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Exosomal miR-21/Let-7a ratio distinguishes non-small cell lung cancer from benign pulmonary diseases

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Abstract

Aim: To assess the exosomal miR-21/Let-7a ratio, a noninvasive method, in distinguishing non-small cell lung cancer (NSCLC) from benign pulmonary diseases.

Methods: The exosomes were extracted from the peripheral blood serum using serum exosomal extraction kit. miR-21 and Let-7a levels were evaluated by quantitative reverse transcription polymerase chain reaction.

Results: We found that miR-21/Let-7a ratio of NSCLC patients was significantly higher than that of healthy people, patients with pulmonary inflammation diseases, and benign pulmonary nodules, respectively. Receiver-operating characteristic analysis revealed that as compared with healthy controls, miR-21/Let-7a produced the area under the curve (AUC) at 0.8029 in patients with NSCLC, which helped to distinguish NSCLC from healthy controls with 81.33% sensitivity and 69.57% specificity. In addition, the AUC of miR-21/Let-7a in NSCLC patients was 0.8196 in comparison to patients with pulmonary inflammation diseases. Meanwhile, the sensitivity and specificity were 56.00% and 100%, respectively. Furthermore, compared with patients with benign pulmonary nodules, the AUC of miR-21/Let-7a in NSCLC patients was 0.7539. The sensitivity and specificity were 56.00% and 82.61%, respectively.

Conclusion: In the present study, our findings revealed that exosomal miR-21/Let-7a ratio holds considerable promise as a noninvasive biomarker for the diagnosis of NSCLC from benign pulmonary diseases.

KEYWORDS

benign pulmonary diseases, differential diagnosis, Let-7a, miR-21, NSCLC

1 | INTRODUCTION

Lung cancer with the highest morbidity and mortality is a great challenge for human health globally. There are nearly one million diagnosed patients with lung cancer and nearly one million deaths each year.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 85% of cases.[2] To date, the first line of NSCLC treatment is still surgical resection with radiotherapy and chemotherapy.[3] However, because the early symptoms of NSCLC are not obvious, most NSCLC patients (about 75%) diagnosed are already in advanced stage, and the overall 5-year survival rate after surgical resection was only 16%.[4,5] Therefore, the early diagnosis of NSCLC is of important clinical value for the treatment of lung cancer patients.

Pathological diagnosis based on biopsy remains the gold criterion for NSCLC diagnosis.[6,7] However, these invasive methods have potential risks to patients. Noninvasive screening tests may

be an important strategy for the detection of early lung cancer and patients' prognosis.[8,9] miRNAs are endogenous noncoding RNAs with 20-25 nucleotides and have a variety of biological functions.[10] miRNA is highly conserved in evolution, and its aberrant expression was contributed to a variety of diseases.[11] miRNAs in peripheral blood have been shown to be promising biomarkers in several diseases.[12-14] Although previous studies have shown that circulating miRNAs are detectable in peripheral blood, miRNA levels are not sufficiently stable due to the presence of RNase.[15,16] However, exosome-encapsulated microRNAs in peripheral blood can resist RNase degradation.[17] Hence, the detection of miRNAs in exosomes in peripheral blood is more stable and can be used for subsequent analysis.

Previous studies have revealed that miR-21[18-20] was significantly upregulated, while Let-7a[21,22] was downregulated in various types of cancer, including NSCLC, and promoted tumor cell proliferation, migration, and invasion. In benign pulmonary diseases, such as pneumonia and chronic obstructive pulmonary disease (COPD), the role of miR-21 and Let-7a is unclear. Additionally, lung lesions are difficult to diagnose correctly by imaging, and the follow-up may delay the optimal treatment opportunity.[23,24] Herein, through comparing the expression levels of miR-21, Let-7a, and miR-21/Let-7a ratio in NSCLC and benign pulmonary nodules, pulmonary inflammation diseases, and healthy controls, our results indicated that miR-21/Let-7a ratio was of clinical diagnostic value, as a new and noninvasive biomarker to distinguish NSCLC patients from benign pulmonary diseases.

2 | METHODS AND MATERIALS

2.1 | Patients and clinical specimens

Seventy-five patients with NSCLC, 23 patients with benign pulmonary nodules, 18 patients with pulmonary inflammation diseases, and 24 healthy donors were enrolled in this study. The pulmonary inflammation and the pathology of the benign pulmonary nodules were diagnosed according to the guidelines of the Chinese Society of Clinical Oncology. All serum samples and patients' clinical information were collected from December 2015 to April 2016 at Fujian Medical University Union Hospital. The study was approved by the hospital ethics committee and the written informed consent was obtained from patients. The selection criteria for NSCLC patients were as below: (a) patients were pathologically confirmed as NSCLC; (b) pulmonary nodule size was less than 3 cm; (c) pathological stage was identified as I-II a; (d) patients with no history of other cancer; and (e) patients had not accepted preoperative treatments. The blood samples from NSCLC patients were collected before surgery. For the benign samples, patients were diagnosed without any cancer, and patients with benign nodules were diagnosed by pathology. Patients with incomplete medical records were excluded. The blood samples were kept at a room temperature for 20 min after collection, and then centrifuged at 500 × *g* for 15 min at 4°C. Afterward, the serum was collected and stored at −80°C for use.

2.2 | Serum exosomes extraction and purification

Serum exosomes were enriched and purified using the SBI ExoQuick kit (Cat. No. EXOQ5A-1, System Biosciences Inc., Palo Alto, CA) according to the manufacturer's guidelines. Briefly, after the frozen serum was thawed, serum samples were centrifuged at 3000 *g* for 15 min at 4°C to remove the precipitate and lipid layer. Next, 500 μL of serum was taken into a new 1.5 EP tube. Meanwhile, 120 μL of SBI precipitant EXOQ5A-1 was added into 500 μL of serum. The samples were mixed up five times using upside down way, and then put into the refrigerator (4°C) for 30 min. The precipitate was collected via centrifugation at 12 000 × *g* for 5 min at 4°C.

2.3 | Exosome microRNA extraction

Exosome miRNA was extracted and purified using Qiagen miRNeasy Mini Kit (Cat. No. 217004, Qiagen, Hilden, Germany) following the manufacturer's instructions. Seven hundred microliters of QIAzol Lysis Reagent was added into the precipitate as mentioned above, followed by thorough mixing until the precipitate was completely dissolved. In ventilation chamber, 100 μL of chloroform was added, and then vortexed for 15 s. Afterward, following 10 minutes equilibration at room temperature. Following the centrifugation at 12 000 × *g* for 15 min at 4°C, the upper aqueous phase (350 μL) was transferred into a new sterile EP tube. 1.5 volumes of 100% ethanol were added and mixed thoroughly by pipetting up and down several times. Next, 700 μL of the above mixture was immediately added into the RNeasy Mini Spin Column, and centrifuged at 12 000 × *g* for 30 s at room temperature. The column was washed with Buffer RWT and Buffer RPE. After drying, total RNA was eluted with 50 μL of RNeasy-free water. RNA concentration was measured by Nano-Drop 2000 (ThermoFisher Scientific Inc., Waltham, MA). The total RNA was stored at −80°C.

2.4 | Serum miRNA quantification by quantitative reverse transcriptase polymerase chain reaction analysis

The reverse transcription reaction was carried out using TransScript® miRNA First-Strand cDNA Synthesis SuperMix (TransGen Biotechnology, Beijing, China) according to the manufacturer's instructions. The RT reaction volume was 20 μL, including 1 μL TransScript® miRNA RT Enzyme Mix, 10 μL 2 × TS miRNA Reaction Mix, 7 μL total RNA, and 2 μL RNase-free water. The reaction procedure was as below: incubated at 42°C for 40 min; and then incubated 70°C for 15 min; refrigerated at 4°C.

Quantitative PCR was performed using TransScript® Green miRNA Two-Step qRT-PCR SuperMix (TransGen Biotechnology). Twenty microliters of complementary DNA (cDNA) was diluted five times with RNase-free water. The qPCR reaction volume was 20 μL containing 0.4 μL Forward Primer (10 μM), 0.4 μL miRNA qPCR Primer (10 μM), 10 μL 2 × TransStart® Top/Tip Green qPCR SuperMix, 8 μL cDNA, and 1.2 μL RNase-free water. qPCR reactions were performed in triplicate in 96-well plates using ABI Prism 7500 (Applied

TABLE 1 Clinicopathological characteristics of all persons enrolled in this study

Clinicopathological characteristics		Healthy controls	Pulmonary inflammation diseases	Benign pulmonary nodule	NSCLC
Age	≤60	13	10	13	33
	>60	11	8	10	42
Gender	Male	15	7	15	50
	Female	9	11	8	25
Smoking status	Yes	13	12	13	45
	No	11	6	10	30
TNM stage	IA	—	—	—	12
	IB	—	—	—	21
	IIA	—	—	—	22
	IIB	—	—	—	15
	IIIA	—	—	—	5
Histological types of NSCLC	AC	—	—	—	39
	SCC	—	—	—	36

Abbreviations: AC, adenocarcinoma; NSCLC, non-small cell lung cancer; SCC, squamous cell cancer.

Biosystems, ThermoFisher Scientific). The thermocycling conditions were as follows: incubated at 95°C for 30 s; 50 cycles of 95°C for 5 s and 60°C for 30 s. After 50 cycles were completed, cooled at 50°C for 30 s.

2.5 | Statistical analyses

Statistical analyses were performed using SPSS 22.0 (IBM Company, Armonk, NY). *T* test was used for univariate analysis of miRNA expression. Logistic regression analysis was used to identify the sensitivity and specificity of miRNAs that distinguished NSCLC from benign lung diseases and healthy controls, and the prediction model was verified by comparing area under the curve (AUC). The sensitivity, specificity, and corresponding cutoff values of miRNA were determined by the receiver-operating characteristic (ROC) curve and the AUC analysis. Data are expressed as mean ± standard deviation. *P* values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Patient characteristics

Seventy-five patients with NSCLC, 23 patients with benign pulmonary nodules, 18 patients with pulmonary inflammation diseases, and 24 healthy controls were enrolled in the present study. All serum samples and patients' clinical information, including age, gender, smoking history, and pathologic types, were collected from December 2015 to April 2016 at Fujian Medical University Union Hospital. The relevant clinical data are presented in Table 1.

3.2 | The levels of serum miR-21 and Let-7 in the study cohort

To detect the levels of serum exosomal miR-21 and Let-7 in patients with NSCLC, benign pulmonary nodules, pulmonary inflammation diseases, and healthy controls, qPCR was conducted. In Figure 1, data revealed that miR-21 level was significantly upregulated only in patients with pulmonary inflammation diseases in comparison to healthy controls ($P = .0485$). Let-7a level in NSCLC patients was dramatically downregulated, as compared with healthy controls ($P = .0268$), patients with pulmonary inflammation diseases ($P < .0001$) or patients with benign pulmonary nodules ($P = .0032$), respectively, while Let-7a levels in patients with pulmonary inflammation diseases were significantly increased compared with healthy controls ($P = .0410$). Furthermore, there was no significant difference in age among different groups (Figures S1 and S2). Additionally, miR-21/Let-7a ratio in NSCLC patients was significantly higher than that in healthy controls ($P = .0002$), patients with pulmonary inflammation diseases ($P = .0007$), as well as in patients with benign pulmonary nodules ($P < .0001$), suggesting that exosomal miR-21/Let-7a ratio holds strong promise as a diagnostic biomarker for NSCLC.

3.3 | Diagnostic accuracy of exosomal miR-21

To test the diagnostic value of miR-21, the ROC curve of the cohort was performed. In Figure 2, for healthy controls and patients with pulmonary inflammation diseases, the AUC value of miR-21 was 0.7002. The sensitivity and specificity were 0.7895 and 0.6522 under an optimal cutoff value, respectively. However, it cannot be used to discriminate between NSCLC and benign pulmonary diseases.

3.4 | Diagnostic accuracy of exosomal Let-7a

To determine the diagnostic value of Let-7a, ROC curve analysis was conducted. In Figure 3, compared with healthy persons, the AUC value of Let-7a in patients with pulmonary inflammation diseases was 0.6693, and the sensitivity and specificity were 0.6842 and 0.6522 under an optimal cutoff value, respectively; the AUC value of Let-7a in NSCLC patients was 0.6730, and the sensitivity and specificity were

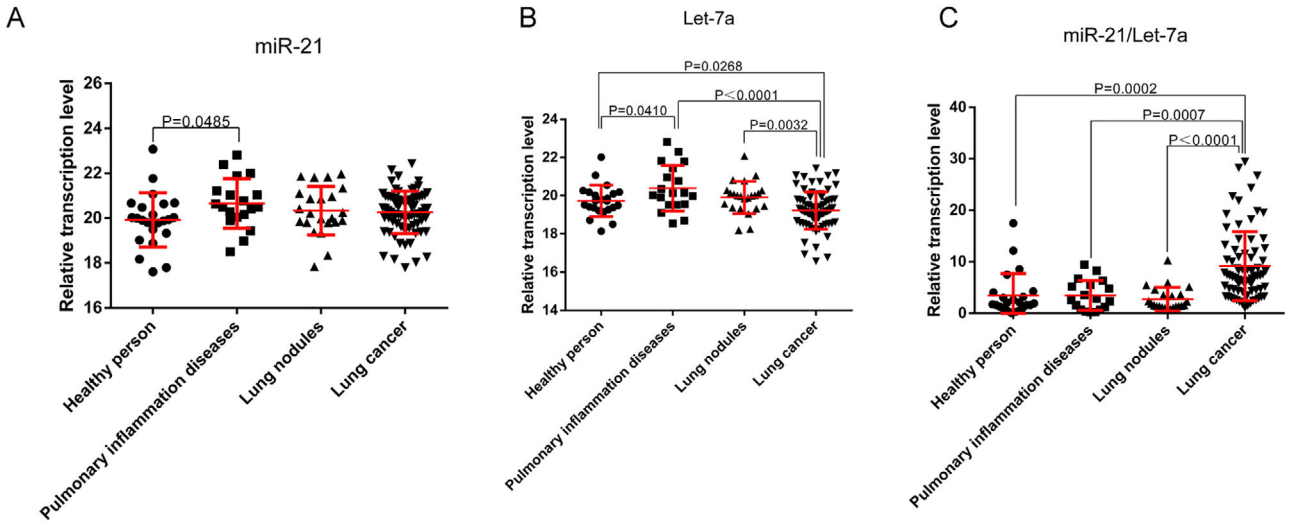

FIGURE 1 Comparison of serum miR-21 and Let-7a expressions in patients with NSCLC, benign pulmonary nodules, pulmonary inflammation diseases, and healthy controls. (A) The miR-21 level and (B) Let-7a level in serum were detected by qPCR. (C) miR-21/Let-7a ratio was analyzed [Colour figure can be viewed at wileyonlinelibrary.com]

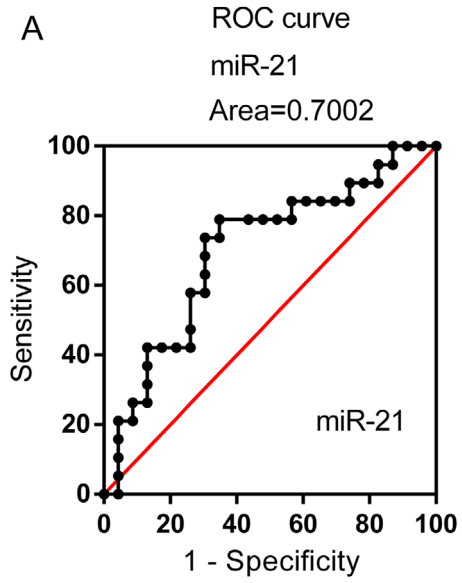

FIGURE 2 ROC curve analysis of serum miR-21 for predicting pulmonary inflammation diseases. The AUC was 0.7002 [Colour figure can be viewed at wileyonlinelibrary.com]

0.6267 and 0.7319, respectively. In addition, the AUC value of Let-7a in NSCLC patients was 0.7828 compared with pulmonary inflammation diseases, and the sensitivity and specificity were 0.6933 and 0.8421; the AUC value of Let-7a in NSCLC patients was 0.7249 compared to benign pulmonary nodules, and the sensitivity and specificity were 0.8000 and 0.6522, respectively, which is not a satisfactory marker for the diagnosis of NSCLC.

3.5 | Diagnostic accuracy of miR-21/Let-7a ratio

To validate the diagnostic value of miR-21/Let-7a ratio in the cohort, the ROC curve analysis was performed. In Figure 4, for NSCLC and healthy controls, the AUC curve of miR-21/Let-7a was 0.8029. The sensitivity and specificity were 0.8133 and 0.6957, respectively. For NSCLC and pulmonary inflammation diseases, the AUC curve of miR-21/Let-7a was 0.8196. The sensitivity and specificity were 0.5600 and 1, respectively. Moreover, for NSCLC and benign pulmonary nodules, the AUC curve of miR-21/Let-7a was 0.7539. The sensitivity and specificity were 0.5600 and 0.8261, respectively. Cumulatively, exosomal miR-21/Let-7a ratio exhibited a better performance in distinguishing NSCLC from benign pulmonary disease than miR-21 and Let-7a.

4 | DISCUSSION

In this study, our findings indicated that exosomal miR-21 level was significantly upregulated only in patients with pulmonary inflammation diseases compared to healthy controls. Exosomal Let-7a levels in patients with pulmonary inflammation diseases and NSCLC patients were markedly elevated compared with healthy controls. Notably, the ROC curve and AUC analyses indicated that exosomal miR-21/Let-7a ratio holds promise as a noninvasive diagnostic marker for the identification of NSCLC and benign pulmonary diseases.

Clinically, because tuberculosis, pneumonia, benign pulmonary lesion, or early stage lung cancer were manifested in computed tomography (CT) imaging as solitary pulmonary nodule, it was difficult to accurately diagnose.[25] Specially, solitary pulmonary nodule is the most common in chest CT scans. A large-scale randomized lung cancer screening test, using low-dose CT (LDCT) screening in high-risk populations, indicates a 6.7% reduction in all-cause mortality in lung cancer and a 20% reduction in lung-specific mortality.[26] However, LDCT does not accurately distinguish malignant pulmonary nodules from benign pulmonary nodules.[27] Therefore, noninvasive methods, such as "liquid biopsy," as an auxiliary examination approaches, have been extensively studied to improve the differential diagnosis of benign and malignant pulmonary nodules.[28,29]

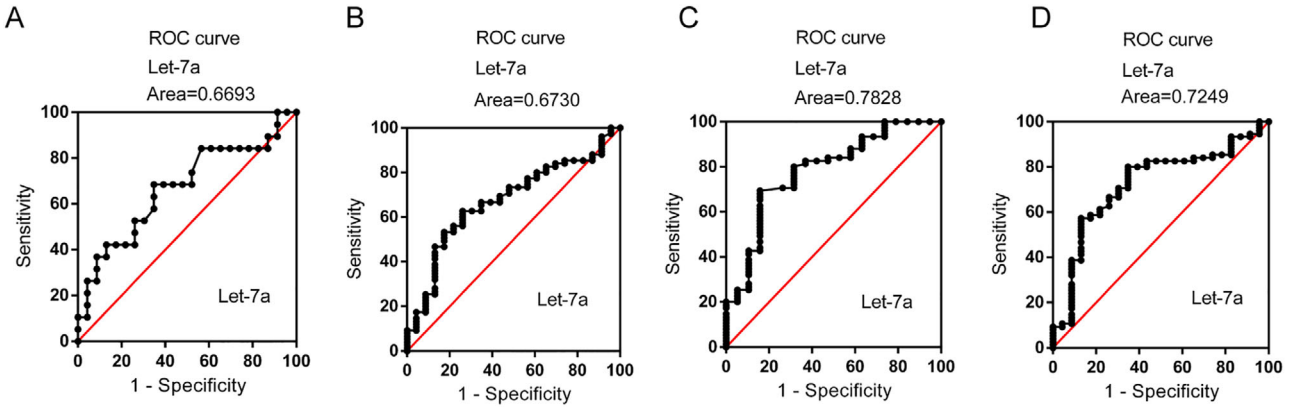

FIGURE 3 Diagnostic accuracy of Let-7a in the validation cohort. (A) The ROC curve and AUC analyses were used to distinguish patients with pulmonary inflammation diseases from healthy controls. And conducted to distinguish NSCLC patients from patients with (B) healthy controls, (C) pulmonary inflammation diseases, and (D) benign pulmonary nodules [Colour figure can be viewed at wileyonlinelibrary.com]

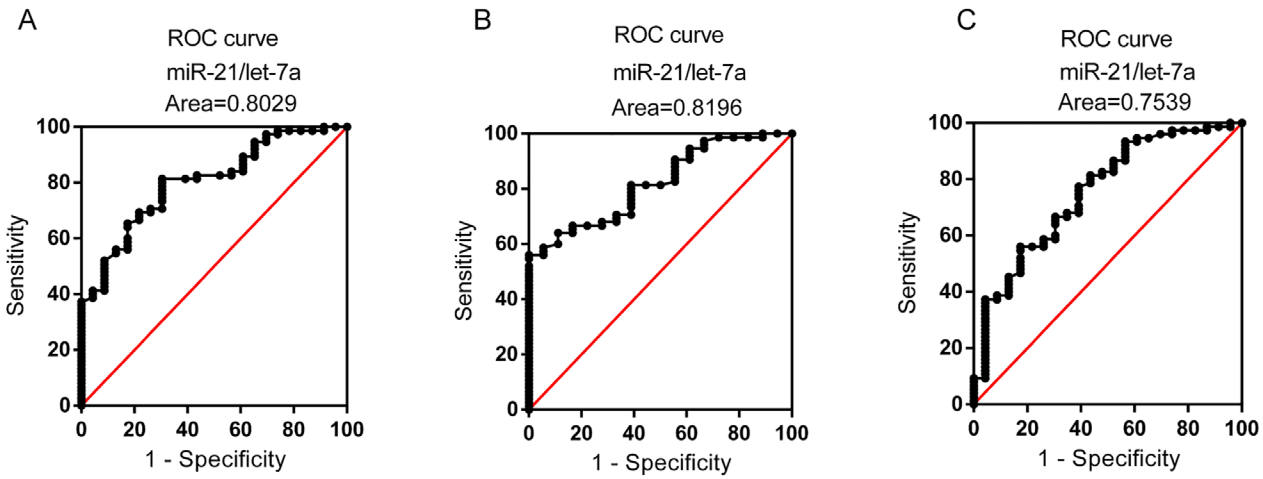

FIGURE 4 Diagnostic accuracy of miR-21/Let-7a ratio in the validation cohort. The ROC curve and AUC analyses were conducted to distinguish NSCLC patients from patients with (A) healthy controls, (B) pulmonary inflammation diseases, and (C) benign pulmonary nodules [Colour figure can be viewed at wileyonlinelibrary.com]

Smoking and inflammation, the main causes of benign nodules in the lung, contribute to the disturbance of microenvironment and the changes of molecular levels.[30] Compared with benign pulmonary nodules, the expression of miRNA in lung cancer is significantly different, which can be used to distinguish malignant tumors from benign lesions.[28,29] Studies have shown that let-7c, miR-1, and miR-146a levels were strikingly reduced in patients with COPD, while miR-15b level was significantly elevated.[31-33]

There are a variety of high-abundance miRNAs in serum and plasma, such as miR-150 in leukocytes and miR-16 in red blood cells and platelets.[13,15] Although circulating miRNAs can be detected in serum, plasma, or whole blood, miRNAs are easily degraded, resulting in the unstable and unreliable results.[16,18] Previous work has suggested that exosomes can protect RNA from degradation by external nucleases.[17,34,35] In the present study, we analyzed the clinical diagnostic values of serum miR-21, Let-7a expression, and miR-21/Let-7a ratio in patients with NSCLC and benign pulmonary nodules, pulmonary inflammation disease, and healthy individuals. miR-21 is abnormally expressed in various cancers,[36] which regulates the expression of phosphatase and tensin homolog[37] and programmed cell death 4,[38] thereby affecting tumor cell proliferation and cancer bone metastasis. Unlike previous studies,[20] exosomal miR-21 level in patients with NSCLC and benign pulmonary nodules has no significant difference as compared to healthy controls in this study, which may be due to the small sample size. In addition, Let-7a is implicated in the proliferation and invasion of lung cancer cells by modulating Aurora-B,[39] K-ras, and HMGA2 expression.[40] Both (miR-21 and Let-7a) were associated with patient prognosis. Here, consistent with the previous work,[41] exosomal Let-7a level was downregulated in NSCLC patients. Although Let-7a has the value of distinguishing pulmonary inflammation disease, benign nodules, and lung cancer, the ratio of miR-21/Let-7a may be a better biomarker for identifying NSCLC and benign pulmonary diseases due to the larger AUC.

However, our research has some limitations. The number of candidate miRNAs is limited; the sensitivity and specificity of serum miRNA in the diagnosis of lung lesions still need to be improved; and

miRNAs derived from different parts of the same patient may have different expression levels. Moreover, further study with larger sample size is still needed.

Taken together, our results suggest that mir-21/Let-7a ratio may be a promising noninvasive biomarker for differential diagnosis of benign and malignant pulmonary nodules.

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AUTHORS' CONTRIBUTIONS

Guofeng Yang conducted the experiments. Tao Wang, Xiangyun Qu, Shuchen Chen, Ziyang Han, Sui Chen, Mingduan Chen, Jihong Lin, Shaobin Yu, Lei Gao, and Kaiming Peng participated in coordination and acquisition of data. Guofeng Yang, Tao Wang, and Xiangyun Qu participated in data analysis. Guofeng Yang and Mingqiang Kang participated in study design, data interpretation, and prepared the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Human Ethics Committee of Fujian Medical University Union Hospital, Fuzhou, Fujian Province. Written informed consent was obtained from individual participants.

FUNDING

No funding was received.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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