



## MINI REVIEW

# Liquid biopsies to distinguish malignant from benign pulmonary nodules

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## Abstract

Over the past decades, low-dose computed tomography (LD-CT) screening has been widely used for the early detection of lung cancer. Increasing numbers of indeterminate pulmonary nodules are now being discovered. However, it remains challenging to distinguish malignant from benign pulmonary nodules, especially those considered to be small or ground-glass (GGN) nodules. Liquid biopsies have been successfully applied in the diagnosis of advanced lung cancer, and the potential value for early detection of lung cancer has made great progress. Recent studies have demonstrated the value of various blood-based tumor biomarkers in determining the nature of pulmonary nodules, including cell-free DNA (cfDNA), microRNAs (miRNAs), circulating tumor cells (CTCs) and tumor-associated autoantibodies (AABs). In this review, we summarize the latest progress of liquid biopsies, and their potential applications and challenges in the diagnosis of malignant pulmonary nodules.

## KEYWORDS

biomarker, cell-free DNA, liquid biopsy, miRNA, pulmonary nodule diagnosis

Rui Tao, Wei Cao, and Feng Zhu contributed equally to this study.

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## INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the world, including China. According to the cancer statistics in 2018, there were 774 000 new cases of lung cancer diagnosed and 691 000 lung cancer-related deaths in China.<sup>1</sup> One of the important reasons for the high mortality of lung cancer is the lack of specific clinical symptoms in the early stages. Most patients with lung cancer are at an advanced stage at the time of first diagnosis. The postoperative five-year survival rate of patients with stage 0 lung cancer can reach 90%, it is reduced to about 60% in patients at stage IA, and is only about 17% in patients with advanced lung cancer.<sup>2</sup>

Low-dose computed tomography (LD-CT) screening has been widely used in the early detection of lung cancer, especially for the >45 age and smoker population. The early stage of lung cancer (especially non-small cell lung cancer [NSCLC]) mainly presents in the form of small nodules in the lung, represented by a lesion with a diameter of less than 3.0 cm on CT scan, which is usually not accompanied by atelectasis, lymphadenopathy and pleural effusion.<sup>3,4</sup> Pulmonary nodules are not the specific manifestations of early lung cancer, and most pulmonary nodules are benign lesions of which the most common benign lesions are granuloma (inflammatory nodules), commonly caused by tuberculosis infection or fungal infections such as spore bacteria. Pneumonia is also a common cause of inflammatory nodules. Pulmonary nodules can also occur in immune-related diseases, including rheumatoid arthritis and granulomatosis with polyangiitis.<sup>5</sup> A previous study of high-risk screening for lung cancer by LD-CT scan has found that pulmonary nodules were detected in 24.2% of 26 722 participants, 96.4% of which were benign.<sup>6</sup> Judging the benign or malignant nature of nodules is a key issue in the early detection of lung cancer. Pulmonary nodules can be generally divided into solid and subsolid nodules, with significantly different morphological features. Ground-glass opacities on CT, including pure ground-glass nodules (pGGNs) and mixed ground-glass nodules (mGGNs), are particularly challenging to diagnose owing to their malignant potential and heterogeneous characteristics.

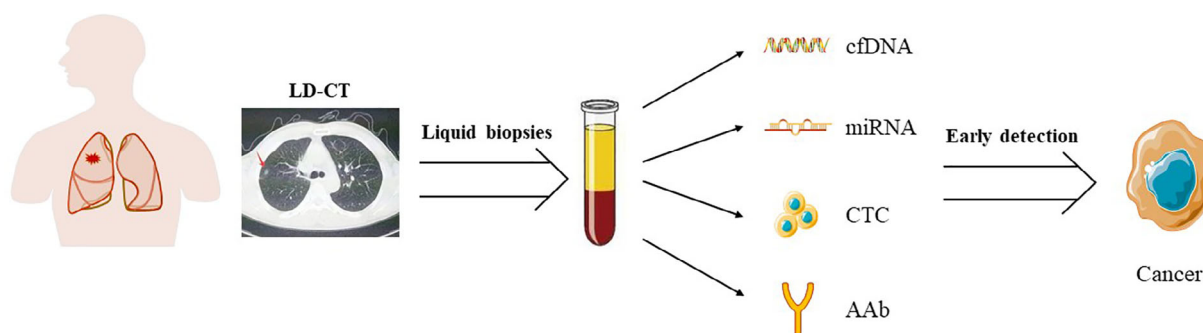
In recent years, liquid biopsies have received increasing attention in the diagnosis of pulmonary nodules by using cell-free DNA (cfDNA), microRNAs (miRNAs), circulating tumor cells (CTCs) and serum biomarkers (Figure 1). The liquid biopsy specimens commonly used for lung cancer are peripheral circulating blood, sputum and bronchoalveolar lavage fluid, which contain tumor cells and tumor-derived products released from lung tumors or precancerous tissues. These specimens are easily obtained with noninvasive and repeatable advantages. As the tissue biopsy is difficult for small pulmonary nodules, liquid biopsy has become a promising clinical strategy. In this review, we summarize the latest progress of liquid biopsies to aid in distinguishing malignant from benign pulmonary nodules.

## CT IMAGING CHARACTERISTICS OF MALIGNANT PULMONARY NODULES

First, the malignant probability of pulmonary nodules can be judged based on the patient's clinical information, including age, smoking history, tumor history and accurate CT imaging characteristics. Some characteristics of CT imaging including nodule size, lobar location, density and margin characteristics, have been used to estimate the risk of malignancy.

### Size of nodules

In general, the risk of malignancy in pulmonary nodules is related to their diameter. There is a higher risk of malignancy in larger nodules. For pulmonary nodules with a diameter of less than 5 mm, the risk of malignancy has been reported to be only 0.4%, and the risk increased to 1.3% in those with a diameter between 5–10 mm.<sup>7</sup> When the diameter of nodules is above 10 mm, the risk of malignancy has been reported to significantly increase from 33% to 60% at 10–20 mm and 64%–82% at greater than 20 mm.<sup>8</sup> Therefore, the diameter of pulmonary nodules is a major consideration in the management of pulmonary nodules.



**FIGURE 1** Schematic representation of liquid biopsies to aid the diagnosis of pulmonary nodules detected by low dose computed tomography (LD-CT). These biomarkers for liquid biopsies include cell-free DNA (cfDNA), microRNAs (miRNAs), circulating tumor cells (CTC) and autoantibodies (AABs)

## Lobar location

The location of pulmonary nodules can also help to estimate the risk of malignancy. A large-scale pulmonary nodule study indicated that a nodule within the upper lobe indicates a higher risk of lung cancer, and 45% of all malignant pulmonary nodules are located in the right upper lobe, which may be associated with the fact that the right upper lobe is the first contact with an external carcinogenic gas.<sup>9</sup> However, the upper lobe is also a site prone to tuberculosis in Chinese patients, so not all nodules located in the upper lobe are malignant.

## Density

Pulmonary nodules can be divided into solid and subsolid nodules according to their density. Subsolid nodules are also called ground-glass nodules (GGNs), which can be classified as pGGN and mGGN based on the presence of a solid component. GGNs have a higher risk of malignancy than solid nodules, while mGGNs have the highest malignant frequency of 63%, followed by pGGN with a malignant frequency of 18%, and solid nodules an incidence of malignancy of only 7%.<sup>10</sup> Moreover, the greater proportion of solid components in mGGN, or the gradual increase of solid components has been reported to indicate a greater probability of malignancy.<sup>8</sup>

## Margin characteristics

Malignant pulmonary nodules are more likely to show rough edges when they spread and invade surrounding lung tissue. Therefore, some identified margin characteristics can increase the probability of malignancy, such as spiculation (a linear shadow of varying length extending from the nodule margin to the surrounding lung tissue) and lobulation (the edge of the lesion characterized by an uneven irregular notch), while benign nodules are usually smooth and round-like with an obvious boundary.<sup>11</sup> In addition, there are some special structural changes in the surrounding structure of malignant pulmonary nodules, including vessel convergence (the thickening of blood vessels near the nodules and the accumulation of blood into the focal areas contributing to the growth of malignant tumors) and pleural indentation (indentation due to subpleural fibrosis or the invasion of malignant tumors to the visceral pleura).<sup>12</sup>

However, CT imaging has several limitations in the diagnosis of malignant lung nodules as follows.<sup>13</sup> (i) False positives. Studies have reported that LD-CT could produce 20% or more false-positive results in baseline screens and 3% or more in subsequent screens.<sup>14</sup> (ii) Overdiagnosis. Studies have reported that 25% of lung cancers found by LD-CT screening were slow growing or indolent, which would not become clinically significant or lead to death if left untreated.<sup>15</sup> (iii) Exposure to excess radiation. Prolonged CT

follow-up can cause exposure to excess radiation, which may lead to a potential carcinogenic risk.<sup>16</sup> (iv) Psychological stress. Most patients with positive results detected by LD-CT had psychological distress, which would affect life and work.

A lung tissue biopsy for small lung nodules is invasive and high risk, and some patients are not considered suitable for this procedure, such as if they suffer from severe emphysema, coagulation disorders, etc. Therefore, noninvasive examinations such as liquid biopsy are needed to aid the diagnosis of aggressive malignant pulmonary nodules.

## LIQUID BIOPSIES FOR PULMONARY NODULES

Recently, the use of liquid biopsies has enabled greater progress in cancer diagnosis for the detection of tumor cells and tumor-derived products in body fluids such as blood, urine, saliva, etc.<sup>17</sup> Compared with traditional tissue biopsies that require invasive surgery, liquid biopsies have an advantage in that they are noninvasive and serial biopsies, which make it possible to monitor the changes of tumor molecules in real time.<sup>18</sup> In addition, liquid biopsies detect the biomarkers released from various tumor sites, which comprehensively reflect the various molecular images of malignant tumors and solve the problem of tumor heterogeneity.<sup>19</sup> Recently, biomarkers of liquid biopsies have been studied for the diagnosis of pulmonary nodules, including cfDNA, miRNAs, CTCs and autoantibodies.<sup>20</sup>

### cfDNA

cfDNA are fragments of double-stranded DNA, usually 150–200 bp, and are released into the blood as a result of cellular apoptosis or necrosis, with a short half-life of 30 min to 2 h in the blood. The content of cfDNA released into blood is very low in healthy people, with an average of 10–15 ng/ml, while the level of cfDNA increases in cancer patients.<sup>21</sup> Several studies have demonstrated that cfDNA from healthy individuals consists of regular short fragments (180–200 bp), produced via enzymatic cleavage during apoptosis. However, cfDNA from patients with different cancer types usually consists of large fragments over 10 000 bp, which could be associated with necrosis.<sup>22</sup> Thus, some studies have used the ratio of shorter to longer cfDNA fragments, called cfDNA integrity, as cancer biomarkers.<sup>23</sup> Furthermore, studies have shown that cfDNA in cancer patients has the same genetic and epigenetic changes with tumors, indicating that they are derived from tumor cells, also called circulating tumor DNA (ctDNA).<sup>24</sup> cfDNA reflects genetic and epigenetic alterations including mutations, deletions, rearrangements, abnormal copy numbers and methylation, thereby making them important biomarkers.<sup>25</sup> Table 1 summarizes the recent studies on cfDNA as a biomarker in pulmonary nodules.

**TABLE 1** Cell-free DNA (cfDNA) as biomarkers for distinguishing malignant from benign pulmonary nodules

Biomarkers	Samples	Genes	Detection methods	Number of patients	Sensitivity (%)	Specificity (%)	References
Mutations	Plasma and PBMC	Whole exome	WES	33	NA	NA	Taylor et al. <sup>28</sup>
Level and integrity	Plasma	NA	qPCR	109	Level (86.4), integrity (91)	Level (61.4), integrity (68.2)	Szpechcinski et al. <sup>26</sup>
Level and integrity	Plasma	NA	qPCR	318	Level (57.5), integrity (55.7)	Level (64.8), integrity (82.9)	Leng et al. <sup>27</sup>
Methylation	Serum, plasma and tissues	APC, RASSF1A	QMSP	89	56.9	90.3	Gao et al. <sup>33</sup>
Methylation	Plasma	Nine markers	NGS	505	79.5	85.2	Liang et al. <sup>13</sup>
Methylation	Tissues and serum	RUNX3, RASSF1A	QMSP	147	NA	NA	Zhang et al. <sup>34</sup>
Methylation	Plasma	SHOX2, PTGER4	QMSP	31	90	91	Chu et al. <sup>35</sup>
Methylation	Plasma	Set 1: CDO1, SOX17, HOXA7 Set 2: CDO1, TAC1, SOX17	QMSP	246	Set 1: 90, Set 2: 89	Set 1: 71, Set 2: 61	Chen et al. <sup>36</sup>

Note: Level, concentration of cfDNA in the blood; Integrity, the ratio of shorter to longer cfDNA fragments in the blood.

Abbreviations: NA, not available; PBMC, peripheral blood mononuclear cells; QMSP, quantitative methylation-specific PCR; qPCR, quantitative real-time PCR; WES, whole exome sequencing.

### cfDNA level and integrity

cfDNA level and integrity have been studied to distinguish malignant from benign nodules. Szpechcinski et al. have reported that the level of cfDNA in the patients with NSCLC was significantly higher compared with that in patients with benign nodules ( $10.76 \pm 13.15$  ng/ml vs.  $6.60 \pm 8.70$  ng/ml). cfDNA integrity was calculated from the Ct values of the 100-bp and 400-bp qPCR products. There was a statistically significant trend ( $p = 0.0584$ ) between the value in patients with benign lung nodules ( $3.06 \pm 1.37$ ) and the value in patients with NSCLC ( $4.00 \pm 1.60$ ). According to the cfDNA concentration (cutoff level: 2.80 ng/ml), the sensitivity and specificity for distinguishing NSCLC cases from patients with benign pulmonary nodules and healthy individuals were 86.4 and 61.4%, respectively. According to the cfDNA integrity (cutoff level: 2.64), the sensitivity and specificity were 91% and 68.2%, respectively.<sup>26</sup> cfDNA level and integrity have also been studied to distinguish NSCLC from tuberculosis. A study has shown that patients with NSCLC had significantly higher cfDNA levels ( $95.67 [51.28, 238.85]$  ng/ $\mu$ l) than those in patients with tuberculosis ( $59.60 [34.25, 102.53]$  ng/ $\mu$ l) and in healthy controls ( $44.66 [24.56, 66.54]$  ng/ $\mu$ l). The integrity of cfDNA in patients with NSCLC was also higher than that in patients with tuberculosis and healthy controls. The study demonstrated that cfDNA level and integrity was used to distinguish NSCLC and tuberculosis with a sensitivity of 57.50 and 55.70% and a specificity of 64.80 and 82.90%, respectively.<sup>27</sup>

### cfDNA mutation

The occurrence and development of tumor is related to its gene mutations. cfDNA contains the same mutations as

its source cells, and the detection of cfDNA mutations can be used to distinguish malignant pulmonary nodules. Chen et al. detected cfDNA and matched peripheral blood mononuclear cells (PBMC) from 33 pulmonary nodules in patients' blood samples by whole exome sequencing (WES), and the results demonstrated that the cfDNA of malignant pulmonary nodules had more mutations than those of benign groups ( $1083 \pm 476$  vs.  $553 \pm 519$ ,  $p < 0.0046$ ).<sup>28</sup> However, WES is not clinically practical to distinguish malignant pulmonary nodules, due to its high cost. A small panel to detect lung cancer specific mutations, such as *EGFR* mutations (pL858R and exon19 del, etc) could be developed in liquid biopsy to distinguish malignant pulmonary nodules.

### cfDNA methylation

In addition to carcinogenic factors causing changes in DNA sequences, abnormal regulatory mechanisms are also common in the course of tumorigenesis, such as DNA methylation.<sup>29</sup> DNA methylation refers to the process of transferring a methyl group to the fifth carbon of cytosine (5-methylcytosine, 5 mC) by DNA methyltransferase (DNMT). DNA methylation often occurs on gene promoter regions and play a key role in regulating the expression of downstream genes.<sup>30</sup> Studies have shown that aberrant DNA methylation often occurs in the early stages of cancer and persists throughout its development, and these aberrant DNA methylations have been found in a variety of tumors.<sup>21,31,32</sup> The methylation of cfDNA as a biomarker in the diagnosis of pulmonary nodules has also been studied.

Li et al. examined the frequency of APC and RASSF1A methylation in the plasma of 89 CT-detected pulmonary nodule patients (58 early stage lung cancers and 31 benign lung diseases) and 23 healthy controls. The results showed

that the frequencies of APC and RASSF1A methylation were 24.1% and 43.1% in the plasma of lung cancer patients; however, the frequencies were 3.2% and 6.5% in benign lung disease. APC and RASSF1A methylation were not detected in healthy controls. The sensitivity and specificity to distinguish lung cancers from benign lung diseases were 56.9% and 90.3%.<sup>33</sup>

Recently, more specific methylation sites on pulmonary nodules have been identified with the development of high-throughput sequencing technology. Liang et al. first identified cancer-specific methylation markers using 230 tissue samples (129 malignant lung cancers and 101 benign lesion samples) by high-throughput bisulfite DNA methylation sequencing. Moreover, using a training set of 66 plasma samples, nine markers (cg19864007\_cg22636429\_cg15542994, cg26970841\_cg03978375\_cg24826.

867, cg04175417, cg21962423, cg23156742, cg06287318, cg21963643, cg07568344 and cg12545252) were selected to build a diagnostic prediction model. From an independent validation set of additional 66 plasma samples, the sensitivity and specificity of the model for differentiating patients with malignant tumors were 79.5 and 85.2%, respectively.<sup>13</sup>

In a study including 147 patients (89 benign and 58 malignant) with small solitary pulmonary nodules  $\leq 10$  mm in diameter, the methylation rate of RUNX3 and RASSF1A gene in the serum cfDNA of patients with malignant solid pulmonary nodules (SPNs) was higher than that in benign SPNs (65.5 and 67.2% vs. 12.3 and 10.1%).<sup>34</sup> Chu et al. detected SHOX2 and PTGER4 gene methylation in the plasma cfDNA of 20 patients with pathologically diagnosed lung cancer, of which 18 were positive for DNA methylation of SHOX2 and PTGER4.<sup>35</sup> Chen et al. found that the methylation frequency of CDO1, TAC1, SOX17 and HOXA7 in patients with malignant pulmonary nodules was significantly higher than that in the benign group. A three-gene combination including the best individual genes (CDO1, SOX17 and HOXA7) had sensitivity and specificity of 90% and 71% in distinguishing malignant from benign pulmonary nodules, while the other three-gene combination (CDO1, TAC1, and SOX17) had sensitivity and specificity of 89% and 61%.<sup>36</sup>

## miRNA

miRNA is a non-coding, short, single-stranded RNA with an average size of 22 nucleotides, which is involved in the pathophysiological processes of the organism by regulating gene transcription and translation.<sup>37</sup> miRNA can regulate gene expression at the post-transcriptional level by binding mRNA 3' UTR, leading to the degradation or transcriptional inhibition of mRNA.<sup>38</sup> Many studies have shown that abnormal expression of miRNA is involved in the proliferation, metastasis, invasion and angiogenesis of tumor cells.<sup>39</sup> miRNA can be secreted into body fluids by normal cells in physiological or pathological conditions, or by apoptotic and necrotic cells.<sup>40</sup> miRNA as a biomarker to judge the nature of pulmonary nodules has also been recently studied.

Many studies have used quantitative PCR to detect miRNA levels in patients' blood or sputum to assist in the diagnosis of benign and malignant pulmonary nodules (Table 2). Recent studies have shown that the sensitivity of miRNA as a biomarker to differentiate malignant pulmonary nodules ranged from 54.8% to 92.9%, and the specificity was 69.2% to 90.9%. The study has shown that the expression levels of plasma miR-21 and miR-210 in the malignant SPN group were higher than those in the benign SPN group and healthy control group, while miR-486-5p had lower expression level in the malignant SPN group than the others. The sensitivity and specificity of three plasma miRNAs (miR-21, miR-210 and miR-486-5p) for differentiating lung tumors from benign SPN were 75.00% and 84.95%.<sup>41</sup> Ten miRNAs (miRNA-17, -146a, -200b, -182, -155, -221, -205, -126, -7, -21) in plasma were reported to aid in nodule diagnosis with the sensitivity ranging from 54.8% to 83.3% and the specificity ranging from 73.3% to 86.7%.<sup>42</sup> Lin et al. developed a classifier with two plasma miRNAs (miRs-205-5p and miRs-126) and the diameter of SPN for distinguishing malignant from benign SPNs, which had 89.9% sensitivity and 90.9% specificity.<sup>43</sup> This study indicates that the combination of plasma biomarkers and radiologic features is more accurate to identify lung cancer from uncertain pulmonary nodules. A recent study also demonstrated that a prediction model containing three miRNAs (miRNA-146a, miRNA-200b, miRNA-7) in plasma and CT features (pleural indentation and speculation) can judge the nature of SPNs with the sensitivity and specificity of 92.9% and 83.3% in the training set as well as 71.8% and 69.2% in the validation set.<sup>44</sup>

Serum can also be used as a medium for detecting miRNA, although studies have showed that the coagulation process may affect miRNA levels in serum.<sup>45</sup> Li et al. used the levels of serum miRNA-21-5p and miRNA-574-5p to determine the nature of pulmonary nodules with a positive predictive value (PPV) of 55%, and a negative predictive value (NPV) of 84.2%.<sup>46</sup> miR-199a-3p, miR-148a-3p, miR-210-3p, miR-378d and miR-138-5p in the serum were also reported to aid in the early diagnosis of pulmonary nodules.<sup>47</sup> Fan et al. found that five miRNA ratios (miR-15b-5p/miR-146b-3p, miR-20a-5p/miR-146b-3p, miR-19a-3p/miR-146b-3p, miR-92a-3p/miR-146b-3p, and miR-16-5p/miR-146b-3p) were higher in the malignant than the benign group.<sup>48</sup>

miRNA in sputum, tissues and peripheral PBMC can also be used as a diagnostic marker for pulmonary nodules. Three miRNA biomarkers (miR-21, 31, and 210) were found in sputum to identify malignant SPNs with 80.52%–82.93% sensitivity and 86.08%–88.41% specificity.<sup>49</sup> Seven miRNAs (miR-199a-3p, chr17\_10932, miR-148a-3p, miR-210-3p, chr1\_1402, miR-378d and miR-138-5p) in tissues and two miRNA (miR-19b-3p, miR-29b-3p) in the PBMC of pulmonary nodule patients have been reported for early lung cancer diagnosis.<sup>50,51</sup> A more recent study also indicated that exosome miRNAs in plasma could aid in the diagnosis of pulmonary nodules, especially GGNs. miR-185-5p/miR-32-5p and miR-140-3p/let-7f-5p showed the highest



**TABLE 2** MicroRNAs (miRNAs) as biomarkers for distinguishing malignant from benign pulmonary nodules

Biomarkers	Samples	Detection methods	Number of patients	Sensitivity (%)	Specificity (%)	References
miR-21, miR-210, miR-486-5p	Plasma	qRT-PCR	250	75.00	84.95	Shen et al. <sup>41</sup>
miR-21, miR-31, miR-210	Sputum	qRT-PCR	413	80.52–82.93	86.08–88.41	Xing et al. <sup>49</sup>
Seven miRNAs	Tissues	NGS, qRT-PCR	208	86.40	60.60	He et al. <sup>50</sup>
miR-21-5p, miR-574-5p	Serum	qRT-PCR	39	55 (PPV)	84.2 (NPV)	Li et al. <sup>46</sup>
miR-205-5p, miRs-126 and diameter	Plasma	microarray and ddPCR	359	89.9	90.9	Lin et al. <sup>43</sup>
miR-19b-3p, miR-29b-3p, spiculation and smoking pack-year	PBMC	qRT-PCR	248	80.3	89.4	Ma et al. <sup>51</sup>
Five miRNA ratios	Serum	qRT-PCR	321	70	90	Fan et al. <sup>48</sup>
miR-199a-3p, miR-148a-3p, miR-210-3p, miR-378d, miR-138-5p	Serum	qRT-PCR	369	34.0	90.2	He et al. <sup>47</sup>
10 miRNAs	Plasma	qRT-PCR	57	54.8–83.3	73.3–86.7	Xi et al. <sup>42</sup>
miRNA-146a, miRNA-200b, miRNA-7	Plasma	qRT-PCR	92	Set 1: 92.9 Set 2: 71.8	Set 1: 83.3 Set 2: 69.2	Xi et al. <sup>44</sup>

Abbreviations: dd PCR, droplet digital PCR; NGS, next generation sequencing; NPV, negative predictive value; PBMC, peripheral blood mononuclear cells; PPV, positive predictive value; qRT-PCR, quantitative reverse-transcription PCR.

diagnostic value, with a sensitivity (59.3%–85.1%) and specificity (75%–100%).<sup>52</sup>

### Circulating tumor cells (CTCs)

CTCs are cancer cells that have entered the blood circulation from primary or metastatic tumor lesions, which are closely related to tumor metastasis and proliferation.<sup>53</sup> As an important biomarker for liquid biopsy, CTC has a good application prospect in cancer diagnosis, management and treatment.<sup>53</sup> The CTC count has been proven to be a promising biomarker for the diagnosis, prognosis and monitoring of therapeutic response in lung cancer.<sup>54–56</sup> The biggest challenge for CTC application in the early detection of cancer is the enrichment of very few CTC cells, which usually exist as single tumor cell in the context of millions of blood cells.<sup>57</sup> The smaller the size of the lesion, the lower the amount of CTCs in the blood, thereby causing low diagnostic sensitivity of CTCs for malignant SPNs. Several techniques dependent on the physical and biological properties of CTCs have been used for CTC enrichment, such as immunomagnetic positive/negative enrichment, microfluidic immunocapture, membrane filtration and so on.<sup>58</sup>

Great progress has been made in the diagnosis of benign and malignant pulmonary nodules by CTC in recent years. The presence of CTC or circulating tumor microemboli (CTM) in the blood enriched by the ScreenCell Cyto filtration method has been used to aid in the diagnosis of suspicious malignant lung nodules in a study containing 75 patients with SPNs, indicating a low sensitivity of 70.1% and high specificity of 100%.<sup>59</sup> Zheng et al. collected CTCs from patients' blood with SPNs by the immunomagnetic positive enrichment and SE i-FISH method, which detected gene copy number in eight chromosomes and tumor-

associated antigen (TAA) CK18. The results demonstrated that CTCs combined with CEA had high diagnostic efficiency for upper, subsolid and  $\geq 8$  mm nodules with a sensitivity of 77.8% and specificity of 90%.<sup>60</sup>

The limitation of the current techniques is the small blood volume available for CTC enrichment, which could result in the low sensitivity of CTC detection. To overcome the limitation, a novel in vivo CTC enrichment device, GILUPI CellCollector has been designed. In the study by Saucedo-Zeni et al., the GILUPI CellCollector functional area was coated with EpCAM antibody and a guidewire was inserted into the patient's veins via an indwelling catheter and maintained for 30 min to isolate the CTCs.<sup>61</sup> Using the CellCollector CTC detection method, He et al. have shown that 15.6% (5/32) of the patients were positive ( $\geq 1$  CTC) in the GGN group, 73.3% of patients were positive in the lung cancer group, while no "CTC-like" events were detected in the healthy group.<sup>62</sup>

The other challenge for CTC application in the early detection of cancer is the accurate identification of CTCs originating from primary tumors. Previous studies have reported that folate receptor (FR) is highly expressed in several types of cancers including lung cancer.<sup>63</sup> Moreover, apart from a rare subgroup of activated macrophages, no cells express FR in the blood, which makes it possible to be a potential biomarker for identifying CTCs in the peripheral blood.<sup>64</sup> Chen et al. have detected FR-positive CTCs from peripheral blood of 200 patients (80 with SPNs and 120 diagnosed with lung cancer) by immunomagnetic negative enrichment and ligand-targeted PCR, and demonstrated that CTC level is an independent risk factor for malignant SPNs.<sup>65</sup> The same method has been used in another two studies to indicate that FR-positive CTCs levels can be used to distinguish malignant from benign pulmonary nodules with a sensitivity of 78.6%–82.7%/80% and a specificity of 68.8%–78.4%/75%.<sup>66,67</sup>

## Autoantibody test

The presence of tumor cells with TAAs can activate the immune system to produce autoantibodies (AAb). AAb can be detected in the early stages of asymptomatic malignancies. In addition, AAb can be found in serum with a long, stable, and high level, so they can be used as biomarkers for differentiating benign and malignant pulmonary nodules.<sup>68</sup> The AAb to lung cancer used in clinic include seven AAb (7AAb) (P53, NY-ESO-1, CAGE, GBU4-5, SOX2, HuD and MAGE A4). The serum of 296 patients with pulmonary nodules were tested with 7AAb in a study, which demonstrated that patients with a positive test result represented a >two-fold risk for the development of lung cancer as compared to a negative result group in 4 mm to 20 mm nodules.<sup>69</sup> Edelsberg et al also indicated that the sensitivity and specificity of 7AAb test for aiding in the diagnosis of intermediate pulmonary nodules were 41 and 93%, and the use of 7AAb test combined with CT examination for pulmonary nodules can reduce the cost of medical treatment.<sup>70</sup> In a recent study, Lastwika et al. defined 11 AAb isolated from B cells of 10 resected lung tumors by high-density protein arrays, and demonstrated that a combination of four AAb (FCGR2A, EPB41L3, LINGO1 and S100A7L2) could detect indeterminate (8–20 mm) pulmonary nodules with a sensitivity and specificity of 91.7 and 57.1%.<sup>71</sup>

## CHALLENGES OF LIQUID BIOPSY IN CLINICAL APPLICATION

It should be noted that the sensitivity and specificity of cfDNA, miRNA, CTC and autoantibodies as biomarkers for the diagnosis of pulmonary nodules are not sufficiently high for clinical use. The possible reasons for this are as follows. First, for malignant pulmonary nodules (early-stage lung cancer), the concentrations of these biomarkers in the blood are extremely low. The detection methods may not be sensitive enough, thereby leading to false negative results. Second, lung tumors have different histological types, which exhibit various genetic and epigenetic alterations. Thus, it is difficult for these biomarkers to cover all tumor-related changes, thereby leading to false negative results. Third, these biomarkers may not be specific enough to lung cancer, thereby leading to false positive results. For example, some biomarkers have been previously reported to be detected in some noncancerous diseases, such as bronchitis, fibrosis, pneumonia, granuloma and scleroderma.<sup>72</sup> Therefore, in order to accurately distinguish malignant pulmonary nodules, the more sensitive detection methods, combination of more biomarkers, and more specific biomarkers need to be developed in the future.

In conclusion, it is difficult to diagnose pulmonary nodules by CT imaging alone, especially for small nodules (<10 mm) of ground-glass nature. Liquid biopsies provide new approaches for noninvasive diagnosis of pulmonary nodules, including ctDNA, miRNAs, CTCs and autoantibodies.

However, the sensitivity and specificity of liquid biopsy in distinguishing malignant nodules still requires improvement. The development of high-throughput sequencing has led to the discovery of more specific mutations, methylation sites and miRNAs in malignant pulmonary nodules, which need large-scale clinical studies to confirm their diagnostic ability. Overall, liquid biopsy is a promising and challenging approach in the clinical diagnosis of pulmonary nodules. It is hoped that in the future the combined strategy of CT imaging with liquid biopsy will significantly improve the diagnostic ability of pulmonary nodules.

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## CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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## REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394–424.
2. Tang Y, Qiao G, Xu E, Xuan Y, Liao M, Yin G. Biomarkers for early diagnosis, prognosis, prediction, and recurrence monitoring of non-small cell lung cancer. *Onco Targets Ther.* 2017;10:4527–34.
3. MacMahon H, Naidich DP, Goo JM, Lee KS, Leung ANC, Mayo JR, et al. Guidelines for management of incidental pulmonary nodules detected on CT images: from the Fleischner Society 2017. *Radiology.* 2017;284(1):228–43.
4. Ettinger DS, Aisner DL, Wood DE, Akerley W, Bauman J, Chang JY, et al. NCCN guidelines insights: non-small cell lung cancer, version 5.2018. *J Natl Compr Canc Netw.* 2018;16(7):807–21.
5. Khan T, Usman Y, Abdo T, Chaudry F, Keddissi JI, Youness HA. Diagnosis and management of peripheral lung nodule. *Ann Transl Med.* 2019;7(15):348.
6. Aberle DR, Adams AM, Berg CD, Black WC, Clapp JD, Fagerstrom RM, et al. Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med.* 2011;365(5):395–409.
7. Horeweg N, van Rosmalen J, Heuvelmans MA, van der Aalst CM, Vliedenthart R, Scholten ET, et al. Lung cancer probability in patients with CT-detected pulmonary nodules: a prespecified analysis of data from the NELSON trial of low-dose CT screening. *Lancet Oncol.* 2014;15(12):1332–41.
8. Nasim F, Ost DE. Management of the solitary pulmonary nodule. *Curr Opin Pulm Med.* 2019;25(4):344–53.
9. Horeweg N, van der Aalst CM, Thunnissen E, Nackaerts K, Weenink C, Groen HJ, et al. Characteristics of lung cancers detected

- by computer tomography screening in the randomized NELSON trial. *Am J Respir Crit Care Med.* 2013;187(8):848–54.
10. Henschke CI, Yankelevitz DF, Mirtcheva R, McGuinness G, McCauley D, Miettinen OS, et al. CT screening for lung cancer: frequency and significance of part-solid and nonsolid nodules. *AJR Am J Roentgenol.* 2002;178(5):1053–7.
  11. Guo HY, Lin JT, Huang HH, Gao Y, Yan MR, Sun M, et al. Development and validation of a (18)F-FDG PET/CT-based clinical prediction model for estimating malignancy in solid pulmonary nodules based on a population with high prevalence of malignancy. *Clin Lung Cancer.* 2020;21(1):47–55.
  12. Wang L, Chen Y, Tang K, Lin J, Zhang H. The value of F-FDG PET/CT mathematical prediction model in diagnosis of solitary pulmonary nodules. *Biomed Res Int.* 2018;2018:9453967.
  13. Liang W, Zhao Y, Huang W, Gao Y, Xu W, Tao J, et al. Non-invasive diagnosis of early-stage lung cancer using high-throughput targeted DNA methylation sequencing of circulating tumor DNA (ctDNA). *Theranostics.* 2019;9(7):2056–70.
  14. Tammemagi M, Lam S. Screening for lung cancer using low dose computed tomography. *BMJ.* 2014;348:g2253.
  15. Veronesi G, Maisonneuve P, Bellomi M, Rampinelli C, Durli I, Bertolotti R, et al. Estimating overdiagnosis in low-dose computed tomography screening for lung cancer: a cohort study. *Ann Intern Med.* 2012;157(11):776–84.
  16. Bach P, Mirkin J, Oliver T, Azzoli C, Berry D, Brawley O, et al. Benefits and harms of CT screening for lung cancer: a systematic review. *JAMA.* 2012;307(22):2418–29.
  17. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol.* 2017;14(9):531–48.
  18. Zheng H, Wu X, Yin J, Wang S, Li Z, You C. Clinical applications of liquid biopsies for early lung cancer detection. *Am J Cancer Res.* 2019;9(12):2567–79.
  19. Zeng H, He B, Yi C, Peng J. Liquid biopsies: DNA methylation analyses in circulating cell-free DNA. *J Genet Genomics.* 2018;45(4):185–92.
  20. Mattox AK, Bettgowda C, Zhou S, Papadopoulos N, Kinzler KW, Vogelstein B. Applications of liquid biopsies for cancer. *Sci Transl Med.* 2019;11(507):eaay1984.
  21. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. *N Engl J Med.* 2018;379(18):1754–65.
  22. Esposito A, Criscitiello C, Trapani D, Curigliano G. The emerging role of "liquid biopsies," circulating tumor cells, and circulating cell-free tumor DNA in lung cancer diagnosis and identification of resistance mutations. *Curr Oncol Rep.* 2017;19(1):1.
  23. Wang B, Huang H, Chen Y, Bristow R, Kassaei K, Cheng C, et al. Increased plasma DNA integrity in cancer patients. *Cancer Res.* 2003;63(14):3966–8.
  24. Bettgowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med.* 2014;6(224):224ra24.
  25. Esposito A, Criscitiello C, Locatelli M, Milano M, Curigliano G. Liquid biopsies for solid tumors: understanding tumor heterogeneity and real time monitoring of early resistance to targeted therapies. *Pharmacol Ther.* 2016;157:120–4.
  26. Szpechcinski A, Rudzinski P, Kupis W, Langfort R, Orłowski T, Chorostowska-Wynimko J. Plasma cell-free DNA levels and integrity in patients with chest radiological findings: NSCLC versus benign lung nodules. *Cancer Lett.* 2016;374(2):202–7.
  27. Leng S, Zheng J, Jin Y, Zhang H, Zhu Y, Wu J, et al. Plasma cell-free DNA level and its integrity as biomarkers to distinguish non-small cell lung cancer from tuberculosis. *Clin Chim Acta.* 2018;477:160–5.
  28. Tailor TD, Rao X, Campa MJ, Wang J, Gregory SG, Patz Jr EF. Whole exome sequencing of cell-free DNA for early lung cancer: a pilot study to differentiate benign from malignant CT-detected pulmonary lesions. *Front Oncol.* 2019;9:317.
  29. Buyschaert I, Schmidt T, Roncal C, Carmeliet P, Lambrechts D. Genetics, epigenetics and pharmaco-(epi)genomics in angiogenesis. *J Cell Mol Med.* 2008;12(6B):2533–51.
  30. Greenberg MVC, Bourc'his D. The diverse roles of DNA methylation in mammalian development and disease. *Nat Rev Mol Cell Biol.* 2019;20(10):590–607.
  31. Sugai T, Yoshida M, Eizuka M, Uesugii N, Habano W, Otsuka K, et al. Analysis of the DNA methylation level of cancer-related genes in colorectal cancer and the surrounding normal mucosa. *Clin Epigenetics.* 2017;9:55.
  32. Salas LA, Lundgren SN, Browne EP, Punska EC, Anderton DL, Karagas MR, et al. Prediagnostic breast milk DNA methylation alterations in women who develop breast cancer. *Hum Mol Genet.* 2020;29(4):662–73.
  33. Gao L, Xie E, Yu T, Chen D, Zhang L, Zhang B, et al. Methylated APC and RASSF1A in multiple specimens contribute to the differential diagnosis of patients with undetermined solitary pulmonary nodules. *J Thorac Dis.* 2015;7(3):422–32.
  34. Zhang Z, Zhao J, Cui X, Huang X, Lu R, Chen P. Methylation of RUNX3 and RASSF1A and the risk of malignancy in small solitary pulmonary nodules. *J Cancer Res Ther.* 2019;15(4):899–903.
  35. Chu X, Zhao W, Wang B. DNA methylation detection of SHOX2 and PTGER4 in plasma contributes to differential diagnosis of pulmonary nodule patients. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi.* 2019;35(4):357–61.
  36. Chen C, Huang X, Yin W, Peng M, Wu F, Wu X, et al. Ultrasensitive DNA hypermethylation detection using plasma for early detection of NSCLC: a study in Chinese patients with very small nodules. *Clin Epigenetics.* 2020;12(1):39.
  37. Bartel DP. Metazoan microRNAs. *Cell.* 2018;173(1):20–51.
  38. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol Med.* 2014;20(8):460–9.
  39. Jiang LP, He CY, Zhu ZT. Role of microRNA-21 in radiosensitivity in non-small cell lung cancer cells by targeting PDCD4 gene. *Oncotarget.* 2017;8(14):23675–89.
  40. Han Y, Li H. miRNAs as biomarkers and for the early detection of non-small cell lung cancer (NSCLC). *J Thorac Dis.* 2018;10(5):3119–31.
  41. Shen J, Liu Z, Todd NW, Zhang H, Liao J, Yu L, et al. Diagnosis of lung cancer in individuals with solitary pulmonary nodules by plasma microRNA biomarkers. *BMC Cancer.* 2011;11:374.
  42. Xi KX, Zhang XW, Yu XY, Wang WD, Xi KX, Chen YQ, et al. The role of plasma miRNAs in the diagnosis of pulmonary nodules. *J Thorac Dis.* 2018;10(7):4032–41.
  43. Lin Y, Leng Q, Jiang Z, Guarnera MA, Zhou Y, Chen X, et al. A classifier integrating plasma biomarkers and radiological characteristics for distinguishing malignant from benign pulmonary nodules. *Int J Cancer.* 2017;141(6):1240–8.
  44. Xi K, Wang W, Wen Y, Chen Y, Zhang X, Wu Y, et al. Combining plasma miRNAs and computed tomography features to differentiate the nature of pulmonary nodules. *Front Oncol.* 2019;9:975.
  45. Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. *PLoS One.* 2012;7(7):e41561.
  46. Li X, Zhang Q, Jin X, Cao L. Combining serum miRNAs, CEA, and CYFRA21-1 with imaging and clinical features to distinguish benign and malignant pulmonary nodules: a pilot study. *World J Surg Oncol.* 2017;15(1):107.
  47. He Y, Ren S, Wang Y, Li X, Zhou C, Hirsch FR. Serum microRNAs improving the diagnostic accuracy in lung cancer presenting with pulmonary nodules. *J Thorac Dis.* 2018;10(8):5080–5.
  48. Fan L, Sha J, Teng J, Li D, Wang C, Xia Q, et al. Evaluation of serum paired microRNA ratios for differential diagnosis of non-small cell lung cancer and benign pulmonary diseases. *Mol Diagn Ther.* 2018;22(4):493–502.
  49. Xing L, Su J, Guarnera MA, Zhang H, Cai L, Zhou R, et al. Sputum microRNA biomarkers for identifying lung cancer in indeterminate solitary pulmonary nodules. *Clin Cancer Res.* 2015;21(2):484–9.
  50. He Y, Yang Y, Kuang P, Ren S, Rozeboom L, Rivard CJ, et al. Seven-microRNA panel for lung adenocarcinoma early diagnosis in patients



- presenting with ground-glass nodules. *Onco Targets Ther.* 2017;10:5915–26.
51. Ma J, Guarnera MA, Zhou W, Fang H, Jiang F. A prediction model based on biomarkers and clinical characteristics for detection of lung cancer in pulmonary nodules. *Transl Oncol.* 2017;10(1):40–5.
  52. Zhang JT, Qin H, Man Cheung FK, Su J, Zhang DD, Liu SY, et al. Plasma extracellular vesicle microRNAs for pulmonary ground-glass nodules. *J Extracell Vesicles.* 2019;8(1):1663666.
  53. Mateo J, Gerlinger M, Rodrigues DN, de Bono JS. The promise of circulating tumor cell analysis in cancer management. *Genome Biol.* 2014;15(8):448.
  54. Duan GC, Zhang XP, Wang HE, Wang ZK, Zhang H, Yu L, et al. Circulating tumor cells as a screening and diagnostic marker for early-stage non-small cell lung cancer. *Onco Targets Ther.* 2020;13:1931–9.
  55. Wu CY, Lee CL, Wu CF, Fu JY, Yang CT, Wen CT, et al. Circulating tumor cells as a tool of minimal residual disease can predict lung cancer recurrence: a longitudinal, prospective trial. *Diagnostics (Basel).* 2020;10(3):144.
  56. Tamminga M, de Wit S, Schuurings E, Timens W, Terstappen L, Hiltermann T, et al. Circulating tumor cells in lung cancer are prognostic and predictive for worse tumor response in both targeted- and chemotherapy. *Transl Lung Cancer Res.* 2019;8(6):854–61.
  57. Hur S, Henderson-MacLennan N, McCabe E, Di Carlo D. Deformability-based cell classification and enrichment using inertial microfluidics. *Lab Chip.* 2011;11(5):912–20.
  58. Rushton A, Nteliopoulos G, Shaw J, Coombes R. A review of circulating tumour cell enrichment technologies. *Cancers.* 2021;13(5):970.
  59. Mascalchi M, Maddau C, Sali L, Bertelli E, Salvianti F, Zuccherelli S, et al. Circulating tumor cells and microemboli can differentiate malignant and benign pulmonary lesions. *J Cancer.* 2017;8(12):2223–30.
  60. Zheng J, Ye X, Liu Y, Zhao Y, He M, Xiao H. The combination of CTCs and CEA can help guide the management of patients with SPNs suspected of being lung cancer. *BMC Cancer.* 2020;20(1):106.
  61. Saucedo-Zeni N, Mewes S, Niestroj R, Gasiorowski L, Murawa D, Nowaczyk P, et al. A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. *Int J Oncol.* 2012;41(4):1241–50.
  62. He Y, Shi J, Shi G, Xu X, Liu Q, Liu C, et al. Using the new CellCollector to capture circulating tumor cells from blood in different groups of pulmonary disease: a cohort study. *Sci Rep.* 2017;7(1):9542.
  63. Parker N, Turk MJ, Westrick E, Lewis JD, Low PS, Leamon CP. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal Biochem.* 2005;338(2):284–93.
  64. He W, Kularatne SA, Kalli KR, Prendergast FG, Amato RJ, Klee GG, et al. Quantitation of circulating tumor cells in blood samples from ovarian and prostate cancer patients using tumor-specific fluorescent ligands. *Int J Cancer.* 2008;123(8):1968–73.
  65. Ding C, Zhou X, Xu C, Chen J, Ju S, Chen T, et al. Circulating tumor cell levels and carcinoembryonic antigen: an improved diagnostic method for lung adenocarcinoma. *Thorac Cancer.* 2018;9(11):1413–20.
  66. Zhou Q, Geng Q, Wang L, Huang J, Liao M, Li Y, et al. Value of folate receptor-positive circulating tumour cells in the clinical management of indeterminate lung nodules: a non-invasive biomarker for predicting malignancy and tumour invasiveness. *EBioMedicine.* 2019;41:236–43.
  67. Xue Y, Cong W, Xie S, Shu J, Feng G, Gao H. Folate-receptor-positive circulating tumor cells as an efficacious biomarker for the diagnosis of small pulmonary nodules. *J Cancer Res Ther.* 2018;14(7):1620–6.
  68. Broodman I, Lindemans J, Van Sten J, Bischoff R, Luider T. Serum protein markers for the early detection of lung cancer: a focus on autoantibodies. *J Proteome Res.* 2017;16(1):3–13.
  69. Massion PP, Healey GF, Peek LJ, Fredericks L, Sewell HF, Murray A, et al. Autoantibody signature enhances the positive predictive power of computed tomography and nodule-based risk models for detection of lung cancer. *J Thorac Oncol.* 2017;12(3):578–84.
  70. Edelsberg J, Weycker D, Atwood M, Hamilton-Fairley G, Jett JR. Cost-effectiveness of an autoantibody test (EarlyCDT-Lung) as an aid to early diagnosis of lung cancer in patients with incidentally detected pulmonary nodules. *PLoS One.* 2018;13(5):e0197826.
  71. Lastwika KJ, Kargl J, Zhang Y, Zhu X, Lo E, Shelley D, et al. Tumor-derived autoantibodies identify malignant pulmonary nodules. *Am J Respir Crit Care Med.* 2019;199(10):1257–66.
  72. Schmidt B, Weickmann S, Witt C, Fleischhacker M. Integrity of cell-free plasma DNA in patients with lung cancer and nonmalignant lung disease. *Ann N Y Acad Sci.* 2008;1137:207–13.

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