Research Article

Serum miR-1228-3p and miR-181a-5p as Noninvasive Biomarkers for Non-Small Cell Lung Cancer Diagnosis and Prognosis

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Background. Lung cancer is the leading cause of cancer-related mortality worldwide, and non-small cell lung cancer (NSCLC) accounts for over 80% of all lung cancers. Serum microRNAs (miRNAs), due to their high stability, have the potential to become valuable noninvasive biomarkers. This present study was aimed to identify the serum miRNAs expression signatures for the diagnosis and prognosis of NSCLC using bioinformatics analysis.

Methods. A total of 12 miRNAs profiling studies have been identified in Pubmed, Gene Expression Omnibus (GEO), and ArrayExpress databases. Differentially expressed miRNAs (DEmiRNAs) were analyzed according to GEO2R online tool and RRA method from R. Then, prediction of DEmiRNAs’ target genes from TargetScan, PicTar, miRDB, Tarbase, and miRanda database. Furthermore, we using reverse transcription–quantitative polymerase chain reaction (RT-qPCR) to evaluate the expression levels of DEmiRNAs in serum samples obtained from NSCLC patients and healthy controls. Subsequently, the clinical significance of the tested miRNAs was determined using receiver operating characteristic (ROC) analysis and Cox regression analysis. Results. A total of 27 DEmiRNAs were identified and 5 of them (miR-1228-3p, miR-1228-5p, miR-133a-3p, miR-1273f, miR-545-3p) were significantly up-regulated and 4 of them (miR-181a-5p, miR-266-5p, miR-361-5p, miR-130a-3p) were significantly down-regulated in NSCLC patients compared with healthy controls. RT-qPCR validated that miR-1228-3p ($P = 0.006$) and miR-181a-5p ($P = 0.030$) were significantly differentially expressed in the serum of NSCLC patients and healthy controls. ROC analysis on miR-1228-3p and miR-181a-5p revealed the area under the curve (AUC) of 0.685 (95% confidence interval [CI], 0.563–0.806; $P = 0.006$) and 0.647 (95% CI, 0.506–0.758; $P = 0.049$). ROC analysis on miR-1228-3p combined miR-181a-5p revealed the AUC of 0.711 (95% CI, 0.593–0.828; $P = 0.002$). Multivariate Cox regression analysis demonstrated that the high serum miR-1228-3p level was an independent factor for the poor prognosis of NSCLC patients. Conclusions. Serum miR-1228-3p and miR-181a-5p are potential noninvasive biomarkers for the diagnosis and prognosis of NSCLC patients.

1. Background

Lung cancer remains the leading cause of cancer-associated mortality worldwide, of which NSCLC accounts for over 80% of lung cancer-related deaths [1, 2]. Despite improvements in the chemotherapeutic drugs used over time, the 5-year survival rate of NSCLC patients is only 18% [3]. Besides, surgical resection is the most effective treatment for NSCLC, but most newly diagnosed patients are at the onset of advanced or metastatic stages and usually lost the chance for operation. Low-dose computed tomography (LDCT) provides excellent anatomic information in the diagnosis of early NSCLC patients. However, LDCT still have a few limitations including high false-positive rates, potential over-diagnosis,
excessive cost and the potential harm related to radiation exposure. Furthermore, the response rates in subsets of NSCLC with tyrosine kinase receptors (mutant EGFR, ALK, and ROS1) were high, drug resistance has been a major challenge [4–6]. Therefore, it is vital to find an early and accurate way to diagnosis and enhance patient’s chances to receive proper treatments.

Currently, considerable studies revealed miRNAs as a new opportunity in the field of noninvasive diagnosis. MiRNAs are endogenous 20–25 nucleotides long, have been found to have a profound impact on several biological and pathological processes like cell development, differentiation, proliferation and apoptosis, which play important roles in the carcinogenesis and progression of lung cancer [7, 8]. DEmiRNAs in NSCLC tissue and adjacent nonmalignant tissues have been reported in a previous study [9]. Circulating miRNAs also could be potential and promising biomarkers for the diagnosis and prognosis of NSCLC. However, the data from different studies are quite variable. Therefore, identification of specific circulating miRNAs reflecting investigated pathological conditions may help to develop novel noninvasive biomarkers and shed a new light on molecular processes involved in cancer and a systematical analysis of miRNA expression signature from multiple platforms and multicenter NSCLC studies is urgently needed.

In this study, due to the presence and stability of cell-free miRNAs have been clearly demonstrated in all body fluid [10, 11], we identified serum and plasma miRNAs related to NSCLC, and then screened and validated miR-1228-3p and miR-181a-5p expression level in the serum of NSCLC patients in comparison to serum of healthy volunteers.

2. Methods

2.1. Data Collection. Up to January 1, 2018, a total of 3 databases including Pubmed (http://www.ncbi.nlm.nih.gov/), GEO (http://www.ncbi.nlm.nih.gov/geo/) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) were used for literature retrieval, and the search terms were (miRNA OR miRNA OR microRNA) AND (lung AND (tumor OR cancer OR carcinoma)). The selection criteria for the literature were: miRNAs detection was microarray or miRNAs sequencing; studies were published in English; patients had pathologically confirmed NSCLC; patients had no history of other cancers; none of the patients received preoperative treatment, such as radiotherapy or chemotherapy; control group was healthy normal controls; the experimental samples were derived from serum or plasma.

2.2. Identification of DEmiRNAs. GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) is a web tool for screening DEmiRNAs by comparing two groups of samples. The procedure of GEO2R is the following: firstly, enter a series accession number in the box. Then, click “Define groups” and enter names (NSCLC and healthy control) for the groups of samples you plan to compare. After samples have been assigned to groups, click “Top 250” to run the test with default parameters. To see more than the top 250 results, or if you want to save the results, the complete results table may be downloaded using the “Save all results” button. The cut-off criterion was set as the P < 0.05 and absolute fold change (FC) >1.5. In addition, the R package ggplot2 package (version 2.2.1, https://cran.r-project.org/web/packages/ggplot2/) was used to perform the volcano plots of all the miRNAs among 12 miRNAs profiling. Moreover, heat maps for the DEmiRNAs was generated using the pheatmap package (version 1.0.8, https://cran.r-project.org/web/packages/pheatmap). For some literatures that did not find original data, we used the miRNAs data listed in the paper or miRNAs information in supplementary data for analysis. All miRNAs names are standardized through miRBase.

2.3. Target Gene Prediction and Functional Enrichment Analysis. Target genes of DEmiRNAs were predicted by 4 different online databases including TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), miRanda (http://www.miranda-mi.org/) and miRDB (http://mirdb.org/). The target genes were screened by the intersection of TargetScan, PicTar, miRanda and miRDB. Then TarBase (http://www.microrna.gr/tarbase/) was used to validate the target genes. Then all of the target genes were sorted from the union of the front genes and the validation genes. Venn Diagram package (version 1.6.17, https://cran.r-project.org/web/packages/VennDiagram/) were applied to identify the overlapping target genes of DEmiRNAs among 12 miRNAs profiling. Furthermore, GeneCodis web tool (http://geneCodis.cnb.csic.es) was used to function enrichment analysis [12–14]. The resulting gene list was submitted to GeneCodis in order to identify the targeted pathway, threshold of FDR was 0.05 and considering enrichment in Panther and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

2.4. Patients and Samples. For the current study, we recruited 50 patients with first diagnosis of NSCLC and previously untreated from the Qilu Hospital of Shandong University, from July 2017 to December, 2017. Moreover, control group consist of thirty healthy volunteers (well matched to the patients according to age and gender) was screened from the Qilu Hospital of Shandong University. This study was approved by the Ethics Committee of Qilu Hospital of Shandong University (KYLL-2013-097; 25 February 2014), and written informed consent was obtained from all patients or their guardians. Once the patient was diagnose with NSCLC, about 5ml of peripheral blood was collected in a sterile tube without anticoagulant before any treatment was performed, and allowed to stand at room temperature for 30 – 60 min to clot, then samples were centrifuged at 4000 rpm for 15 min at the room temperature and for another 10 min at 12000 rpm at 4°C to completely remove the cell debris. Finally, the resultant serum was stored at -80°C, samples with visual evidence of hemolysis were excluded from further analysis.

2.5. Total miRNA Isolation and miRNAs Expression Analysis by RT-qPCR. Total miRNA was extracted from 200 μL of serum samples using miRcute serum/plasma miRNA isolation kit (Tiangen Biotech) according to the manufacturer's
Study design

Identification of DEmiRNAs

- Screening miRNA expression profiles
- GEO2R online tool analysis

Bioinformatics analysis

- Predict target genes of DEmiRNAs
- Functional enrichment analysis
- GO and pathway analysis

Clinical validation

- NSCLC patients ($n=30$) Healthy controls ($n=30$)
- qRT-RCR

Noninvasive biomarkers of NSCLC

**Figure 1:** The general overview of study design.

**Table 1:** The basic information of the 12 studies.

| Study | References | Region         | MiRNA numbers | Tumor type | Sample resource | Sample numbers | Time     | Database          |
|-------|------------|----------------|---------------|------------|-----------------|----------------|---------|-------------------|
| 1     | Lodes [12] | North America  | 547           | Various    | Serum           | 2 NSCLC, 14 normal | 2009.07 | Pubmed, ArrayExpress, GSE16512 |
| 2     | Wang [13]  | China          | 427           | Various    | Serum           | 88 NSCLC, 17 normal | 2011.06 | Pubmed            |
| 3     | Foss [14]  | Italy          | 880           | Various    | Serum           | 11 NSCLC, 11 normal | 2011.03 | Pubmed            |
| 4     | Roth [16]  | Germany        | 1158          | Various    | Serum           | 21 NSCLC, 11 normal | 2012.06 | Pubmed            |
| 5     | Rani [17]  | Ireland        | 667 ADC       | ADC        | Serum           | 40 NSCLC, 40 normal | 2013.12 | Pubmed            |
| 6     | Hu [18]    | China          | 723           | Various    | Plasma          | 73 NSCLC, 34 normal | 2014.02 | Pubmed            |
| 7     | Wang [19]  | China          | 754           | Various    | Serum           | 31 NSCLC, 31 normal | 2015.08 | Pubmed            |
| 8     | Nadal [20] | North America  | 334           | Various    | Serum           | 70 NSCLC, 22 normal | 2015.06 | Pubmed            |
| 9     | Halvorsen AR| Norway        | 272           | Various    | Serum           | 38 NSCLC, 16 normal | 2016.10 | GSE70080          |
| 10    | Qu LL [21] | China          | 5915          | ADC        | Plasma          | 9 ADC, 4 normal   | 2017.01 | GSE93300          |
| 11    | Liu X      | China          | 5915          | Various    | Plasma          | 6 NSCLC, 3 normal  | 2017.02 | GSE94536          |
| 12    | Xu ZL [22] | USA            | 7815          | Various    | NSCLC serum, Normal plasma | 24 NSCLC, 24 normal | 2017.05 | GSE46729          |
The concentration of miRNA was measured using the NanoDLite. According to the manufacturer’s instructions, miRNA profiling was performed with RT-qPCR instrument StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) using miDETECT A Track™ miRNART-qPCR Starter Kit (RiboBio). The primers of these miRNAs and cel-miR-39 were obtained from RiboBio Corporation (Guangzhou, China). After the reactions, the $\Delta$Ct values were determined. The fold change of each miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [15].

2.6. Statistical Analysis. The serum miRNA level was expressed as $2^{-\Delta\Delta Ct}$ to maintain the normal distribution of the parameter and assure a positive correlation with the miRNA level of expression and student’s t test was used to analyze miRNA expression level. Mann–Whitney tests were used to check associations between miRNA expression levels and clinicopathological features of the patients. The survival rates were estimated by the Kaplan-Meier analysis and the significance of differences was examined by log-rank test. We also performed overall survival (OS) to investigate survival outcome. OS was defined as the time between the date of surgery and the date of death or last followup. The diagnostic performance of miRNAs was assessed by the ROC curve analysis and calculated the AUC to evaluate the predictive power of candidate miRNAs for NSCLC. Multivariate analysis of the prognostic factors was performed with Cox regression model. Data was presented as mean ± standard deviation (SD) and $P<0.05$ were considered statistically significant. All statistical analysis was performed using the SPSS version 20.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Identification of DEmiRNAs. The general overview of the study design is shown in Figure 1. According to the selection criteria, 12 full-text studies were retrieved from July 2009 to January 2018 (Table 1) [11–18]. All these 12 studies including 413 NSCLC patients and 513 healthy controls were used to screen miRNA signature and a total of 2381 significantly up-expressed and 513 significantly down-expressed miRNAs were extracted. Then 5 GEO datasets (GSE16512, GSE70080, GSE93300, GSE94536 and GSE46729) were used to perform volcano maps and to get the DEmiRNAs ($P<0.05$ and absolute FC $>1.5$, Figure 2). According to RRA method 5 up-regulated miRNAs and 27 down-regulated miRNAs were screened ($FDR<0.05$). Finally we selected 9 DEmiRNAs including the top 4 down-regulated miRNAs (hsa-miR-181a-5p, hsa-miR-26b-5p, hsa-miR-361-5p and hsa-miR-130a-3p) and all 5 up-regulated miRNAs (hsa-miR-1228-3p hsa-miR-1228-5p hsa-miR-133a-3p hsa-miR-1273f hsa-miR-545-3p) for the further study based on the FDR value.
3.2. Target Gene Prediction and Functional Enrichment Analysis. TargetScan, miRanda, miRDB and PicTar were used to predict target genes of 9 DEmiRNAs, overlap predicted target genes from 4 databases (Figure 3) and Tarbase was used to validate target genes. Then, the overlapped genes plus validated genes was defined as target genes. Furthermore, removing 108 repeated target genes, we gained a total of 8002 target genes of DEmiRNAs (Table 2). 8002 target genes were used to perform functional enrichment analysis. The gene ontology (GO) analysis showed that these target genes were mainly involved in the regulation of transcription, DNA-dependent (GO:0006355), Nucleus (GO:0005634) and Protein binding (GO:0005515) (Table 3). Further Panther and KEGG pathway analysis were performed to investigate the significance of target genes in the development of NSCLC, the results showed that these genes were significantly enriched in Pathways in cancer and Wnt signaling pathway (Figure 4).

3.3. Association between Expression Levels of Serum miRNAs and Clinicopathological Characteristics. Among the 50 NSCLC patients, there were 34 males and 16 females, 37 adenocarcinomas (ADCs) and 13 squamous cell carcinomas (SCCs), 14 patients are at stage I, 7 at stage II, 10 at stage III and 19 at stage IV. The clinicopathological characteristics of patients with NSCLC and healthy volunteers are presented in Table 4.

The current study revealed that miR-1228-3p, miR-133a-3p and miR-545-3p were significantly up-regulated ($P=0.006$, $P=0.043$ and $P=0.047$, respectively), while miR-181a-5p and miR-361-5p were significantly down-regulated ($P=0.029$ and $P=0.006$) in NSCLC patients compared with healthy controls (Figure 5(a)-5(e)). Among NSCLC patients, miR-1228-3p expression level ($P=0.009$) in ADC patients was higher compared with healthy controls, while the expression levels of miR-181a-5p ($P=0.031$) and miR-361-5p ($P=0.006$) were lower than healthy controls (Figure 5(f)-5(h)). In SCC patients, miR-545-3p expression level ($P=0.034$) was higher compared with healthy controls (Figure 5(i)).

As for TNM stage, the expression level of miR-181a-5p and were significantly lower than healthy controls in TNM stage I ($P=0.028$), as well as miR-361-5p in both TNM stage II ($P=0.016$) and IV ($P=0.048$). On the contrary, the
Table 2: Target gene prediction of 9 DEmiRNAs.

| miRNA       | TargetScan | 4 target gene prediction databases | 1 verified database | Target gene numbers |
|-------------|------------|-----------------------------------|---------------------|---------------------|
|             |            | Pic tar | mirDB | miRanda | Overlap | Tarbase | Union |
| miR-1228-3p | 4529       | 224    | 214   | 7103    | 51      | 3       | 54    |
| miR-1228-5p | 1360       | 139    | 34    | 3218    | 5       | 15      | 20    |
| miR-133a-3p | 571        | 339    | 310   | 5314    | 85      | 298     | 383   |
| miR-1273f   | 5400       | 274    | 401   | 1445    | 3       | 0       | 3     |
| miR-545-3p  | 5435       | 472    | 794   | 4678    | 48      | 499     | 547   |
| miR-181a-5p | 1365       | 508    | 887   | 7846    | 186     | 1884    | 1884  |
| miR-26b-5p  | 1042       | 543    | 508   | 7465    | 145     | 2949    | 2949  |
| miR-361-5p  | 295        | 218    | 340   | 5615    | 41      | 685     | 726   |
| miR-130a-3p | 1026       | 578    | 652   | 7751    | 173     | 1543    | 1544  |
| Total       | 8110       |        |       |         |         |         |       |

Table 3: GO analysis of target genes.

| Function enrichment | FDR         | Target |
|---------------------|-------------|--------|
| **Go biological process (BP)** |             |        |
| GO:0006355: Regulation of transcription, DNA-dependent | 4.51E-119 | 621    |
| GO:0045893: Positive regulation of transcription, DNA-dependent | 7.41E-46 | 204    |
| GO:0045944: Positive regulation of transcription from RNA polymerase II promoter | 3.83E-45 | 232    |
| GO:0006915: Apoptotic process | 3.61E-41 | 229    |
| GO:0045892: Negative regulation of transcription, DNA-dependent | 9.24E-39 | 174    |
| GO:0000122: Negative regulation of transcription from RNA polymerase II promoter | 1.65E-37 | 176    |
| GO:0007165: Signal transduction | 2.73E-36 | 353    |
| GO:0010467: Gene expression | 5.03E-34 | 168    |
| GO:0006468: Protein phosphorylation | 2.91E-33 | 164    |
| GO:0044419: Interspecies interaction between organisms | 1.12E-32 | 144    |
| **Go cellular component (CC)** |             |        |
| GO:00005634: Nucleus | 0          | 2024   |
| GO:0005737: Cytoplasm | 0          | 1859   |
| GO:00005737: Nucleus, cytoplasm | 5.851E-210 | 975    |
| GO:0005829: Cytosol | 5.38E-155 | 813    |
| GO:0005730: Nucleolus | 5.43E-134 | 606    |
| GO:0005634: Nucleus, nucleolus | 2.29E-129 | 584    |
| GO:0005737: Cytosol, cytoplasm | 9.11E-123 | 606    |
| GO:0005622: Intracellular | 1.24E-108 | 685    |
| GO:0016020: Membrane | 9.19E-97 | 1104   |
| GO:0005654: Nucleoplasm | 1.86E-95 | 394    |
| **Go molecular function (MF)** |             |        |
| GO:0005515: Protein binding | 0          | 1770   |
| GO:0046872: Metal ion binding | 9.92E-150 | 975    |
| GO:0000166: Nucleotide binding | 1.10E-134 | 771    |
| GO:0003677: DNA binding | 7.10E-134 | 688    |
| GO:0008270: Zinc ion binding | 1.24E-115 | 693    |
| GO:0046872: Metal ion binding, zinc ion binding | 3.22E-108 | 648    |
| GO:0005524: ATP binding | 8.41E-97 | 550    |
| GO:0005515: Protein binding, nucleotide binding | 6.55E-95 | 363    |
| GO:0005524: ATP binding, nucleotide binding | 4.73E-89 | 506    |
| GO:0003677: DNA binding, protein binding | 8.64E-80 | 278    |
expression level of miR-1228-3p in TNM stage III ($P=0.007$) and IV ($P=0.026$) were significantly higher compared with healthy controls, as well as miR-545-3p in TNM stage IV ($P=0.013$) (Figure 6(a)). When the tumor diameter $<3$ cm, the expression levels of miR-1228-3p, miR-133a-3p and miR-545-3p were significantly higher compared with healthy controls ($P=0.020$, $P=0.005$ and $P=0.037$) and miR-1228-3p expression level in tumor diameter $>3$ cm group was significantly higher compared with healthy controls ($P=0.017$). In contrast, the expression level of miR-361-5p in tumor diameter $>3$ cm group was significantly lower compared with healthy controls ($P=0.006$) (Figure 6(b)). The high expression level of miR-1228-3p and low expression level of miR-181a-5p were related to lymph node metastasis ($P=0.005$, $P=0.003$, respectively), while the high expression level of miR-545-3p was related to no lymph node metastasis ($P=0.045$). As for miR-361-5p, its expression level in no lymph node metastasis group was lower compared with healthy controls ($P=0.027$), as well as lymph node metastasis group compared with healthy controls ($P=0.015$) (Figure 6(C)).

### 3.4. Diagnostic Value of Serum miRNAs NSCLC Patients.

ROC curve analysis was used to investigate the diagnostic value of miR-1228-3p, miR-133a-3p, miR-545-3p, miR-181a-5p and miR-361-5p in distinguishing NSCLC patients from normal controls. Expression levels of 5 serum miRNAs were measured from NSCLC patients and healthy controls. The diagnostic relevance of each miRNA, both single and...
combination, were analyzed (Table 5). The results showed that ROC analysis revealed the AUC for miR-1228-3p was 0.685 (95% confidence interval [CI], 0.563-0.806; \(P = 0.006\)), for miR-133a -3p was 0.636 (95% CI, 0.512-0.760; \(P = 0.043\)), for miR-545-3p was 0.635 (95% CI, 0.514-0.756; \(P = 0.045\)), for miR-181a-5p was 0.647 (95% CI, 0.506-0.758; \(P = 0.049\)) and the AUC for miR-361-5p was 0.635 (95% CI, 0.508-0.761; \(P = 0.045\)). ROC analysis indicated that the combination of miR-1228-3p and miR-181a-5p provided best diagnostic discriminant with an AUC of 0.711(95% CI 0.593-0.828; \(P = 0.002\)).

3.5. Associations of Serum miRNAs Expression Levels with OS.

To explore whether serum miRNAs expression levels will affect the clinical outcomes, we constructed a prognostic classifier using Kaplan-Meier analysis on 50 NSCLC patients. It showed that miR-1228-3p and miR-181a-5p expression levels were significantly associated with the OS of NSCLC patients (both \(P = 0.041\)) (Figure 7). As for miR-133a -3p, miR-545-3p and miR-361-5p, the expression levels of all these 3 miRNAs have no significance with OS statistically (\(P = 0.236, P = 0.709, P = 0.199\), respectively). The median OS in miR-133a -3p, miR-545-3p and miR-361-5p low expression group were both 8 months whereas in high expression group were all 7 months. The multivariate Cox hazard regression analysis demonstrated that expression level of serum miR-1228-3p were an independent prognostic indicator of NSCLC (hazard ratio(HR) 1.487, 95% CI 1.130-1.958; \(P = 0.005\)).

4. Discussion

In the current study, we integrated expression profiles of 413 NSCLC patients and 513 healthy controls in 5 datasets from GEO database and identified a panel of 32 DEMiRNAs. According to FDR value, we finally identified 9 DEMiRNAs for further study. Then we used 5 online databases and screened a total of 8002 target genes of these 9 DEMiRNAs, functional enrichment analysis showed that these target genes were mainly involved in the regulation of transcription, DNA-dependent, Nucleus, Protein binding and significantly enriched in Pathways in cancer, especially in Wnt signaling pathway. The high expression levels of miR-1228-3p, miR-133a-5p and miR-545-3p and low expression levels of miR-181a-5p and miR-361-5p were also validated via an independent NSCLC cohort from Qilu Hospital of Shandong
The result indicated that the expression level of miR-1228-3p was related to TNM stage, tumor diameter and lymph node metastasis, the expression level of miR-545-3p was related with TNM stage and tumor diameter, the expression levels of miR-181a-5p and miR-361-5p were related to TNM stage and lymph node metastasis, the expression level of miR-133a-3p was related with tumor diameter only. Furthermore, the expression levels of miR-1228-3p and miR-181a-5p were significantly associated with the OS of NSCLC patients.

The incidence of lung cancer is the leading factor in malignant tumors. Up to date, the gold standard in diagnosing NSCLC is pathologic evidence of malignant cells, which typically requires a surgical procedure or an invasive examination. It is mostly at advanced stage as long as lung cancer is diagnosed. The 5-year survival rate of advanced lung cancer is less than 20%, but the 5-year survival rate of stage IA lung cancer can reach 60% [21]. Early diagnosis is the key strategy to improve the outcome of lung cancer. Current methods including CEA level and CT screening cannot predict the risk of NSCLC for patients who have small lung nodules accurately. Therefore, specific and sensitive biomarkers for the detection of malignancies are urgently required to reduce the worldwide morbidity and mortality caused by NSCLC.

MiRNAs have been identified as potential biomarkers for lung cancer, it can be used to evaluate the invasion, metastasis, treatment response and prognosis of cancer. Although tumor sample miRNAs have been demonstrated to be associated with the development of tumors in many studies, it is
difficult to obtain tumor samples in clinical practice. Recent studies have supported that circulating miRNAs have potential diagnostic effects for NSCLC. Studies [22, 23] have shown that there is a significant difference between serum miRNAs and blood cell miRNAs in patients with lung cancer, and blood cells can affect the detection rate of whole blood miRNAs [18, 24]. So that we choose serum miRNAs as the source of hematology of the subjects. Numerous circulating miRNA signatures have been reported for the detection of NSCLC, but the miRNAs signature identified by different groups vary from one another because of the inconsistencies platforms, it is necessary to find a better way to screen different miRNAs. The RRA approach is as good way to eliminate differences among various platforms, by which reordered miRNAs according to the FDR value.

| miRNAs | AUC  | P value | Lower  | 95% CI  | Upper  |
|--------|------|---------|--------|---------|--------|
| miR-1228-3p | 0.685* | 0.006 | 0.563 | 0.806 |
| miR-133a-3p | 0.636* | 0.043 | 0.512 | 0.760 |
| miR-545-3p | 0.635* | 0.045 | 0.514 | 0.756 |
| miR-181a-5p | 0.647* | 0.049 | 0.506 | 0.758 |
| miR-361-5p | 0.635* | 0.045 | 0.508 | 0.761 |
| miR-1228-3p + miR-133a-3p | 0.615 | 0.087 | 0.490 | 0.739 |
| miR-1228-3p + miR-545-3p | 0.622 | 0.069 | 0.500 | 0.744 |
| miR-1228-3p + miR-181a-5p | 0.711* | 0.002 | 0.593 | 0.828 |
| miR-1228-3p + miR-361-5p | 0.651* | 0.025 | 0.520 | 0.781 |
| miR-133a-3p + miR-545-3p | 0.705* | 0.002 | 0.592 | 0.818 |
| miR-133a-3p + miR-181a-5p | 0.679* | 0.008 | 0.554 | 0.804 |
| miR-133a-3p + miR-361-5p | 0.637* | 0.041 | 0.510 | 0.764 |
| miR-545-3p + miR-181a-5p | 0.585 | 0.207 | 0.460 | 0.710 |
| miR-545-3p + miR-361-5p | 0.611 | 0.097 | 0.485 | 0.738 |
| miR-181a-5p + miR-361-5p | 0.646* | 0.030 | 0.520 | 0.772 |
| miR-1228-3p + miR-133a-3p + miR-545-3p + miR-181a-5p | 0.698* | 0.003 | 0.585 | 0.811 |
| miR-1228-3p + miR-133a-3p + miR-181a-5p + miR-361-5p | 0.661* | 0.017 | 0.535 | 0.787 |
| miR-1228-3p + miR-133a-3p + miR-361-5p | 0.616 | 0.084 | 0.489 | 0.743 |
| miR-1228-3p + miR-545-3p + miR-181a-5p | 0.588 | 0.19 | 0.463 | 0.713 |
| miR-1228-3p + miR-545-3p + miR-361-5p | 0.609 | 0.105 | 0.483 | 0.735 |
| miR-1228-3p + miR-181a-5p + miR-361-5p | 0.647* | 0.029 | 0.517 | 0.776 |
| miR-133a-3p + miR-545-3p + miR-181a-5p | 0.609 | 0.103 | 0.486 | 0.733 |
| miR-133a-3p + miR-545-3p + miR-361-5p | 0.643* | 0.033 | 0.519 | 0.768 |
| miR-133a-3p + miR-181a-5p + miR-361-5p | 0.663* | 0.015 | 0.538 | 0.789 |
| miR-545-3p + miR-181a-5p + miR-361-5p | 0.605 | 0.116 | 0.475 | 0.736 |
| miR-1228-3p + miR-133a-3p + miR-545-3p + miR-181a-5p + miR-361-5p | 0.614 | 0.089 | 0.491 | 0.737 |
| miR-1228-3p + miR-133a-3p + miR-545-3p + miR-361-5p | 0.640* | 0.037 | 0.516 | 0.764 |
| miR-1228-3p + miR-133a-3p + miR-181a-5p + miR-361-5p | 0.663* | 0.015 | 0.538 | 0.789 |
| miR-1228-3p + miR-545-3p + miR-181a-5p + miR-361-5p | 0.607 | 0.112 | 0.477 | 0.737 |
| miR-133a-3p + miR-545-3p + miR-181a-5p + miR-361-5p | 0.623 | 0.067 | 0.493 | 0.753 |
| miR-1228-3p + miR-133a-3p + miR-545-3p + miR-181a-5p + miR-361-5p | 0.623 | 0.067 | 0.493 | 0.753 |

Note: * P < 0.05, ** P < 0.01.
There are many researches of miR-1228-3p in various diseases. It has been reported that miR-1228-3p expression level was involved in drug resistant of breast cancer, chronic heart failure, endometrial carcinoma, it can be expressed steadily in the prostate cancer, colorectal cancer and secretions of hepatocellular carcinoma. There are another two studies about miR-1228-3p on NSCLC. One is about miR-1228-3p differentially expressed in NSCLC exocrine and another suggested that miR-1228-3p can be used as an endogenous reference gene. It means that miR-1228-3p can be stable in the exocrine and circulatory and further confirms that it can be released to the cell through the exocrine.

The miR-181 family includes miR-181a, miR-181b, miR-181c and miR-181d, contains the same seed sequence, which can display the functional redundancy of the gene in mRNA. The role of miR-181a-5p as a tumor suppressor has been confirmed in previous studies. For example, lower expression level of miR-181a-5p was associated with a worse survival rate in colorectal cancer. In gastric cancer and lung cancer, the expression of miR-181a-5p through target BCL2 increased the sensitivity of cancer cells to cisplatin and vincristine, which further induced the apoptosis of cancer cells. In addition, miR-181a-5p can reduce the metastasis in breast and colon cancer cells. All the results suggested that miR-181a-5p can affect the survival, invasion and metastasis of tumor cells, and even the therapeutic response to chemotherapeutic drugs, while the further role of miR-181a-5p in NSCLC remains to further explore.

5. Conclusion

In conclusion, our study indicated that miR-181a-5p play an important role in the early diagnosis of NSCLC and the combined expression levels of miR-1228-3p and miR-181a-5p have certain diagnosis efficacy for NSCLC. Furthermore, high expression level of miR-1228-3p and low expression level of miR-181a-5p have a shorter survival time, which indicated that miR-1228-3p and miR-181a-5p can be used as noninvasive diagnostic and prognostic biomarkers for NSCLC. However, it is vital to conduct more in-depth studies to explore the molecular roles of serum miR-1228-3p and miR-181a-5p in the future.

Abbreviations
NSCLC: Non-small cell lung cancer
miRNAs: microRNAs
GEO: Gene expression omnibus
DEmiRNAs: Differentially expressed miRNAs
The data used to support the findings of this study are available from the corresponding author upon request.

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