Non-invasive diagnostic platforms in management of non-small cell lung cancer: opportunities and challenges

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Abstract: Several non-invasive diagnostic platforms are already being incorporated in routine clinical practice in the work up and monitoring of patients with lung cancer. These approaches have great potential to improve patient selection and monitor patients while on therapy, however several challenges exist in clinical validation and standardization of such platforms. In this review, we summarize the current technologies available for non-invasive diagnostic evaluation from the blood of patients with non-small cell lung cancer (NSCLC), and discuss the technical and logistical challenges associated incorporating such testing in clinical practice.

Keywords: Non-invasive biopsies; biomarkers; non-small cell lung cancer (NSCLC)

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Introduction

Genomic analysis of non-small cell lung cancer (NSCLC) revealed high genomic mutational burden and recurrent alterations in several oncogenes (1,2). These driver oncogenic alterations are key molecular events and often have therapeutic implications. Testing for oncogenic alterations in EGFR, ALK, ROS1 and BRAF are considered standard of care for all patients with non-squamous NSCLC and in addition, more comprehensive genomic profiling including MET, ERBB2, RET, and TRK is often considered (3). There have been remarkable advances in our understanding of the genomic heterogeneity among patients with NSCLC leading to opportunities for targeted therapy for patients with clinically actionable genomic alterations. Interrogation of the genomic alterations in the tumor is thus critical in clinical decision-making for patients with NSCLC. Tissue biopsy is the gold standard for tumor genotyping. However, the challenges of tissue based biomarker analysis include complexity of molecular alterations and issues with adequate tumor tissue acquisition. In addition, spatial and temporal intra-patient heterogeneity of the genomic alterations can make tissue testing and interpretation of the results challenging. Efforts to understand the complex biology of cancers is largely limited by the inability to adequately capture the intra-patient heterogeneity of tumors. Moreover, exposure to treatments both cytotoxic chemotherapy and targeted therapy can create an adaptive biological process in the tumor and such clonal evolution can have therapeutic implications (4). Such key molecular insights are hard to obtain through tissue biopsies. Non-invasive diagnostic platforms popularly known as "liquid biopsies" can potentially provide useful insights into the genetic and epigenetic make-up of the tumor and can be used as complementary diagnostics in addition to the tissue biomarker analysis. Liquid biopsies can also address some of the issues around tumor heterogeneity and clonal evolution of the tumors particularly in the setting of acquired resistance to targeted therapies.

Liquid biopsies generally refer to genomic analysis

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Table 1 Comparison of circulating tumor cells and circulating-tumor DNA platforms

	Advantages	Disadvantages
Circulating tumor DNA	ctDNA is easier to isolate; long-term storage for subsequent analysis feasible; analysis with real-time PCR or digital PCR for known point mutations and very low allele fractions, or targeted deep sequencing	Limited pre-analytical/analytical assay validation with some platforms; short fragment DNA from necrotic or apoptotic cancer cells; limited possibilities for downstream analysis (DNA only)
Circulating tumor cells	Evaluation of intact cancer cells is invaluable for downstream analyses allowing interrogation of the DNA, RNA or at the protein level; some CTC live cell isolation platforms can allow ex-vivo expansion and creating in-vitro and patient-derived xenograft models to investigate drug susceptibility and allow deeper understanding the molecular profiles; single-cell analysis technologies allow for new insights into the genetic make-up of CTCs	Complex detection and enrichment steps required and often require sophisticated technologies; low CTC numbers in non-metastatic and low disease burden settings

of circulating tumor cells (CTCs) or circulating tumor nucleic acids (ctDNA and ctRNA). However, several novel techniques using microRNA, platelet-microvesicles, serum metabolites and exosomes are currently being validated. In this review, we summarize the liquid biopsy platforms currently available for clinical use in patients with NSCLC. We discuss the general approach, the clinical applications and methodological challenges of liquid biopsy testing in lung cancer.

Liquid biopsies use peripheral blood samples as a source of the bioanalytics like ctDNA, ctRNA, CTCs, exosomes, platelets or serum proteome. Liquid biopsies allow for rapid biomarker work up, can be used to follow emergence of acquired resistance to treatments and could potentially identify early response or lack of response.

Circulating tumor nucleic acids

Circulating tumor nucleic acid technologies were first developed to detect circulating fetal DNA in the circulation of pregnant women to develop non-invasive prenatal tests (5). Patients with cancer have higher levels of circulating free DNA (cfDNA) compared to normal healthy individuals (6). Recent technological innovations in massively parallel sequencing led to developments of molecular assays with ability to detect minute allelic imbalances in circulating nucleic acid. These platforms can now provide opportunity for interrogating the cfDNA with robust sensitivity and specificity.

Detection and analysis

Sample preparation for ctDNA analysis is very critical and is typically done using collection kits like cell-free DNA BCT® and PAXgene Blood DNA tubes that contain formaldehyde-free preservative reagents that prevent the nuclease-mediated degradation of ctDNA. These tubes also help stabilize nucleated blood cells, thus reducing the release of cellular germline cfDNA (7). A majority of the cfDNA in patients with cancer is not tumor DNA (ctDNA). There are varying amounts [0.01–10%, minor allele frequency (MAF)] of cfDNA in patients with cancer and only a fraction of the DNA represents tumor DNA (ctDNA). The proportion of the ctDNA is a factor of the tumor burden and the tumor biology (8). The clinical utility and reproducibility of the ctDNA assay is thus contingent upon adequately differentiating ctDNA and appropriate normalization. Recent studies suggest that ctDNA have shorter cell-free DNA fragment lengths compared to normal cell-free DNA (9-11). Several kits capturing the smaller fragment lengths from cell-free DNA are available and this may optimize the detection and amplification of ctDNA.

There are multiple sequencing platforms that are available for analysis of ctDNA and these platforms all have some various advantages and disadvantages (12). Platforms such has real-time quantitative PCR (qPCR), the Scorpion Amplification-Refractory Mutation System (ARMS) and droplet digital PCR (dPCR) can provide high sensitivity for detection of known genomic alterations and relatively easy to integrate into the clinical workflow with easy bioinformatic burden. Beads, Emulsion, Amplification and Magnetics (BEAMing) is another recent approach that can provide an extremely sensitive detection threshold of low MAF known genomic alterations in a high background of wild type cfDNA (13). Both BEAMing and dPCR use

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emulsion PCR based methodology with individual DNA fragments are in droplets allowing for DNA fragments to be amplified independently. Using fluorescent labeled probes mutant and wild-type alleles can be distinguished allowing more accurate quantification of the mutant allele fractions compared to RT-PCR (14,15).

The methods discussed so far allow for detection and quantification of known genomic alterations. However, sequencing using next generation sequencing (NGS) platforms allows for detecting novel alterations at a frequency as low as one mutant copy in several thousand wild-type copies. NGS based approach can have high sensitivity and can screen not only for known mutations, but entire breadth of the targeted genome for previously unknown mutations and alterations. In addition to mutations gene rearrangements and copy number alterations can also be detected. Using hybrid capture methodology portions of the genomic areas of interest are identified among the amplified cfDNA libraries by hybridization capture with oligonucleotides or "baits" complementary to these regions for enrichment and repeat PCR. In addition, tagged-amplicon deep NGS using multiplexed PCR can be used for a lager panel of target regions of interest. Using these approaches adequate sensitivity required for clinical multiplexing can be ensured with ctDNA profiling (16-18). However, NGS based approach is expensive, and is time intensive requiring more sophisticated bioinformatic support in interpretation of the results (*Table 1*).

Clinical utility of ctDNA assays

There are several potential opportunities for incorporating ctDNA assays into clinical practice. The most important indications for ctDNA assays would be in patients who have inadequate tissue for molecular diagnostic work up for predicting response to targeted treatment, and monitoring for the development of acquired resistance to targeted therapy. In addition, recent studies suggest possible role in early response monitoring (19) and monitoring minimal residual disease after definitive oncologic treatment (8,20-23).

Comprehensive genomic profiling using ctDNA assays can cover all forms of genomic alterations namely indels, point mutations, gene amplifications and rearrangements. This is invaluable for patients where biopsy at diagnosis or progression is not feasible or tissue is insufficient. In addition, ctDNA may capture a more comprehensive molecular summary involving all the metastatic burden of the patients' cancer compared to a small biopsy which may not reflect the tumor heterogeneity (24).

Identification of clinically actionable genomic alterations

Most patients with advanced stage NSCLC have diagnostic tissue from bronchoscopic or CT-guided biopsies and often patients have limited material for molecular work up (25). Recent advances in NGS technology and optimizing these newer platforms for ctDNA assays, allow for multiplex screening of large panels of genes with high sensitivity often over 75% and high concordance with tissue based testing particularly when the tissue biopsy and the ctDNA testing are temporally concurrent (<1 month) to the ctDNA testing (18,26). In another meta-analysis of 20 studies ctDNA based platforms for detecting EGFR mutations had a pooled sensitivity of 62% and specificity of 95% when compared to tissue based testing (27). ctDNA is an effective and efficient method to detect EGFR mutation status in NSCLC and could complement tissue based testing to improve diagnostic accuracy and yield in conjunction with tissue based testing.

Detecting acquired resistance mechanism and defining sequencing of targeted therapies based on emergence of resistant clones

Acquired resistance to targeted therapy occurs through clonal evolution and dynamic changes in molecular make-up of the tumors. Treatment with targeted drugs causes selective pressures leading to temporal molecular heterogeneity which can have clinical and biological implications (28). There is increasing evidence that some of these acquired molecular events following therapeutic interventions have clinical implications. Most notable example is the emergence of a gatekeeper mutation in EGFR kinase domain, namely T790M in patients with EGFR mutant NSCLC on treatment with a first-generation EGFR targeted therapy. These secondary EGFR T790M mutations are seen in approximately 50% of EGFR-mutant tumors upon progression on a first-line EGFR inhibitor and predict response to newer generation EGFR inhibitors with higher specificity and potency against the T790M mutation (29,30). Unlike EGFR mutant NSCLC patients with ALK rearranged NSCLC appear to have a much broader array of on-target gatekeeper mutations (L1196M, G1269A, C1156Y, L1152R, I1151Tins, F1174C/L/V etc.) in the ALK domain and amplification of ALK (PMID, PMID, PMID, PMID) (31-34). In addition, on-target acquired genomic alterations tumors can develop alternate pathway

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activation like MET and KIT amplifications, BRAF, NRAS, FGFR2, PIK3CA, IGF1R and ERBB family of receptor mutations (35-38). This degree of heterogeneity, dynamic shifts in clonal cellular populations and polyclonal resistance mechanisms makes molecular work up for patients progressing on targeted therapy complicated. Despite advances in sequencing technologies for interrogating tissue biopsies there are operational challenges in acquiring biopsies at multiple time points during therapy. Moreover, the tissue based approaches do not capture the temporal or spatial heterogeneity accurately (39). Recently plasma ctDNA based assays for EGFR T790M were demonstrated to have high tissue concordance for EGFR T790M and shown to be predictive of response to osimertinib (40-42). In addition, EGFR C797S and L798I were identified as novel mechanisms of acquired resistance to osimertinib (43). Using more sensitive ctDNA NGS platforms one can identify sub-clonal mutations (like MET, PIK3CA, HER2, BRAF etc.) that may evolve into the dominate clone at progression. This could aide in designing potentially novel strategies for sequential targeted therapies targeting these resistant sub-clonal populations by using ctDNA monitoring resulting in prolonged benefit with lower risk of toxicity from combination approaches.

CTCs

Tumors shed cells into circulation and these cells have the potential to disseminate widely through the venous and arterial circulation with a potential to initiate a metastatic focus. These CTCs persist in circulation withstanding a variety of stresses both mechanical and metabolic. Generally epithelial cells in circulation lacking anchorage undergo apoptosis by a process called anoikis (44). Complex integrin-dependent mechanisms orchestrated by receptor tyrosine kinases like TrkB are essential to suppress anoikis and permit tumor invasion and metastasis (45). In addition the large size of the CTCs (20–30 μm) relative to the capillary lumens (~8 μm) allow for CTCs to get trapped in tissues and escape the circulation on their first pass (46). Recent advances in technology allow for accurate detection of CTCs with potential for clinical utility (47,48).

Detection and analysis

Two broad methodologies for CTC detection are label-based positive selection using tumor epithelial cell surface markers or label-independent negative selection approach based on biophysical or functional properties of CTCs (49). Label-based capture assumes that CTCs have the same phenotypic characteristics as the primary tumor. Specific markers like cytokeratin and EpCAM are used for positive selection techniques (50). CellSearch® system (Veridex, Raritan, NJ, USA) is a Food and Drug Administration (FDA) approved platform utilizing the positive selection approach for CTC enumeration for patients with castration-resistant prostate cancer, breast cancer and colon cancer (51-53). This method uses enrichment of cytokeratin and EpCAM positive cells by immunofluorescence staining. However, in NSCLC some of the CTCs may transform to mesenchymal cells and lack cytokeratin staining (54,55). There are no reliable surface markers that can be used to comprehensively sort and select NSCLC CTCs. Label-independent isolation of CTCs using biophysical and functional characteristics of CTCs appears to be an attractive alternative for patients with NSCLC. Isolation by Size of Epithelial Tumor cell (ISET®) platform allows for separation of CTCs using a filtration device which sorts the cells and permits cytological phenotyping of the cells (56,57). Some other recent platforms allow not just sorting of the morphologically distinct CTC populations but allow for live and functional CTC separation allowing opportunities for further molecular studies (58-61). In another non-labelled approach using micro-fluidic chip based platform CTCs are separated in laminar flow conditions due to physical interactions with EpCAM-coated microposts in the chip (47). These non-labelled approaches have superior CTC detection rates patients with NSCLC compared to EpCAM/Cytokeratin labelled sorting approaches. In addition to increased detection rates these platforms allow for several post-isolation downstream applications. These platforms that retain CTC viability can allow ex-vivo expansion and creating in-vitro and patient-derived xenograft models to investigate drug susceptibility and allow deeper understanding the molecular profiles and biologic behavior in response to therapeutic intervention (59,60). In addition to sequencing and gene expression studies such platforms have recently been used to evaluate expression of PD-L1 and could potentially be used as a biomarker and monitoring tool for PD1/PDL1 targeted drugs (61,62). However, these assays need further clinical validation for reproducibility and therapeutic utility prior to routine clinical use.

Clinical utility of CTC assays

Due to the lack of standardization of the methods, varying

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detection thresholds and lack of clear definition of CTCs across platforms the clinical utilization of CTC based assays has been somewhat limited.

Role as a prognostic biomarker

In a few studies done in patients with early stage NSCLC increased CTC were found to correlate with poor outcomes (63-65). This correlation with adverse outcomes was also seen in patients with advanced stage NSCLC having systemic chemotherapy (66,67). Though some studies failed to show the correlation with prognosis (67) a meta-analysis of 20 studies evaluating CTCs in NSCLC demonstrated a correlation with stage, lymph node status and the outcome (68). Despite these encouraging findings the use of CTCs enumeration in clinical practice is limited because of lack of proper prospective validation and need for standardization of cutoff values and isolation methods of CTCs.

Prediction of response to treatment

Like ctDNA based approaches CTC assays can be useful tools to detect and monitor oncogenic driver mutations particularly EGFR and ALK fusion genes. The concordance of EGFR mutations using CTC with tissue was reported to be over 80% (69,70). Beyond NGS sequencing CTCs have the potential for mRNA characterization and array-based comparative genomic hybridization studies (58,59).

Though both ctDNA and CTC platforms appear to be comparable and with equal clinical potential in serving the role of a "liquid biopsy" these platforms have some distinct differences in the clinical applications. The logistics of implementation of the liquid biopsy platforms are simpler with ctDNA based approaches because of ease of collection and storage for biobanking. Most importantly CTC approaches require specialized instrumentation for capture of the target cells and cut-offs of minimal CTC for the assays are yet to be standardized. The newer NGS platforms optimized for ctDNA assays are highly efficient with great degree of sensitivity in monitoring low levels of disease. In addition to potential role in complementing tissue based genotyping for patients with advanced stage NSCLC and ctDNA can be a very valuable tool for monitoring for minimal residual disease especially after surgical resection (8,20,22). However, utility of liquid biopsies in monitoring minimal residual disease in early stage NSCLC is yet to be demonstrated in a prospective trial. In addition to the technical differences with implementation of the ctDNA and CTC platforms there may be some differences in the biological distinctions that need to be noted. ctDNA could likely is derived from tumor cells actively shedding ctDNA or necrotic cells undergoing apoptosis compared to CTC which are tumor derived intact cells (71,72). Unlike ctDNA CTCs can provide more information regarding clonal evolution and allow single cell interrogation and truly representative of the level of intra-patient heterogeneity. Depending on the platform used CTCs could be intact viable tumor cells that can be invaluable for downstream analyses, allowing interrogation of the DNA, RNA or at the protein level (58-61). In addition, recent work demonstrating the utility of CTCs in developing models of in-vitro and in-vivo (patient derived xenografts) drug sensitivity hold great promise (59,60).

Conclusions

Despite significant advances in our understanding of the biology of lung cancer and improved scope of personalized therapy the overall improvement in outcomes of patients with NSCLC has been modest. There were significant advances in liquid biopsy technologies to interrogate the clonal evolution and heterogeneity of tumors. Having effective diagnostic tools like liquid biopsies can help monitor tumor clonal evolution of tumors during treatment and will enable more rationale disease monitoring and sequencing of targeted therapy.

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Footnote

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

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