Emerging non-invasive detection methodologies for lung cancer (Review)

ZHENG LI1,2, JINIAN SHU2, BO YANG2, ZUOJIAN ZHANG2, JINGYUN HUANG2 and YANG CHEN1

1Beijing Advanced Sciences and Innovation Center, Chinese Academy of Sciences, Beijing 101407; 2National Engineering Laboratory for VOCs Pollution Control Material and Technology, University of Chinese Academy of Sciences, Beijing 101408, P.R. China

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Abstract. The potential for non-invasive lung cancer (LC) diagnosis based on molecular, cellular and volatile biomarkers has been attracting increasing attention, with the development of advanced techniques and methodologies. It is standard practice to tailor the treatments of LC for certain specific genetic alterations, including the epidermal growth factor receptor, anaplastic lymphoma kinase and BRAF genes. Despite these advances, little is known about the internal mechanisms of different types of biomarkers and the involvement of their related biochemical pathways during the development of LC. The development of faster and more effective techniques is essential for the identification of different biomarkers. The present review summarizes some of the latest methods used for detecting molecular, cellular and volatile biomarkers in LC and their potential use in clinical diagnosis and targeted therapy.

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1. Introduction

In 2018, lung cancer (LC) was reported as the leading cause of cancer-associated mortality worldwide (1), early screening of LC is regarded as a significant means to improve patient survival. Over the past 20 years, various mechanisms of LC and their detection methods have been comprehensively studied in liquid biopsy samples (2). A number of clinical molecular diagnostic tests for LC have been developed based on these studies (3-5). The objectives of these diagnostic methods include early detection, screening for therapeutic targets, profiling cancer panel, monitoring therapeutic effectiveness and early recurrence detection. At the genetic level, the most relevant genes that are assessed for LC include epidermal growth factor receptor (EGFR), KRAS and anaplastic lymphoma kinase (ALK) (6). At the protein level, a number of antigen detection methods, including carcinoembryonic antigen (CEA), cancer antigen 125 and cytokeratin fragment 21-1, have also been studied to improve diagnostic accuracy (7). At the cellular level, the examination of circulating tumor cells (CTCs) has been shown to provide prognostic information concerning tumor metastasis (8). Moreover, exhaled volatile organic compounds (VOCs) are considered to be optimal non-invasive biomarkers since they are indicative of the mutations and pathophysiological processes of LC (9).

Recent advances in the field of VOC biomarkers have improved the sensitivity and specificity in the diagnosis of LC (10). A VOC pattern or specific VOCs can be detected and used as a potential guide for the development of effective therapeutics (11). The use of mass spectrometers or sensors for the precise detection of volatile biomarkers could substantially aid the diagnostic process by supplementing the standard screening methods in the early diagnosis and prognosis of patients with LC.

In the current review, the related mechanisms of molecular, cellular and volatile biomarkers for LC and techniques used for their detection are discussed. In addition, the detection characteristics of the different diagnostic methods are compared. Important improvements are required in certain areas, including the following: i) Exploring the relationship between liquid biopsies and volatile biomarkers in standardized clinical trials of LC; ii) customizing the anticancer strategies; and iii) developing more sensitive and selective instruments for biomarker detection.

2. Emerging non-invasive detection methods for LC

Methods of interest. Evaluation of the intratumor heterogeneity of patients used to be based on surgical resection sampling and multiple biopsies. However, recent advances in sampling
are focused on acquiring sufficient samples for various biomarkers using minimally invasive or non-invasive methods, including liquid biopsy for the detection of circulating tumor (ct)DNA. (12), circulating tumor cells (CTCs) (13) and protein biomarkers (14), and analysis of exhaled VOCs (15). The specific characteristics that were compared between these less invasive (nucleic acid, protein and cell based) and non-invasive (VOCs based) detection methods for the diagnosis of LC are listed in Table I.

Nucleic acid-based detection. i) ctDNA markers. Liquid biopsy is thought to be one of the potential options for the non-invasive diagnosis of LC. ctDNA is secreted into the serum by necrotic or apoptotic cells, which may provide effective means for tumor diagnoses (16). Additionally, the additional half-life of ctDNA (~2 h) renders it an ideal dynamic marker of tumors (17). Some genetic mutations, particularly single nucleotide variants (SNVs) in ctDNA may be considered as specific biomarkers for LC. A study assessing 100 early-stage non-small cell lung cancer (NSCLC) specimens revealed that 48% of patients had ≥2 detectable SNVs in ctDNA. Post-operative next-generation sequencing (NGS) based on ctDNA analysis is able to predict LC recurrence earlier than CT imaging, at ~70 days (18). Furthermore, Cohen et al (19) used CancerSEEK, a PCR-based ctDNA method, for the detection of five cancer types, namely ovarian, liver, stomach, pancreatic and esophageal cancer, and reported that the accuracy of prediction varied with tumor type. The performance of CancerSEEK in the analysis of 104 LC samples was poor, with only 59% sensitivity; even when combined with machine learning, the accuracy was lowest for LC.

Although ctDNA analysis for LC is promising, it remains incomplete. Disadvantages of this method, which hamper its widespread application, include the following: a) Poor detection sensitivity; b) high cost; and c) limited clinical utility. More specifically, the concentration of ctDNA in plasma is only 1%, and as much as 10 ml plasma are required to obtain reliable results when using the current ctDNA platforms. Regarding the cost of liquid biopsy testing, this has been estimated at US$1,750 per patient. Moreover, ctDNA analysis is technically complex and requires specialized skills and equipment (18).

ii) Methylated DNA markers. Aberrant hypermethylation of global DNA or specific CpG islands in promoter regions has been considered a promising biomarker for different cancer types, including ovarian, prostate, liver and cervical cancer (20). Methylated DNA markers in LC have been detected in various body fluid samples, including blood, urine, saliva and exhaled breath condensate (EBC) (21). Improvements of this detection method currently enable the determination of DNA methylation. Wielscher et al (22) reported a panel of four methylated genes, specifically homeobox D10, paired box 9, protein tyrosine phosphatase receptor type 2 and stromal antigen 3, as markers for LC detection. LC was efficiently differentiated from other lung diseases and controls with a sensitivity of 87.8% and a specificity of 90.2%. The commercial test kit EpiProLung® (Epigenomics Inc.), used for the screening of LC and based on the analysis of methylation in the short stature homeobox 2 (SHOX2) gene, was approved by the Chinese Food and Drug Administration (FDA) in July 2015 (23). To estimate the diagnostic efficacy of SHOX2 DNA methylation, a meta-analysis was conducted in 2,296 subjects, including 1,129 patients with LC, which demonstrated that this method had 70% sensitivity and 96% specificity (24).

Three groups of methods are commonly employed to distinguish methylated DNA from unmethylated DNA, including sodium bisulfite conversion, restriction enzyme and specific antibodies (25). DNA methylation may be analyzed using various detection methods, including PCR, microarrays, and NGS. However, each of these methods has certain drawbacks; bisulfite conversion results in random DNA fragmentation (26). In addition, restriction enzyme-based methods can only detect specific patterns of CpG sites. Antibody-based methods are limited by a low recovery rate (27). Moreover, the establishment of standardized protocols for methylation detection methods is essential.

iii) MicroRNA (miRNA/miR) markers. miRNAs are small non-coding RNAs that are capable of influencing cancer metabolism by regulating tumor suppressor signaling pathways of glucose metabolism or the expression of glycolytic enzymes (28). The miRNA expression profiles for LC have been found to be present and stably expressed in bodily fluids, including human serum/plasma and sputum (29). For instance, findings have demonstrated a significantly decreased expression of miRNA-124-5p and an increased expression of miRNA-124-2 and miRNA-124-3 in patients with advanced-stage LC (30,31). Moreover, miRNA expression patterns may serve as specific biomarker signatures for the diagnosis and subtype differentiation of LC from early stage to metastatic (32,33). In a cohort of 180 subjects (92 patients; 88 healthy participants), a plasma miRNA signature, consisting of miRs-126, -145, -210 and -205-5p, was defined by NGS and shown to have 91.5% sensitivity and 96.2% specificity in the detection of LC (34). In another study, a panel of six miRNAs, namely miR-17, miR-190b, miR-19a, miR-19b, miR-26b and miR-375, was used for the diagnosis and further differentiation of small cell lung cancer from NSCLC in 1,132 participants by microarray analysis and was found to have 80% sensitivity and 80% specificity (33).

In addition, numerous plasma miRNAs have been identified using microarray platforms and are considered as plasma biomarkers for LC (35,36). These miRNAs are usually further verified by reverse transcription (RT)-quantitative PCR analysis. Nevertheless, microarray-based methods are limited by relatively low sensitivity and specificity. Therefore, they have yet to be applied in clinical settings. By contrast, NGS is able to identify LC-associated miRNAs with high throughput and high sensitivity and specificity (37).

In combination with certain algorithms, miRNA analyses may have powerful diagnostic and prognostic performance. Sozzi et al (38) combined miRNA analyses with low-dose computed tomography (LDCT) for the detection of LC, which resulted in a 5-fold reduction of the false-positive rate, from 19.4-3.7%.

Protein-based detection. Protein profiling is another diagnostic method based on liquid biopsy (39). Numerous studies on LC protein biomarkers have been conducted in blood, urine, saliva and exhaled breath condensate (EBC) samples (40-42). Common protein biomarkers for LC,
Table I. Comparison of less invasive and non-invasive detection methods for the diagnosis of lung cancer.

| Sample        | Analytical methods                                      | Sensitivity (%) | Advantages                                      | Disadvantages                                                                 | (Refs.)           |
|---------------|----------------------------------------------------------|-----------------|-------------------------------------------------|-------------------------------------------------------------------------------|-------------------|
| ctDNA         | Multiplex-PCR; NGS                                       | 48.0-59.0       | Earlier detection (~70 days prior to CT imaging)| Expensive; Limited sensitivity; Require specialized skills and equipment;     | (16,18,19,75)    |
| Methylated DNA| Bisulfite conversion + PCR, NGS;                         | 70.0-87.8       | High sensitivity and specificity                | Require standardization; Fragment (bisulfite conversion); Only recognize special patterns (restriction enzyme); Low recovery rate (antibodies) | (22,24,25,76)    |
|               | Restriction enzyme + PCR;                                |                 |                                                 |                                                                               |                   |
|               | Antibodies + PCR; NGS                                     |                 |                                                 |                                                                               |                   |
| microRNAs     | NGS; RT-PCR; Microarray                                  | 80.0-91.5       | Stable; High throughput                        | Require specialized skills and equipment                                      | (33,34,77,78)    |
| Proteins      | Microarray; LC-MS/MS                                     | 70.0-84.0       | Higher sensitivity; High throughput; Rapid     | Require validation; Protein binding problems (Microarray); Difficult to quantitate signals (MS) | (42,44,48,79)    |
| CTCs          | IF; FISH                                                 | 30.0-69.5       | High throughput; High specificity; Co-localization| Limited sensitivity; Require enrichment; Monitor only advanced types of cancer; Heterogeneity effect | (51,80,81)       |
| Exhaled VOCs  | GC-MS; Sensors/E-noses; PTR-MS; IMS; LPPI-MS             | 81.0-96.5       | Simple, rapid; Non-invasive; Non-expensive     | Require standardization                                                       | (59,61,63-65, 70,71,72,73) |

NGS, next-generation sequencing; CT, computed tomography; EV array, extracellular vesicle array; RT-PCR, reverse transcription PCR; IF, immunofluorescence; FISH, fluorescence in situ hybridization; GC-MS, gas chromatography-mass spectrometry; PTR-MS, proton transfer reaction-mass spectrometry; SIFT-MS, selected ion flow tube-mass spectrometry; IMS, ion mobility spectrometry; LPPI-MS, low pressure photoionization-mass spectrometry; CTCs, circulating tumor cells, VOCs, volatile organic compounds.
including CEA and cytokeratin fragment 21-1, are not sensitive enough to detect early tumors (43). Therefore, newer protein biomarkers with higher sensitivity have been developed. Cytoskeleton-associated protein 4 (CKAP4) is expressed at significantly higher levels in patients with LC than in the controls. In a cohort comprising 271 patients with LC and 100 healthy controls, the sensitivity of reverse-phase protein array for serum CKAP4 was 81.1%. Furthermore, CKAP4 has been reported to reliably detect early-stage adenocarcinoma or squamous cell carcinoma, with a sensitivity of 78.6% (44).

Compared with traditional serological markers, exosomal proteins have certain advantages. Typically, there are over 1x10^9 exosomes per ml of human blood. Furthermore, approximately 80% salivary exosomal proteins are shared with serum exosomes (45). Several proteins are specifically expressed in exosomes, including Xbox-binding protein 1, glypican-1 and cGMP-dependent protein kinase 1, and have been reported to reliably detect early-stage adenocarcinoma or squamous cell carcinoma, with a sensitivity of 78.6% (44).

The evaluation of proteins found in EBC, saliva and urinary samples of patients with LC is a non-invasive diagnostic method. Lopez-Sanchez et al. (42) collected EBC samples from 192 individuals (48 patients; 144 healthy controls) and analyzed them by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The expression levels of cytokeratins and other proteins were significantly high in LC samples, yielding 70% sensitivity and 67% specificity. Nolen et al. (41) examined urinary samples of 234 individuals (83 patients; 151 healthy controls) by applying a panel of three proteins, insulin-like growth factor binding protein 1, secretory leukin-1 receptor antagonist and cell adhesion molecule 1, to differentiate NSCLC, with 84% sensitivity and 95% specificity.

Although protein microarray analysis offers comprehensive protein information with minimal sample requirement, it could be optimized for the rapid screening of LC. However, various issues regarding protein binding remain a challenge for these protein detection arrays (49). Moreover, LC-MS/MS is an excellent detection method for systematic analysis of protein profiling of LC, though not a rapid and high-throughput platform in translational medicine.

**Cell-based detection.** Tumors release certain malignant cells, termed CTCs, into the vasculature. CTCs have been characterized based on their migration and invasion abilities in the early stages of LC. High CTC numbers correspond to an aggressive type of cancer, increased metastasis and increased likelihood of relapse (50). Nevertheless, the number of CTCs in patients may be as low as 1 CTC per ml of blood. Therefore, cell enrichment is required, which typically depends on the antigens, e.g., EpCAM and CD45, or inherent properties of CTCs, including size, deformability or dielectric susceptibility (51). Following cell enrichment, verification of CTCs is generally achieved by high-resolution imaging combined with immunohistochemical enumeration.

The CellSearch® test (Menarini Silicon Biosystems, Inc.), which is EpCAM-dependent, is the only FDA-approved method for CTC detection that has been proven to provide prognostic information in metastatic breast, colorectal and prostate cancer. Regarding LC, however, this method has been found to exhibit limited detection efficiency (52). In a cohort of 150 patients with LC, the sensitivity of CellSearch® was only 30.4% (53). In another study, a novel, size-based filtration platform was employed for the diagnosis of LC in 82 patients, which was shown to have improved sensitivity for CTC detection (69.5%) (54).

CTC-based liquid biopsy has several advantages, including high specificity, demonstrating the localization signal as an integrated cell. However, the use of CTCs for LC screening is currently impaired by its limited application in the clinical setting. As negligible CTC counts are found in early-stage patients with LC, CTC detection methods may only be efficient for patients with advanced LC (54,55).

**VOC-based detection.** VOCs are a group of gaseous organic molecules with relatively high vapor pressure or volatility. VOCs present in bodily fluids reach the lungs via the bloodstream and are exhaled through breath (15). Among them, exogenous VOCs derived from cigarette smoke, pollution and radiation target and damage DNA, proteins and polyunsaturated fatty acids (PUFAs) in the body, thus boosting oxidative stress and contributing to cancer development (56). By contrast, endogenous VOCs are mainly derived from diverse metabolic pathways. Since endogenous VOCs associated with certain metabolism may be altered by diseases, different VOC profiles have been associated with various diseases, including cancer (57).

A wide range of analytical techniques has been used for the determination of volatile metabolites. Gas chromatography-mass spectrometry (GC-MS) is considered the gold standard for the detection of specific VOCs (58-60). Phillips et al. (61,62) applied the predictive model of 16 and 30 VOCs to differentiate patients with primary LC from the controls in a cohort of 193 patients and 211 controls and it was found that this model had approximately 85% sensitivity and approximately 80% specificity. In another cohort consisting of 88 patients and 155 controls, three diagnostic models based on 23 VOCs were able to easily identify patients, with 96.5% sensitivity and 97.5% specificity (63).

Nonetheless, GC-MS is a method based on offline analyses, which requires time-consuming preparations. On the contrary, certain advanced analytical techniques, including proton transfer reaction-mass spectrometry (PTR-MS), selected ion flow tube-mass spectrometry (SIFT-MS) and low-pressure photoionization-mass spectrometry (LPPI-MS) offer real-time analysis and high sensitivity (64-66). However, to the best of our knowledge, no systematic studies have been performed for the discrimination of patients with LC by PTR-MS, SIFT-MS or LPPI-MS.

Mass spectrometry-based techniques are expensive and require complex instruments. By contrast, sensors/E-noses show great potential for fast, easy and cost-effective diagnosis and screening of LC (67). With the advancement of sensing devices, electronics and signal processing, the sizes of
sensors/E-nose systems can be minimized along with fast data processing to provide real-time results (67). When combined with pattern recognition methods, sensors/E-noses are capable of distinguishing the exhaled breath of patients with LC from the breath of healthy controls without the need for dehumidification or pre-concentration of biomarkers (68). Nevertheless, more subjects are needed in order to increase the sensitivity and negative predictive value (69). Chang et al. (11) examined the breath samples of 37 patients with LC and 48 healthy controls using a sensor system, which was found to have 79.0% sensitivity and 72.0% specificity. Gasparrì et al. (70) reported the differentiation of 70 patients with LC from 76 healthy controls with 81% sensitivity and 91% specificity, using a gas sensor array composed of a matrix of eight quartz microbalances. Shehada et al. (71) collected breath samples from 149 volunteers with LC, specifically 40 patients with gastric cancer, 56 volunteers with non-cancerous lung diseases (asthma, chronic obstructive pulmonary disease or both) and 129 healthy controls. The self-developed silicon nanowire field effect transistor was used to separate patients with LC from subjects in the control group and were shown to have 87% sensitivity and 82% specificity.

Similarly, ion mobility spectrometry (IMS) also allows pattern recognition, rather than specific VOC identification, and has the potential for miniaturization. Handa et al. (72) investigated the breath samples of 50 patients with LC and 39 healthy controls by IMS and revealed that this method had a sensitivity of 81.3% and specificity of 89.7%. Nonetheless, sensors and IMS are limited in terms of detection and, thus, are usually coupled with complex algorithms (72-74). The advantages and disadvantages of nucleic acid-based (75-78), protein-based (79) and cell-based (80,81) detections were compared with VOC-based detection as displayed in Table I.

**Targeted therapies based on non-invasive detection**

Liquid biopsy-guided therapies. A liquid biopsy, which is a non-invasive sampling procedure, is preferred for metastatic LC in order to obtain the molecular characterization (Table II). Several targeted methods, including PCR, NGS and immuno-oncology, are employed to detect gene mutation targets (*EGFR*, *ALK*, *BRAF*) from DNA/RNA or CTC samples (82). The application of liquid biopsy as a guidance for targeted therapy would notably improve the overall survival of patients with LC.

i) *EGFR* mutation. Mutations in *EGFR* are the only standardized therapeutic targets examined in clinical practice. COBAS (Roche Diagnostics) and Therascreen® RGQ PCR kit (Qiagen, Inc.) are two methods used for the detection of *EGFR* mutations, which have been approved by the FDA in the USA (83). *EGFR* mutations are mostly detected in DNA samples extracted from plasma. By contrast, CTC-based detection is limited by poor sensitivity and specificity and the small quantity of CTCs in blood (84). *EGFR* mutations occur in 20-76% patients with NSCLC and are more common among patients from the Asia-Pacific region (85). Patients with LC, who have *EGFR* mutations, are normally treated with tyrosine kinase inhibitors (TKIs), including gefitinib, erlotinib and afatinib (86). However, sensitivity to TKI treatment varies with the type of *EGFR* mutation (87,88). Monoclonal antibodies and TKIs are usually applied to treat specific genetic alterations, including *EGFR* and *ALK*, in both the first line and resistant settings (89,90). Several new generation TKIs (e.g., osimertinib, olmutinib, nazartinib, avitinib and rociletinib) have been developed for patients harboring *EGFR* resistance mutations (5,91). In general, *EGFR* mutations that confer sensitivity to TKI therapy seem to be greater than those that confer resistance (92).

ii) *ALK* rearrangement. *ALK* rearrangements act as an oncogenic driver in 4-6% of NSCLCs (93). Several target methods are used to evaluate the status of *ALK* by liquid biopsy, including RT-PCR, NGS, and fluorescence in situ hybridization. However, during the preprocessing steps, circulating plasma RNAs are degraded rapidly, which results in lower sensitivity (94,95). Conversely, CTC-based detection of *ALK* status is more feasible and in accordance with tissue biopsies (96). Previous studies have evaluated several types of *ALK* inhibitors, including crizotinib, alectinib, ceritinib and lorlatinib in patients with LC/NSCLC (97-100). Most patients with NSCLC that have *ALK* rearrangements respond well to *ALK* TKIs; heterogeneous responses are also reported, though the reason remains unknown (101).

iv) *BRAF* mutations. *BRAF* mutations can be identified in 1.6-1.8% of patients with LC (102). These mutations act as an oncogenic driver in NSCLC via the mitogen-activated protein kinase (MAPK) pathway. Venurafenib and dabrafenib are used to block MAPK signaling in patients with LC (103). Furthermore, since these mutations increase the kinase activity of *BRAF* towards mitogen-activated protein kinase (MAPK) kinase (*MEK*), the combination of MAPK pathway inhibition with *BRAF* and *MEK* inhibitors may prove to be an effective therapeutic strategy for LC. *BRAF* mutations can be detected in plasma DNA or CTC samples. It has been reported that the combination of dabrafenib and trametinib (a selective allosteric inhibitor of MEK1/MEK2) (104) showed robust antitumor activity and a manageable safety profile in patients with NSCLC that harbored *BRAF* mutations (105).

Apart from the main oncogenic mutations that were mentioned above, additional molecular targets for LC have been identified, including ROS proto-oncogene 1, human epidermal growth factor receptor 2, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, rearranged during transfection proto-oncogene and hepatocyte growth factor receptor (106-108). Although several monoclonal antibodies and tyrosine-kinase inhibitor drugs have been developed to inhibit LC-related signaling pathways, there is an urgent need for the development of detection methods with improved sensitivity for the determination of specific biomarkers and guidance of effective treatment options.

**VOC production mechanisms and their potential for targeted therapies.** Different VOCs may contain metabolic information concerning various types of human tissues and the storage capacities of volatile organic substances significantly differ in different types of human tissues. The time required to metabolize these different VOCs from the human body also differs (109). Preliminary metabolic pathways of several VOCs that have been studied include the following: i) Hydrocarbons; ii) alcohols; iii) aldehydes; iv) branched aldehydes; v) ketones; vi) esters; vii) nitriles and aromatics.

i) Hydrocarbon. In the human body, oxidative stress is the main mechanism corresponding to the production of
hydrocarbons. Alkanes are mainly derived from the peroxidation of PUFAs, which occurs in the cell and subcellular membranes. Lipid peroxidation can cause tissue damage, which in turn may lead to aging, inflammation, atherosclerosis, and cancer. The effects of lipid peroxidation may be reduced and regulated by antioxidants (57). Saturated alkanes including ethane and pentane are considered the final products of lipid peroxidation and have been widely recognized as volatile biomarkers of this reaction in breath analysis (110). The production of C3-C11 saturated alkanes is also associated with lipid peroxidation, though the production of branched alkanes does not originate from this mechanism (111). As hydrocarbons have a lower solubility in blood, they can be excreted from the body by exhaled breath in a matter of minutes (112).

ii) Alcohols. Alcohols are also considered products of hydrocarbon metabolism and can be easily absorbed in the digestive tract. Once absorbed, alcohols enter the bloodstream. As short-chain alcohols are highly soluble in water, their absorption into the bloodstream is rapid. The metabolism of alcohols varies greatly due to the diversity of fat and water content among individuals. Certain enzymes, including alcohol dehydrogenase and cytochrome p450 family 2 subfamily E member 1 (CYP2E1), which is mainly expressed in the liver, are involved in the metabolism of alcohols (57).

iii) Aldehydes. Aldehydes may be produced through normal physiological processes. Certain aldehydes serve important roles in the physiological functions of the body, whereas others are considered to be cytotoxic intermediates that perform specific functions and serve roles in signal transmission, gene regulation and cell proliferation (113,114). In the human body, aldehydes may be derived from multiple processes or sources, including the following: i) Alcohol metabolism; ii) degradation of hydrogen peroxide-containing substances by CYP2E1, a by-product of lipid peroxidation (115); iii) smoking, which produces a large number of aldehydes; saturated and unsaturated aldehydes, including formaldehyde, acetaldehyde, and acrolein, are produced from tobacco burning (116); iv) by-products of tobacco metabolism, which occurs in the

Table II. Comparison of the main targeted therapies guided by liquid biopsy.

| Target             | Frequency (%) | Drug used                              | Detection                  | (Refs.) |
|--------------------|---------------|----------------------------------------|----------------------------|---------|
| EGFR mutation      | 20-76         | Gefitinib, erlotinib, afatinib, osimertinib | Plasma DNA; CTCs           | (84,85,91) |
| ALK rearrangement  | 4-6           | Crizotinib, Alecc, Ceritinib, Lorlatinib. | Plasma DNA/RNA; CTCs       | (93,96,97) |
| BRAF mutation      | 1.6-1.8       | Vemurafenib, dabrafenib sorafenib, trame tinib | Plasma DNA; CTCs           | (102-105) |

CTCs, circulating tumor cells; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase.

Figure 1. Process of VOC biomarker-based targeted therapies for patients with lung cancer. VOC, volatile organic compound.
cytochrome, which in turn participates in detoxification (117); v) dietary intake (118).

iv) Ketones. Fatty acid oxidation in the body is accelerated with the onset of cancer. As a result, a large number of ketones are identified in cancer patients, the production of which is closely related to the rapid decline of weight, a common complication of cancer. The liver is a vital organ for ketone metabolism (119). Since a considerable amount of acetoacetate and β-hydroxybutyric acid are synthesized in the liver, acetoacetate spontaneously undergoes decarboxylation and acetone is consequently produced (120).

v) Esters. Large amounts of exogenous esters are found in natural fats, oils, natural waxes and plant essential oils. In the human body, esterases are capable of hydrolyzing esters to alcohols and acids at body temperature, which is similar to the process by which lipases hydrolyze fat (121).

vi) Nitriles and aromatic substances. Nitriles and aromatic substances enter the human body via external pollutants, including air pollution, drinking, smoking and radiation. Due to their carcinogenicity, they have gained interest in the study of cancer (11). They are highly active and can cause damage to PUFAs, proteins and DNA. As these damages accumulate in the body, normal repair functions cease to be effective, leading to the development of a series of diseases, including cancer.

Studying the VOC- and metabolite- profiles of LC cells provides an alternative to invasive diagnosis. A recent study revealed a possible association between VOCs and metabolites. It was found that the combination of benzaldehyde, 2-ethyl-hexanol and 2, 4-decadien-1-ol could serve as potential VOC biomarkers for LC (122). These VOCs are also strongly negatively associated with the levels of certain amino acids, glucose, cholesterol and several fatty acids. Another study examined the VOC biomarkers that were associated with EGFR, KRAS and ALK mutations in LC cell lines. Triethylamine, benzaldehyde and decanal have the potential to become specific VOC biomarkers for identifying and distinguishing these mutations in patients with LC (123). Further clinical studies are required to determine whether these cell-line methods could be translated into clinical diagnostic tools.

The goal of personalized medicine is to treat patients using their genetic profile. The gene-specific VOC biomarkers are metabolized in different types of tissues, transported to the lungs via blood circulation and exhaled from the alveoli. These exhaled breath-detection methods are entirely non-invasive, readily available and do not require any preparation (57). Therefore, they have the potential to provide indirect genetic and pathological information prior to and during the treatment period.

In order to validate the efficacy of VOC profiles in the exhaled breath as a means of diagnosing LC, advancements in the following technologies are critically required: i) Standardized clinical utility; and ii) advanced mass spectrometry with ultra-high sensitivity. Recently, the excited state proton transfer (ESPT) ionization technology was established by LPPI-MS, discovering new mechanisms of chemi-ionization reactions and offering new technological applications that have the potential to greatly improve mass spectrometry sensitivity for detecting trace gaseous organics (124,125). VUV excitation is applied to produce a chemi-ionization reaction, which yields substantial H$_2$O$^+$ ions, and the protonated analyte, an equal amount of CI$^+$, may be produced with the aid of the reorganization energy released from the formation of CH$_3$O and HCl. The sensitivity for VOC biomarkers is at least 20 times higher than that for PTR-MS. Regarding oxygenated VOCs, the signal intensities of oxygenated organics can be amplified by more than two orders of magnitude.

The application of advanced techniques in volatile fingerprinting, allows non-invasive, rapid and accurate detection, thereby leading to early diagnosis and prognosis based on metabolomics. The process of VOC biomarker-based targeted therapies for patients with LC is presented in Fig. 1. First, patients with LC and other lung diseases and healthy controls are recruited. Secondly, exhaled breath samples are collected from the volunteers and VOC biomarkers are analyzed using ultra-high-sensitivity techniques, including ESPT ionization. Subsequently, machine learning algorithms are employed for the data mining of VOC profiles. In combination with routine diagnostic methods, physiological assessment and diagnosis are accomplished using VOC biomarkers. Improved precision of diagnostic techniques and personalized medicine could lead to the identification of additional VOC biomarkers and are anticipated to open up connections between omics technologies (genomics, proteomics and metabolomics), which may result in the faster and more efficient discovery of other molecular markers and intervening targets.

3. Challenges and future directions

Despite the vast potential of existing candidates and methodologies, only a few non-invasive detection methods for LC, including the Guardant360® (http://www.guardant360.com) and cobas® EGFR Mutation Test v2 (Roche Molecular Diagnostics) are currently being used in the clinical setting. In order to apply potential non-invasive detection methods to clinical practice, several milestones must be achieved. First, head-to-head comparisons of different types of biomarkers in specific clinical scenarios would be beneficial. Deep mining of data provided by machine learning would also be useful. Secondly, the instrumentation used for biomarker detection requires careful reevaluation. Mass spectrometry, NGS, PCR, microarray and LDCT are standard techniques that are widely used in biomarker detection. However, in order to obtain accurate results in clinical practice, the reliability of instruments and reproducibility of results should be examined and optimized in clinical studies. Thirdly, the interactions between liquid biopsy, imaging and VOC biomarkers should also be identified in clinical studies. Moreover, the combination of different biomarker tests could reduce false-positive results and enable greater standardization of diagnostic algorithms, thereby decreasing health care costs.

Although recent advances in the field of oncogenic gene mutations have made it possible to realize molecular-targeted therapy, monitoring the VOC signatures associated with cancer-specific genetic mutations may be a faster and easier method than conventional gene profiling. This technique would aid the improvement of drug selection and detection of resistance, thereby increasing the clinical benefits for patients with LC through safer, more timely and effective interventions that could improve their overall survival and quality of life.
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ZL contributed to the design and writing of the manuscript, with support from ZZ and JH. BY and YC drafted the initial manuscript and revised it critically for important intellectual content. JS made substantial contributions to conception and design, and gave final approval of the version to be published. All authors read and approved the final manuscript.

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Patient consent for publication

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Competing interests

The authors declare that they have no competing interests.

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EMERGING NON-INVASIVE DETECTION METHODOLOGIES FOR LUNG CANCER

INTRODUCTION
Lung cancer is the leading cause of cancer-related deaths worldwide, with an estimated 1.7 million deaths in 2018. Early detection is crucial for improving outcomes, as non-small cell lung cancer (NSCLC) often presents at an advanced stage, leading to poor survival rates. There is a growing interest in non-invasive detection methodologies to improve early diagnosis and tailor treatment strategies.

METHODS

1. Liquid biopsy: Analysis of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs)
   - ctDNA is a non-invasive biomarker that reflects the molecular profile of the primary tumor and metastases.
   - CTCs are tumor cells that have shed from the primary tumor and are present in the peripheral blood.

2. Breath analysis: Detection of volatile organic compounds (VOCs) and breath biomarkers
   - VOCs can be analyzed for their potential as non-invasive biomarkers of cancer.
   - Breath analysis can be performed using mass spectrometry or gas chromatography.

3. Biomarkers: Identification of specific genetic mutations and protein expression
   - EGFR, KRAS, and ALK mutations are common in NSCLC.
   - Biomarker testing is integral to selecting appropriate therapy.

4. Imaging: Functional imaging techniques like positron emission tomography (PET) and diffusion-weighted MRI
   - PET-CT is used for staging and monitoring response to treatment.
   - Diffusion-weighted MRI can detect early tumor progression.

5. Nanotechnology: Development of nanomaterials for targeted delivery and imaging
   - Nanomaterials offer the potential for highly specific and minimally invasive detection of cancer.

6. Artificial intelligence (AI): Image analysis and pattern recognition for improved diagnostic accuracy
   - AI algorithms can analyze large datasets of medical images and predict outcomes.

RESULTS

1. Liquid biopsy
   - ctDNA analysis can detect early stage NSCLC with high sensitivity.
   - CTCs can provide insights into tumor heterogeneity and drug resistance.

2. Breath analysis
   - VOC analysis has shown promise in identifying cancer patients.
   - Breath biomarkers are being studied for their potential as non-invasive diagnostic tools.

3. Biomarkers
   - EGFR mutations are sensitive indicators of response to EGFR inhibitors.
   - ALK rearrangements predict sensitivity to ALK inhibitors.

4. Imaging
   - PET-CT is a valuable tool for staging and monitoring treatment response.
   - Diffusion-weighted MRI can detect early tumor progression.

5. Nanotechnology
   - Nanomaterials offer the potential for highly specific and minimally invasive detection of cancer.

6. Artificial intelligence
   - AI can analyze medical images and predict outcomes with high accuracy.

CONCLUSIONS
Non-invasive detection methodologies offer promising avenues for improving early detection and targeted treatment of lung cancer. Further research is needed to validate these techniques and integrate them into clinical practice.

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