OncoTargets and Therapy

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REVIEW

Update on liquid biopsy in clinical management of non-small cell lung cancer

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Abstract: Lung cancer, a leading cause of cancer-related mortality, has a low rate of early diagnosis and a poor prognosis for advanced stages. Recent advances in further mastery of the biology of tumors promote the diagnosis and therapy, especially for non-small cell lung cancer (NSCLC). However, tumor tissue-based information is often not available in most cases due to the invasive and high risk nature of the tumor biopsy procedures. Liquid biopsy, based on the multiple liquid samples including circulating tumor cells (CTC), circulating tumor DNA (ctDNA), and tumor-derived exosome obtained from blood or urine as well as other body fluids, can also provide valuable tumor-related information, playing an important role in management of NSCLC in clinical practice. It is widely believed that concordance of detection for tumor by liquid samples in comparison with tissue biopsy for both early and advanced stage NSCLC patients is optimistic. We herein review the current and future clinical application of liquid biopsy, including early diagnosis and management of precise personalized treatment in lung cancer. The future directions of development for liquid biopsy are also discussed in this review.

Keywords: liquid biopsy, non-small cell lung cancer, early diagnosis, molecular targeted therapy, immunotherapy

Introduction

Lung cancer is the most common cause of cancer-related mortality in the world, with 154,050 related new deaths in America every year reported in 2018.[1,2] Extremely low rates of early diagnosis lead to most patients being diagnosed with advanced stage, and the 5-year relative survival rate remaining at only 18%, which contribute to the high mortality of lung cancer.[1] Therefore, it is important to promote pre-screening among the general population to detect lung cancer in earlier stages. A low-dose CT scan has been recommended in screening for lung cancer since 2011 as it can reduce the mortality rate of 20% from lung cancer and the mortality rate of 6.7% from any cause.[3] However, its clinical practice value is limited by the false positive caused by image detection, leading to unnecessary invasive operations in the healthy population.[3] Also, long-term annual CT scanning inevitably increases the risk of radiation exposure. There is an urgent need for more specific and less invasive biomarkers that can be used as complementary or alternatives to radiological approaches to better select the right risk cohort. Liquid biopsy, based on body fluids including plasma, urine, and other liquids, can detect tumor-related biomarkers to diagnose lung cancer earlier and safer.[4] Moreover, the combination of liquid biopsy and radiology examination would be able to increase the accuracy and cost-effectiveness of screening and early detection for lung cancer.

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submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/OTT.S203070

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The individual precise targeted treatment based on molecular classification has increasingly improved the overall survival of lung cancer, especially the NSCLC, significantly.[5,6] For example, EGFR tyrosine kinase inhibitors have increased the overall survival of patients with EGFR mutated significantly.[5] Except the molecular targeted therapy, immune therapy is also increasingly important among advanced lung cancer and promotes the outcomes of advanced NSCLC. The selection criterion for immune therapy is recommended to be based on the molecular status of PD-L1 expression.[7] Other potential factors, including tumor mutation burden (TMB), are also analyzed to predict the prognosis of patients receiving immune therapy according to the genomic profiles of the tumor.[8,9] Tissue biopsy was regarded as the "standard procedure" for molecular detection and was indispensable in decision-making concerning treatment for advanced NSCLC patients before 2016.[10] However, tumor tissue is often not available due to the invasiveness and the failure of getting enough tumor tissue for further detection of gene variations.[11] Especially for those targeted-therapy resistant patients, re-biopsy is extremely difficult due to the suboptimal clinical condition.[12] Liquid biopsy, characterized by non-invasiveness, easy accessibility, and good repeatability, was increasingly used to conduct molecular profiling testing and monitoring drug-resistance dynamically.[13,14] A large variety of surrogates in human liquid are available to make the individual therapy more efficientand precise.

In this paper, we will focus on clinical applications and future directions of liquid biopsy in management of NSCLC.

Early diagnosis of lung cancer by liquid biopsy

It has been determined that liquid biopsy is likely to detect tumor-specific biomarkers to diagnose lung cancer at early stage in previous research. Actually, different tumor-derived elements, including ctDNA, cfRNA, CTC, exosomes, and protein can be isolated from body fluids to reveal the molecular landscape of the tumor. In previous research, it was found that the level of plasma cfDNA in cancer patients is higher in comparison with healthy controls.[15] In addition, it was not only confirmed that the concentration of cfDNA in plasma was higher in NSCLC patients compared to benign lung tumors and healthy controls, but also demonstrated that cfDNA integrity obtained from Ct values of 100-bp qPCR products divided by ones in 400-bp, showed great power (91% sensitivity, 68.2% specificity) to discriminate NSCLC and benign lung tumors.[16] It was shown that mutations in ctDNA only has 50% sensitivity of early detection for lung cancer due to the fewer detectable amounts of ctDNA released by early-stage tumors, while proteins in plasma have also been described to be useful to detect and diagnose lung cancer at early stages. Therefore, an evaluation of a combination of 16 driver genes mutation in ctDNA and eight circulating proteins, including carcinoembryonic antigen (CEA), cancer antigen 125 (CA-125), cancer antigen 19-9 (CA19-9), hepatocyte growth factor (HGF), tissue inhibitor of metalloproteinases 1 (TIMP-1) protein levels, pro-lactin (PRL), osteopontin (OPN), and myeloperoxidase (MPO), called CancerSEEK, was conducted to improve the early detection for multiple cancers, including lung cancer. The results presented that CancerSEEK had the ability not only to diagnose the presence of relatively early cancers with higher sensitivity, 70% for median and 60% for lung cancer, but also to differentiate the original organ of cancers effectively.[17]

Non-coding RNAs in biologic fluids have been investigated to assess its potential to detect lung cancer at the early stage. MiRNAs are the most adequate cfRNA molecules in the fluids samples, and various studies have proposed this as a useful and non-invasive tool for cancer diagnosis. Different expression of a panel of miRNAs in sputum, including miR-145, miR-126, and miR-7, generated 90% sensitivity and specificity to distinguish NSCLC from the controls.[18] In addition to identifying malignant or benign, miRNAs in plasma are also used to distinguish the histological classification of lung cancer, whether it is small cell lung cancer or non-small cell lung cancer. Plasma panel A, consisting of six miRNAs (miR-17, miR-190b, miR-19a, miR-19b, miR-26b, and miR-375), demonstrated high power to discriminate the lung cancer from healthy donors, while plasma panel B, involving three miRNAs (miR-17, miR-190b, and miR-375) from panel A determined high diagnostic accuracy in distinguishing between small cell lung cancer (SCLC) and NSCLC.[19] This means that non-invasive biomarkers could play a crucial role in diagnosing as well as determining optimal treatment based on benign and malignant differentiation and histological judgment.

Epigenetic biomarkers, including cfDNA/RNA methylation, have gained increasing attention as a non-invasive material for lung cancer early diagnosis. Abnormal DNA

methylation was often found to be associated with cancer, while hypermethylation was usually discovered in tumor-suppressor genes and hypomethylation in proto-oncogenes.[20] It has been reported that DNA methylation can happen at an early stage of lung cancer and be used for the screening and early diagnosis of lung cancer. Methylation of SHOX2 and PTGER4 were demonstrated to be able to distinguish lung cancer from healthy control, with 91–98% area under curve (AUC).[21] Based on the specific enrichment of methylated fragments from cfDNA and its tumor-specific patterns, a high AUC of 0.971 was produced to discriminate between early stage lung cancer and healthy controls.[22] CfRNA methylation was demonstrated to mediate miRNA expression and cancer cell migration.[23] Serum miR-34b/c methylation was applied to diagnose malignant pleural mesothelioma,[24] and cfRNA methylation for early diagnosis of lung cancer needs further study to prove.

Metabolites, including amino acids, carboxylic acids, and tocopherols are often downregulated in cancer samples, which can be adopted to diagnosis cancer at an early stage. A classifier of nine serum metabolites allowed us to distinguish cancer with control samples with a sensitivity of 100% and specificity of 95%.[25] It has been proved that phosphatidylethanolamines (PE) can be used to distinguish benign and malignant nodules within a CT screening trial.[26]

Imaging examinations, like CT scans, were identified as a useful way to screen and detect lung cancer at an early stage. However, the application was limited by the higher false positive rate and radiation exposure. Given non-invasive biomarkers' wide applications in screening of lung cancer, a combination of those non-invasive biomarkers and radiologic screening, like a CT scan, is more valuable and acceptable than using the CT scan alone. A panel of three miRNAs selected from the TCGA database demonstrated a sensitivity of 81.2% to discriminate lung cancer from healthy controls, while two miRNAs from the panel combining the measurement of size of pulmonary nodules can reach 89.9% diagnostic sensitivity.[27] In addition to panels of miRNAs, autoantibody assays have also been determined to be capable of complementing CT scanning in lung cancer diagnosis as they can be found in plasma 5 years before the initial detection of lung cancer.[28] A panel of seven autoantibodies (7-AABs) was determined to have 61% sensitivity and 90% specificity in patients with solid and/or nodules ground glasses GGNs), which were higher than traditional biomarkers such as CEA. The positive predictive values (PPVs) in the 7-AABs panel and CT scanning were 85.2% and 69.0%, respectively. The combination of CT scanning and 7-AABs panel significantly elevated the PPV in comparison to a CT scan alone (95.0% vs 69.0%; $P<0.001$) or 7-AABs panel alone (95.0% vs 85.2%; $P<0.001$). Moreover, the false positive rates in patients with defined size and GGNs and/or nodules decreased significantly by combining CT scanning and the 7-AABs panel together.[29]

To summarize, those non-invasive biomarkers analyzed by liquid biopsy provide an efficient way to screen and find lung cancer at an early stage. Moreover, when those biomarkers are combined with imaging examination, there would be more lung cancers detected at early stages than before. Therefore, all of these deserve a wide range of applications in clinical practice.

Utility of liquid biopsy in management of molecule targeted therapy

One of the latest breakthroughs in NSCLC is represented by the molecule-targeted therapy in specific molecular status of this disease. Also, the most important targeted therapies are EFGR tyrosine kinase inhibitors (EGFR-TKIs) and ALK receptor tyrosine kinase (ALK-TKIs), which have improved the survival of advanced NSCLC significantly in comparison with chemotherapy.[5,6,30,31] However, drug-resistance is inevitable during the course of management of these targeted therapies.[32] The next-generation drugs able to overcome those resistance mechanisms have also been developed rapidly. According to the NCCN guidelines, testing gene variations is required before both choosing proper treatment for newly-diagnosed and drug resistant NSCLC patients.[10] However, many patients have no access to receive tumor biopsy due to the invasiveness of the procedure or the tumor tissue obtained is not sufficient for detection of gene alterations.[33] Especially for drug resistant patients, 20% of these have no access to successful re-biopsy.[34] Moreover, tumor heterogeneity makes the tumor biopsy in one site not able to cover the comprehensive genomic profiles and which can be complemented by plasma genotyping as it is circulating throughout the body and evenly distributed.[35] Finally, it is difficult to achieve dynamic monitoring during the treatment process due to the invasive nature of tissue testing. Previous researchers have determined that liquid samples, including plasma and urine, can be used as a surrogate for tissue to test gene variations,

and can dynamically monitor changes to analyze drug resistance earlier during the course of treatment, as part of the circulating nucleic acid in it is tumor-related and easy to be obtained and operated.[36–38] As previously mentioned, EGFR and ALK are the major targeted molecules for precise targeted therapy, and the majority of liquid biopsies involved research concerns these two molecules.

Effective detection of genotype to select proper patients for targeted therapy

Gene mutation based on tumor tissue-testing can predict tumor response to targeted treatment, and is often viewed as gold standard, while liquid biopsy can also provide an efficient way to analyze gene mutation for proper therapy when tissue biopsy is not available. Recent progress in the sensitivity and accuracy of DNA detection has enabled the genotyping of ctDNA, mostly consistent with genomic variations found in tumors. Since ctDNA has been discovered in the plasma of NSCLC patients, there were increasing numbers of researchers detecting gene variations via plasma: it was found for the first time that 79.7% of EGFR mutation in plasma can be detected in matched tumor DNA by the method of denaturing high-performance liquid chromatography (DHPLC) in advanced NSCLC patients.[39] Using a more sensitive amplification refractory mutation system (ARMS) to quantitate ctDNA in advanced NSCLC patients, it has been proved that plasma could be a suitable surrogate for EGFR mutation detection, since high concordance, specificity, and sensitivity have been achieved.[40] It was also recommended by the official organizations to detect the targeted molecular by cobas@ (a common method of ARMS) when tissue is not accessible.[10] Concordance between ctDNA and tumor DNA in terms of genetic detection is increasing with the advance in testing methods. In comparison with tumor biopsy, sensitivity of detection for EGFR exon19 deletions and exon21 L858R were 81.82% and 80.00% through droplet digital PCR assays, respectively.[41] Liquid biopsy can not only detect the gene mutations non-invasively, but also evaluate the prognosis for targeted therapy. Studies have demonstrated the EGFR mutation in plasma could predict outcomes independently for patients receiving the therapy of gefitinib, since the results of ORR and PFS, which were similar to previous results presented by tumor tissue.[42]

In contrast to EGFR, the applications of liquid biopsy in ALK mutations are limited. Almost all these tests were based on the NGS method because it was difficult to measure the multiple rearrangements through ctDNA with PCR-based methods. NGS reached an acceptable level of sensitivity of 79.2% and specificity of 100% for detection of ALK mutations in plasma ctDNA of newly-diagnosed patients with confirmed ALK rearrangements in their tissue biopsies.[43] Besides the optimal sensitivity, capture-based NGS can also detect rare novel ALK rearrangements like FAM179A-ALK and COL25A1-ALK in plasma ctDNA.[44] There are also other driver genes like MET, ROS1, and BRAF in NSCLC, but fewer studies existed to detect these genetic aberrations in peripheral blood samples. With the presence of various methods for liquid biopsy and the elevation in diagnostic accuracy of ctDNA, reliable technology allows for detection of multiple genes in parallel for an increasing number of gene targets identified as a therapeutic focus. Multiple genes variations can be analyzed with optimal sensitivity in plasma at the same time by using next generation sequencing (NGS). It has been found that overall sensitivity of alterations in plasma cfDNA can achieve 94% by using a NGS panel of 35 cancer-related genes.[45]

In addition to being consistent with tumor biopsy, some of the results discovered by ctDNA but not in tumor tissue were also likely to be demonstrated as meaningful in some cases. For example, drug resistant mutation T790M can be found in plasma but not in tumor, and the outcome of following therapy reflected the accuracy of this result provided by plasma ctDNA only.[46] It was believed that existing tumor heterogeneity generated such a discrepancy between ctDNA and tumor tissue DNA, which meant the individual biopsy of a single site could not cover the whole genomic profile of the tumor itself. Besides the analysis of targeted molecular, gene alterations detected by plasma ctDNA can also predict the outcome of therapy.

Dynamic follow-up to predict the efficacy and outcome of targeted therapy

Clinical use of dynamic evaluation of driver gene alterations qualitatively or quantitatively was able to predict the survival for targeted therapy. It is generally believed that the status or frequency of EGFR mutations in plasma is associated with therapeutic efficacy in people with positive sensitizing EGFR and receiving 1st EGFR-TKI. There were reports on the relationship between EGFR mutation status at baseline and the overall prognosis of EGFR-TKI therapy. It was proved by Zhu et al[47] that lower plasma EGFR mutation concentrations in baseline were correlated

with longer progression-free survival (16.23 months vs 8.43 months; *P*=0.0019). However, there also existed the opposite results. Scientists found that EGFR mutation (+) can predict a better progress free survival (PFS) than EGFR(-) in plasma (18.8 vs 9.4 months, *P*=0.003).[48] Patients with EGFR(+) in ctDNA superior PFS (12.6 vs 6.7 months, *P*<0.001) and OS (35.6 vs 23.8 months, *P*=0.028) were compared with those with EGFR(-) in ctDNA. Moreover, it was also shown that patients harboring high EGFR-mutated abundance (>5.15%) in ctDNA presented longer PFS than those having low EGFR mutated abundance (≤5.15%) in plasma (15.4 vs 11.1 months, *P*=0.021).[49] There are corresponding reasons accounting for these different results. For the former ones, the presence of EGFR mutation of higher allelic frequency suggested a heavy tumor burden compared to the negative and lower allelic frequency for EGFR alterations in plasma. The extensive tumor burden may lead to progress at a faster rate, which lead to a shorter survival for 1st EGFR-TKI in patients with EGFR(+) in plasma.[47] For the latter, the relatively high allelic frequency of intra-tumor EGFR mutant could suggest that TKIs-sensitive mutated clones accounted for the majority of whole tumor clones, and took a longer time to develop drug resistance compared to those harboring low frequency of intra-tumor EGFR mutations.[48,49] Nevertheless, the mechanism of ctDNA released into the circulatory system is not clear. The detection assays used in these studies to analyze the EGFR mutation qualitative and quantitatively were also different, which maybe influence the comparison among these results concerning the prognosis value of EGFR mutation. Therefore, the relationship between EGFR mutation in ctDNA and outcomes of EGFR-TKI still needs further exploration.

Dynamic following-up of the mutated genes such as EGFR during the course of targeted therapy appears to be associated with outcome of involved treatment. It has been observed that PFS was 10.1 months for patients with undetectable EGFR mutation in plasma after receiving EGFR-TKI for 2 months, while it was only 6.3 months for those with detectable EGFR mutation (HR=3.88, 95% CI=1.48–10.19, *P*=0.006).[50] A prospective study by Zhou et al[51] found two dynamic types of quantitative changes of L858R during the course of EGFR-TKI treatment, one that rose to its highest level (ascend type) while the other maintained its stable level (stable type) when disease progressed. They found that median PFS in the ascend type was higher than in the stable type (11.1 vs 7.5months, *P*=0.023). The methylated ctDNA has also been investigated to be related with the prognosis of EGFR-TKI. Salazar et al[52] proved that patients with methylated CHFR promoter survived for a shorter period when receiving EGFR-TKI as the second-line therapy, compared to conventional chemotherapy. Wang et al[43] also discovered that the rate of ALK rearrangements drops to 69.2% after treatment with Crizotinib. Decreased allele frequency of BRAF V600E by dynamic monitoring in plasma ctDNA was correlated closely to corresponding targeted treatment response.[53]

In conclusion, dynamic monitoring of quantitative and qualitative changes in variations is mainly for prediction of outcomes for targeted treatment in patients with positive driver gene alterations. It can also provide the chance of finding a drug resistant mechanism in advance during the process of surveillance.

Early finding and exploring mechanism of drug resistance in targeted therapy

There is no doubt that drug resistance usually happens after long-term use of targeted therapy. Many common mechanisms of resistance have been identified, including T790M for first and second line of EGFR-TKI and C797S for third line of EGFR-TKI. The selection for appropriate therapy after drug resistance is based on the best known of these mechanisms. However, many advanced patients have no access to re-biopsy for analysis of drug resistant mechanism due to the poor health condition. The merge of liquid biopsy enables these resistant mechanisms to be analyzed easily and earlier. Previous research demonstrated that 45% of patients with T790M could have this variation detected before progression of the disease.[54] It has been determined that T790M can be found in plasma ctDNA prior to radiological progression as much as 2–12 months earlier.[50] T790M can also be detected in urine ctDNA 3 months before disease progression confirmed by radiologic examination.[55] In addition to early detection, the concordance between cfDNA and tumor tissue in the analysis of T790M is also considerable to guide the next generation line of therapy. T790M was detected in cfDNA with sensitivity from 70–83% when compared with tumor re-biopsy.[56–58]

Moreover, patients with T790M(+) in plasma could predict an optimal survival for osimertinib. Research has also shown that patients with T790M positive in plasma have equivalent outcomes with patients positive by tumor biopsy after receiving osimertinib.[56] However, in the group of patients with T790M(+) in tumor tissue and receiving TKI treatment at the 2nd line or later, further analysis showed that T790M positivity in plasma would be a prognostic factor for the worse OS during

receiving the 2nd line or later TKI treatment after 1st PD, and the group with T790M positive in ctDNA had significantly shorter overall survival (OS) than the negative group (26.9 months vs NA, *P*=0.0489).[54] Dynamic monitoring of T790M can also be beneficial to guide an efficient management of treatment. The complete clearance of T790M during the treatment with osimertinib would predict a worse PFS (6.1 months vs 15.2 months).[59] Thress et al.[60] also found that the continual appearance of T790M after 6 weeks' treatment with osimertinib would predict a shorter PFS (5.5 months vs 10.9 months) and decreased ORR (35% vs 70%). These results suggest the emergence of pre-existing distinct resistant clones after drug resistance of osimertinib, which lead to a worse prognosis.

Similarly, resistant alterations in patients proceeding with osimertinib are continually emerging. MET amplification and C797S, the most common mechanism for drug resistance for third generation EGFR-TKI – osimertinib, were also discovered through analyzing ctDNA in plasma.[32,61] In addition to these, a growing number of mechanisms were found in ctDNA from multiple biologic liquid samples. Point mutations like EGFR G796/C797, L792, and L718/G719 were detected in the plasma of drug-resistant patients.[62,63] BRAF V600E has been demonstrated as a resistant mechanism via pleural effusion in patients found to be drug resistant with osimertinib.[64]

There was also evidence to demonstrate that resistant mutations of crizotinib appeared 2months before radiographic disease progression.[65] The drug resistant mutation for ALK-TKI, including L1196M and G1269A, can also be analyzed by ctDNA in biologic fluids. Also, dynamic changes in these resistant mutations are also consistent with the efficacy of the 2nd line of ALK-TKI.[66] Other mechanisms, like I1171T and V1180L mutations, were also detected in ctDNA by NGS.[67] Although acquired resistance mutations for ALK-TKI are not required to change primary therapy into another different ALK-TKI forwards, it is important to determine the next generation of TKIs based on the distinct mutations. When the re-biopsy is not available, the genomic profile obtained from ctDNA by NGS panel is preferred as it no only can provide the known mechanisms but also provide the other unknown and potential mechanisms of resistance for which proper treatment may be acquired from clinical trial or expanded access.

Evaluation for immunotherapy via liquid biopsy

In recent years, immune checkpoint inhibitor (ICI) therapy has achieved great success in multiple malignant solid tumors, especially for NSCLC. It not only revolutionizes the treatment strategy for NSCLC, but also prolongs the survival for advanced patients. However, the ORR of ICI therapy only remains at 20% or less in second line treatment. Identification of immune-related biomarkers applied for diagnosis, prognosis, and monitoring during immunotherapies is urgently required. How to choose patients appropriately for ICI therapy has become a hot issue nowadays. Researchers have shown that the high expression of PD-L1 protein presented in tumor cells was determined to be related with efficacy of ICI treatment.[68] Easy access to body fluids makes it preferred to tumor biopsy concerning the detection of biomarkers as it has the ability to cover molecular landscape of tumors non-invasively and dynamically. And the CTCs could represent a surrogate for analysis of PD-L1 expression. It was found that expression of PD-L1 on the surface of CTCs both at baseline and 3 months after treatment were found to be associated with poor patients' outcome.[69] Also, PD-L1 expression in CTCs and peripheral WBCs were correlated with PD-L1 expression in tumor tissue, with concordances of 93% and 80%, respectively.[70] Ilie et al [70] also confirmed that analysis of PD-L1 on CTCs is highly feasible, but the expression of PD-L1 on CTCs has no significant impact on the prognosis for immunotherapy. Therefore, there remain conflicting results in different cohorts for the lack of standardized methodology for detection on PD-L1 expression.[71] Further exploration needs to illustrate the relationship between PD-L1 expression on CTCs and prognosis for immunotherapy.

In addition to PD-L1 expression on tumor cells, tumor mutation burden (TMB), measured by mutations per megabase (mb) with hybrid based NGS, might be another response biomarker for outcomes of immunotherapy. An analysis from the trial of CheckMate-026 determined a relationship between high tissue-based TMB and better clinical benefit with nivolumab in NSCLC in the first-line.[9] Aaron et al[8] also provided evidence to show that high TMB (≥20 mutations/mb) predicted better outcomes, including ORR (46% vs 14%; *P*=0.0025), PFS (10 vs 2.2 months; *P*=0.0005), and OS(11.1 months vs not reached, *P*=0.0557) in comparison with low (1–5 mutations/mb) to intermediate (6–19 mutations/mb) TMB after receiving treatment with anti-PD1/PD-L1 monotherapy. However, obtaining the tumor tissue needed for mutations detection is always a challenge facing the advanced NSCLC patients. Some researchers have determined that blood also can be considered as a source of molecular testing. The number of mutations in variants of unknown

significance (VUS) in plasma ctDNA was shown to be related with outcomes of checkpoint inhibitor-based immunotherapy. Khagi et al[72] presented that PFS and disease control rate (DCR) were improved in patients with VUS >3 compared to those with VUS ≤3. Also, responders had a higher median PFS than non-responders with VUS >3 (23 vs 2.3 months, *P*=0.0004). With the deep exploration in alteration detection by cfDNA, calculation of TMB from blood (bTMB) cfDNA has been developed through deep sequencing. Research has shown that bTMB is correlated to clinical prognosis for immune checkpoint inhibitor therapy. Analysis found bTMB analyzed by the NGS panel with a robust cut-point ≥16 performed in POPLAR samples and confirmed in OAK samples predicted an increased PFS benefit from atezolizumab. Also, bTMB was determined to be associated with tissue TMB (tTMB) not the PD-L1 expression.[73] All of these suggest that the value of this bTMB assay to conduct molecular diagnostic and therapeutic algorithms for immunotherapy is promising, and the prospective validation of bTMB assay in the first line of NSCLC is warranted.

Dynamic monitoring of identical molecules was also crucial during the process of immunotherapy. The changes of ctDNA levels predict the efficacy of immunotherapy. It was presented that PFS was longer in ctDNA responders (>50% decrease at mutant allele fraction in comparison with baseline) than non-responders (205.5 vs 69 days; *P*<0.001).[74] It has also been proved that the patients with ctDNA detected at 8 weeks after ICI means poor PFS and OS outcomes than patients with undetected ctDNA at the same time.[75] An imaging scan is insufficient to evaluate the efficacy and analyze the drug resistance, since the pseudo-progression-appearance of larger or new tumor lesions often happen before the tumor actually shrinks. Evidence has been provided to show that dynamic monitoring of ctDNA can be applied to distinguish real progression of disease from pseudo-progression, since the ctDNA levels decrease rapidly in pseudo-progression, while patients with real progression shows significant increases in ctDNA levels.[76] Monitoring the efficacy and disease through liquid biopsy enables the ICI more efficiently and successfully.

More broadly, an integrated and comprehensive bio-sampling has been approached to find non-invasive biomarkers for administration of immunotherapy. However, the way to detect expression of PD-L1 on CTCs still remains the big issue, and the method to measure the bTMB is distinct from tTMB and has not come to a conclusion yet. Standardization of methods and rigorous validation are required to better understand the relationship between biomarkers and immunotherapy.

Future directions

Liquid biopsy is an increasingly developing field in oncology, especially for lung cancer. However, there still remain some challenges for the sensitivity and utility of liquid biopsy in clinical practice, as the percent of circulating tumor related molecules is scarce. The accurate mechanism of the releasing of ctDNA has not been revealed exactly, and the percentage of ctDNA in circulating free DNA (cfDNA) is often as low as 1% in most cases. Therefore, the technology needed to evaluate and analyze the molecular profiles in liquid biopsy must be sensitive and standardized. With the development of accurate detection methods such as Next-Generation Sequencing (NGS); Magnetics (BEAMing); and digital driplet PCR (ddPCR), liquid biopsy has made it possible to detect the driver gene mutations at extreme rarity and cover the cancer-related driver genes as much as tumor tissue can.[36,77,78]

Although plasma is the most popular fluid sample for liquid biopsy, it is not always preferred in all cases. There are also many other specimens available, like urine, sputum, cerebrospinal fluid (CSF), or pleural effusion, to address liquid biopsy under different situations.[79,80] Easy access to liquid samples has also ensured the dynamic monitoring of the efficacy and drug resistance during the process of treatment.[50,81] Each of these modalities is able to present novel diagnostic information, and their role of exploration is highly valued.

Tumor heterogeneity is another issue needed to be considered during the whole management of lung cancer. A single type of sample, whether tumor tissue or liquid fluid, might not be able to cover all relevant distinct subclones. Various mediums, including ctDNA, CTC, and exosomes, allow for tumor analysis from different aspects. A variety of biologic liquid samples are different and complementary to illustrate the molecular profiles comprehensively. Here, we summarize the most important aspects of liquid biopsy which would be progressed in the future.

Advanced methods developed for liquid biopsy

Various analytic methods have been developed to identify molecular alterations through liquid biopsy. Sensitivity is the most important factor to be considered, as the percentages of tumor related ctDNA vary from patient to patient and are often extremely limited in most cases. ddPCR, an absolute

quantification assay of molecular identification, makes the concordance of detection of mutation skyrocket in comparison with tumor biopsy.[41,82] It was determined that ddPCR was more sensitive than conventional PCR based assays through a technology to part the sample to a single droplet with 1 or 0 DNA molecules to be amplified. The results were confirmed by analyzing the signal from each positive or negative droplet.[83]

In addition, the covered range of molecules to be detected is another key factor to be measured. As we all know, classic PCR-based assays are only able to target the known driver mutations. It is recommended that cobas@ test the exon 19 deletions, L858R and T790M mutations from ctDNA. Recent studies have frequently applied more accurate analyses both in depth and breadth to cover tumor-related driver gene mutations as much as possible, from a panel of targeted regions of exons or introns in various genes, whole exomes sequencing, to whole genomes sequencing. Especially for drug resistant mechanism exploring, only NGS can make it possible to detect the potential mechanisms. A prospective study performed by Thompson et al[36] showed that over 50 therapeutically targetable driver mutations and 12 resistance mutations can be detected simultaneously in ctDNA by NGS, and the unique mutation identified can also guide an effective therapy when tissue biopsy is not feasible. With the emphasis on co-mutant variations, along with therapy-targeted molecules including EGFR and ALK, NGS enables comprehensive evaluation of precise treatment with the detection of multiple genes alterations at the same time.[84] Especially with additional barcoding technology, the sensitivity would be increasing to lower the limit of mutation frequency for detection.[85,86] Many researchers and statements recommend the NGS supporting the use of NGS in clinical practice.[87,88] Except for the developments in methods aimed at assay detection, more efforts have been made to improve the efficacy of ctDNA accumulation in liquid samples. Mouliere et al[89] revealed the size difference between non-mutant and mutant DNA by a genome-wide and pancancer scale. Enrichment of ctDNA exists in fragment sizes between 90 and 150 bp. Choosing fragments between 90 and 150 bp increases the detection of alterations, including the actionable mutations and copy number variations, with a area under the curve (AUC) >0.99 in comparison with AUC <0.80 without fragmentation selection.

Various mediums available for liquid biopsy

At present, the most common and mature biologic element for liquid biopsy is ctDNA, which is released into blood circulation through apoptosis or necrosis by cancer cells[90,91] and is widely used in many aspects of clinical diagnosis and treatment in NSCLC.[54,92] There are also many other biologic mediums available for liquid biopsy. Circulating tumor cells (CTCs) in the bloodstream were released from primary and metastatic tumor sites and have been demonstrated to be used to screen for lung cancer, detect genotyping of individual tumors, and be associated with the outcome of treatment in NSCLC.[93,94] The tumor-derived exosome, a new medium used for liquid biopsy, is secreted by living cells and contains nucleic acids including DNA and RNA which might reflect the tumor genomic profile and correlate with tumor dynamic in cancer patients.[95–97] Except those common mediums, there are also some optimal elements including cfRNA and circulating proteins available for liquid biopsy to better guide the diagnosis and treatment selection.[17,98] All of these accurate detection methods, along with the easy-to-obtain body fluid samples and those abundant mediums, will make liquid biopsy an increasingly trendy approach in the clinical management of lung cancer.

CTCs, formed by cell detachment from the primary tumor mass, and presented in the blood of many solid cancer patients, have also ever been prevalence of accurate diagnosis, prognostic, and predictive evaluation of treatment in NSCLC. Although detection of CTCs requires complex and sensitive method due to the difficulty in capturing the CTCs in circulation,[99,100] the advance in technology also makes an increasing understanding of the potentials of CTCs in management of NSCLC.

Previous study provided evidence to show that CTCs detection can act as diagnostic markers in NSCLC. CTCs labeled by a folate receptor (FR) conjugate presented a sensitivity of 73.2% (67.2% in stage I) and specificity of 84.1% to diagnose NSCLC. Moreover, CTCs also have the potential to detect genetic alterations with the accurate assay. It has been demonstrated that 92% of expected EGFR activating mutation can be detected by CTC before the course of treatment.[101] Furthermore, CTCs provide another effective way to detect fusion gene identification through liquid biopsy. Tan et al[102] have examined the use of FISH to analyze EML4-ALK rearrangements in comparison with matched tissue biopsies, and demonstrated 100% sensitivity. It is believed that CTCs are also capable of detecting the resistant alterations in advanced NSCLC. The agreement of detection for T790M was 74% between CTCs and tumor biopsy.[35] Extra T790M and MET amplification were identified in CTCs, but not in tumor biopsy

from patients with PD in 1st EGFR-TKI.[103] As mentioned previously, analysis of single-site tissue biopsy cannot cover the overall genomic profile related to treatment due to the inter-tumor heterogeneous.[104] The same as ctDNA, CTCs can also reduce the difference in genomic profiles caused by tumor heterogeneity, as genomic analysis from a few CTCs included the overall SNV/INDEL profiles, which are shown in the different metastatic tumor sites during the course of treatment.[105] Also, ctDNA combined with CTC have been proved to be complementary for assessment of acquired drug-resistance to first-line EGFR-TKIs and may increase the number of patients receiving the next line precise therapy.[103] As mentioned above, CTCs also provided a convenient way to evaluate the PD-L1 expression to select the proper candidates for immunotherapy. However, the rather low detection rate of CTCs limits its application for early diagnosis and variation detection. With the advance in detection methods, CTCs will be improved to a wide range of applications in clinical practice.

As a new surrogate to tumor tissue, a tumor-derived exosome has been identified that the nucleic acid in which is more stable and abundant in analysis of genetic alterations based on which seems to be more promising. It has been revealed that miRNAs isolated from exosomes enable us to distinguish between lung cancer patients and healthy individuals with a specificity and sensitivity of 92.3% and 80.3%, respectively.[106] The detection for common EGFR, KRAS, and BRAF mutations has higher sensitivity in plasma exoNA (exosome DNA and RNA) by NGS compared with testing of plasma ctDNA by ddPCR.[107] EGFR T790M mutation was screened for both in exoNA and ctDNA of advanced NSCLC in both retrospective and prospective cohorts (N=210), and achieved 92% sensitivity, which is higher than in historical cohorts using ctDNA alone, and decreased the rate of unnecessary re-biopsy from 42% to 8% after getting drug resistance.[108] Therefore, exosomal DNA and RNA may be used to detect mutations and explore the mechanism of resistance in NSCLC patients for its high concordance with tumor biopsy.

Circulating-free RNA has recently emerged as an important role in cancer diagnosis and response to tumor therapy. As mentioned previously, miRNA in plasma or other biologic samples can be used as biomarkers to diagnose lung cancer with accuracy. It was determined that cfRNA has the potential to predict outcomes for various therapy in NSCLC. Li et al show that increased levels of miR-660-5p in plasma after crizotinib treatment could predict a good tumor response (*P*=0.012).[109] Moreover, a special type of cfRNA, called tumor educated platelets (TED) related RNA and generated by confrontation between platelets and tumor cells, presented as a suitable source of non-invasive detection of cancer. A panel of RNAs was selected from platelet RNA-sequencing libraries to discriminate the early stage NSCLC and late stage NSCLC with accuracy of 81% and 88%, respectively.[98] The presence of cfRNA also enables the better examination of ALK rearrangements with the sensitivity of 65% sensitivity and 100% specificity by PCR based assays.[65] Collectively, these evidences suggest that cfRNA may provide a robust biologic material for liquid biopsy-based diagnosis and treatment selection.

Various mediums available for liquid biopsy needs further prospective explorations and validations to determine the appropriate ones for practical situations. Particularly, the most promising biomarkers for cancer screening and the most consistent genomic profiles compared with tumors seems to be the most important factors when considered.

Multiple liquid samples feasible for liquid biopsy

Although plasma is the most popular fluid sample for liquid biopsy, there are also many other specimens available like urine, sputum, cerebrospinal fluid or pleural effusion can be obtained to address it as ctDNA and other mediums can also be found in those samples. The sensitivity of EGFR mutation in urine can reach approximately the same level as plasma test when tissue result as a reference,[58] while the combination of plasma, urine, and sputum can increase the concordance of driver gene mutations in comparison with plasma only.[110] The utility of the ctDNA or CTC from cerebrospinal fluid (CSF) make it possible to study the genetic alterations, and monitor treatment response as it is difficult to test the tumor site in patients with brain metastasis. CTCs are more likely to be found in CSF than in peripheral blood which provided an effective way to diagnose brain metastases in NSCLC and genetic profiles detected by CSF-CTCs were highly concordant with which in the primary tumor (17/19, 89.5%).[111] Moreover, it has been illustrated that ctDNA in the CSF is more representative than plasma ctDNA and cellular CSF DNA since driver gene mutations including EGFR can be detected in all ctDNA from CSF, and the allele fractions is much higher in CSF ctDNA than in the other two media.[79] Husain et al[112] also indicated that driver gene mutations identified by ctDNA from ascites and pleural effusions may

also present additional useful information not detected by tumor DNA and plasma cell-free DNA genomic profile to guide effective therapy in advanced NSCLC. This evidence was provided to reveal that unique biologic fluid samples are distinct in genomic profiles and should be applied into matched situations properly for the tumor heterogeneity.[22]

In spite of the appearance of considerable medium and samples for liquid biopsy, ctDNA in plasma is still the leading choice for the detection of biomarkers due to the easy access and appreciable concordance in comparison with the standard process – tumor biopsy. In order to adapt the liquid biopsy to practice efficiently, distinct liquid samples should be considered at different situations, and the differences in characters between various liquid samples needs to be clarified clearly.

Conclusion

The clinical utility of liquid sample-derived biomarkers to detect early cancer, predict tumor response and explore therapy resistance are certainly wanted and urgently required. Liquid biopsy, as an alternative way to analyze variations, not only provide a non-invasive way to detect alterations to diagnose lung cancer in advance, but also complement the result detected by tissue biopsy to allow more cancer patients to receive the precise therapy. With the increasing accurate technology, more liquid samples and mediums are available to perform comprehensive diagnosis and provide accurate treatment management for both early and advanced lung cancer.

Disclosure

The authors declare no potential conflicts of interest in this work.

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