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Analysis of differential expression profile of miRNA in peripheral blood of patients with lung cancer

Qingfang He[1] | Yirong Fang[2] | Feng Lu[1] | Jin Pan[1] | Lixin Wang[1] | Weiwei Gong[1] | Fangrong Fei[1] | Jingjie Cui[3] | Jieming Zhong[1] | Ruying Hu[1] | Mingbin Liang[1] | Le Fang[1] | Hao Wang[1] | Min Yu[1] | Zuo-Feng Zhang[4]

[1]Zhejiang Provincial Center for Disease Prevention and Control, Hangzhou, China
[2]Shaoxing Center for Disease Control and Prevention, Shaoxing, China
[3]Hangzhou Dianzi University, Hangzhou, China
[4]Department of Epidemiology, Fielding School of Public Health, University of California, Los Angeles, CA, USA

Correspondence
Min Yu, Zhejiang Provincial Center for Disease Prevention and Control, Hangzhou 310051, China.
Email: myu@cdc.zj.cn

Zuo-Feng Zhang, Professor of Epidemiology and Medicine, UCLA Fielding School of Public Health, Los Angeles, CA 90095, USA.
Email: zfzhang@ucla.edu

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Abstract

Purpose: To identify potential molecular targets for lung cancer intervention and diagnosis, we analyzed the differential miRNA expression of peripheral blood between lung cancer patients and healthy controls.

Methods: Three pairs of cases' and controls' peripheral blood samples were evaluated for miRNA expression by microarray. 12 miRNAs were selected for RT-PCR validation and target genes prediction. In addition, 4 miRNAs were selected for future validation by RT-PCR in a large sample of 145 cases and 55 frequency-matched healthy controls.

Results: A total of 338 differentially expressed miRNAs were screened and identified by microarray. According to the fold changes, the top ten upregulated miRNAs were hsa-miR-124-3p, hsa-miR-379-5p, hsa-miR-3655, hsa-miR-450b-5p, hsa-miR-29a-5p, hsa-miR-200a-3p, hsa-miR-542-3p, hsa-miR-138-5p, hsa-miR-219a-2-3p, and hsa-miR-4701-3p, and the top ten downregulated miRNAs were hsa-miR-34c-5p, hsa-miR-135a-5p, hsa-miR-132-3p, hsa-miR-3178, hsa-miR-4449, hsa-miR-4999-3p, hsa-miR-1246, hsa-miR-4424, hsa-miR-1252-5p, and hsa-miR-24-2-5p. RT-PCR verification of the 12 miRNAs revealed that 5 of 8 upregulated miRNAs, 2 of 4 downregulated miRNAs showed a significant difference between the cases and controls ($P < .05$). A large number of target genes and their functional set showed overlapping among the 453 predicted target genes of the 12 miRNAs ($P < .01$). RT-PCR in the large sample confirmed the significant differential expression level of hsa-miR-29a-5p, hsa-miR-135a-5p, hsa-miR-542-3p, and hsa-miR-4491 between cases and controls ($P < .05$), and three of these microRNA, except hsa-miR-29a-5p, were significant after Bonferroni correction for adjustment of multiple comparisons.

Conclusion: There was a significant difference in miRNAs expression in the peripheral blood between lung cancer patients and healthy controls, and 4 miRNAs were validated by a large-size sample.

KEYWORDS
lung cancer, miRNA array chip, real-time fluorescent quantitative PCR (RT-PCR)

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1 | INTRODUCTION

Lung cancer is a common malignant tumor. According to the Chronic Disease Monitoring Report of the Zhejiang Province (2017), the incidence rate of lung cancer was 80.51/100 000, and 84.72/100 000 in the city, and 78.16/100 000 in the countryside, as well as 99.37/100 000 among males, and 61.33/100 000 among females.[1] Lung cancer is ranked as the first cause of cancer-related death in the Zhejiang Province. A total of 26 788 deaths were reported in 2017, and the reported mortality rate was 55.65/100 000. The reported mortality rates in the city and the countryside were 52.04/100 000 and 58.03/100 000, respectively. The mortality of lung cancer increases with age; the mortality rate among the elderly (>65 years) reached 299.2/100 000.[1] These alarming incidence rate and mortality of lung cancer statistics may due to the lack of effective early diagnostic methods of lung cancer. Currently, surgical resection is the main effective therapy for lung cancer. However, most lung cancer patients have lost the chances for surgery for quite of them are not diagnosed until at metastatic or even advanced stages.[2] Therefore, it is crucial to explore potential molecular targets for early detection or intervention/treatment of lung cancer, thereby reducing the mortality of lung cancer.

MicroRNA (miRNA) is a widespread class of non-coding small RNA, and its mature state is a kind of single-chain small molecule RNA with a length of about 19-23 nucleotides. Mature miRNA mainly inhibits the translation of target mRNA by the complementary base pairing of 3-untranslated region (UTR), 5-UTR and the coding region of target mRNA, which regulates the expression of target genes at the post-transcriptional level. The study of bioinformatics has shown that single miRNA molecules can bind to hundreds of target mRNAs with different functions and play regulatory roles in almost all mammalian pathological and physiological activities, such as individual development, tissue differentiation, cell apoptosis, and energy metabolism and are closely associated with the occurrence and development of diseases. In recent years, some studies reported that serum/plasma miRNA expression profile could effectively distinguish between cancer patients and healthy individuals and might be related to the development of lung cancer[3-5] or might have potential clinical value in the early diagnosis, prognostic, and therapeutic of lung cancer.[6-10] However, the functions of miRNA remain unclear. Therefore, we used a miRNA chip screening system for lung cancer, and then, real-time fluorescent quantitative PCR (RT-PCR) verification method to investigate the difference of plasma miRNA expression profile between lung cancer patients and healthy controls to predict target genes and to analyze the signal pathways, and to explore the relationship between miRNA and the genesis and the development of lung cancer.

2 | SUBJECTS AND METHODS

2.1 | Subjects

A total of 145 lung adenocarcinoma patients were recruited from various county-level hospitals in the Zhejiang Province, diagnosed with first primary lung cancer (confirmed by histopathology), Most of the stages for the lung cancer cases are either T1 or T2. Not all the stages for the lung cancer cases are available, although we made great efforts to collect. The reason might be that some patients' histology and stages were diagnosed in hospitals of other provinces like Shanghai, but treated at their local hospitals without diagnostic materials in medical records.

Patients did not receive any surgery, chemotherapy, or radiotherapy before fasting venous blood collection. Healthy individuals (without any kind of cancer, frequency matched with cases by age [±5 years] and gender) for chronic disease screening were recruited from the community during the same period and fasting venous blood were collected. Participants were asked about their smoking status. Those who responded as abstained smoking means they smoked but abstained for more than 1 year. Occasionally smoking was defined as those who occasionally smokes but does not meet the frequently smoking standard. Frequently smoking was defined as smoking every day for more than 1 year. People who did not smoke were defined as never smokers.

For microarray chip screening, we first randomly selected three patients, who were frequency matched with three randomly selected healthy individuals from the community during the same period (matched with age [±5 years] and gender). The six blood samples of the three patients and three healthy individuals were used first for microarray chip screening of differentially expressed miRNAs, then for validation by RT-PCR. The information on the three cases and three controls is as follows: (a) all three cases were diagnosed as having adenocarcinoma of the lung; (b) in the three patients and three healthy individuals, the male/female rations are both 2/1, and the ages are 61 ± 12 and 62 ± 10 years for cases and controls, respectively; (c) one lung cancer patients were smoker matched with one smoking healthy control, the other 4 participants are all never smokers. The differences in the age and gender between the two groups were not significant.

In the second stage, all the blood samples of the 145 lung adenocarcinoma patients' and frequency-matched 55 healthy individuals' were used for a larger sample validation of differentially expressed miRNAs by RT-PCR. The age, gender distribution, family history of lung cancer and smoking status of participants are shown in Table 1, and no significant differences were observed between the two groups.

2.2 | Experimental procedures

2.2.1 | RNA extraction and quality control

Plasma RNA was extracted by the TRIzol method and purified with RNasey Mini Kit (Qiagen). NanoDrop ND-1000 (Thermo) was used to measure the concentration of purified RNA and detect the quality of RNA. The integrity of the RNA was detected by denaturing agarose gel electrophoresis.

TABLE 1 General information of the study subjects for verification by using fluorescent quantitative PCR in large sample

Subjects	Lung cancer patients (145)	Healthy controls (55)	ORc (95% CI)
Gender (M/F)	100/45	30/25	1.852 (0.980-3.501)
Age (year)	62 ± 11	65 ± 13	1.022 (0.993-1.051)
Family history of lung cancer			
No = 0	135	54	1.00
Yes = 1	10	1	0.250 (0.031-2.001)
Smoking			
Never	63	44	1.00
Abstained	63	0	1.187 (0.497-2.836)
Occasionally	2	1	0.000 (0.000)
Frequently	17	10	0.850 (0.068-10.610)

Abbreviation: ORc: Crude OR.

2.2.2 | RNA labeling and microarray chip hybridization

RNA labeling and microarray chip hybridization were performed according to the method and instructions provided by Exiqon (Exiqon A/S Skelstedet 162950).

2.2.3 | Microarray chip scanning

Microarray chip scanning was performed by using Axon GenePix 4000B microarray chip scanner (Molecular Devices) according to the manufacturer's instructions.

2.2.4 | Real-time fluorescent quantitative PCR verification (RT-PCR)

The cDNA synthesis of both target miRNA and reference miRNA (hsa-miR-93-5p) was performed after quality inspection and used for RT-PCR, respectively. The volume of reaction was 10 µL. The detection of target miRNA and reference hsa-miR-93-5p was performed according to the following procedure: 95°C for 10 minutes; then, 40 cycles of each (95°C for 10 seconds, 60°C for 60 seconds [fluorescence collection]). The melting curve of RT-PCR products was established according to the following procedure: 95°C for 10 seconds, 60°C for 60 seconds, and 95°C for 15 seconds after amplification. Then slowly heated from 60 to 99°C (Ramp Rate was automatically set to 0.05°C/s by the instrument).

The expression of target miRNA was corrected with reference miRNA. The volume of 2 µL was used for each sample for RT-PCR. The hsa-miR-93-5p (the expression level is constant among different samples) was used as a reference. The sequences of related primers are shown in Table 2.

2.3 | Target gene prediction

Target gene prediction was performed by using the information of three database tools including miRbase, miRanda, and TargetScan. TopGO analysis was performed for the target genes.

2.4 | The methods for data analysis

All data were analyzed using SPSS 13.0 software. The continuous variables were presented as the means ± SD. The univariate logistic regression analysis was performed to assess associations with gender, age, family history of lung cancer and smoking (multi-categorical variables transformed into dummy variables) between the cases and controls, and group variable (cases = 1, controls = 0) was used as the dependent variable (sls = 0.1, sle = 0.05).

2.5 | Analysis of miRNA expression profiling

The microarray chip scanning image was read by using GenePix Pro 6.0 (Molecular Devices), and the signal value of the probes was extracted. The Exiqon miRCURY LNA™ microRNA microarray chip contains 3100 probes, covering all human, mouse, and rat microRNA annotated by miRbase 18.0 and also the viral microRNA related to these species.

Each probe has four repeats on the microarray chip, retaining the probes with value ≥30.0 in all samples and calculating the median value. All of the probes with the signal value ≥30 were screened for median value standardization among the microarray chips to obtain the standardized data, and the differentially expressed probes were screened. Standardization can minimize the system error among microarray chips so that different microarray chips are comparable. Fold change and *P*-value were used in this experiment for miRNAs selection with Fold Change ≥2.0, *P*-value ≤ .05.

2.6 | Results analysis of the RT-PCR for verification

The results of the RT-PCR for verification were analyzed by using $2^{-\Delta\Delta CT}$ method. Lung cancer cases group $2^{-\Delta\Delta CT}$/Healthy control group $2^{-\Delta\Delta CT} > 1$ was considered as upregulation. Lung cancer cases group $2^{-\Delta\Delta CT}$/Healthy control group $2^{-\Delta\Delta CT} < 1$ was considered as down-regulation. $P \leq .05$ was considered as significant.

TABLE 2 List of primers for real-time quantitative PCR and the results of fluorescent quantitative PCR verification

Genes	Primers	Annealing temperature (°C)	Product length (bp)	Fold change (lung cancer case group vs healthy control group)	*P* value
hsa-miR-93-5p	GSP:5′GGCAAAGTGCTGTTCGTG3′ R:5′CAGTGCGTGTCGTGGAGT3′	60	65		
Upregulated miRNAs					
hsa-miR-3655	GSP:5′AGGCTTGTCGCTGCGGT3′ R:5′GTGCGTGTCGTGGAGTCG3′	60	61	0.77	.320
hsa-miR-450b-5p	GSP:5′GGGGTTTTGCAATATGTTCC3′ R:5′GTGCGTGTCGTGGAGTCG3′	60	64	1.53	.003*
hsa-miR-29a-5p	GSP:5′GGGACTGATTTCTTTTGGT3′ R:5′CAGTGCGTGTCGTGGA3′	60	65	4.08	.003*
hsa-miR-542-3p	GSP: 5′GGGGTGTGACAGATTGATAA3′ R:5′CAGTGCGTGTCGTGGAGT3′	60	66	1.79	.014*
hsa-miR-138-5p	GSP:5′GGGGCTGGTGTTGTGAATC3′ R:5′GTGCGTGTCGTGGAGTCG3′	60	63	1.56	.045*
hsa-miR-502-5p	GSP:5′GGGGAATCCTTGCTATCTGG3′ R:5′GTGCGTGTCGTGGAGTCG3′	60	64	0.99	.946
hsa-miR-4491	GSP:5′GGGGAATGTGGACTGGTGTG3′ R:5′GTGCGTGTCGTGGAGTCG3′	60	64	2.21	.034*
hsa-miR-192-5p	GSP:5′GGGGCTGACCTATGAATTG3′ R:5′CAGTGCGTGTCGTGGAGT3′	60	65	1.78	.097
Downregulated miRNA					
hsa-miR-34c-5p	GSP:5′GGGAGGCAGTGTAGTTAGC3′ R:5′CAGTGCGTGTCGTGGAGT3′	60	66	0.69	.148
hsa-miR-135a-5p	GSP:5′GGGGTATGGCTTTTTATTCCT3′ R:5′GTGCGTGTCGTGGAGTCG3′	60	65	0.23	.002*
hsa-miR-1246	GSP:5′GGGGAATGGATTTTTGG3′ R:5′CAGTGCGTGTCGTGGAG3′	60	63	0.23	.016*
hsa-miR-125a-5p	GSP:5′GCTCCCTGAGACCCTTTA3′ R:5′CAGTGCGTGTCGTGGAGT3′	60	66	0.15	.058

Note: GSP is the specific primer corresponding to miRNA; R is the primer that matched with RT primer. The fold change of lung cancer case group vs healthy control group >1 was considered as upregulation, and <1 was considered as downregulation.
*Indicates $P \le .05$ (significant).

2.7 | Target gene prediction and signaling pathway analysis

2.7.1 | Target gene prediction

Target gene prediction was performed by using the information of three databases including miRbase, miRanda, and TargetScan. The overlapping part of the predictive results derived from the three databases was defined as the final prediction results. The association strength of the Overlap number >3 and Overlap-coefficient >0.5 was used as the default screening index.

2.7.2 | Gene ontology (GO) analysis

The GO project provides a controlled vocabulary to describe gene and gene product attributes in any organism.[11] The ontology covers three domains: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). Fisher's exact test is used to find if there is more overlap between the DE list and the GO annotation list than would be expected by chance. The *P*-value denotes the significance of GO terms enrichment in the DE genes. The lower the *P*-value, the more significant the GO Term (to our above described 12 miRNAs, *P*- value ≤ .01 was selected).

2.7.3 | Signaling pathway analysis

Pathway analysis is a functional analysis of mapping genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The *P*-value was calculated by the Fisher Exact Test that denotes the significance of the pathway correlated to the conditions. Lower the *P*-value, more significant is the pathway. $P < .05$ was used in our study as the threshold to obtain the high-frequency annotation, signaling transduction, and disease-related pathway of the gene set. miRNA_GO_Network map was drawn by using gene sets correlation analysis from the miRNA and BP enrichment analysis in GO.

3 | RESULTS

3.1 | Preliminary screening of miRNA Array chip

Three hundred and thirty-eight differentially expressed miRNAs were identified through initial microarray chip analysis. Among them, 204 were significantly upregulated, and 134 were significantly downregulated (Fold Change ≥2.0, *P*-value ≤ .05). According to the fold changes, the top ten upregulated miRNAs were hsa-miR-124-3p, hsa-miR-379-5p, hsa-miR-3655, hsa-miR-450b-5p, hsa-miR-29a-5p, hsa-miR-200a-3p, hsa-miR-542-3p, hsa-miR-138-5p, hsa-miR-219a-2-3p, and hsa-miR-4701-3p, and the top ten downregulated miRNAs were hsa-miR-34c-5p, hsa-miR-135a-5p, hsa-miR-132-3p, hsa-miR-3178, hsa-miR-4449, hsa-miR-4999-3p, hsa-miR-1246, hsa-miR-4424, hsa-miR-1252-5p, and hsa-miR-24-2-5p.

3.2 | RT-PCR verification

According to the microarray chip screening results and literature reports, 12 miRNAs (Table 2) were then selected for the verification by using RT-PCR.

The results of the RT-PCR verification showed that among the chosen 12 miRNAs, 5 of 8 significantly upregulated miRNAs showed significant difference between the lung cancer group and the healthy control group ($P < .05$, all of them were upregulated); 2 of 4 significantly downregulated miRNAs showed significant difference between the two groups ($P < .05$, both of them kept downregulated) (Table 2). The amplification and melting curves (not shown in paper) showed that the RT-PCR reaction had good amplification efficiency and specificity.

3.3 | Target gene prediction

The overlapping part of the predictive results derived from three databases including miRbase, miRanda, and TargetScan were selected, which showed a total of eight miRNAs in the overlapping part of predictive results derived from the three databases. The eight miRNAs and the number of their predictive target genes were as follows: hsa-miR-125a-5p with 102 target genes, hsa-miR-135a-5p with 91 target genes, hsa-miR-138-5p with 45 target genes, hsa-miR-192-5p with 22 target genes, hsa-miR-34c-5p with 64 target genes, hsa-miR-450b-5p with 67 target genes, hsa-miR-502-5p with 27 target genes, and hsa-miR-542-3p with 35 target genes, for a total of 453 target genes (Figure not shown). GO and signaling pathway enrichment analysis of the predictive 453 target genes were performed.

3.4 | GO analysis and GO cluster enrichment analysis of predictive target genes

The GO analysis showed a large amount of functional overlapping among the 453 predicted target genes. In addition, they were enriched in BP including cellular macromolecule metabolic process, cell cycle regulation, cellular protein metabolic process, positive regulation of the BP, cellular protein modification process, and intracellular signal transduction; in the CC including intracellular, intercellular, cytoplasm, organelle part, ion channel complex, and stress fiber; and in the MF including protein binding, nucleotide binding, and protein kinase activity, ($P < .01$) (Tables 3).

3.5 | Enrichment analysis of signaling transduction pathway

Pathway enrichment analysis based on KEGG was performed on the predictive target genes set through DAVID database and the signaling pathways with $P < .05$ were selected (Figure 1). The results showed a large amount of overlapping among the signaling transduction pathways of predictive target genes set. The KEGG pathway analysis involved several disease-related pathways including transcriptional misregulation in cancer, proteoglycans in cancer, mRNA surveillance pathway, RNA degradation, cAMP signaling pathway, dopaminergic synapses, and vascular smooth muscle contraction ($P < .05$) (Table 4).

3.6 | Validation by RT-PCR

According to the screening results by microarray, the RT-PCR verification results of microarray chip samples and the literature reports, four miRNAs including hsa-miR-29a-5p, hsa-miR-135a-5p, hsa-miR-542-3p, and hsa-miR-4491 were selected for differentially expressed miRNAs verification by RT-PCR in a large sample of 145 first-episode lung cancer patients and 55 healthy controls. The experimental approaches, reference genes, and data analysis methods were the same as the RT-PCR validation experiments in the microarray chip samples. The results showed that the difference of expression level of hsa-miR-29a-5p, hsa-miR-135a-5p, hsa-miR-542-3p, and hsa-miR-4491 between the lung cancer group and the control group showed significance ($P < .05$), which were in accordance with the upregulation/downregulation trend of the screening results by microarray and the RT-PCR verification results of microarray chip samples (Figure 2).

Multiple comparisons might lead to false positives. We conducted multistage of validations among large samples of cases and controls in order to reduce potential multiple comparison

TABLE 3 The Gene Ontology (GO) biological process (BP), cellular component (CC), and molecular function (MF) analysis of target genes

GO.ID	Term	Category	The number of target genes	*P* value
GO:0044260	Cellular macromolecule metabolic process	BP	237	1.53207E-06
GO:0007049	Cell cycle	BP	70	1.61085E-06
GO:0048522	Positive regulation of cellular process	BP	148	1.63651E-06
GO:0044267	Cellular protein metabolic process	BP	153	2.91601E-06
GO:0048518	Positive regulation of biological process	BP	165	4.43874E-06
GO:0006464	Cellular protein modification process	BP	123	5.37341E-06
GO:0036211	Protein modification process	BP	123	5.37341E-06
GO:0035556	Intracellular signal transduction	BP	94	1.08515E-05
GO:0022402	Cell cycle process	BP	54	1.64587E-05
GO:0006793	Phosphorus metabolic process	BP	103	1.85407E-05
GO:0005622	Intracellular	CC	346	1.24301E-06
GO:0044424	Intracellular part	CC	338	3.26228E-06
GO:0005623	Cell	CC	379	3.83014E-05
GO:0044464	Cell part	CC	377	0.000112113
GO:0005737	Cytoplasm	CC	269	0.000131442
GO:0005829	Cytosol	CC	99	0.00020604
GO:0044422	Organelle part	CC	212	0.000559938
GO:0043229	Intracellular organelle	CC	290	0.000657913
GO:0034702	Ion channel complex	CC	16	0.000695189
GO:0001725	Stress fiber	CC	6	0.000864451
GO:0032553	Ribonucleotide binding	MF	68	4.09233E-05
GO:0005515	Protein binding	MF	272	4.68665E-05
GO:0032555	Purine ribonucleotide binding	MF	67	5.7016E-05
GO:0017076	Purine nucleotide binding	MF	67	6.72523E-05
GO:0000166	Nucleotide binding	MF	78	0.000151141
GO:1901265	Nucleoside phosphate binding	MF	78	0.000153272
GO:0004672	Protein kinase activity	MF	28	0.000184924
GO:0032550	Purine ribonucleoside binding	MF	64	0.000193572
GO:0001883	Purine nucleoside binding	MF	64	0.000202749
GO:0032549	Ribonucleoside binding	MF	64	0.000202749

false-positive issues. We have attempted using a 2-stage approach. In the discovery stage, we identified 338 differentially expressed microRNAs, but only 12 microRNAs were identified in the further PCR confirmation. We were not able to adjust for multiple comparisons because of relatively small sample size. In the confirmation/validation stage, among 12 microRNAs, we eventually identified four microRNAs with *P*-value <.05. If we use Bonferroni correction (0.05/12 = 0.00417), three microRNAs are statistically significant hsa-miR-542-3p, hsa-miR-4491, and hsa-miR-135a-5p.

4 | DISCUSSION

Studies have shown that miRNAs are abnormally expressed in a variety of tumors such as lung cancer,[5-7,10,12] breast cancer,[13] and liver cancer.[14] More than 50% of the miRNA genes have been located in tumor-related genomic regions or fragile sites.[15] The miRNAs can act not only as tumor suppressor genes to downregulate the activity oncogenes but also to downregulate the activity of tumor suppressor genes. miRNAs can also affect the development and progression of tumors by intervening cell cycle, apoptosis, angiogenesis, and metastasis.[9,16,17] So far, only a very few of the miRNAs and their regulating target genes had been identified, and the functions of most miRNAs were still unclear. Further studies are needed on the functions of miRNAs to search for new miRNAs to accurately predict their target genes and correctly understand the interactions of miRNA and its target genes.

To date, the total number of miRNAs that was included in the latest version of miRbase22.1 database[11] had exceeded 38 000 precursor sequences, and a total of mature miR and miR* products of

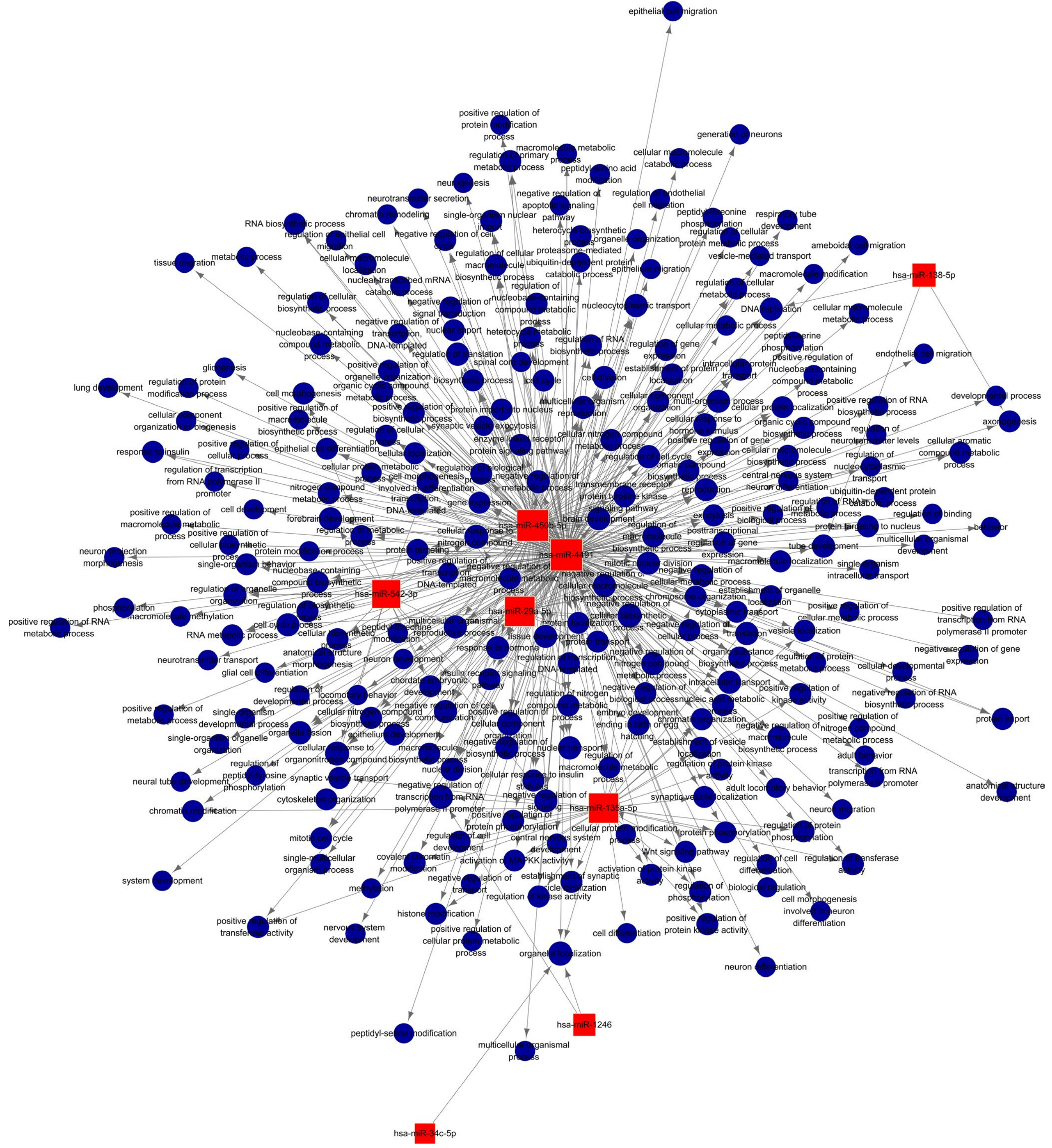

FIGURE 1 miRNA_GO_Network analysis

48 885 covering a total of 168 species in mirbase18.0 that are mentioned in this article. With the development of a high-throughput detection technology of miRNA, an increasing number of new miRNA have been discovered. The ambiguous and incorrectly commented sequences in miRbase database were constantly cleared, and the reliability of the database was continuously improved. MiRNA microarray is the most commonly used method for high-throughput detection of large amount of miRNA specific expression in tumor samples.[18] Compared with traditional RNA analysis methods, it has the following advantages: (a) high-throughput joint detection; (b) the detection efficiency and sensitivity are high; and (c) sample demanded is small, and only 1 μg of total RNA can complete all the tests, which has great significance for tumor samples that are extremely difficult to obtain.

TABLE 4 The Enrichment analysis of signaling transduction pathway

PathwayID	Definition	The number of target genes	*P* value
hsa03015	mRNA surveillance pathway—Homo sapiens (human)	8	.00173546
hsa04728	Dopaminergic synapse—Homo sapiens (human)	9	.004934847
hsa04270	Vascular smooth muscle contraction—Homo sapiens (human)	8	.009863016
hsa04261	Adrenergic signaling in cardiomyocytes—Homo sapiens (human)	9	.0111802
hsa05205	Proteoglycans in cancer—Homo sapiens (human)	11	.01188643
hsa05202	Transcriptional misregulation in cancer—Homo sapiens (human)	10	.01285791
hsa04360	Axon guidance—Homo sapiens (human)	8	.01298216
hsa03013	RNA transport—Homo sapiens (human)	9	.02525796
hsa04024	cAMP signaling pathway—Homo sapiens (human)	10	.02583363
hsa04713	Circadian entrainment—Homo sapiens (human)	6	.0322689
hsa00052	Galactose metabolism—Homo sapiens (human)	3	.0367944
hsa03018	RNA degradation—Homo sapiens (human)	5	.04100519

As a research method of expression spectroscopy, it is difficult to avoid false-positive results for the miRNA chip. Therefore, it is necessary to verify the chip screened miRNA. Although RT-PCR is a low-throughput detection method, it has high detection sensitivity and can faithfully reflect the abundance of miRNA in samples. Further, RT-PCR verification of large samples can more truly reflect the actual expression of miRNA. Accordingly, the quality controls in the RT-PCR analysis are very important, as in our study, we (a) measured the concentration of purified RNA and detected the quality of RNA before RT-PCR analysis, (b) used hsa-miR-93-5p as a reference and the expression of target miRNA was corrected with reference miRNA, (c) The negative template control was performed in the RT-PCR analysis, (d) melt curve and application curve are used to determine the specificity of the products, (e) the results of the RT-PCR for verification were analyzed by using $2^{-\Delta\Delta CT}$ method and the differentially expressed probes were screened.

General human tissue could express miRNA, studies also showed that miRNA might not be degraded by endogenous RNase in serum and plasma and could still exist in a very stable form in harsh conditions (such as high temperature, low or high pH environment, and multiple freeze-thaw). Moreover, the expression profiles of miRNA in the serum and plasma of normal people and patients with different diseases would be specifically changing with the physiological status, disease type, and disease courses. Based on this, this study used microRNA chip with 3100 probes and screened out a total of 338 miRNAs which are differentially expressed in lung cancer patients and healthy controls. Among them, 204 miRNAs were significantly increased, and 134 miRNAs were significantly downregulated. In the 12 miRNAs which were verified by RT-PCR, seven miRNAs were confirmed. RT-PCR validation results of large samples suggested hsa-miR-29a-5p, hsa-miR-135a-5p, hsa-miR-542-3p, and hsa-miR-4491 could be used as candidate genes for early screening of lung cancer, and three of these microRNAs, except hsa-miR-29a-5p, were significant after Bonferroni correction for adjustment of multiple comparisons. These results illustrated that RT-PCR verification results were highly consistent with miRNA chip results, suggesting that miRNA chip detection is an efficient, rapid, and high-throughput method for the analysis of miRNA differential expression between lung cancer patients and healthy controls, while RT-PCR is suitable for the study of individual gene expression changes. The two methods could complement and confirm each other.

Previous studies showed that miRNA family is an important part of the gene expression regulatory network and might be involved in the regulation of multiple signaling pathways.[19-21] The determination of miRNA target gene was the key to study the biological function of miRNA, and the functional analysis of target gene was helpful to understand the functional mechanism of miRNA. Bioinformatics prediction showed that miRNAs could regulate more than 30% of protein-coding genes.[22] An individual miRNA could act on a number of target genes to regulate biological processes, while multiple miRNAs could also regulate some of the target genes. Through the analysis, 12 differentially expressed miRNAs that we selected participated in the regulation of 37 281 predicted target genes and functioned in a variety of physiological processes. Further analysis showed that

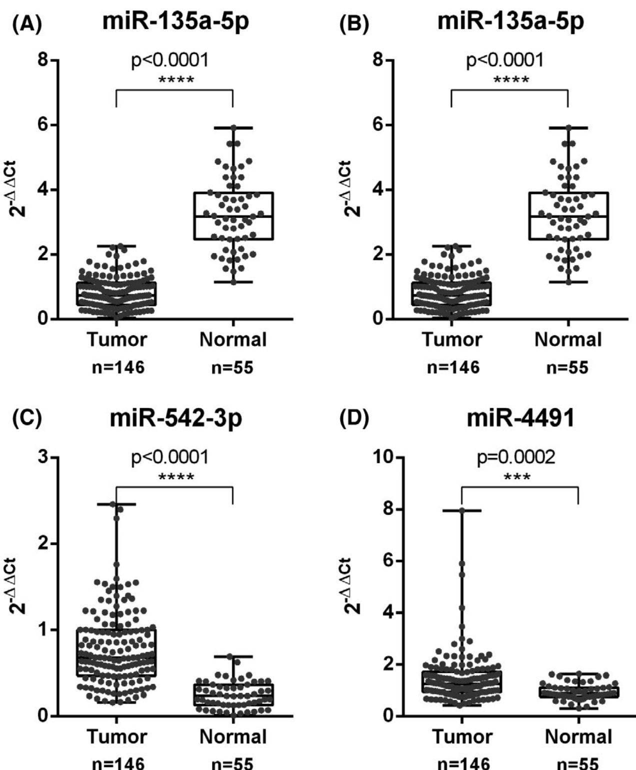

FIGURE 2 Results of the larger sample validation by real-time fluorescence quantitative PCR (RT-PCR). *Note*: tumor = lung cancer patients; normal = healthy controls. The fold change of the lung cancer group vs the healthy control group >1 was considered as upregulation, <1 was considered as downregulation; $^{*}P \le .05$ (significant), $^{***}P \le .001$ (significant), $^{****}P \le .0001$ (significant)

the 12 miRNAs had 453 overlapped predicted target genes; through the analysis of target genes, we found that the collection functions of target genes were also highly coincident suggesting that the regulation of target genes by miRNA had a synergistic effect. When a few miRNAs were commonly upregulated in tissues, the inhibition of a target gene would be strengthened, and vice versa.

The KEGG pathway analysis showed a large amount of overlapping among the signal transduction pathway of target gene sets, which are involved in cancer transcriptional dysregulation pathway, proteoglycan, and cancer pathway, suggesting that it might be closely related to the cancers such as lung cancer. Studies suggested that hsa-miR-29a family could inhibit the protein expression of B7-H3 and perform inhibition effects on a variety of B7-H3 protein-mediated solid tumors, including lung cancer, and hsa-miR-29a mutation could promote the growth of tumor cells[23]; hsa-miR-29a in the peripheral circulation in lung cancer patients could bind and activate toll-like receptors (TLR) in immune cells around tumors. Activation of TLRs could trigger NF-κB pathway, leading to an increase in the expressions of IL-6 and TNF-α in immune cells and increasing the potential for tumor growth and metastasis,[24] which was consistent with the results of this study, suggesting that hsa-miR-29a was involved in the pathological process of lung cancer. Although the relationship between hsa-miR-4491 and lung cancer has not been reported, our study suggested that it might play an important role in the pathogenesis of lung cancer. According to another report, miR-542-3p was located in Xq26.3 and was downregulated in non–small-cell lung cancer and breast cancer. It could directly target survival protein or angiopoietin-2, respectively, resulting in growth arrest and angiogenesis inhibition. miR-542-3p acts as a tumor suppressor gene,[25,26] which is inconsistent with the high expression of miR-542-3p in lung cancer cases in this study. Zhang Hongyan et al[27] investigated miR-135a expression and clinical pathology of lung cancer and found that miR-135a was closely related to the clinical stage and pathological grade of lung cancer. The serum miR-135a level was downregulated in non–small-cell lung carcinoma (NSCLC) patients and was associated with poor prognosis. It can be used as a biomarker for NSCLC prognosis.[28] Zhou's findings indicated that miR-135a promotes cell apoptosis and inhibits cell proliferation, migration, invasion, and tumor angiogenesis by targeting IGF-1 gene through the IGF-1/PI3K/Akt signaling pathway in NSCLC,[29] and MiR-135a inhibits its migration and invasion and regulates EMT-related marker genes by targeting KLF8 in lung cancer cells,[30] which is consistent with our study, while Zhang's study showed that MiR-135a overexpression promoted viability, migration, and invasion, but inhibited apoptosis of NCI-H1650 and NCI-H1975 cells.[31] Our subsequent cell biology experiments supported our results that the overexpression of mir-135a-5p could inhibit the invasion of adenocarcinoma cells, and the overexpression of mir-542-3p could promote the proliferation of adenocarcinoma cells (data not shown). Taken together, the results suggest that there are divergences in the expression and function of various miRNAs in lung cancer, which might be due to that the upregulation or downregulation of miRNA mainly dependent on the types of tissue and the target proteins of miRNA. We cannot simply define a specific miRNA as a tumor suppressor gene or an oncogene. In one cell system, it may be an oncogene, while in another it may be a tumor suppressor gene. And these areas need further studies.

In summary, we screened the miRNA expression profile which was closely related to lung cancer by using miRNA microarray and bioinformatics approaches. In addition, target prediction showed that lung cancer-related miRNA targets were very extensive, involving the genes including oncogenes, tumor suppressor genes, signal transduction genes, and cell cycle regulation related genes, which would provide a new experimental basis and ideas for the further research on the effect of miRNA on the occurrence and development of lung cancer in future.

CONFLICT OF INTEREST

None declared.

ORCID

Qingfang He https://orcid.org/0000-0002-2021-4431

Feng Lu https://orcid.org/0000-0002-0185-9445

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