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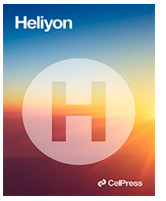

Research article

A novel microRNA miR-4433a-3p as a potential diagnostic biomarker for lung adenocarcinoma

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ABSTRACT

Background: Lung adenocarcinoma is one of the leading causes of cancer-related deaths because of the lack of early specific clinical indicators. MicroRNAs (miRNAs) have become the focus in lung cancer diagnosis. Further studies are required to explore miRNA expression in the serum of lung adenocarcinoma patients and their correlation with therapy and analyse specific messenger RNA targets to improve the specificity and sensitivity of early diagnosis.

Methods: The Toray 3D-Gene miRNA array was used to compare the expression levels of various miRNAs in the sera of patients with lung adenocarcinoma and healthy volunteers. Highly expressed miRNAs were selected for further analysis. To verify the screening results, serum and pleural fluid samples were analysed using qRT-PCR. Serum levels of the miRNAs and their correlation with the clinical information of patients with lung adenocarcinoma were analysed. The functions of miRNAs were further analysed using the Kyoto Encyclopedia of Gene and Genomes and Gene Ontology databases.

Results: Microarray analysis identified 60 and 50 miRNAs with upregulated and downregulated expressions, respectively, in the serum of patients with lung adenocarcinoma compared to those in healthy individuals. Using qRT-qPCR to detection of miRNAs expression in the serum or pleural effusion of patients with early and advanced lung adenocarcinoma, we found that miR-4433a-3p could be used as a diagnostic marker and therapeutic evaluation indicator for lung adenocarcinoma. Serum of miR-4433a-3p levels significantly correlated with the clinical stage. miR-4433a-3p may be more suitable than other tumour markers for the early diagnosis and evaluation of therapeutic effects in lung adenocarcinoma. miR-4433a-3p may affect tumour growth and

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metastasis by acting on target genes (*PIK3CD, UBE2J2, ICMT, PRDM16* and others) and regulating tumour-related signalling pathways (MAPK signal pathway, Ras signalling pathway and others). *Conclusion:* miR-4433a-3p may serve as a biomarker for the early diagnosis of lung adenocarcinoma and monitoring of therapeutic effects.

1. Introduction

Lung cancer is one of the leading causes of cancer-related death, and lung adenocarcinoma is one of the main types of cancer [1]. Various forms of medical technology, including surgical therapy, chemotherapy, radiotherapy, and targeted therapy, have been applied for the treatment of lung adenocarcinoma [2–4]. However, owing to the lack of early specific clinical indicators and the characteristics of the tumour, such as recurrence, metastasis, and drug resistance, the survival of lung adenocarcinoma patients has not been significantly prolonged [5,6]. Early diagnosis has been shown to significantly improve the 5-year survival of patients with lung adenocarcinoma [7]. Therefore, it is necessary to explore new prognostic biomarkers or therapeutic targets to improve the early diagnosis and treatment of lung adenocarcinoma.

Traditional diagnostic methods have employed sputum, pleural fluid, bronchoscopy, and lung tissue biopsy [8]. These methods are highly accurate; however, most are invasive operations with a risk of delayed detection and repeated testing. Therefore, new detection methods are required [9,10]. Early screening for lung cancer using low-dose computed tomography (CT) has drawbacks such as overdiagnosis, false-positive results, and radiation exposure [11–13]. As blood and serum are among the most accessible and low-cost biological samples, biomarkers based on blood and serum (such as cfDNA, miRNA, and circulating tumour cells) have become the focus of research for the early diagnosis of lung cancer [14–16]. However, these markers have not been widely used in the diagnosis of early lung adenocarcinoma. These biomarkers require advancements in bioengineering techniques to address their toxicity, side effects, and low efficacy at high doses, which entails higher economic expenditure and more rigorous clinical trials [17].

MicroRNAs (miRNAs) are small non-coding RNA molecules that downregulate the expression of key genes regulating cellular processes by inhibiting or degrading messenger RNA (mRNA) targets [18,19]. Many studies have shown that miRNAs play a prominent role in tumourigenesis by regulating various signalling pathways involved in tumour development, such as cell proliferation, metastasis, angiogenesis, metabolism, and apoptosis [20–22]. For example, miR-197-3p can promote tumour growth and angiogenesis by targeting TIMP2 and TIMP3 and increasing the metastasis of lung adenocarcinoma [23]. This could be used as a prognostic biomarker and therapeutic target for anti-angiogenic therapy in lung adenocarcinoma. Studies have also found that miR-143-3p induces the expression of SOX5, promoting the epithelialisation and progression of NSCLC [24]. Therefore, miRNAs can regulate different tumour-related mRNAs and affect tumour development. The high relative stability of miRNAs in common clinical tissues and biological fluids, as well as the close correlation between miRNA expression profiles and disease status, make them new tools for basic diagnosis [25]. Many miRNAs have been found in the serum of lung cancer patients, which predict the histological type and prognosis of lung cancer, providing a theoretical basis for the individualised treatment of lung cancer patients [26,27]. However, there are few studies on the role of miRNAs and miRNA–mRNA regulatory networks in the early diagnosis and treatment of lung cancer, which are currently mainly based on chest CT and histopathological examinations.

To detect lung adenocarcinoma at an early stage, thereby reducing the use of chemotherapeutic drugs and molecular targeted drugs in lung cancer and avoiding the adverse consequences of advanced lung adenocarcinoma, a sensitive diagnostic method is important to improve the survival rate and prognosis of patients with lung adenocarcinoma. In this study, we explored the expression of miRNAs in the serum of these patients, their relevance to therapy, and specific mRNA targets to improve the specificity and sensitivity of early diagnosis in lung adenocarcinoma patients.

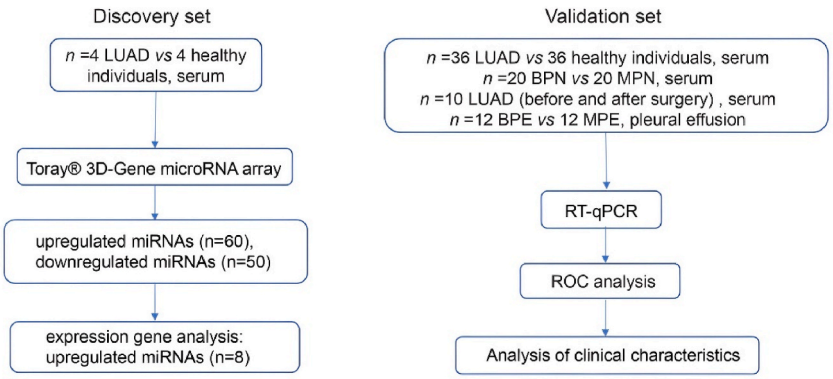

Fig. 1. The flow chart of the study design. LUAD, lung adenocarcinoma; miRNA, microRNA; BPN, Benign pulmonary nodules; MPN, Malignant pulmonary nodules; BPE, Benign pleural effusion; MPE, Malignant pleural effusion; RT–qPCR, quantitative reverse transcription–polymerase chain reaction; ROC, receiver operating characteristic.

2. Results

2.1. Identification of tumour-associated miRNAs in the serum of healthy volunteers and patients with lung adenocarcinoma

Fig. 2A shows the identification of miRNAs with different expression levels in the sera of normal subjects and patients with lung adenocarcinoma using microarray analysis. We identified 60 and 50 miRNAs with upregulated and downregulated expressions, respectively, in the serum of patients with lung adenocarcinoma compared to those in healthy individuals (Fig. 2B). miRNAs with a multiple change value greater than 10 and a p-value less than 0.05 were selected for further analysis based on differential gene expression analysis. As shown in Fig. 2C, eight miRNAs upregulated in patients with adenocarcinoma were selected for further validation.

2.2. Verification of miRNA expression in the serum and pleural effusion of lung adenocarcinoma patients using qRT-PCR analysis

We verified the stability of related miRNA expression in the serum of patients with lung adenocarcinoma compared to that in healthy subjects. Five miRNAs with high expression were identified: miR-6721-5p, miR-10527-5p, miR-4433a-3p, miR-3184-3p, and miR-181a-2-3p. As shown in Fig. 3A–E, the levels of these five miRNAs in the serum of patients with lung adenocarcinoma were significantly higher than those in normal serum. The area under the ROC curve (AUC) values for miR-6721-5p, miR-10527-5p, miR-4433a-3p, miR-3184-3p, and miR-181a-2-3p were between 0.5 and 1 (Fig. 4A–E). Therefore, we predicted that these five miRNAs may be useful for the diagnosis of lung adenocarcinoma. To further detect the correlation between these five miRNAs and lung adenocarcinoma, we measured their expression in pleural fluid. As shown in Fig. 5A–E, all five miRNAs were highly expressed in the pleural fluid of patients with lung adenocarcinoma. Receiver operating characteristic (ROC) curve analysis showed that these five miRNAs could be used to diagnose lung adenocarcinoma (Fig. 6A–E). Thus, miR-6721-5p, miR-10527-5p, miR-4433a-3p, miR-3184-3p, and miR-181a-2-3p can be used as serum and pleural fluid markers for intermediate and advanced lung adenocarcinoma.

2.3. miR-4433a-3p can be used for the diagnosis of early-stage lung cancer

qRT-PCR analysis revealed that only the expression of miR-4433a-3p was statistically significant, and the remaining miRNAs without statistical significance were excluded. As shown in Fig. 7A, miR-4433a-3p was more highly expressed in the serum of patients with malignant pulmonary nodules than in patients with benign pulmonary nodules. ROC curve analysis showed that miR-4433a-3p had high sensitivity and specificity for the diagnosis of early lung adenocarcinoma (Fig. 7B). Therefore, miR-4433a-3p alone can be used as a highly sensitive and specific indicator for the diagnosis of early lung adenocarcinoma.

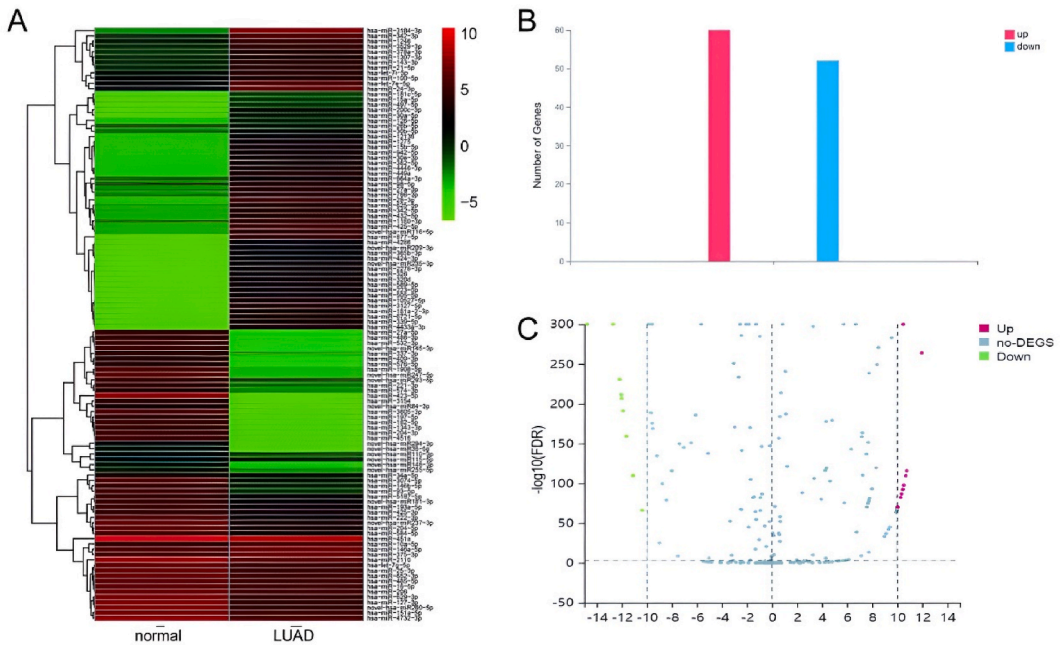

Fig. 2. Identification of tumour-associated miRNAs in the serum of healthy volunteers and patients with lung adenocarcinoma. (A–B) Heatmap of miRNA microarray expression between LUAD group and healthy control group. 60 upregulated and 50 downregulated microRNAs were identified. (C) Multiple micrornas with change values greater than 10 and p value less than 0.05 were screened based on differential expression gene analysis.

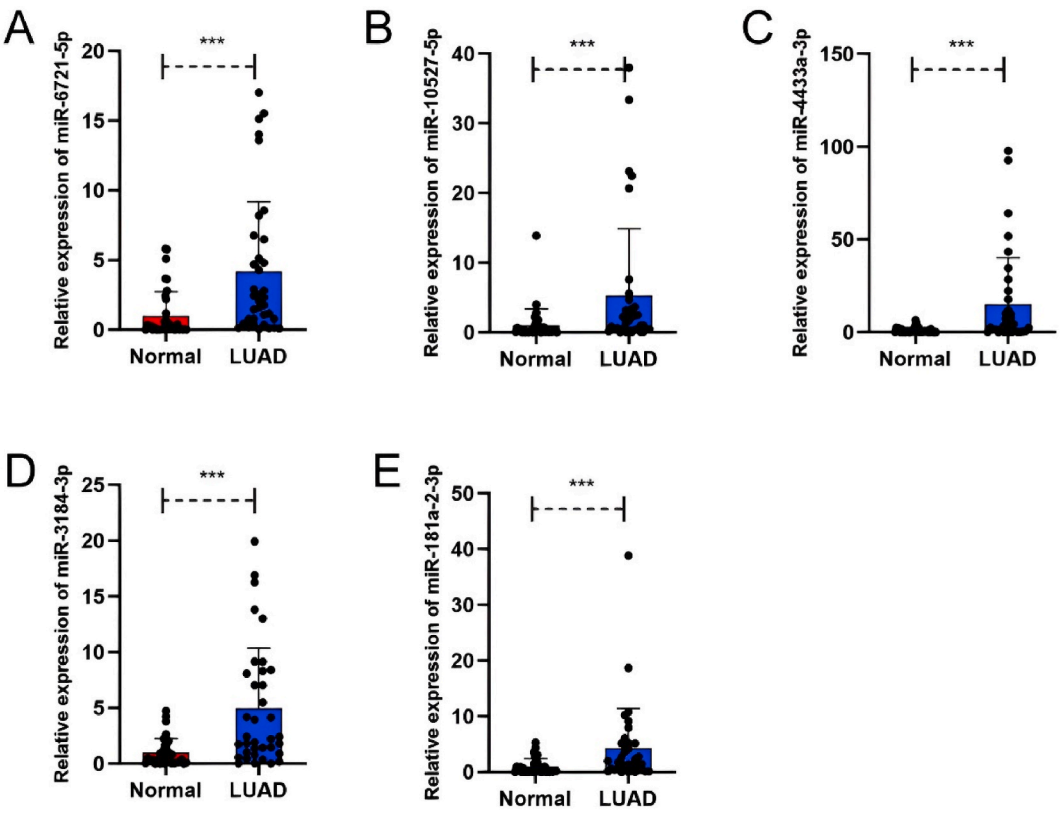

Fig. 3. Verification of microRNA expression in the serum of lung adenocarcinoma patients using quantitative qRT-PCR analysis. (A) The expression of miR-6721-5p in serum was measured by qRT-PCR. (B) The expression of miR-10527-5p in serum was measured by qRT-PCR. (C) The expression of miR-4433a-3p in serum was measured by qRT-PCR. (D) The expression of miR-3184-3p in serum was measured by qRT-PCR. (E) The expression of miR-181a-2-3p in serum was measured by qRT-PCR. ***, $P < 0.001$.

2.4. Related miRNAs as indicators for evaluating the efficacy of surgical treatment for early lung cancer

We evaluated the diagnostic role of these miRNAs in the serum of patients with early-stage lung cancer before and after surgery. As shown in Fig. 8A–E, the expression levels of miR-6721-5p, miR-4433a-3p, miR-3184-3p, and miR-181a-2-3p were significantly reduced in patients with early-stage lung cancer after surgery. Therefore, these four miRNAs could be used to evaluate the efficacy of surgical treatment for lung adenocarcinoma.

2.5. Correlation between miR-4433a-3p levels and clinical information in patients with lung adenocarcinoma

The patients with lung adenocarcinoma were divided into two groups for analysis. The cutoff value (8.0) was used as the median relative expression level of miR-4433a-3p in all serum samples. As shown in Table 7, the serum level of miR-4433a-3p was significantly correlated with the clinical stage ($P = 0.004$) and tended to be associated with age ($P = 0.084$). There was no significant correlation between the other clinicopathological factors and the serum expression levels of miR-4433a-3p. As shown in Table 8, the pleural fluid levels of miR-4433a-3p tended to be associated with CEA levels ($P = 0.111$). The presence of miR-4433a-3p in pleural fluid was not significantly correlated with clinicopathological factors. The expression levels of CEA, CA19-9, CA125, cyfra21-1, proGRP, NSE, and LDH were not significantly reduced in patients with early-stage lung cancer after surgery (Table 9). Based on previous findings, we observed increased expression of miR-4433a-3p in early-stage lung adenocarcinoma. Therefore, miR-4433a-3p may be more suitable than other tumour markers for the early diagnosis and evaluation of therapeutic effects in lung adenocarcinoma.

2.6. The selected miRNAs and their targets were analysed by bioinformatics

To further analyse the role of these miRNAs in lung adenocarcinoma, we identified their related target genes, such as PIK3CD, UBE2J2, ICMT, and PRDM16 (Fig. 9). We conducted a Gene Ontology (GO) enrichment analysis to study the cellular components, biological processes, and molecular functions of these miRNAs (Fig. 10A–C). These results are related to the occurrence and development of tumours, including the regulation of protein phosphorylation, protein binding, and other processes. We used Kyoto Encyclopedia of Genes (KEGG) analysis to predict the signalling pathways in which these miRNAs may participate, such as the MAPK

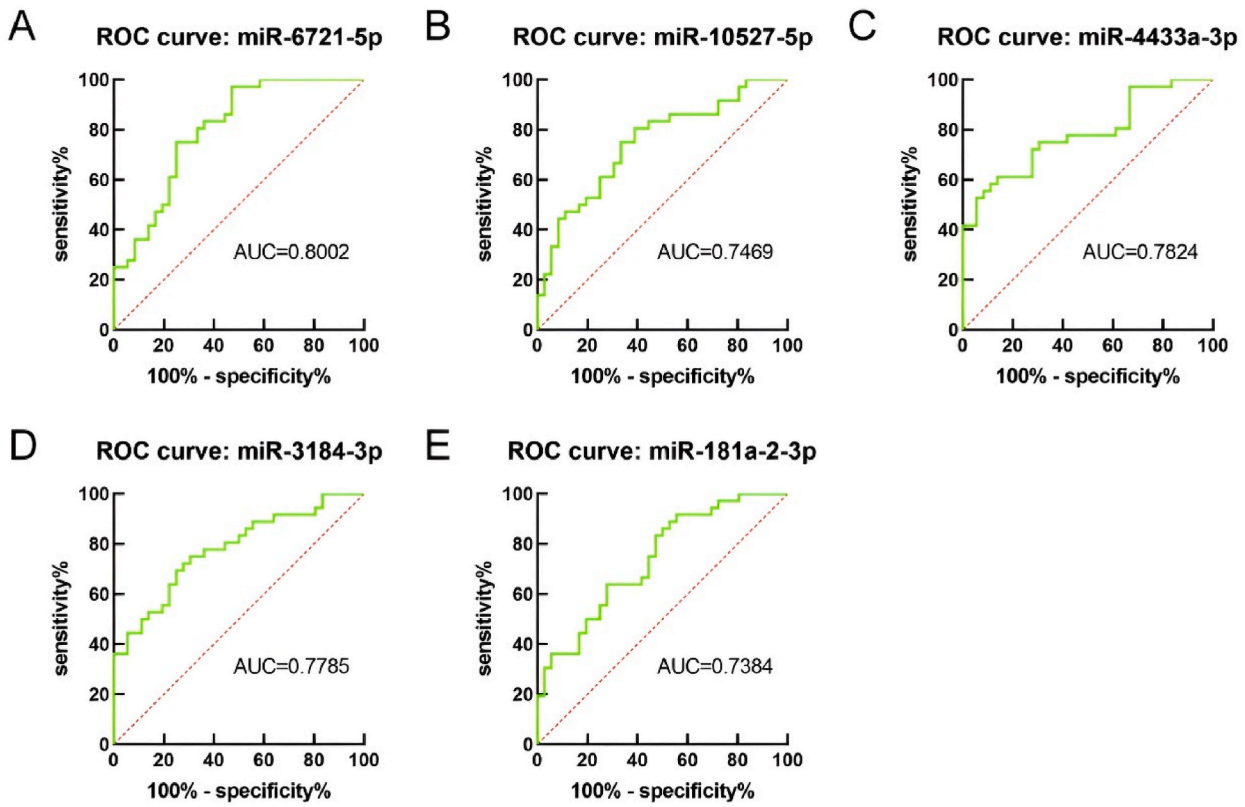

Fig. 4. Diagnostic values of microRNA in the serum of lung adenocarcinoma patients using quantitative RT-PCR analysis. (A) The ROC curve analysis of miR-6721-5p in serum produced an AUC value of 0.8002. (B) The ROC curve analysis of miR-10527-5p in serum produced an AUC value of 0.7469. (C) The ROC curve analysis of miR-4433a-3p in serum produced an AUC value of 0.7824. (D) The ROC curve analysis of miR-3184-3p in serum produced an AUC value of 0.7785. (E) The ROC curve analysis of miR-181a-2-3p in serum produced an AUC value of 0.7384.

and Ras signalling pathways (Fig. 10D). These miRNAs may affect tumour growth and metastasis by acting on target genes and regulating tumour-related signalling pathways.

3. Discussion

The treatment of lung adenocarcinoma is mainly determined by the clinical stage, morphological diagnosis, and the patient's own state, and the treatment of lung adenocarcinoma is helpful in prolonging the quality of life of patients; therefore, it is urgent to find a sensitive diagnostic marker of lung adenocarcinoma [28,29]. The detection of miRNAs has long been predicted to be an important method for cancer-based blood tests because they exist in a very stable form in human serum and are easily detected [30–32]. For example, the plasma level of miR-21 in patients with breast cancer is higher than that in healthy controls and patients with benign breast tumours. Inhibition of miR-21 inhibits the proliferation and metastasis of breast cancer cells; therefore, miR-21 levels are important plasma markers for the diagnosis of breast cancer [33]. An increasing number of studies have found that miRNAs in the blood can also be used for the diagnosis and detection of oesophageal, gastric, colorectal, cervical, and other cancers [34–37]. Plasma levels of miR-340 and miR-450b-5p have been found to improve diagnostic accuracy in non-small cell lung cancer studies [38]. In our study, only five miRNAs were significantly upregulated in the serum of patients with lung adenocarcinoma. Subsequently, we expanded our analysis from blood to pleural fluid. miR-6721-5p, miR-10527-5p, miR-4433a-3p, miR-3184-3p, and miR-181a-2-3p were highly expressed in the pleural water of patients with lung adenocarcinoma. ROC curve analysis revealed that these five miRNAs could be used as biomarkers for the diagnosis of lung adenocarcinoma. Therefore, our findings reveal that these five miRNAs are highly expressed in both pleural fluid and blood and are significant in the diagnosis of lung adenocarcinoma.

With the application of CT technology in the early screening of lung cancer, early lung adenocarcinoma is found in the form of lung nodules, and benign and malignant nodules are preliminarily judged according to the features of CT images [39]. A definite diagnosis of the nature of pulmonary nodules requires puncture biopsy, which is difficult to perform, expensive, invasive, and has other disadvantages. In recent years, exploring rapid, simple, and low-cost methods (such as cfDNA, miRNA, and circulating tumour cells) to screen for benign and malignant pulmonary nodules has attracted increasing attention, providing a diagnostic basis for further treatment [40,41]. Although miRNA detection has been shown in many studies to be an important non-invasive diagnostic method for lung cancer, the detection of miRNAs in lung cancer is mostly limited to the blood, and they are rarely detected in the early stages [42].

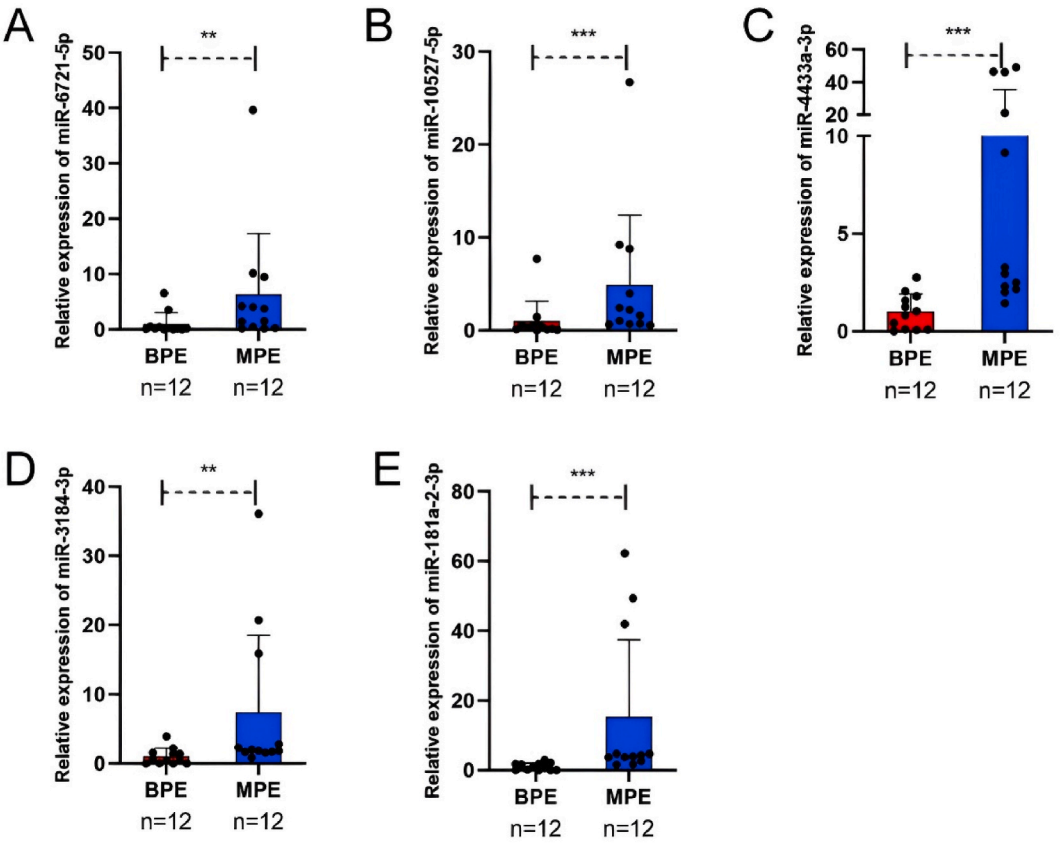

Fig. 5. Verification of microRNA expression in the pleural effusion of lung adenocarcinoma patients using quantitative qRT-PCR analysis. (A) The expression of miR-6721-5p in pleural effusion was measured by qRT-PCR. (B) The expression of miR-10527-5p in pleural effusion was measured by qRT-PCR. (C) The expression of miR-4433a-3p in pleural effusion was measured by qRT-PCR. (D) The expression of miR-3184-3p in pleural effusion was measured by qRT-PCR. (E) The expression of miR-181a-2-3p in pleural effusion was measured by qRT-PCR. **, $P < 0.01$; ***,$P < 0.001$.

However, the above results were based on a clear diagnosis of tumours, most of which were in the middle and late stages; thus, our results could not reflect the significance of these miRNAs in the early diagnosis of lung adenocarcinoma. In our study, the test samples ranged from middle to advanced lung cancer, as well as lung nodules indicative of early-stage lung adenocarcinoma. Serum was collected from several patients with early adenocarcinoma who showed pulmonary nodules on CT and no other signs of metastasis. Compared to patients with benign pulmonary nodules, only miR-4433a-3p was highly expressed in the serum of patients with malignant pulmonary nodules. In conclusion, miR-4433a-3p can be used for the early diagnosis of lung adenocarcinoma.

Simultaneously, our study found that miR-6721-5p, miR-4433a-3p, miR-3184-3p, and miR-181a-2-3p were of great significance in evaluating surgical efficacy in early lung adenocarcinoma, and the expression of these miRNAs in the serum of patients with lung adenocarcinoma after surgery was significantly decreased. miR-4433a-3p was more sensitive than other tumour markers (CEA, CA19-9, CA125, cyfra21-1, proGRP, NSE, and LDH) in evaluating the efficacy of surgical treatment. miR-4433a-3p can not only be used for the diagnosis of early lung adenocarcinoma but also to evaluate therapeutic effects in lung adenocarcinoma, including surgical treatment and other treatments such as chemotherapy and targeted therapy. We also found that the screened miRNAs were closely related to many signalling pathways associated with tumour progression, further verifying that these miRNAs may play an important role in the occurrence and development of lung adenocarcinoma. Previous studies have found that miRNAs can promote the progression and drug resistance of lung cancer through a variety of signalling pathways, such as the IL-6/STAT3, MAPK/ERK, and PI3K/AKT signalling pathways, leading to poor prognosis [43–45]. Our current study was limited to surgical treatment. Future research should focus on the role of miR-4433a-3p in evaluating the efficacy of treatment and drug resistance in lung adenocarcinoma.

The current body of research has provided ample evidence for the regulatory role of miRNAs in tumour chemotherapy resistance [46]. Silencing of miR-200b enhances the resistance of lung adenocarcinoma cells to docetaxel and attenuates chemoresistance by inhibiting histone acetylation of the miR-200b promoter [47]. Hence, the investigation of drug resistance in lung cancer should be the primary focus of future research. The overexpression of CPSF6-mediated XBP1 3′UTR shortening enhances DNA damage repair in lung adenocarcinoma cells and confers resistance to cisplatin treatment [48]. LOC85009 has the potential to counteract docetaxel resistance in lung adenocarcinoma by regulating ATG5-induced autophagy, suggesting that it is a promising target for overcoming chemotherapy resistance [49]. Therefore, identifying potential therapeutic targets to combat drug resistance in lung adenocarcinoma has emerged as

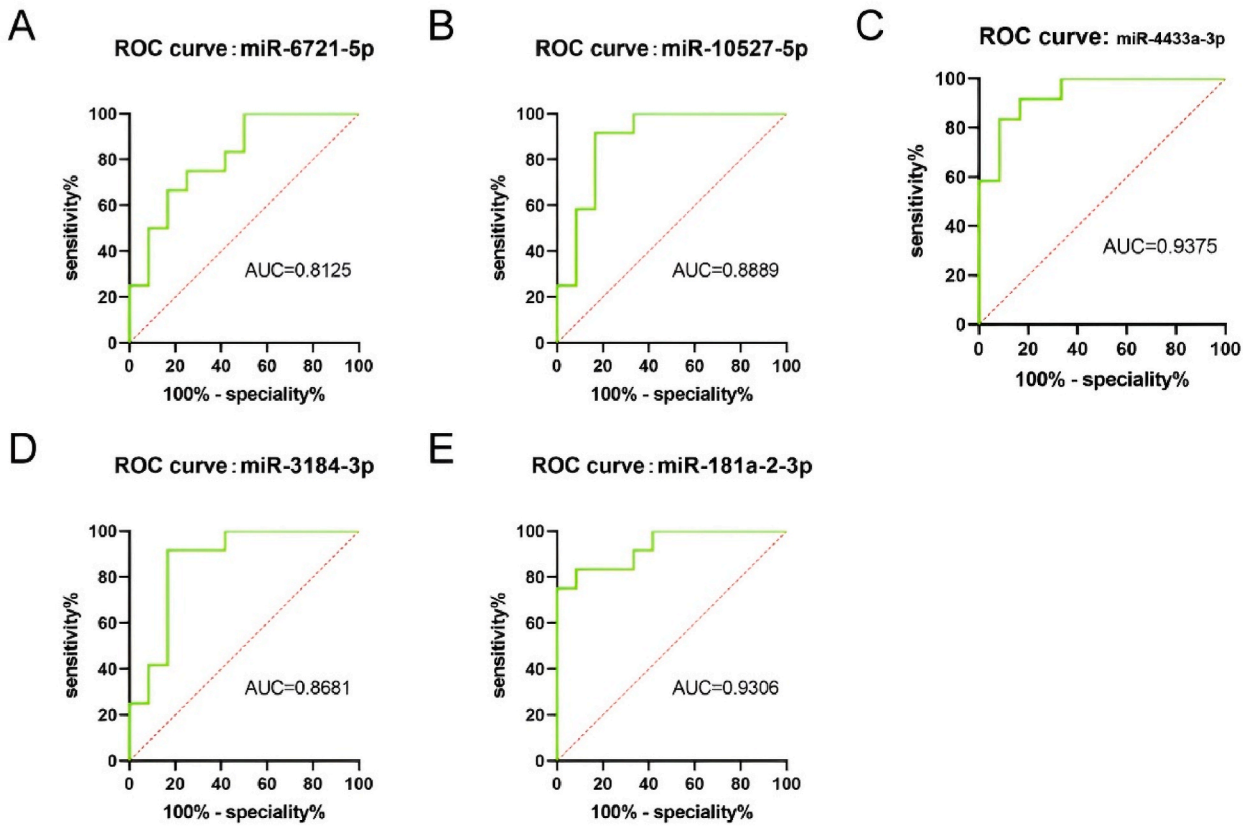

Fig. 6. Diagnostic values of microRNA in the pleural effusion of lung adenocarcinoma patients using quantitative qRT-PCR analysis. (A) The ROC curve analysis of miR-6721-5p in pleural effusion produced an AUC value of 0.8125. (B) The ROC curve analysis of miR-10527-5p in pleural effusion produced an AUC value of 0.8889. (C) The ROC curve analysis of miR-4433a-3p in pleural effusion produced an AUC value of 0.9375. (D) The ROC curve analysis of miR-3184-3p in pleural effusion produced an AUC value of 0.8681. (E) The ROC curve analysis of miR-181a-2-3p in pleural effusion produced an AUC value of 0.9306.

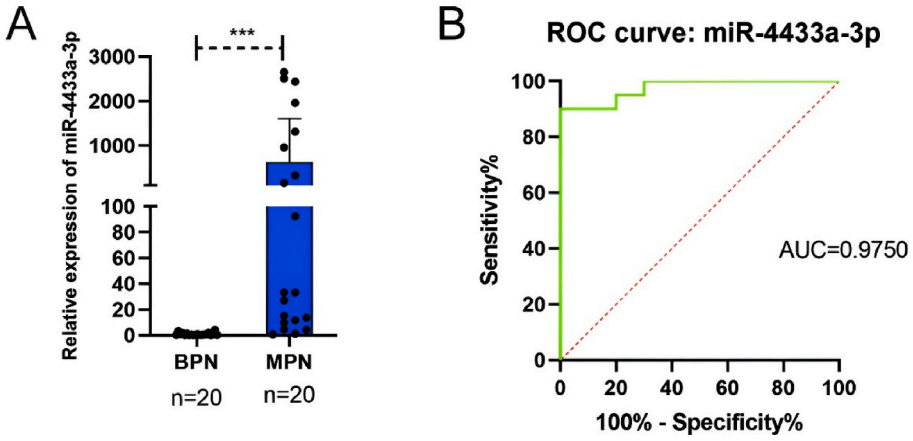

Fig. 7. miR-4433a-3p can be used for the diagnosis of early-stage lung cancer. (A) The expression of miR-4433a-3p in serum was measured by qRT-PCR. (B) The ROC curve analysis of miR-4433a-3p in serum produced an AUC value of 0.9750.

a prominent area of research.

The miRNAs bind to the 3′UTR region of their corresponding mRNA molecules, thereby exerting inhibitory effects on gene expression and playing a crucial role in cancer development [50]. In our study, we found that PIK3CD, UBE2J2, ICMT, PRDM16, and other miRNAs may form a regulatory network with miR-4433a-3p. PIK3CD affects the migration, invasion, proliferation, and growth of

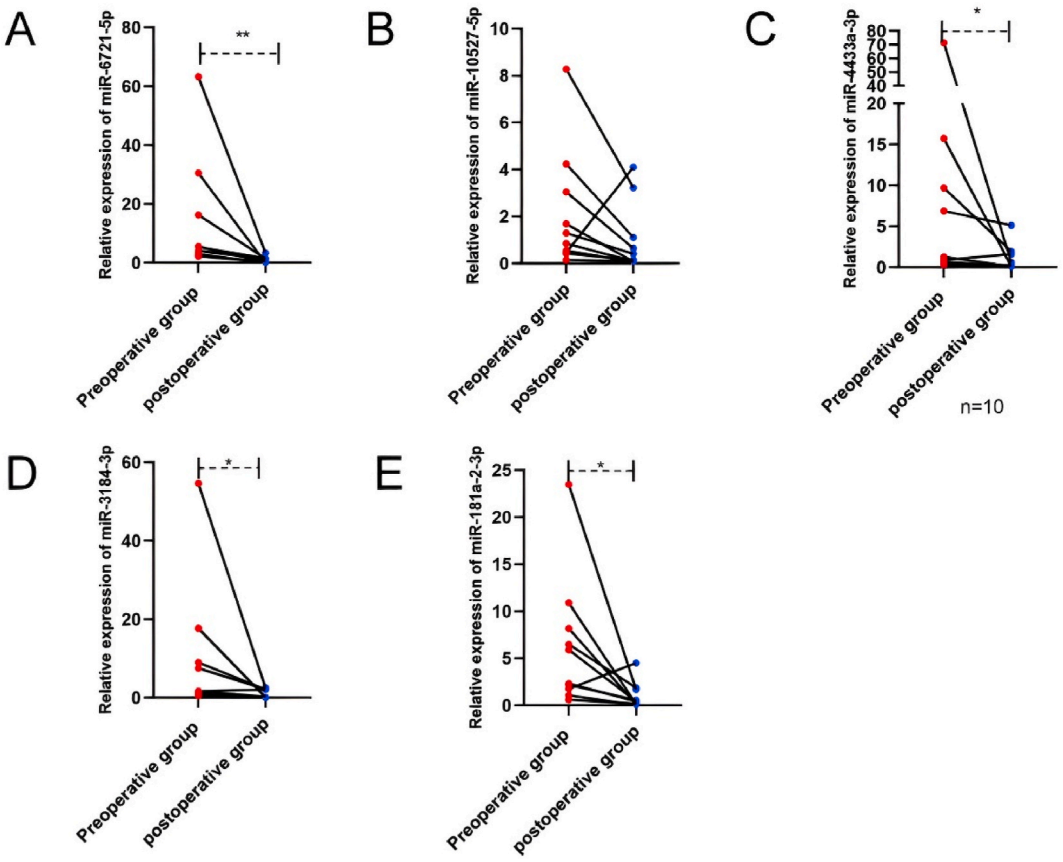

Fig. 8. Related miRNAs as indicators for evaluating the efficacy of surgical treatment for early lung cancer. (A) The expression of miR-6721-5p in serum was measured by qRT-PCR. (B) The expression of miR-10527-5p in serum was measured by qRT-PCR. (C) The expression of miR-4433a-3p in serum was measured by qRT-PCR. (D) The expression of miR-3184-3p in serum was measured by qRT-PCR. (E) The expression of miR-181a-2-3p in serum was measured by qRT-PCR. *P $<$ 0.05; **P $<$ 0.01.

glioblastomas by regulating the activity of P21-activated kinase (PPAK3) and plecstrin 2 (PLEK2) [51]. UBE2J2 is a ubiquitin proteasome component that promotes epithelial mesenchymalisation and accelerates tumour invasion [52,53]. ICMT affects tumour cell proliferation and apoptosis through Ras/Raf/Mek/Erk signalling and epithelial–mesenchymal transition [54,55]. PRDM16 activates adipocytes, leading to obesity, glucose intolerance, insulin resistance, and dyslipidaemia, which affect various metabolic diseases [56,57]. Investigations of these target genes in lung adenocarcinoma are limited. Therefore, miR-4433a-3p may modulate epithelialisation, apoptosis, and metabolism in lung adenocarcinoma by targeting these genes.

Increasing evidence indicates that miRNAs are strongly associated not only with tumour diseases but also with other conditions, such as diabetic nephropathy and Alzheimer's disease. This poses a challenge for utilising miRNAs in diagnosis and treatment because of the impact of human physiological and pathological states [58]. In this study, directly verifying that miR-4433a-3p remains unaffected by the physiological and pathological status of patients was challenging. The diagnostic role of miR-4433a-3p in lung adenocarcinoma could only be substantiated through the analysis of diverse samples. During early tumourigenesis, malignant transformation arises from a cascade of genetic alterations triggered by hypoxia and the generation of reactive oxygen and nitrogen species resulting from a persistent inflammatory response in the absence of pathogens [59]. The secretion of abundant miRNAs by non-small cell lung cancer cells triggers the activation of Toll-like receptors, leading to the initiation of primary inflammation and facilitation of tumour growth and metastasis [60]. Thus, the relationship between miR-4433a-3p and the pathophysiological processes of early lung adenocarcinoma warrants further investigation.

4. Conclusion

In conclusion, several screened miRNAs can be used for the haematological diagnosis of lung adenocarcinoma, among which only miR-4433a-3p can be used for the diagnosis of malignant lung nodules (early lung cancer). Meanwhile, miR-4433a-3p can also be used as an evaluation index for therapeutic effects in lung adenocarcinoma. However, miR-6721-5p, miR-4433a-3p, miR-3184-3p, and miR-181a-2-3p can be used to evaluate the efficacy of surgical treatment for early lung cancer.

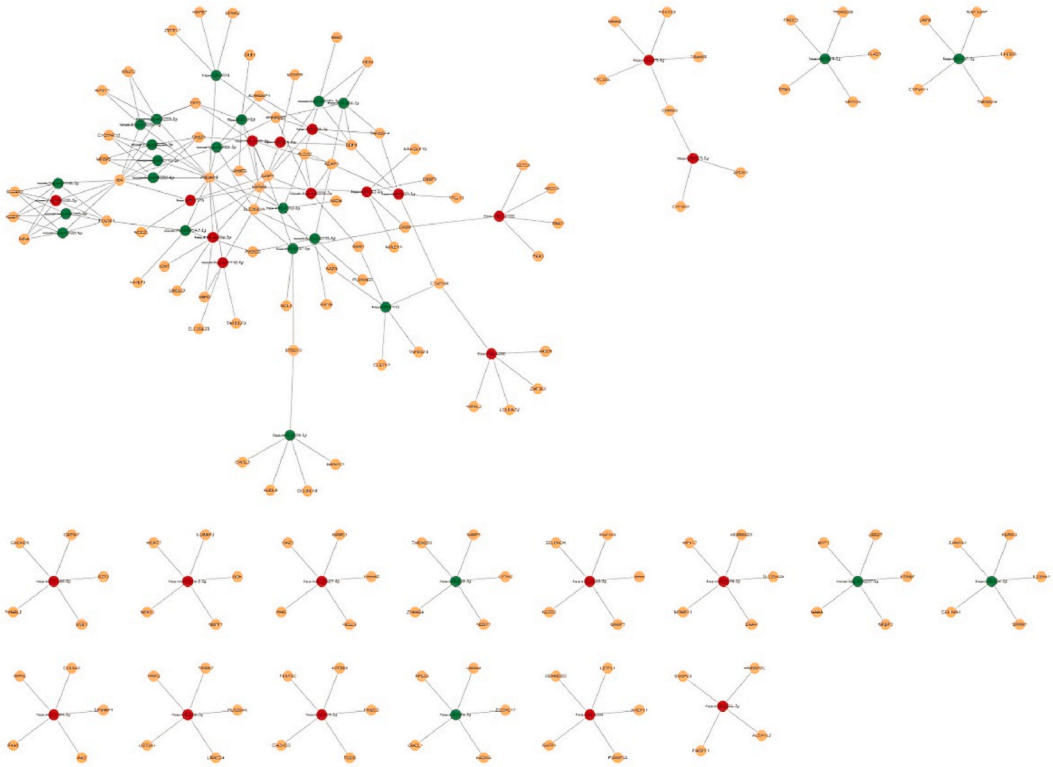

Fig. 9. The microRNA–mRNA regulatory network. Red represents the upregulated expression of microRNA; Green represents the downregulated expression of microRNA.

5. Methods

5.1. Patients and study design

This study was reviewed and approved by the Research Ethics Committee of Yancheng First People's Hospital (Yancheng, Jiangsu, China; ethical approval number: [2021]-K061). From October 2020 to March 2023, serum from 36 healthy individuals, 36 patients with lung adenocarcinoma, 20 patients with benign pulmonary nodules, 20 patients with malignant pulmonary nodules, and 10 patients with lung adenocarcinoma (before and after surgery) were collected for the study. Pleural fluid samples were collected from 12 patients with inflammatory disease and 12 patients with lung adenocarcinoma. All patients were of Han Chinese descent.

The inclusion criteria for all patients with lung adenocarcinoma and benign and malignant lung nodules were based on the histopathological diagnosis, excluding other tumours, with no radiotherapy or chemotherapy having been performed. In addition, the patient with malignant pulmonary nodules was confirmed to have no metastatic tumours through systemic evaluation and was diagnosed with early lung adenocarcinoma. All healthy individuals were excluded from various medical histories, such as tumours and connective tissue diseases. Written informed consent was obtained from all patients. All specific clinical information is presented in Tables 1–5. There were no significant differences in age or sex between the control and lung adenocarcinoma groups. All serum samples were collected in a vacuum collection vessel containing anticoagulants, centrifuged, and stored at −80 °C. All pleural fluid samples were collected and stored at −80 °C.

This study was designed as follows: (1) Using the Toray® 3D-Gene miRNA array, the expression levels of various miRNAs in the serum of lung adenocarcinoma patients and healthy volunteers were compared, and the appropriate miRNAs were selected for further analysis. (2) To verify the screening of highly expressed miRNAs, serum and pleural fluid samples were analysed using qRT-PCR. (3) The serum levels of screened miRNAs and their correlation with the clinicopathologic features of patients with lung adenocarcinoma were analysed. (4) The function of miRNAs was further analysed using the KEGG and GO databases. A flowchart of the study design is shown in Fig. 1.

5.2. RNA extraction

The collected serum was incubated in TRIzol reagent for 5 min and then left on ice for 5 min after the addition of chloroform. The mixture was centrifuged at 16000×*g* for 20 min at 4 °C to collect the supernatant. The supernatant was mixed with an equal volume of isopropyl alcohol, kept at −20 °C overnight, and then centrifuged at 16000×*g* for 20 min at 4 °C. After discarding the supernatant, the

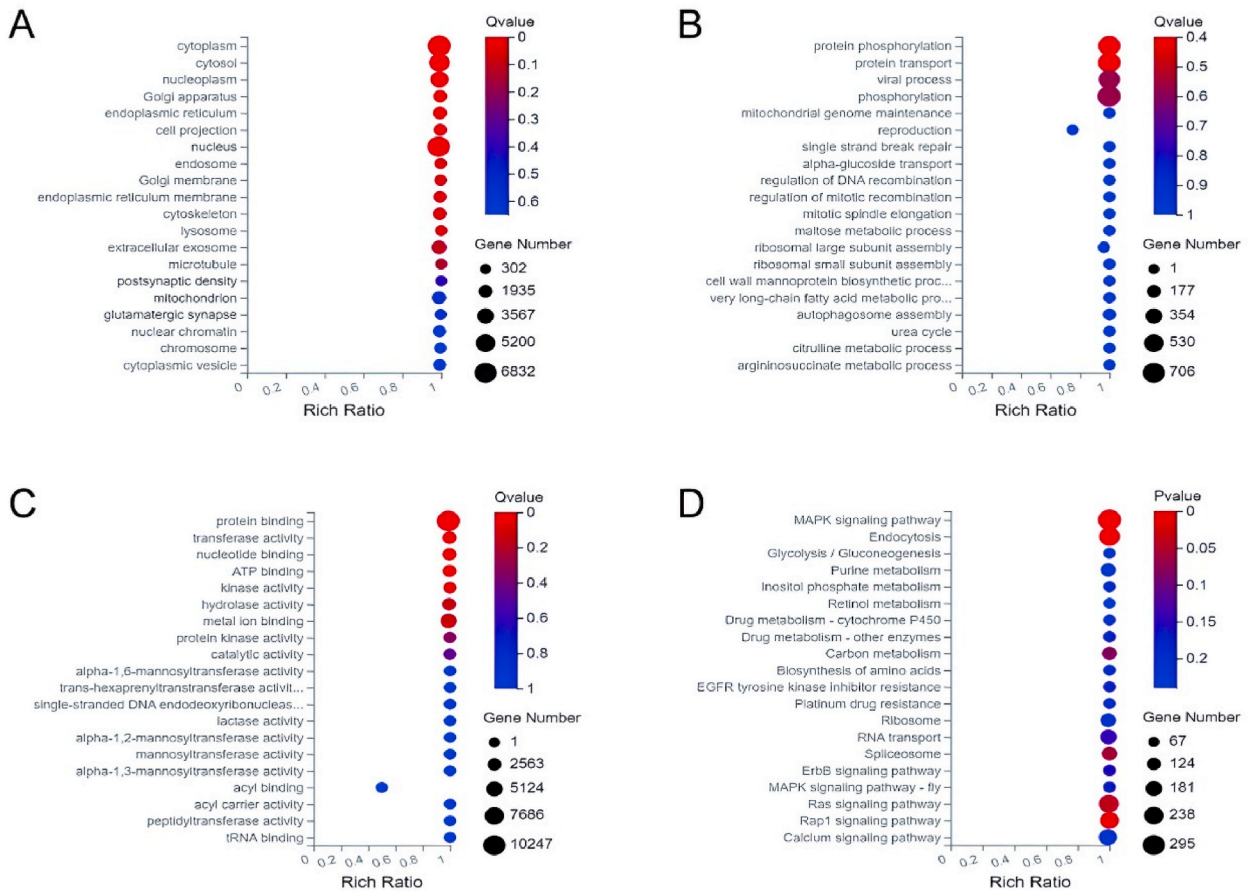

Fig. 10. The selected microRNAs and their targets were analysed by bioinformatics. (A) GO enrichment analysis of related cell components. (B) GO enrichment analysis of related biological processes. (C) GO enrichment analysis of related molecular functions. (D) KEGG analysis of correlated signal pathways.

Table 1
Clinical characteristics of samples used in microRNA array.

Characteristic	Healthy individuals (n = 4)	lung adenocarcinoma (n = 4)	p value
Age, years			
Median	69 ± 11.16	63.25 + 5.90	0.408
Range	57–79	55–69	
Sex			
Female	2	2	1
Male	2	2	

There were no significant differences in age and sex between the healthy individuals and patients with lung adenocarcinoma. These samples were used for microarray analysis. A value of $P \geq 0.05$ was not considered statistically significant.

Table 2
Clinical characteristics of 36 healthy individuals and 36 patients with lung adenocarcinoma.

Characteristic	Healthy individuals (n = 36)	lung adenocarcinoma (n = 36)	p value
Age, years			
Median	59.58 ± 10.31	62.91 + 10.57	0.18
Range	45–82	42–81	
Sex			
Female	20	18	0.814
Male	16	18	

There were no significant differences in age and sex between the healthy individuals and patients with lung adenocarcinoma. These samples were used for RT-qPCR analysis. A value of $P \geq 0.05$ was not considered statistically significant.

Table 3
Clinical characteristics of patients with pulmonary nodule.

Characteristic	BPN (n = 20)	MPN (n = 20)	p value
Age, years			
Median	61.75 ± 11.7	62.3 + 7.46	0.72
Range	36–76	51–77	
Sex			
Female	9	12	0.314
Male	11	8	

There were no significant differences in age and sex between the patients with benign pulmonary nodules and patients with malignant pulmonary nodules. These samples were used for RT-qPCR analysis. A value of $P \geq 0.05$ was not considered statistically significant.

Table 4
Clinical characteristics of patients with lung adenocarcinoma (before and after surgery).

Characteristic	n = 10
Age, years	
Median	63.6 ± 8.93
Range	42–77
Sex	
Female	7
Male	3

Table 5
Clinical characteristics of patients with pleural fluid.

Characteristic	BPE (n = 12)	MPE (n = 12)	p value
Age, years			
Median	68.92 ± 13.98	65.75 + 10.44	0.537
Range	47–93	50–85	
Sex			
Female	4	4	1
Male	8	8	

There were no significant differences in age and sex between the patients with benign pleural effusion and patients with malignant pleural effusion. These samples were used for RT-qPCR analysis. A value of $P \geq 0.05$ was not considered statistically significant.

Table 6
Primer sequence.

Gene	primer	sequence
hsa-miR-6721-5p	RT-primer	5′- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCCTA -3′
	F-primer	5′- GTGGGCAGGGGCTTATTG -3′
	R-primer	5′- AGTGCAGGGTCCGAGGTATT -3′
hsa-miR-10527-5p	RT-primer	5′- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCGTT -3′
	F-primer	5′- GCGAAAGCAAATGTTGGGTG -3′
	R-primer	5′- AGTGCAGGGTCCGAGGTATT -3′
hsa-miR-3184-3p	RT-primer	5′- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGAGGG -3′
	F-primer	5′- GCGAAAGTCTCGCTCTCTGC -3′
	R-primer	5′- AGTGCAGGGTCCGAGGTATT -3′
hsa-miR-4433a-3p	RT-primer	5′- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGTCC -3′
	F-primer	5′- GCGACAGGAGTGGGGGTG -3′
	R-primer	5′- AGTGCAGGGTCCGAGGTATT -3′
hsa-miR-181a-2-3p	RT-primer	5′- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGTACA -3′
	F-primer	5′- GCGACCACTGACCGTTGAC -3′
	R-primer	5′- AGTGCAGGGTCCGAGGTATT -3′
U6	F-primer	5′- CTCGCTTCGGCAGCACA -3′
	R-primer	5′- AACGCTTCACGAATTTGCGT -3′

Table 7
Correlation between the miR-4433a-3p serum level and clinical information in patients with lung adenocarcinoma.

		serum miR-4433a-3p concentration		*P*-value
		high	low	
Total	68	34	34	
Age				
< 65	40	24	16	0.084
≥65	28	10	18	
Sex				
Female	34	18	16	0.809
Male	34	16	18	
Clinical stage				
I-II	22	17	5	0.004
III-IV	46	17	29	
CEA(ng/ml)		11.51 ± 27.61	35.81 ± 108.91	0.259
CA19–9(U/ml)		19.42 ± 21.99	47.86 ± 194.41	0.465
CA125(U/ml)		36.57 ± 75.75	47.46 ± 67.23	0.574
cyfra21–1(ug/L)		3.65 ± 2.91	4.93 ± 5.68	0.281
proGRP(pg/ml)		40.84 ± 19.29	38.76 ± 16.88	0.672
NSE(ng/ml)		21.23 ± 29.43	15.69 ± 4.55	0.309
LDH(U/L)		207.56 ± 59.57	229.85 ± 76.49	0.187

The correlation between the miR-4433a-3p serum level and clinicopathological factors (age, sex, clinical stage, the expressions of CEA, CA19-9, CA125, cyfra21-1, proGRP, NSE and LDH) in patients with lung adenocarcinoma was compared in all patients with lung adenocarcinoma. A value of $P < 0.05$ was considered statistically significant. A value of $P \geq 0.05$ was not considered statistically significant.

Table 8
Correlation between the miR-4433a-3p pleural fluid level and clinical information in patients with lung adenocarcinoma.

		pleural effusion miR-4433a-3p concentration		*P*-value
		high	low	
Total	12	5	7	
Age				
< 65	6	3	3	1
≥65	6	2	4	
Sex				
Female	4	1	3	0.576
Male	8	4	4	
Clinical stage				
I-II	0	0	0	1
III-IV	12	6	6	
CEA(ng/ml)		157.08 ± 213.61	517.28 ± 474.52	0.111
cyfra21–1(ug/L)		134.81 ± 206.53	168.37 ± 178.09	0.79
proGRP(pg/ml)		62.58 ± 21.06	39.66 ± 11.33	0.115
NSE(ng/ml)		22.86 ± 26.0	11.05 ± 6.21	0.374
LDH(U/L)		643.46 ± 805.42	420.27 ± 188.52	0.187

The correlation between the miR-4433a-3p pleural level and clinicopathological factors (age, sex, clinical stage, the expressions of CEA, cyfra21-1, proGRP, NSE and LDH) in patients with lung adenocarcinoma was compared in all patients with lung adenocarcinoma. A value of $P \geq 0.05$ was not considered statistically significant.

Table 9
Serum tumor marker levels in patients with lung adenocarcinoma (before and after surgery).

	before surgery	after surgery	*P*-value
CEA(ng/ml)	2.3117 ± 1.48606	1.89 ± 0.89543	0.213
CA19–9(U/ml)	14.195 ± 14.67232	12.4317 ± 10.65561	0.372
CA125(U/ml)	29.3967 ± 27.26562	44.8917 ± 37.82687	0.04
cyfra21–1(ug/L)	4.42 ± 4.70209	3.162 ± 0.50559	0.582
proGRP(pg/ml)	34.44 ± 8.66963	31.144 ± 10.18759	0.532
NSE(ng/ml)	13.822 ± 3.48366	16.244 ± 5.24745	0.526
LDH(U/L)	196.5714 ± 26.27735	175.4714 ± 42.33225	0.357

The serum tumor markers in patients with lung adenocarcinoma showed no significant difference before and after surgery.

RNA was washed with 75 % ethanol and then dissolved by adding 20 μL of DEPC-treated water. RNA concentration was measured using a NanoDrop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA).

5.3. miRNA library construction

Four patients with lung adenocarcinoma and four healthy individuals were selected as the test samples. Total RNA was isolated and purified by electrophoresis on a 15 % urea-modified polyacrylamide gel, and small RNA regions corresponding to 18–30 nt bands in the 14–30 ssRNA Ladder marker (TAKARA) were removed and recovered. The small RNAs were then attached to adenylated 3′ adapters annealed to unique molecular identifiers, followed by the ligation of 5′ adapters. SuperScript II reverse transcriptase (Invitrogen, USA) was used to transcribe these small RNAs into cDNA, and several rounds of PCR amplification were performed with a PCR Primer Cocktail and PCR Mix to enrich the cDNA fragments. Agarose gel electrophoresis was used to screen target fragments of 110–130 bp, which were then purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). Fragment size distribution was examined using an Agilent 2100 bioanalyzer, and the library was quantitatively analysed using qRT-PCR (TaqMan Probe).

Raw data processing required the removal of low-quality tags, tags with 5′ primer contaminants, tags without 3′ primers, tags without insertions, tags with poly(A), and tags shorter than 18 nucleotides. The filtered data were mapped to the reference genome and another sRNA database [61]. The Dr. Tom Multiomics Data Mining System (https://biosys.bgi.com) was used for data analysis, mapping, and mining. The miRDeep2 software was used to predict the secondary structure [62]. MiRanda, RNAhybrid, and TargetScan were used to predict miRNA target genes [63–65]. The miRNA–mRNA regulatory network was mapped using the tidyverse package in R4.1.2 software. PossionDis was used for differential expression analysis [66], and FDR $\leq$0.001 and Log2Ratio absolute value $\geq$ 1 were selected as default thresholds to judge the significance of differential expression. Gene functions were annotated using the KEGG and GO databases.

5.4. qRT-PCR of miRNA expression levels

Total RNA extracted from serum was used to synthesise cDNA using the miRNA 1st Strand cDNA Synthesis Kit (stem-loop) MR101 (Vazyme, China). The expression of specific RNAs was quantified using the miRNA Universal SYBR qPCR Master Mix on a StepOnePlus Real-Time PCR System (ABI, USA). Primer sequences for reverse transcription and qRT-PCR were synthesised using GenScript. The primer sequences are listed in Table 6.

5.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (La Jolla, CA, USA) and IBM SPSS Statistics version 20.0. The data are displayed as means ± SD. To assess the statistical significance, the chi-square test, Student's t-test, or paired *t*-test was used. ROC curves and the AUC were used to evaluate the feasibility of using miRNA levels for diagnosis. Verification of miRNA expression in the serum and pleural effusion of patients with lung adenocarcinoma and early-stage lung cancer was performed using Student's t-test. Differential expression of related miRNAs in early-stage lung adenocarcinoma before and after surgery was analysed using Student's t-test. All specific clinical information was analysed using the chi-square test and Student's t-test. The cutoff value (8.0) was used as the median relative expression level of miR-4433a-3p in all serum samples. The correlation between miR-4433a-3p levels and the clinical information of patients with lung adenocarcinoma was analysed using the chi-square test and Student's t-test. The chi-square test was used to analyse statistical differences in the age, sex, and clinical stage of all participants. A value of $P < 0.05$ was considered statistically significant (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

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Data availability statement

Data included in article/supp. material/.

Ethical declarations

This study was reviewed and approved by the Research Ethics Committee of Yancheng First People's Hospital (Yancheng, Jiangsu, China; ethic number: [2021]-K061). All participants (or their proxies or legal guardians) provided informed consent to participate in the study.

CRediT authorship contribution statement

Zhixiao Sun: Writing – review & editing, Writing – original draft, Validation. **Jian Sun:** Validation, Supervision. **Hang Hu:** Validation, Supervision. **Shuhua Han:** Data curation. **Panpan Ma:** Data curation. **Bingqing Zuo:** Data curation. **Wang Zheng:**

Supervision, Project administration. **Zhongxiang Liu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Zhongxiang Liu reports financial support was provided by the Elderly Health Research Project of Jiangsu Province. Zhongxiang Liu has patent pending to Guan Sun. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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