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Emerging platforms using liquid biopsy to detect *EGFR* mutations in lung cancer

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Summary

Advances in target therapies for lung cancer have enabled detection of gene mutations, specifically those of *EGFR*. Assays largely depend on the acquisition of tumor tissue biopsy, which is invasive and may not reflect the genomic profile of the tumor at treatment due to tumor heterogeneity or changes that occur during treatment through acquired resistance. Liquid biopsy, a blood test that detects evidence of cancer cells or tumor DNA, has generated considerable interest for its ability to detect *EGFR* mutations, however, its clinical application is limited by complicated collection methods and the need for technique-dependent platforms. Recently, simpler techniques for *EGFR* mutant detection in in urine or saliva samples have been developed. This review focuses on advances in liquid biopsy and discusses its potential for clinical implementation in lung cancer.

Keywords

Liquid biopsy; saliva; lung cancer; *EGFR*; target therapy

Lung cancer, *EGFR* mutations, and liquid biopsy

Lung cancer is the leading cause of cancer-related deaths worldwide. It is estimated that in 2015, approximately 158,040 deaths will be due to lung cancer in the USA alone [1]. In recent years, we have developed a better understanding of the molecular abnormalities that define lung cancer subsets and have developed better therapeutics. In particular, the discovery of driver oncogenes has led to therapies that target specific gene alterations responsible for aberrant oncogenic signaling and proliferation. The most important driver oncogene in lung cancer is epidermal growth factor receptor (*EGFR*), a component of the molecular signaling pathway that controls the proliferation and growth of cells. Since the discovery of *EGFR* mutations in lung cancer ten years ago, *EGFR*-targeted therapies have become a key component of lung cancer therapy and are superior to chemotherapy in terms of overall response rate, progression-free survival, and quality of life for patients with untreated non-small cell lung cancer (NSCLC) with sensitizing *EGFR* mutations [2–6].

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EGFR mutation analysis is performed on tumor cells from biopsy or cytology specimens obtained from bronchoscopy, computed tomography-guided biopsy, surgical resection, or drainage from malignant pleural effusions. However, methods other than surgical resection for sampling tumor tissue have significant limitations because the harvested tumor tissue represents a single snapshot in time, the tissue is subject to selection bias due to tumor heterogeneity, and it is difficult to obtain enough DNA for *EGFR* mutation analysis if the number of tumor cells is insufficient [7]. Because of the invasive procedure and the progressive development of drug-resistant *EGFR* mutations, the initial detection and continuous monitoring of *EGFR* mutations is a substantial challenge.

Liquid biopsies are non-invasive blood tests that detect circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) which come from passive release from apoptotic or necrotic cells or actively secreted exosomes shed into the blood. Unlike traditional biopsies, liquid biopsies are non-invasive and can capture multiple sites of tumor growth simultaneously. Ultra-sensitive methods to capture CTCs or methods that permit the sensitive analysis of cell-free ctDNA allow testing for *EGFR* mutations. Combining these ultra-sensitive methods with the detection of *EGFR* mutations in urine and saliva means that liquid biopsies can complement tissue biopsies, especially when the number of tumor cells is insufficient to allow for DNA extraction. In addition, liquid biopsies enable clinicians to adjust therapeutic strategies in a timely fashion, consequently improving the clinical outcomes of *EGFR*-targeted therapy.

Circulating tumor DNA (ctDNA)

The correlation between cell-free nucleic acid levels (including circulating free DNA or cfDNA), which were first discovered in 1948, in plasma and cancer was first reported in 1977 when it was first demonstrated that plasma levels of cfDNA were much higher in cancer patients than in healthy controls [7]. Moreover, variations in ctDNA level and microsatellite changes are correlated with the clinical status of unaffected individuals or early relapse in patients with lung cancer who were receiving surgical resection during follow-up [8]. CtDNA genotyping is more efficient than many CTC capture technologies. CTCs must be separated from the more abundant hematologic cells in the blood, which requires a significant laboratory facility, while ctDNA genotyping methods only require special handling. CTCs in circulation encounter substantial apoptosis and are fragile, leading to variability between different CTC assays. In contrast, current ctDNA technologies are sensitive enough to detect tumor-specific somatic mutations, even if the ctDNA fragments represent only a minority of all DNA fragments in the circulation.

Source and biology of ctDNA

Apoptotic and Necrotic Cells—Tumor cells constantly release cfDNA into circulation by a variety of mechanisms (Figure 1) including rapid tumor cell turnover, apoptosis, and necrosis. The double-stranded ctDNA in plasma can be separated and visualized by gel electrophoresis, with the fragments ranging between 180 and 1000 bp likely formed by apoptosis. In contrast, DNA released by necrosis is non-specifically digested and smears with a fragment size of about 10,000 bp [9].

Extracellular Vesicles—In addition to passive release from apoptotic or necrotic cells, living tumor cells can also actively secrete ctDNA. Cells release extracellular vesicles (EVs) into the extracellular environment. EVs can originate from the endosomal or plasma membrane and are called exosomes and microvesicles, respectively [10]. EVs play important roles in intercellular communication by transferring cytosolic proteins, lipids, RNA, and DNA between cells [11]. Thus, DNA actively transferred via EVs might be from a tumor. In addition, EVs also carry large (>10 kb) double-stranded DNA such as mutated KRAS, p53, and *EGFR* sequences [12,13]. EV nucleic acids are attractive candidates for markers since EVs are stable and protect the contained nucleic acids against degradation and denaturation. Furthermore, EVs can be collected from complex plasma samples or in cancer-associated body fluids such as pleural effusion, ascites, saliva, and urine, via various methods of isolation such as ultracentrifugation and immunoaffinity isolation based on specific EV surface markers. The co-isolation of exoRNA and cfDNA from the plasma of cancer patients allows for a comprehensive analysis of oncogene mutations [14].

Assays for EGFR mutations using ctDNA in plasma samples

Since ctDNA often represents only a small fraction (< 1.0%) of total cfDNA, detection of ctDNA remains challenging [7]. Thus, direct sequencing approaches such as Sanger sequencing or pyrosequencing are not suitable for detecting *EGFR* mutations using ctDNA. Many of the currently available ctDNA genotyping methods require only minimal special handling and do not depend on special equipment. These technologies are sensitive enough to detect tumor-specific somatic mutations in circulation with plasma DNA from normal cells. Several different types of PCR-based assays have been developed for ctDNA genotyping, including the amplification-refractory mutation system (ARMS)/Scorpion assay, mutant-enriched PCR, peptide nucleic acid (PNA)-mediated PCR, PNA-locked nucleic acid (LNA) PCR clamp, BEAMing (beads, emulsions, amplification, and magnetics), and digital PCR. In addition to these PCR-based assays, high-resolution melting (HRM) analysis, denaturing high performance liquid chromatography (DHPLC), mass spectrometry genotyping, and next generation sequencing (NGS) have also shown potential in detecting *EGFR* mutations from plasma (Table 1).

ARMS/Scorpion assay—ARMS is a reliable method for detecting single base mutations or small deletions on the basis of using of sequence-specific PCR primers, which allow the amplification of only the target allele. Because Taq DNA polymerase can effectively distinguish a match from a mismatch, especially at the 3′ end of a primer, specific mutated sequences are selectively amplified. The amplification proceeds at full speed with a fully matched primer, however only low-level background amplification occurs when the 3′ base is mismatched. Scorpion is a tailed primer containing a PCR primer covalently linked to a probe. The fluorophore in the probe interacts with a quencher, which is also incorporated in the probe, and reduces its fluorescence. The fluorophore and quencher become separated when the probe binds to the amplicon during PCR, resulting in an increase in fluorescence [15]. Specific Scorpion ARMS primers have been designed and optimized for detecting various *EGFR* mutations. ARMS is one of the most widely used methods for ctDNA based *EGFR* mutation assays [16–19].

Mutant-enriched PCR—Mutant-enriched PCR is a sensitive assay that can detect one mutant gene among up to 10^4 copies of the wild-type gene. This sensitivity is achieved by selective PCR amplification of mutant gene sequences with a two-stage procedure. The first stage is amplification of both mutant and wild-type sequences, followed by selective digestion of the wild-type DNA with thermostable restriction enzymes during PCR. A subsequent step then amplifies the undigested fragments, which are enriched for mutant sequences [20]. Thus, mutant-enriched PCR can detect the mutation status directly and can be combined with other methods to improve detection sensitivity. This method has been shown to detect *EGFR* mutations in serum from patients with NSCLC [21,22].

PNA-mediated PCR and PNA-LNA PCR clamp—The PNA-mediated PCR and PNA-LNA PCR clamp assay uses peptide nucleic acid (PNA), a synthetic DNA analog in which the phosphodiester backbone is replaced by a peptide-like repeat, which acts as both a PCR clamp and sensor probe [23]. The PNA probe can selectively inhibit the amplification of wild-type DNA but not mutant DNA because the binding between PNA/DNA is stronger than that between DNA/DNA with extremely high specificity. Moreover, PNA oligomers are not recognized by DNA polymerases and therefore not utilized as primers in the subsequent PCR reaction. A fluorescent tag allows the PNA probe to generate unambiguous melting curves for real-time monitoring. Oligonucleotides containing PNA-locked nucleic acid (LNA) hybridize to complementary DNA with higher affinity and have higher melting temperature compared to oligonucleotide DNA. Thus, shorter LNA probes can be used as allele-specific tools in genotyping [24]. In PNA clamp PCR, amplification of the wild-type sequences is suppressed, and only amplification of the mutant sequences is enhanced. In combination, LNA probes selectively detect mutant sequences in the presence of wild-type sequences, which largely increases the specificity of the assay. Because PNA clamp primers have wild-type sequences and LNA probes have mutant sequences, they are located in the same position. PNA clamp primers competitively inhibit mutant LNA probes to bind to the wild-type and further increase the specificity of detection. In this way, *EGFR* mutations can be detected in the presence of 100–1,000-fold higher wild-type *EGFR* background. Because of its high sensitivity and specificity, the PNA-LNA PCR clamp is suitable to detect *EGFR* mutations in histological samples, such as surgical specimens, and in cytological samples, such as sputum and pleural effusions [25,26].

BEAMing—BEAMing was developed on the basis of four principal components: beads, emulsion, amplification, and magnetics. BEAMing relies on single-molecule PCR at a massively parallel scale so that millions of individual DNA molecules can be assessed with standard laboratory equipment, similar to NGS technologies [27]. Briefly, BEAMing starts with conventional PCR of a predetermined locus. The resulting product is added to millions of oligonucleotide-coupled beads in oil. An emulsion is created in which most of the beads bind to only a single DNA molecule and is followed by a second round PCR. After the magnetic capture step, single-base primer extension or hybridization with mutant-specific probes is performed with different fluorescent probes. Finally, detection and quantification is performed by flow cytometry analysis. Moreover, specific variants can be isolated by sorting and used for further analysis. Because BEAMing analyzes one allele at a time, it is highly sensitive and can detect rare mutant alleles. BEAMing can also detect mutations (such as

PIK3CA and *EGFR)* using ctDNA samples [28,29]. Furthermore, this concept is used in some digital PCR platforms.

Digital PCR—Digital PCR can be used to directly quantify and clonally amplify nucleic acids [30]. A single DNA template is amplified from minimally diluted samples, resulting in amplicons exclusively from one template. Different fluorophores are used to distinguish different alleles or for further analysis by sequencing. Thus, the exponential analog nature of conventional PCR is transformed into a linear digital signal suitable for statistical analysis of rare events such as mutant ctDNA. In addition, because only small amounts of DNA are needed, samples from saliva or urine can be used. Digital PCR has been used to quantify *EGFR* mutations in clinical specimens and is a promising molecular diagnostic tool with high sensitivity and specificity [31,32].

High-resolution melting (HRM) analysis—HRM analysis is a powerful technique for the detection of mutations, polymorphisms, and epigenetic differences in double-stranded DNA samples. The HRM process involves precisely warming DNA amplicons so that the two strands of DNA separate when the melting temperature of the amplicon is reached. HRM analysis monitors this melting process in real-time by using fluorescent dyes that bind specifically to double-stranded DNA. These dyes fluoresce brightly when they are bound and have reduced fluorescence in the absence of double-stranded DNA. The melting temperature is influenced by several factors such as the length, GC content, and sequence of the DNA. Thus, differences in DNA sequences of various mutants have distinct HRM signatures. HRM has been used to screen for *EGFR* mutations in serum samples for patients with NSCLC [33]. However, because large amounts of DNA are required, its application in ctDNA based assays is limited.

DHPLC—Denaturing high performance liquid chromatography (DHPLC) identifies mutations by detecting differences between heteroduplex formation by wild-type/mutated DNA strands and homoduplex formation by two wild-type DNA strands. Heteroduplex and homoduplex molecules can be separated by ion-pair, reverse-phase liquid chromatography on a special column matrix with partial heat denaturation of the DNA strands. To increase specificity for *EGFR* mutation analysis, mutations in exons 18–21 can be analyzed in combination with the DNA endonuclease SURVEYOR assay, which selectively cleaves the mismatched heteroduplex [34]. For these analyses, DNA can be prepared from both frozen and formalin-fixed, paraffin-embedded tumor specimens. A partially denaturing HPLC assay is a useful approach for the routine detection of *EGFR* variants due to its high sensitivity and low detection limits for minority alleles [35].

Mass spectrometry—Mass spectrometry combined with base extension after PCR allows for the detection of ctDNA with single base specificity and single DNA molecule sensitivity [36]. In this assay, DNA is amplified by PCR and then linearly amplified with a base extension reaction, which is designed to anneal to the region upstream of a mutation site. A few bases are added to the extension primer to produce different extension products from wild-type and mutant DNA. Mass spectrometry has recently been used to detect *EGFR* mutations for which the detection rate was around 60% [37,38].

Next generation sequencing (NGS)—Recently, there has been a shift from automated Sanger sequencing to NGS for genetic analysis [39]. NGS consists of a number of methods including template preparation, sequencing and imaging, and data analysis. The combination of specific protocols determines the data output as well as the quality and cost of different NGS platforms [40]. NGS has been introduced into clinical analysis to detect oncogenic mutations and has been developed into streamlined commercial products with targeted panels that include the main genetic alterations with predictive value, including *EGFR* mutations [41-43]. In addition, due to its sequence based backbone and high sensitivity, NGS is suitable for identifying uncommon *EGFR* mutations in ctDNA samples.

Circulating tumor cells (CTCs)

Circulating tumor cells (CTCs) originate from solid tumors and are involved in hematogenous metastatic spread. CTCs are shed from primary cancers, migrate to distant sites, and establish other foci of disease even at an early stage [44]. The first description of circulating tumor-like cells was made in 1869 during the autopsy of a metastatic cancer patient [45]. CTCs occur at very low concentrations (1–10 cells per 10 mL in the peripheral blood of most cancer patients), which is a challenge for any analytical platform. For several decades, many translational and clinical lung cancer research projects have been conducted on CTCs. However, only recently has the development of modern equipment permitted the reliable capture and characterization of CTCs [46]. Studies of CTCs have demonstrated that they are heterogeneous, which emphasizes the need for multiplex approaches to capture relevant CTC subsets. Compared with other minimally invasive assays, the main advantage of liquid biopsies of CTCs is their potential in clinical and biological applications. In several clinical studies, the number of CTCs was used as a biomarker for prognostic stratification, for the evaluation of disease response during therapy, to identify patients with early metastasis, and during follow-up in order to detect relapse [47,48]. Compared to ctDNA, CTCs can be used in a functional assay since *ex vivo* cultures of CTCs can be used to individualize testing of drug susceptibility [49]. The molecular characterization of CTCs can help to facilitate the detection of biomarkers for targeted therapy for personalized cancer treatment.

Platform of CTC enrichment

The platform of CTC enrichment includes a panel of advanced technologies based on the specific biological or physical properties of CTCs that distinguish them from surrounding normal hematopoietic cells. Immunomagnetic assays (CellSearch) and microfluid assays (CTC-Chip) are based on affinity binding. Antibodies that bind to cell surfaces that are expressing specific antigens are used to capture epithelial cell adhesion molecule (EpCAM) positive cells (positive selection), or to remove cells expressing the leukocytic antigen CD45 (negative selection). EpCAM is a pan-epithelial marker and is abundantly expressed on the surface of tumor cells of epithelial cell origin. However, aggressive tumor cells may undergo epithelial-mesenchymal transition and lose the expression of epithelial markers, which can disturb enrichment when using the EpCAM antibody-based technique [50]. Other CTC capture platforms are based on physical properties such as the size, density, and electrical properties of CTCs when compared with erythrocytes and leukocytes. Due to limitations in

enrichment and the elimination of contaminant leukocytes, the application of methods based on physical properties to detect *EGFR* mutations in lung cancer remains rare. The current strategies for CTC enrichment are summarized in Figure 1.

Immunomagnetic assays—Immunomagnetic assays target an antigen using an antibody conjugated to a magnetic bead, and the antigen-antibody complex is subsequently isolated under a magnetic field. Of the immunomagnetic assays currently in use, the CellSearch system is most commonly used in clinical studies. In this system, EpCAM is used to capture CTCs. The results of the CellSearch system are reproducible across different laboratories and is therefore the only standardized technology approved by the US Food and Drug Administration (FDA) for the detection of CTCs in patients with metastatic breast, colon, and prostate cancer [46]. However the application of the CellSearch system in detecting *EGFR* mutations in a clinical setting remains limited due to variable sensitivity. In a phase II clinical trial of pertuzumab and erlotinib (tyrosine kinase inhibitors or TKIs), a higher number of CTCs was associated with a response to treatment, and a decreased number of CTCs during treatment was correlated with 18-Fluoro-deoxyglucose positron emission tomography (FDG-PET) and Response Evaluation Criteria In Solid Tumors (RECIST) finding in evaluating treatment responses and a longer progression-free survival [51]. However, mutational analysis of CTCs captured on the CellSearch platform is challenging, as only one of eight *EGFR* mutations was identified in archival tissue and in an available matching blood sample for CTC evaluation [51]. In another study using NGS to assess *EGFR* mutations in circulating tumor cell preparations from NSCLC patients, *EGFR* mutations were identified in CTC preparations of 31 (84%) patients, corresponding to those present in matching tumor tissue [52].

Microfluidic-based (CTC-Chip)—The microfluidic-based CTC capture device, also known as the CTC-Chip, involves flowing whole blood through a chamber embedded with 80,000 microposts that have been coated with an antibody. The first CTC-Chip was composed of an array of anti-EpCAM antibody-coated microposts. The chip has since been developed to contain a herringbone structure [53]. Flow kinetics are adjusted to minimize shear stress and to enhance the chance of collisions between CTCs and antibody-coated microposts. This technique can capture large numbers of CTCs and can be used in lung, prostate, pancreatic, breast, and colon cancers. In patients with metastatic disease, the CTC detection rate reaches 99% with a purity of approximately 50%. Maheswaran *et al.* first used CTC-Chips to perform a molecular analysis of DNA from CTCs [54]. CTCs were isolated from 27 patients, and molecular analysis was performed in 12 patients using specimens of the primary tumor. A Scorpion Amplification Refractory Mutation System (SARM), standard sequencing, or both were used for *EGFR* mutational analysis. *EGFR* activating mutations were found in the CTCs from 92% of the patients who were expected to be positive, including those treated with *EGFR* TKIs and those harboring the T790M mutation, which is the most common mutation associated with acquired resistance to *EGFR* TKIs [55]. New technology has enabled rare CTCs to be isolated from blood samples using tumor antigen-independent microfluidic CTC-iChip technology. Using two-stage magnetophoresis and antibodies against leukocytes, more white blood cells are depleted and more rare cells

are isolated [56]. However, further studies using this methodology are needed to validate their application in detecting *EGFR* mutations.

Cell filtration based on cell size—Although most CTCs derived from epithelial cancers are larger than leukocytes, they have a wide variation in size, ranging from 17 to 52 μm [57]. Several pore and filter-based approaches have been developed to prevent blocking and to accelerate the retrieval of CTCs [58,59]. Lin *et al.* increased the size of CTCs by tagging them with a large number of microbeads conjugated with anti-EpCAM antibodies and then used a microfluidic filtration device for separation [60]. These approaches show significant improvement in capturing cancer cells, however further validation with clinical specimens is required, in which both heterogeneous CTC size and resistance to filtering shear stress are likely to be disturbing variables.

Cell separation based on density gradient centrifugation—Density-based gradient centrifugation separates CTCs into a mononucleocyte fraction of blood away from the more dense cells present in the erythrocyte and granulocyte fractions. By blending silicone oils for CTC enrichment, Seal *et al.* developed a quantitative, inexpensive, and simple floatation method that detects CTCs in samples obtained from patients with a variety of cancers with cytological staining after a filtration step [61]. Ficoll-Paque solution, a synthetic polysaccharide, is used for gradient centrifugation separation of mononuclear cells from blood and has been used to enrich CTCs. This technique detected CTCs in 41% of patients undergoing surgical resection for colorectal cancer [62]. Combined with a porous barrier allowing erythrocytes and some leukocytes to pass through while retaining CTCs with density based centrifugation, the OncoQuick system provides effective enrichment [63]. The OncoQuick system was used to enrich CTCs in blood samples obtained from 30% of 37 gastrointestinal cancer patients [63] and 40% of 63 advanced breast cancer patients [64]. In a comparative study with CellSearch, CTCs were found in 23% of 61 patients after OncoQuick enrichment compared to 54% with the CellSearch system [65]. Although centrifugation is widely employed for CTC enrichment as an inexpensive and reliable technique, the elimination of contaminant leukocytes is limited and therefore it is commonly used as an initial step combined with further enrichment techniques.

Cell separation based on dielectrophoresis (DEP)—Different concentrations of molecules on inner and outer membranes create a cell membrane charge, which is altered during tumorigenesis due to abnormal metabolic transformations [66]. Dielectrophoresis (DEP) has been used to separate cells based on their electrical properties. In DEP, tumor cells are attracted towards an electric field generated by electrodes, while other cells are flushed away. ApoStream was the first commercial system for continuous flow DEP enrichment of CTCs and has a capture efficiency of over 70% and a viability greater than 97% from cell lines spiked in whole blood after an initial Ficoll gradient centrifugation step [67]. DEP has unique advantages over other methods because of the excellent viability of and minimal disruption to capture cells in test cell lines. However, unlike the CTC-Chips flowing whole blood through a herringbone structure coated with an antibody directly, an initial centrifugation enrichment step is needed in DEP and whole blood cannot be processed

directly [68]. The capture purity can be improved with additional enrichment stages, however this increases the risk of reducing the capture efficiency.

Clinical application of genotyping of circulating tumor cells

For *EGFR* genotyping, whole genome amplification is used to increase the amount of DNA from isolated single CTCs and to identify specific mutations using conventional sequencing or NGS platforms [46]. Pooling of individual CTCs from the same patient may decrease false findings and increase reproducibility, however it leads to a loss of information on intra-patient heterogeneity. Although single cell technologies decrease intra-patient heterogeneity, a larger volume of blood or highly sensitive mutation analysis technologies such as digital PCR or BEAMing are needed for further analysis [46].

Saliva and the electric field-induced release and measurement (EFIRM) platform

Saliva contains a variety of biomolecules including DNA, mRNA, miRNA, protein, metabolites, and microbiota. Changes in concentrations of these components can be used as biomarkers for the early detection of oral and systemic diseases including oral, lung, and ovary cancer as well as to evaluate disease prognosis and monitor the response to treatment [69]. The salivary genome consists of both human (70%) and microbial (30%) DNA. The quality of salivary DNA is sufficient such that 72–96% of samples can be genotyped, 84% can be amplified, and 67% can be sequenced [70]. In addition, saliva can be stored for a long time without significant degradation [71]. Recently, we explored the clinical utility of saliva to detect *EGFR* mutations in NSCLC patients by developing a core technology, electric field-induced release and measurement (EFIRM) [72]. We termed the saliva-based EFIRM detection of *EGFR* mutations as SABER (SAliva-Based *EGFR* Mutation Detection). The core technology in EFIRM is a polymer-based electrochemical chip with an array of 16 bare gold electrode chips as a sensor. Each unit of the array has a working electrode, a counter electrode, and a reference electrode. A 16-channel electrochemical reader controls the electrical field applied to the 16 array sensors and simultaneously reports the amperometric current. Paired probes were designed for EFIRM including detector probes and capture probes specific for TKI-sensitive mutations. The detector probes are labeled with fluorescein isothiocyanate, and the capture probes were first copolymerized with pyrrole onto the bare gold electrodes by applying a cyclic square wave electric field. After polymerization, samples mixed with the detector probes are transferred onto the electrodes for hybridization. After adding anti-fluorescein antibodies conjugated to horseradish peroxidase, interactions between the 3,3′,5,5′-tetramethylbenzidine substrate and horseradish peroxidase occur and the amperometric signal is measured. The total detection time is less than 10 minutes and requires only 20–40 μL of plasma or saliva. The detection of *EGFR* mutations with the SABER system was developed using cell lines and validated in a lung cancer xenograft model and clinical samples. In cancer cell lines, as little as 0.1–1% of mutant DNA was detected in the presence of wild-type DNA, and in an animal model, we observed a positive linear relationship between the electrochemical current and tumor size ($R = 0.86–0.98$). Furthermore, a blinded test was performed on saliva samples from 40 late-stage NSCLC patients. Receiver operating characteristic analysis indicated that EFIRM detected exon 19

deletions with an area under the curve (AUC) of 0.94 and the L858R mutation with an AUC of 0.96. The mechanism underlying the existence of tumor-specific oncogenic mutations in saliva remains unclear. However, we found that the amperometric currents of the EFIRM signals from plasma were highly correlated with those from saliva, implying that *EGFR* mutant DNA in saliva may come from plasma.

Detection of *EGFR* mutations in urine

Compared to serum collection, urine collection (as with saliva collection) is noninvasive and does not require any special equipment apart from sterile collection containers. Using DNA agarose gel electrophoresis analysis of total urine DNA, two distinct sizes of DNA can be identified. High molecular weight DNA is likely derived from urinary tract cells, and low molecular weight is likely derived from the circulatory system [73]. Previously, detecting urine tumor DNA was used only for tumors located in the urinary tract such as bladder or kidney tumors. Until recently, short-length, tumor-derived DNA other than that from the urinary tract was thought to be filtered through the kidney barrier and excreted into urine. The detection of mutant *KRAS* in the urine from patients with colon cancer led to the usage of urine DNA biomarkers for noninvasive disease screening, diagnosis and prognosis [74]. Similarly, in patients with systemic histiocytic disorders oncogenic DNA that contains *BRAF*V600E mutations can be detected from urine samples. Langerhans cell histiocytosis and Erdheim-Chester disease are heterogeneous systemic histiocytic disorders with a high frequency of somatic *BRAF*V600E mutations (40–60%), and these patients exhibit a dramatic response to the BRAF inhibitor vemurafenib [75,76]. Unfortunately, the scant histiocyte content often has stromal contamination, which limits accuracy, while lesions in the brain, orbits, and right atrium make it difficult to isolate material for *BRAF* genotyping [77]. Hyman *et al.* used a droplet-digital PCR (ddPCR) assay to quantitatively detect *BRAF* (V600E) mutations in plasma and urine ctDNA and performed a prospective, blinded study including 30 patients with Langerhans cell histiocytosis and Erdheim-Chester disease. There was a 100% concordance rate between tissue and urinary cfDNA genotypes in treatment-naïve samples [78]. At the European Lung Cancer Conference in 2015, Husain *et al.* demonstrated that *EGFR* mutant DNA can be detected in patients with metastatic lung cancer [79]. In this study, urine samples were obtained from six metastatic NSCLC patients who progressed on treatment with erlotinib. CtDNA was extracted and quantified by ddPCR assay. *EGFR* status was analyzed using a PCR method that amplified short target DNA fragments using kinetically favorable binding conditions for a wild-type blocking oligonucleotide followed by massively parallel deep sequencing. The results showed a 100% concordance rate and suggest that testing for ctDNA *EGFR* mutations in urine is a novel, non-invasive method that may allow for the dynamic monitoring of responses to anti-EGFR therapy.

Clinical implementation of detecting *EGFR* mutations using liquid biopsy

EGFR genotyping

When liquid biopsy was first used to detect *EGFR* mutations in NSCLC patients, the key concern was whether or not genetic variations within ctDNA were consistent with tumor

tissues and whether the results could be used to complement biopsy results. Many studies have demonstrated that blood samples reflect genetic changes in tumors of NSCLC patients using CTCs or ctDNA (Table 1). Two recent meta-analyses included 20 and 27 eligible studies to investigate the diagnostic value of ctDNA compared with tumor tissues. The pooled sensitivity, specificity, and diagnostic odds ratio of the 20 studies were 0.674, 0.935, and 29.582, respectively, and the AUC was 0.93 [80]. Similar results were observed for the 27 pooled studies with sensitivity, specificity, and diagnostic odds ratio of 0.620, 0.959, and 38.270, respectively [81]. ARMS/Scorpion is the most commonly used method and provides very high specificity but varying sensitivity in many studies. However, due to differences in the sample cohorts recruited, the results should be further verified by more comprehensive comparison studies. Another study also emphasized that different stage and different differentiation of cancer cells may affect the sensitivity [22]. The sensitivity for *EGFR* mutation analysis using blood cfDNA tends to be higher in late-stage lung cancer or poorly differentiated tumors. Two studies have compared the detection ability of CTCs and ctDNA. By comparing the detection rate of the CTC-Chip to ctDNA using the SARM assay, Maheswaran *et al.* found that the CTC-Chip was more sensitive than ctDNA (92% versus 33%) [54]. Punnoose *et al.* compared the detection ability of ctDNA and CTCs by CellSearch and found greater sensitivity in detecting mutations with ctDNA than with CTCs (50% versus 12.5%) [51]. Another study demonstrated that the detection rate of *EGFR* mutations with ctDNA depended on the number of CTCs. The *EGFR* mutation detection rates with cfDNA were significantly higher in patients with ≥ 2 CTCs per 7.5 ml (100%) than in those with <2 CTCs per 7.5 ml (10%) [82]. With regards to detecting *EGFR* DNA in saliva or urine, prospective clinical trials are needed to estimate the sensitivity and specificity for *EGFR* genotyping.

Monitoring TKI resistance by second mutations

Despite good responses to EGFR TKIs in the majority of lung cancer patients carrying sensitive *EGFR* mutations, most of these patients eventually become resistant to EGFR TKIs within one year [83]. Several studies report that the acquired resistance in approximately 50–60% of cases is due to the acquisition of a second site T790M “gatekeeper” mutation in the kinase domain of *EGFR* [55,84]. Recently, AZD9291, an oral, potent, and irreversible EGFR TKI, was developed. AZD9291 selects EGFR TKI sensitizing mutations and the T790M resistance mutation. According to the results of recent clinical trials, AZD9291 is highly active in patients with lung cancer with the *EGFR* T790M mutation with a median progression-free survival of 9.6 months in *EGFR* T790M-positive patients compared to 2.8 months in *EGFR* T790M-negative patients [85]. However, a new tumor biopsy is required to assess disease progression after the last regimen of T790M targeted TKI treatment. Since patients at this stage are often too weak to receive a second biopsy, a non-invasive method for detecting the T790M mutation is needed. The T790M mutation was identified in 2005 [83,86] and in 2008 it was confirmed that this mutation can be identified from CTCs or plasma DNA in patients who received EGFR TKIs [54,87]. The concordance rate based on liquid and tumor biopsies differ. Using CTC-Chip to analyze the T790 mutation in 23 patients, Maheswaran *et al.* reported a concordance rate of 69% [54]. In another study using ctDNA, the *EGFR* T790M mutation was identified in 70% (5 of 7) of patients with known tumor *EGFR* T790M mutations [87]. The detection rate of T790M seems to correlate with

treatment status. Using CTC-Chip, Maheswaran *et al.* detected the T790M mutation in CTCs from 2 of 6 patients (33%) who had a response to TKIs, and in 9 of 14 patients (64%) who had clinical progression [54]. In another study, *EGFR* T790M was identified from plasma DNA in 54% (15 of 28) of patients with a prior clinical response to gefitinib/erlotinib (EGFR TKIs), 29% (4 of 14) of patients with prior stable disease, and in no (0 of 12) patients who had primary progressive disease or in those who were not treated with gefitinib/erlotinib [87]. In other studies, the T790M mutation was detected in 72.7% and 28% of plasma DNA using different methods [28,38]. The progression-free survival of T790M-positive patients is significantly shorter than that for T790M-negative patients [78]. However, since AZD9291 is highly active in patients with lung cancer with the *EGFR* T790M mutation, the early detection of the T790M mutation may help to identify patients who will benefit from AZD9291 treatment but not in predicting a response to TKI treatment. Since T790M can be successfully detected from urinary ctDNA before disease progression in patients receiving EGFR TKIs, dynamic assessment of the response and progression from a completely non-invasive sample is possible [79]. Recent studies have focused on detecting the T790M mutation in pretreatment lung cancer patients. Using a ddPCR method, T790M mutation was detected in 79.9% (n = 373) of samples, which is a higher frequency than in previous reports. The T790M mutation was detected more frequently in patients with a larger tumor and those with common *EGFR*-activating mutations [88]. In order to identify the most sensitive platform, these different platforms need to be standardized so that a direct comparison can be made.

Early detection of *EGFR*-mutated lung cancer

Surgery is the most effective treatment for lung cancer, however only one third of lung cancer patients are diagnosed at an early enough stage to have surgery. Detection in the early stages could reduce mortality from lung cancer. Recently, the National Lung Screening Trial demonstrated that low-dose computed tomography screening (LDCT) is a less expensive [90] and more effective method to detect early lung cancer and thereby reduce lung cancer mortality [89] compared to conventional chest X-ray imaging. However, this trial also raised two major limitations, including the inefficiency in identification of non-smokers who carry the highest likelihood of developing lung cancer, and which nodules are likely to be cancerous before surgery. In addition, the eligibility criteria for the National Lung Screening Trial did not identify those who would be most likely to benefit from LDCT, and false-positive findings could cause harm due to unnecessary interventions and undue anxiety for the patients [91]. In resectable NSCLC, CTCs are only detected in 19–50% of cases using immunomagnetic assays. The lack of a good antibody panel for CTCs and low sensitivity limit the use of CTCs as a routine diagnostic screening tool for the detection of early lung cancer [92]. In a recent study using an ultrasensitive method for personalized cancer profiling by deep sequencing, ctDNA was detected in 100% of patients with stage II to IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions down to ~0.02% [93]. Another study also reported that *EGFR* DNA could be detected in early-stage lung cancer in 10–81% of cases. However, the application of circulating *EGFR* DNA to screen for lung cancer should be limited to certain ethnic groups. According to studies investigating *EGFR* gene mutation status in early [94] or advanced [95] lung adenocarcinoma in Asian countries, around 50% of the patients were found to be positive for

EGFR mutations. Although the detection rate of *EGFR* DNA in early-stage lung cancer is lower than late-stage, it remains a promising tool for screening lung cancer in combination with LDCT for Asian patients.

Expert commentary

Use of ctDNA and CTCs to detect *EGFR* mutations has received increasing attention since a feasible, reliable, and minimally invasive approach is needed for clinical research and practice. CtDNA analysis is an attractive option for genotyping, monitoring treatment response, and early detection because there is no need to enrich and isolate a rare population of cells. However, optimizing and standardizing new technologies with appropriate analytical and clinical validity remain challenging. In addition, other biofluids such as saliva and urine that can be used with less-invasive sampling methods also have the potential for use in detection of *EGFR* mutations. However, large prospective clinical trials are needed to establish the sensitivity and specificity of this technique for clinical application.

Five-year view

The term “liquid biopsy” was originally introduced for the analysis of CTCs, but is now also used for ctDNA analysis from plasma, urine, and saliva. The usage of new equipment such as NGS for the detection of tumor-associated mutations in the blood opens up exciting possibilities not only for *EGFR* mutations but also for other driver mutations of lung cancer. In September 2014, the Committee for Medicinal Products for Human Use of the European Medicines Agency approved the use of ctDNA to assess *EGFR* mutation status when selecting EGFR-TKIs for the patients in which obtaining a tumor sample is not an option [96]. This update is applicable in all European Union member countries and will benefit patients with locally advanced or metastatic NSCLC who do not have an available or evaluable tumor sample for *EGFR* mutation analysis. The development of irreversible EGFR TKIs such as AZD9291, which targets T790M, may facilitate the advancement and clinical application of liquid biopsy, since not all patients can tolerate repeat biopsies. However, much work remains to be done to optimize the different technologies and their application, standardize these across different platforms, and enable their broad application from cancer research to point of care.

In contrast to these labor-intensive and time-consuming methods requiring specialized or costly facilities, saliva offers several benefits in identifying *EGFR* mutations compared with blood-based platforms (Table 2). For example, collecting saliva samples poses a minimal risk of blood transmitted infectious diseases and the processing of saliva samples for EFIRM analysis is less complicated than methods for extracting DNA or isolating CTCs from blood. Finally, the complicated, technique-dependent molecular methods and equipment needed to detect ctDNA are not needed for EFIRM. However, the EFIRM platform is limited to detecting the common *EGFR* mutations *EGFR* L858R and exon 19 deletion but not yet for other uncommon EGFR -TKI sensitive mutations or the T790M mutation. Comprehensive probe design and prospective clinical trials are needed to validate the clinical application of EFIRM. Although special equipment such as digital PCR or NGS are needed to analyze *EGFR* mutations in urine DNA, the collection of urine is completely non-invasive and

cDNA can be extracted rapidly. Both saliva and urine platforms are user-friendly and may be closer to the point of care than circulating DNA or tumor cells since they can identify certain *EGFR* mutations rapidly.

Although the development of TKIs contributes to the improvements in progressive-free survival and quality of life of patients with lung cancer, identifying early-stage lung cancer and surgical intervention remain the best treatment strategies for lung cancer. The detection of ctDNA in early-stage lung cancer by NGS-based methods [93] could lead to its application in screening for early-stage lung cancer. Large multicenter trials are needed to validate whether this can improve the efficacy of screening and diagnosis in combination with LDCT.

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** of considerable interest

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Key issues

- EGFR targeted therapies are superior to chemotherapy in terms of overall response rate, progression-free survival, and quality of life in patients with untreated non-small cell lung cancer with sensitizing EGFR mutations as determined by invasive biopsies.
- Liquid biopsies are non-invasive and have the potential to complement tissue biopsies and monitor treatment response and drug resistance.
- Several platforms are available to detect EGFR mutations from ctDNA; meta-analysis studies have reported low sensitivity and high specificity.
- CellSearch is the only standardized technology approved by the FDA for the detection of CTCs in cancer patients, however its application in lung cancer is still limited.
- Saliva-based EFIRM detection offers a point of care platform for the detection of EGFR mutations since there is no need to extract DNA or for complicated equipment to detect the EGFR mutations. Prospective clinical trials are needed to determine the sensitivity and specificity.
- Urine-based platforms to detect EGFR mutations may be a novel method for the dynamic monitoring of anti-EGFR therapy and drug resistance.
- The development of oral irreversible EGFR TKIs specifically targeting *EGFR* T790M will accelerate the application of liquid biopsies in a clinical setting. However, optimization and standardization of these methods are needed before their clinical application.

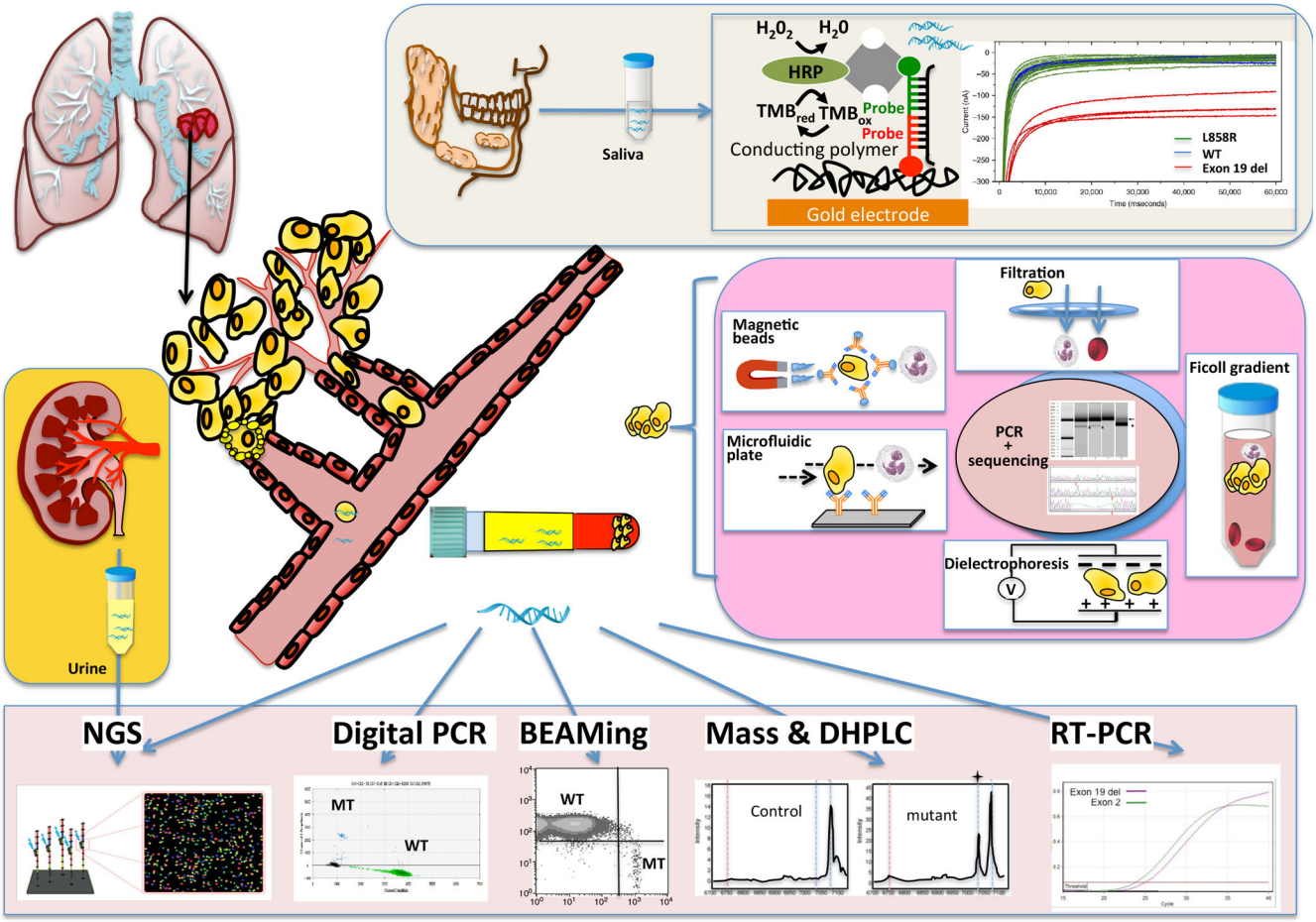

Figure 1. Different platforms of liquid biopsy in detecting *EGFR* mutations in patients with lung cancer. 1) Electric field-induced release and measurement (EFIRM) is a conducting polymer-based electrochemical chip with an array of 16 bare gold electrode chips. Each unit of the array consisted of a working electrode, a counter electrode, and a reference electrode, which were all bare gold prior to the treatment. Conjugations between sample DNA, detector probe (green), capture probe (red) cause a reaction between horseradish peroxidase (HRP)-labeled reporter probe and the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and generates amperometric signals. 2) Circulating tumor cells can be enriched according to their specific properties from the bloodstream of cancer patients; magnetic bead (CellSearch) and CTC chips are based on the biological properties using antibodies binding to the surface of cells expressing specific antigen-(EpCAM) positive cells. Filtration, Ficoll gradient and dielectrophoresis are based on the physical properties of tumor cell including size, density and electrical properties, respectively, compared to erythrocytes and leukocytes. 3) Circulating tumor DNA originates from cell necrosis, apoptosis or exosomes, and the *EGFR* genotype can be determined by PCR-based assays including the real-time PCR amplification-refractory mutation system (ARMS)/Scorpion assay, mutant-enriched-PCR, peptide nucleic acid (PNA)-mediated PCR, PNA-locked nucleic acid (LNA) PCR clamp, BEAMing (beads, emulsions, amplification and magnetics), and digital PCR. Other non-PCR based methods including denaturing high

performance liquid chromatography (DHPLC), mass spectrometry genotyping, and next generation sequencing (NGS) have also been used to detect *EGFR* mutations from plasma. 4) Urine ctDNA is first purified using resin exchange, a filter and exclusion column and then isolated using a standard circulating DNA kit. Circulating tumor DNA is quantified using digital PCR, and *EGFR* mutation status is analyzed using a PCR method that amplifies short target DNA fragments with wild-type blocking followed by massively parallel deep sequencing.

Table 1

Studies using liquid biopsy to detect *EGFR* mutations of lung cancer patient within the past 5 years

Sample	Method	Sample size	Conclusion
blood			
CTC	CellSearch/real time-PCR	41(relapsed)	Detection rate 12.5% [51]
	CellSearch/NGS	31(III/IV)	Detection rate 84% [52]
CtDNA	ARMS/Scorpion assay	86(III/IV)	Sensitivity 43.1%, Specificity 100%, PPV 100%, NPV 54.7%, Detection rate 66.3% [16]
		803(III/IV)	Detection rate 94.3% [97]
		145(III/IV)	Sensitivity (plasma, 48.2%; serum, 39.6%) and specificity (plasma, 95.4%; serum, 95.5%) [98]
		68(III/IV)	Sensitivity 22.06%, specificity 96.97%, PPV 88.24% and NPV 54.70% [18]
		54(III/IV)	Detecting T790M in 54% of patients with prior clinical response to TKI and in 29% patients of prior stable disease [87]
	Digital PCR	12(IV)	Detection rate 41.7% for T790M [99]
		373(I/II/III/IV)	Detection rate 79.9% for T790M [88]
	Mass spectrometry genotyping,	34(III/IV)	Detection rate 61% [37]
	High-resolution melting analysis	24(I/II/III/IV)	Sensitivity 91.67%, specificity 100% [33].
	Mutant-enriched-PCR/ Mutant-enriched sequencing	111(I/II/III/IV)	Detection rate 71.2%, sensitivity 35.6% and specificity 95.5%. Varied with the disease stage and pathological differentiation [22].
		58(III/IV)	Sensitivity 77.8%, specificity 100% and detection rate 93.1% [21].
	DHPLC	296(III/IV)	Detection rate 63.5% [100]
	PNA-mediated PCR	35(III/IV)	Detection rate 17% [25].
		57(III/IV)	Detection rate 87.7% [26].
	BEAMing	44 (III/IV)	Detection rate 72.7% [28]
	SABER (single allele base extension reaction)	75(III/IV)	Detection rate 28% [38].
Platform Comparison	ARMS/Scorpion assay	51(III/IV)	Sensitivity 50.0% and specificity 100%
	Mutant-enriched liquidchip		Sensitivity 25.0% and specificity 96.2%
	DHPLC		Sensitivity 25.0% and specificity 92.3% [19]
	Direct sequencing versus Mutant-enriched PCR	60(III/IV)	Sensitivity 18.3% versus 55.0% [101]
Meta-analysis			
	20 studies involving 2012 patients		Sensitivity 67.4%, specificity 93.5%, PLR 10.307, NLR 0.348 and DOR 29.582 [80]

Sample	Method	Sample size	Conclusion
	27 studies involving 3,110 patients		Sensitivity 62%, specificity 95.9%, DOR 38.3 and AUSROC 0.91[81]
Saliva			
DNA	EFIRM	40(III/IV)	Exon 19 Del (AUSROC 0.94) and L858R (AUSROC 0.96) [72]
Urine			
DNA	NGS	10(IV)	Detection rate 100% for the T790M mutation [79]

ARMS: Amplification refractory mutation system.

DHPLC: Denaturing high performance liquid chromatography

PNA: Peptide Nucleic Acid

PNA-LNA: Peptide nucleic acid-locked nucleic acid

BEAMing: Beads, emulsions, amplification and magnetics

EFIRM: Electric field-induced release and measurement

NGS: Next Generation Sequencing

PPV: positive predictive value, NPV: negative predictive value, PLR: positive likelihood ratio, NLR: negative likelihood ratio, DOR: diagnostic odds ratio, AUSROC: the area under the summary ROC curve.

Table 2

Differences between different platforms of liquid biopsy in detecting *EGFR* mutations

	CTC	CtDNA	Saliva	Urine
Equipment for isolation of CTC or ctDNA	Special instruments for isolation and identification	Simple blood sampling and standard preparation of plasma DNA	Saliva instead of blood sampling, no need to prepare CtDNA	Urine instead of blood sampling, ion-exchange resin/silica-based resin and standard preparation Compliment biopsy
Mutation analysis	Real-time PCR, NGS	Multiple platform	EFIRM	NGS
Analysis for heterogeneity and clonality	Yes, if enough CTCs are captured	No, results are the average from all cells shedding tumor DNA into the circulation	No, results are the average from all cells shedding tumor DNA into the circulation	No, results are the average from all cells shedding tumor DNA into the circulation
Applicability for functional assay	Yes, cell-by-cell analyses, generation of cell lines and analyses in animal models	No	No	No
Applicability for diagnostic or monitoring purposes	Compliment biopsy and monitor treatment response	Compliment biopsy, monitor treatment response, detecting resistance and potential in screening early stage lung cancer	Compliment biopsy	Monitoring treatment response, detecting resistance