequency genetic alterations. In this methodology, target molecules are separated into a large number of partitions so that each partition receives a number of molecules (usually ranging from 0–2) theoretically following a Poisson distribution. In consequence, target molecules are amplified mostly on an individual basis and measurements rely on counting the total of positive partitions containing one or more target molecules and negative partitions where no amplification is detected. dPCR allows for the detection of mutated ctDNA in a high background of wild type cfDNA and for the quantification of small fold change differences. Several studies have assessed the feasibility of dPCR for biomarker testing and cancer monitoring and have evidenced that dPCR is indeed an adequate technology for such purpose (44-48).

BEAMing: this technique is based on a combination of emulsion dPCR with magnetic beads and flow cytometry for detection and quantification of mutant tumor DNA molecules (49). BEAMing is a highly sensitive but complex method to detect genetic mutations at very low levels (50). This methodology has been compared to mutation analysis of tumor tissue in a recent publication that tested 21 mutations in BRAF, *EGFR*, KRAS and PIK3CA in samples from different cancer types (13.8% of NSCLC). Results were concordant for archival tissue and plasma cfDNA in 91% cases for BRAF mutations, in 99% cases for *EGFR* mutations, in 83% cases for KRAS mutations and in 91% cases for PIK3CA mutations, demonstrating that BEAMing is feasible and highly concordant with tumor tissue analysis (51). Parallely, Taniguchi *et al.* used this technology for plasma testing of *EGFR* mutations (52) in 44 patients with *EGFR* mutant NSCLC (23 with progressive disease after TKI and 21 TKI-naïve). Activating mutations were detected in the cfDNA of 72.7% of the patients, while the T790M was found in 43.5% of the cases with a progressive disease.

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To assess the ability of different technologies to detect *EGFR* mutations in ctDNA, including T790M, a comparison of multiple platforms was undertaken (53). It included two non-digital (cobas™ *EGFR* mutation Test and therascreen™ *EGFR* amplification refractory mutation system assay) and two digital platforms (Droplet Digital ™ PCR and BEAMing digital PCR). This study used samples from a trial that investigated the safety and efficacy of AZD9291, a third-generation *EGFR*-TKI, in patients with a sensitizing mutation whose disease progressed after a TKI. For *EGFR*-TKI-sensitizing mutations, high sensitivity (78–100%) and specificity (93–100%) versus tissue was observed using the four platforms. Regarding the T790M mutation, the digital platforms outperformed the non-digital platforms. Subsequent assessment using 72 additional baseline plasma samples was conducted using the cobas *EGFR* mutation Test and BEAMing dPCR and using a tissue test result as a non-reference standard. For the T790M detection, the sensitivity and specificity were 73% and 67% for the first technology and 81% and 58%, respectively, with a concordance between the platforms >90%. Another recent publication comparing BEAMing and cobas test showed a higher concordance between BEAMing plasma and tumor EGFR results than between cobas *EGFR* plasma and tumor (82% for activating mutations and 73% for T790M using BEAMing *vs*. 73% and 64%, respectively, using cobas plasma test) (54).

NGS: the emerging of new drugs that target other genomic alterations than *EGFR* mutations have significantly increased the complexity of biomarker testing. In the context of a growing number of driver mutations to test, NGS presents itself as a suitable technology since it allows simultaneous detection of multiple alterations in a very efficient manner. However, the use of this technology might be limited by a modest sensitivity. Using deep sequencing as a detection system for *EGFR* mutation in the ctDNA of stage I–IV NSCLC patients, Uchida *et al*. reported a diagnostic sensitivity and specificity for exon 19 deletions of 50.9% and 98%, respectively; and for the L858R mutation of 51.9% and 94.1%,. Although overall sensitivity was 54.4% for all cases; it dropped to 22.2% in stages IA-IIIA, compared to 72.7% in stages IIIB–IV, highlighting that NGS might be restricted to advanced disease (55). Similarly, Couraud *et al*. used the IonTorrent Personal Genome Machine for the deep sequencing of the most relevant hotspot somatic mutations (*EGFR*, BRAF, KRAS, HER2, and PIK3CA) in tumor and plasma cfDNA of never-smoker NSCLC patients. In ctDNA, 50 mutations (36 *EGFR*, 5 HER2, 4 KRAS, 3 BRAF, and 2 PIK3CA) were identified. Sensitivity of the test was 58% and the estimated specificity was 87% (56). Because sensitivity appears to be compromised in early stages, several strategies to solve this issue are being tested. Newman *et al*. have developed an ultrasensitive method for quantifying ctDNA that can detect ctDNA in 100% of patients with stage II–IV NSCLC and 50% of patients with stage I, with 96% specificity for mutant allele fractions down to 0.02%. Researchers communicated that ctDNA levels correlated with tumor volume and distinguished between residual disease and treatment-related imaging changes. Measurement of ctDNA levels allowed for earlier response assessment compared to radiographic approaches, facilitating personalized cancer therapy (57). However, low quantities of ctDNA in blood and sequencing artifacts could limit the analytical sensitivity of this methodology. To overcome these limitations, the same groups have recently developed other technological approaches for improved detection of ctDNA (58).

Finally, two meta-analyses exploring the diagnostic value of cfDNA for the detection of *EGFR* mutation status have been published (59,60). Luo *et al.* [2014] reviewed 20 studies involving 2012 cases to assess the diagnostic performance of cfDNA compared with tumor tissue and concluded that detection of *EGFR* mutations in cfDNA has an adequate diagnostic accuracy. Likewise, Qiu *et al.* [2015] analysed 27 studies involving 3,110 patients and reported effectiveness similar to Luo *et al.*, with overall sensitivity of 62%, specificity of 96% and diagnostic odds ratio of 38.270.

Exosomes

Exosomes are vesicles of endocytic origin with a diameter of 40–100 nm that transfer information to the target cells (including proteins, DNA, mRNA, as well as non-coding RNAs) through at least three mechanisms: fusion with the plasma membrane, receptor-ligand interaction or endocytosis by phagocytosis (61). Thakur *et al*. provided also evidence that tumor-derived exosomes carry dsDNA, showing that exosomal DNA could reflect the mutational status of parental tumor cells (62).

Exosomes are critically involved in tumor initiation, growth, progression, metastasis, and drug resistance by transferring oncogenic proteins and nucleic acids. The role of exosomes in modulating the pathways that lead to the development of resistance to both RT and chemotherapy is an emerging area of intense research. Therefore, exosomes and their load could be biomarkers with value in diagnosis,

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prognosis and prediction of therapeutic responses (63).

Exosomes have been isolated and characterized from distinct cells under normal and stressed conditions. Several methods have been used for exosome isolation including ultracentrifugation, combined with sucrose gradient, and the immune-bead isolation [e.g., magnetic activated cell sorting (MACS)]. In addition, different commercial kits are available for their isolation and purification. Transmission electron microscopy (TEM), Western blot, and FACS are frequently used to characterize the isolated exosomes based on their biochemical properties, such as morphology, size or exosomal markers. However, there are not accurate methods to determine the concentration of exosomes and researchers have to rely on inaccurate measurements of protein concentration or nanoparticle tracking analysis. Quantitative RT-PCR, nucleic acid sequencing, Western blot, or ELISA has been used for exosome RNA and protein identification (64).

In patients with NSCLC, the circulating levels of tumor exosomes, exosomal small RNA and specific exosomal miRNA have been tested in order to validate them as a potential marker for diagnosis and prognosis in patients with adenocarcinoma (65).

Rabinowits *et al*. (66) reported significant differences in circulating exosomes and miRNA concentrations between lung adenocarcinoma patients and a control group. In addition, the similarity found between the circulating exosomal miRNA and the tumor-derived miRNA patterns led the authors to suggest that circulating exosomal miRNA might be useful as a screening test for lung adenocarcinoma. Similarly, another recent work reported a model based on microRNAs derived from circulating exosomes capable to discriminate between lung adenocarcinoma and granuloma (67).

Finally, the identification of large rearrangements in liquid biopsies such as the EML4-ALK translocation, that determines eligibility for treatment with FDA-approved ALK-kinase inhibitors, remains challenging. It appears reasonable to approach this issue by detecting the EML4-ALK fusion transcript. To this aim, the RNA protected in vesicles as the exosomal RNA appears to be a promising source for this kind of analyses and some diagnostic tests designed to identify the predictive biomarker EML4-ALK in liquid biopsy are based on EML4-ALK fusion transcripts detection using exosomal RNA (68).

TEPs

Blood platelets have recently emerged as important players in cancer development and responses to tumor growth. The confrontation of these platelets with tumor cells via transfer of tumor-associated biomolecules has been named "education". This interaction induces, among other processes, specific splicing of pre-mRNAs (69). In addition, platelets can ingest circulating RNA (70). Taken together, it appears that TEP could be a suitable source of material for liquid biopsy-based analyses. A recent publication by Best *et al*. (71) developed a classifier based on RNA-Seq of TEPs. Across six primary tumor types (NSCLC, colorectal carcinoma, glioblastoma, pancreatic cancer, hepatobiliary cancer, and breast cancer), the location of the primary tumor was correctly identified with 71% accuracy. Moreover, they identified several genetic alterations or biomarkers that could guide the use of drugs targeting those mutations. In addition to exosomes, TEPs seem to be an adequate source for fusion gene identification in liquid biopsies. Nilsson *et al*. have examined the use of RT-PCR to detect EML4-ALK rearrangements in platelets and plasma compared with matched tissue biopsies. RT-PCR demonstrated 65% sensitivity and 100% specificity for the detection of EML4-ALK rearrangements in platelets. Intriguingly, in a subset of 29 patients treated with crizotinib, the detection of these rearrangements in platelets was found to be correlated with lower PFS and OS. Authors also concluded that monitoring of EML4-ALK rearrangements in the platelets of one patient over a period of 30 months revealed crizotinib resistance two months prior to radiographic disease progression (72).

Collectively, these data suggest that TEP may provide a RNA BioSource for liquid biopsy based diagnosis (73).

Conclusions

Personalized medicine in oncology relies on the customization of healthcare using molecular analyses. In this context, diagnostic testing is used to select appropriate and optimal therapies based on a patient's cancer genome and to obtain molecular biomarkers in an easily accessible, minimally invasive way in order to follow the molecular profile of a patient's tumor longitudinally. The clinical application of liquid biopsy in NSCLC is progressively proving a pivotal tool for screening and early detection of cancer, real-time monitoring of therapy or risk of relapse (prognosis) and identification of therapeutic targets and resistance mechanisms.

ctDNA and CTCs in NSCLC patients are being intensively investigated, as they have proven to provide a

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dynamic picture of molecular disease changes and can be used to identify molecular alterations emerging during the course of treatment in a noninvasive manner. It is becoming increasingly clear that metastasis and tumors are extremely heterogeneous, and therefore each stage should be treated as a different condition. It has also been recently demonstrated that plasma ctDNA is suitable for monitoring treatment response and also for the early detection of acquired resistance mechanisms (e.g., T790M in NSCLC) (74). These and other studies are laying the foundations for the development of cfDNA as a new and non-invasive source of material that can be used routinely when testing for predictive biomarker for targeted therapies.

The value of CTCs analysis in lung cancer is currently mainly related to prognosis, but there is an increasing interest in the development of new techniques for molecular characterization of CTCs. It is not fully understood whether CTCSs are the only cause of metastasis, but it has been reported that their counts correlate with a poorer prognosis in lung cancer and in other tumor types, although there is still a need for clinical validation of these findings, particularly in lung cancer.

The information obtained from liquid biopsies, together with imaging analysis, replacing invasive tissue biopsies, will probably guide treatment decision and hopefully will improve the outcome for lung cancer patients, having also de potential for early diagnosis of tumors that are not yet visible on imaging.

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Footnote

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