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Expression profile of miRNA in NSCLC tissues in middle-altitude area

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Abstract. Micro ribonucleic acid (miRNA) expression profile in non-small cell lung cancer (NSCLC) tissues in middle-altitude area was analyzed using the Affymetrix chip technique, to predict the target genes of abnormally-expressed miRNAs, and to analyze the target gene-related signaling pathways and cell biological functions regulated by them. The difference in miRNA expression profile in NSCLC tissues was analyzed using the Affymetrix chip technique. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed for the verification of some differentially-expressed miRNAs. The genes predicted by at least 6 out of 12 commonly used prediction methods of miRNA target genes, based on miRWalk2.0, were considered as target genes. The functions of differentially-expressed miRNA target genes were analyzed via Gene Ontology (GO) enrichment analysis, and the main signaling pathways involving target genes were analyzed via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. There was abnormal expression of miRNAs in NSCLC tissues in the middle-altitude area. There were 140,405 target genes predicted for differentially-expressed miRNAs. The GO enrichment analysis of the functions of the target genes of differentially expressed miRNAs revealed that they mainly influence the binding process of intracellular components to protein, the positive regulation of biological process and the regulation of metabolic process. Moreover, these target genes were mainly enriched in the immunity, gene expression, metabolism and signal transduction, among which signal transduction was enriched with the most genes. The expression levels of miRNA-139-5p and miRNA-150-5p in lung cancer group were lower than those in the control group. The expression of miRNAs in NSCLC tissues in the middle-altitude area is abnormal, and most miRNAs are downregulated.

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Introduction

There are numerous diagnostic methods for lung cancer, such as sputum pathology, tumor markers, imaging, CT scan, percutaneous lung puncture, and fiberoptic bronchoscopic and surgical tissue biopsy. Sputum cytologic culture is a traditional diagnostic method with high diagnostic specificity of >98%, but the sensitivity is only 66% (1). A trend toward lower sensitivity was noted for lesions that were <2 cm in diameter. However, sensitivity is higher for central lesions than for peripheral lesions. Therefore, for lesions <2 cm in diameter, CT is better than sputum cytology, especially in peripheral lung cancer. Although its diagnostic sensitivity is improved by cytologic smears, the arrangement mode of cancer cells is often changed during the smear process, harming the pathological diagnosis of lung cancer. There is a number of studies on the abnormal expression of micro ribonucleic acid (miRNA) in lung cancer tissues. However, the results are inconsistent or the biological functions remain unclear (1-3). The abnormal expression of miRNA is also related to environment and genetics, which also have prognostic risks (4). Whether the expression of miRNAs is inconsistent in lung cancer tissues and serum is unknown, and it has been reported that the miR-133 expression is increased in both lung cancer tissues and serum (5). According to the MeDIP-chip microarray analysis, there is methylation of miRNAs (miR-10b, miR-1179, miR-137, miR-572, miR-3150b and miR-129-2) in primary lung tumor, and miR-1179 mimics prevent cell growth through inhibiting the target gene CCNE1 (6).

miRNA is a kind of non-coding small-molecule RNA, which can target a variety of genes. miRNA is involved in regulating various biological processes, including the cell signal expression, proliferation, differentiation and apoptosis. Each miRNA can regulate hundreds of messenger RNAs (mRNAs) in a parallel and targeted manner, and any change in its expression level may produce significant influences on biological processes and lead to pathophysiological changes (7). Tumor cells are in a special hypoxic microenvironment, and hypoxia will occur once the tumor diameter becomes more than several

hundred microns (8). The production of hypoxic environment and the activation of its major effector, hypoxia-inducible factor-1 (HIF-1), are common features of advanced cancer (9). Currently, there are few studies on whether the *in vitro* hypoxic state further aggravates the hypoxia in the microenvironment of lung cancer cells, and leads to abnormal expression of some miRNAs in lung cancer cells, and whether it is involved in the process of carcinogenesis, invasion and metastasis of lung cancer. A recent study found a significant correlation between Tibetan EGLN1/PHD2 haplotypes (D4E and C127S) and lung cancer, corresponding to a 2-fold increase of lung cancer risk in high altitude, and a ≥2-fold increased risk for rs117813469 and rs142764723 of the ten EPAS1/HIF-2α variants (10), although the expression of miRNA is regulated by multiple factors. The tumor microenvironment, especially the effect of hypoxia on the biological characteristics of the tumor is clear. In the present study, lung cancer patients in middle-altitude area were enrolled to observe the effect of environmental hypoxia and tumor on the expression of miRNA. GeneChip scanning was performed on lung cancer tissues of 4 patients with non-small cell lung cancer (NSCLC) and 5 patients of the control group in the middle-altitude area. The differentially expressed miRNAs in cancer tissues were screened, the target genes of differentially expressed miRNAs were predicted, and the target gene-related signaling pathways and cell biological functions regulated by them were analyzed.

Patients and methods

Study subjects. A total of 30 patients admitted to the Respiratory and Critical Disease Department and Oncology Department of the Qinghai Provincial People's Hospital (Xining, China) from October 2016 to October 2017, who were definitely diagnosed with NSCLC via pathological biopsy of lung tissues (Fig. 1), were selected as the lung cancer group. There were 22 males and 8 females enrolled, with an average age of 64.58±12.56 years and age range of 41-77 years. The cancer tissues of all lung cancer patients were obtained by surgical resection or percutaneous lung puncture, and were confirmed by immunohistochemistry. There were 17 cases of squamous cell carcinoma and 13 cases of adenocarcinoma, according to pathological classification. The clinical stage of the above NSCLC patients was $T_{1\text{-}2}N_0M_0$ for all the patients according to the WHO classification (11). The patients of the lung cancer group lived permanently in a middle-altitude area (altitude: 1,500-2,500 m), they were diagnosed initially with primary tumor and did not receive any treatment (chemoradiotherapy, molecular targeted therapy, or surgical resection), and had no malignant tumors in other organs. Further 34 non-tumor patients, admitted to the Qinghai Provincial People's Hospital during the same period and living permanently in a middle-altitude area, were selected as the control group, which included 24 males and 10 females with an average age of 59.36±14.08 years and age range of 39-75 years. Samples were collected from marginal normal lung tissue obtained from non-tumor patients with pneumothorax by surgical resection. There were no significant differences in sex (χ^2=0.0594, P=0.8074) and age (t=1.556, P=0.1247) between the two groups (P>0.05), and thus, they were comparable. The patients of this study and/or their guardians were informed and signed an informed consent form. All study processes met the ethical requirements and were reviewed and approved by the Ethics Committee of the Qinghai Provincial People's Hospital (approval no. 2015-07).

RNA extraction, miRNA reverse transcription and miRNA polymerase chain reaction (PCR). Total RNA was extracted from cells using the TRIzol reagent (Ambion; Thermo Fisher Scientific, Inc.). miRNA reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems Life Technologies; Thermo Fisher Scientific, Inc.), and PCR primers corresponding to miRNAs were synthesized by Applied Biosystems (Thermo Fisher Scientific, Inc.). miRNA-16 was used as an internal reference. Sequences of miR-139-5p and miR-150-5p primers were: miRNA-139-5p forward, GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACactgga and reverse, TCT ACAGTGCACGTGTCTCC; miRNA-150-5p forward, GTC GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACcactgg and reverse, TCTCCCAACCCTTGTACC; miRNA-16 forward, GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACcgccaa and reverse, GCTTGT AGCAGCACGTAAATATTG; miRNA-1 U6 forward, CTC GCTTCGGCAGCACA and reverse, AACGCTTCACGAATT TGCG. PCR was performed with QuantStudio™ 7 Flex Real-Time PCR system (Thermo Fisher Scientific, Inc.) by using the $2^{-\Delta\Delta Cq}$ method (12). Reaction system (15 μl): 7 μl Master Mix I (0.15 μl of 100 mM dNTPs with dTTP, 1.00 μl of 50 U/μl MultiScribe™ Reverse Transcriptase, 1.5 μl 10X Reverse Transcriptase Buffer, 0.19 μl of 20 U/μl RNase Inhibitor, and 4.16 μl nuclease-free water), 3 μl 5X RT primer and 5 μl RNA sample. Master Mix (MultiScribe™ Reverse Transcriptase, Reverse Transcriptase Buffer, RNase Inhibitor, nuclease-free water) was derived from TaqMan® MicroRNA Reverse Transcription kit. The reverse transcription conditions were: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. RT-qPCR amplification system (20 μl): 1.00 μl TaqMan Small Assay (20X; Applied Biosystems Life Technologies; Thermo Fisher Scientific, Inc.), 1.33 μl product from RT reaction, 10 μl TaqMan Universal PCR master mix II (2X; Applied Biosystems Life Technologies; Thermo Fisher Scientific, Inc.) and 7.67 μl nuclease-free water. Amplification conditions were as follows: Option AmpErase UNG activity at 50˚C for 2 min, enzyme activation at 95˚C for 10 min, a total of 40 cycles, denaturation at 95˚C for 15 sec, and annealing/extension at 60˚C for 60 sec.

Immunohistochemistry. EnVision system (Dako; Agilent Technologies, Inc.) was used to detect the expression of TTF-1 (MAB-0599), NapsinA (MAB-0704), CK7 (MAB-0166), P40 (MAB-0666), CK5/6 (MAB-0276), and P63 (MAB-0365) proteins. All protein antibodies were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. Tissues were fixed in 4% neutral buffered formaldehyde for 24 h at room temperature. The experimental procedure was as follows: Paraffin-embedded tissues were cut into 3-4 μm and heated overnight at 65˚C. The tissue sections were deparaffinized and rehydrated. Inactivation of endogenous peroxidase (3% H_2O_2, 10 min) and washing with PBS for 3 times, every 2 min, were carried out. The antigen hot fix was EDTA, pH 9.0, 20 min. The tissues were washed with PBS 3 times, every 2 min in distilled water prior to being blocked at room temperature

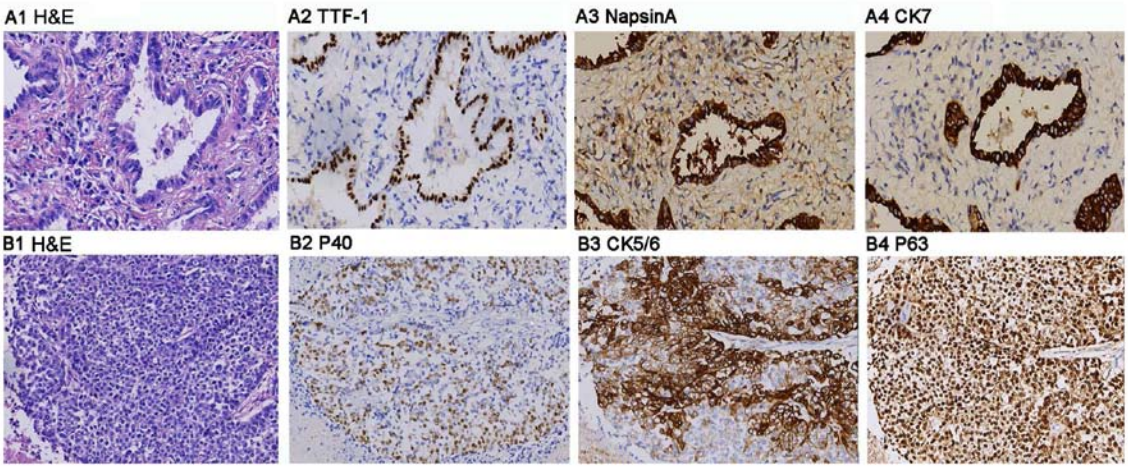

Figure 1. Immunohistochemistry of lung adenocarcinoma and lung squamous cell carcinoma tissues. (A-1) H&E staining, (A-2) TTF-1 expression, (A-3) NapsinA expression, and (A-4) CK7 expression in lung adenocarcinoma tissues (x200). (B-1) H&E staining, (B-2) P40 expression, (B-3) CK5/6 expression, and (B-4) P63 expression in lung squamous cell carcinoma tissues (x200).

for 10 min (goat serum working solution; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Incubation with the primary antibody was carried out for 1 h at room temperature (with TTF-1 1:50, NapsinA 1:50, CK7 1:100, P40 1:50, CK5/6 1:100, P63 1:50) and then the tissues were washed with PBS 3 times, every 2 min. Secondary antibody was added using MaxVision™ HRP-Polymer anti-Mouse/Rabbit IHC kit (cat no. 5010; Fuzhou Maixin Biotechnology Development Co., Ltd.) at room temperature for 25 min. Washing with PBS for 3 times, every 2 min followed. DAB chromogenic reagent was used to detect the protein expression for 3-10 min (ready-to-use DAB color liquid, microscopic control). The slides were subsequently stained with hematoxylin and bluing was carried out. Sliced tissues were conducted with graded alcohol dehydration and xylene following the manufacturer's instructions. Leica DM2500 optical microscope (Leica Microsystems, Ltd.) was used for observation. H&E staining in serial sections of each paraffin block specimens was performed for immunohistochemistry and expression analysis (Fig. 1).

GeneChip scanning of lung cancer tissues. Four patients diagnosed with NSCLC via pathological biopsy (E8, E2-t, E6-t and E9; Fig. 2A), and 5 non-tumor patients (C6, C8, C5, C1 and C3; Fig. 2A) were selected. The cancer and non-tumor tissues were collected and stored at -80˚C for Affymetrix GeneChip scanning (Affymetrix: Thermo Fisher Scientific, Inc.). Selection criteria for the lung cancer patients: i) Long-term residence in the middle-altitude area; ii) initial diagnosis of patients who did not receive any treatment (chemotherapy, molecular targeted therapy, or surgical resection); iii) patients with primary tumor; and iv) no other organ malignancy. Selection criteria for control patients: i) Patients with pneumothorax; and ii) no lung or other organ tumors. miRNAs were selected to expand the sample for verification, according to the following conditions: i) miRNAs consistently expressed according to the results of microarray and the published literature; ii) miRNAs with downregulated expression; and iii) miRNAs reported to be associated with lung cancer. miR-139-5p and miR-150-5p with obvious differential expression were verified by RT-qPCR with expanded sample size.

Statistical analysis

Chip difference analysis. The chip image information was converted into digital signal using the Affymetrix® GeneChip® Console® software (Affymetrix: Thermo Fisher Scientific, Inc.). The probe signal was integrated into the probeset signal, and the inter-sample variation caused by non-biological factors was removed via inter-chip normalization. The data were preprocessed using Range Migration Algorithm (RMA) (13). Differential genes were analyzed by Significance Analysis of Microarrays (SAM) R software package (https://www.r-project.org/), and the significant difference of chip data was analyzed. Differential genes were screened based on $P<0.05$ and fold change >2 or <0.5.

Cluster analysis. Cluster analysis was performed for the differentially expressed miRNAs in the lung cancer and control groups using cluster software. Differentially expressed miRNAs were screened based on $P<0.05$ and fold change >2 or <0.5.

Prediction and analysis of target genes of differentially expressed miRNAs. The genes predicted by at least 6 out of 12 commonly used prediction methods of miRNA target genes (miRWalk, DIANA-microTv4.0, miRanda-rel2010, mirBridge, miRDB4.0, miRmap, miRNAMap, PicTar2, PITA, RNA22v2, RNAhybrid2 and Targetscan6.2), based on miRWalk2.0 (14), were considered as target genes.

Functional enrichment analysis. Functional enrichment analysis was performed for the target genes of differentially expressed miRNAs using DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/), including Gene Ontology (GO) enrichment analysis and pathway enrichment analysis (15). GO enrichment includes biological process, cell component and molecular function. The functional regulation image was plotted using the CytoScape software (https://cytoscape.org/) and Bingo plug-in, followed by input of the target genes. Pathway enrichment analysis mainly referred to Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (16).

Data analysis. Experimental measurement data were expressed as the mean ± SD. SPSS 17.0 software (SPSS, Inc.) was used for statistical analysis. The log-rank P-values were obtained from a univariate Cox analysis, whereby miRNA expression was evaluated in response to patient survival time. Univariate Cox

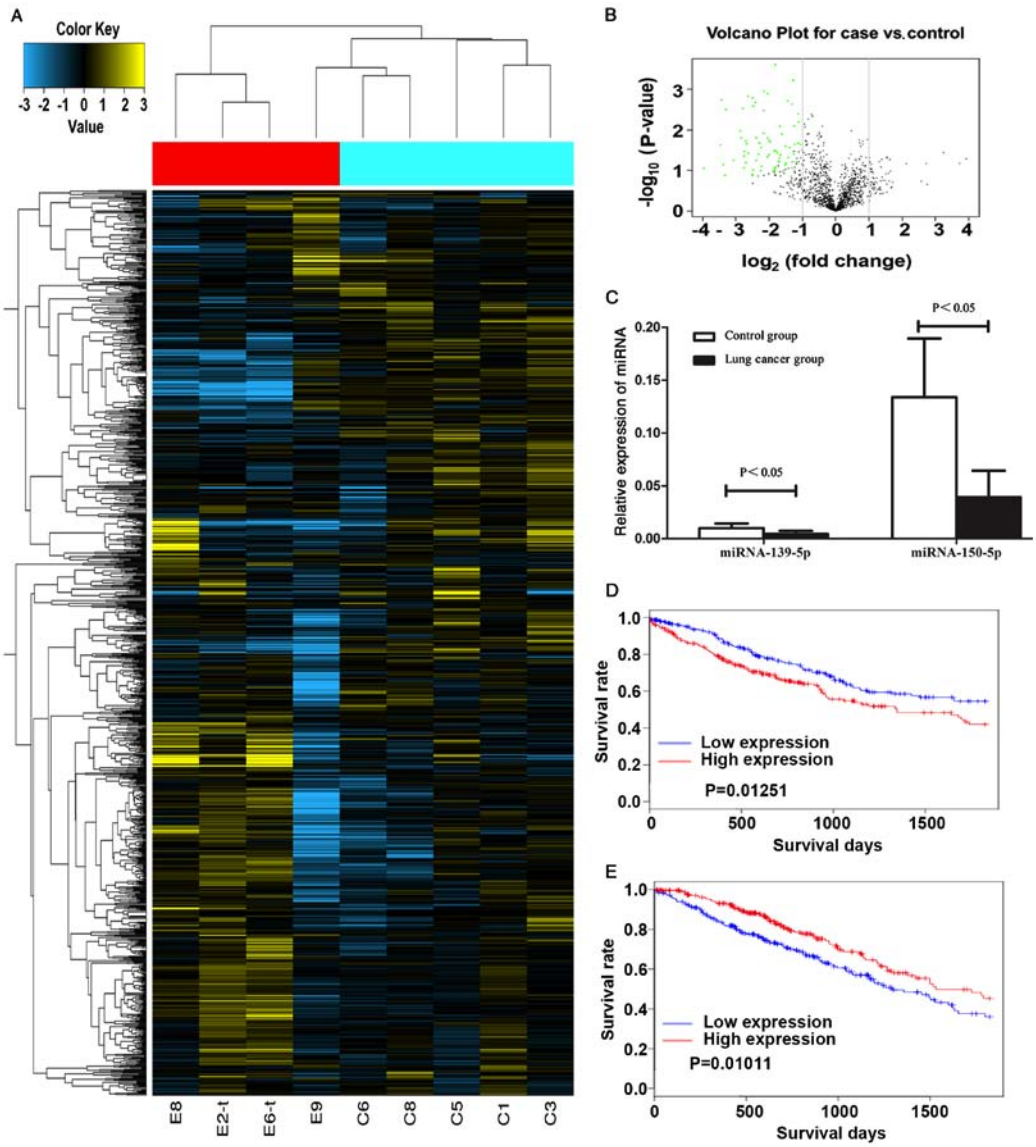

Figure 2. Global view of miRNA profile of NSCLC. miRNA expression profile of NSCLC was determined using Affymetrix oligoarrays. (A) Comparison of cluster data between NSCLC and non-tumor patients. (B) Volcano plot of differential miRNAs of NSCLC and non-tumor patients. The green dots on the left of the graph show the 76 downregulated miRNAs with fold change <0.5 in patients with NSCLC. (C) miRNA-139-5p and miRNA-150-5p in the lung cancer group (black column) were lower than those in the control group (white column), in accordance to the GeneChip results, displaying statistically significant differences (P<0.05). (D) Value of miR-139-5p low expression and miR-150-5p high expression for survival prognosis in lung squamous cell carcinoma. The low expression of miRNA-139-5p in lung squamous cell carcinoma shows good survival prognosis. (E) Value of miR-139-5p low expression and miR-150-5p high expression for survival prognosis in lung adenocarcinoma. The high expression of miRNA-150-5p in lung adenocarcinoma shows good survival prognosis. miRNA, micro ribonucleic acid; NSCLC, non-small cell lung cancer.

analysis also provided the Z-scores, which weighed the importance of the miRNA in response to these parameters. The t-test P-values were obtained from the Student's t-test, based on the patient vital status. Log_2 mean expression of the miRNA in the two survival cohorts was also obtained. The log-rank P-values were obtained from a univariate Cox analysis and survival characteristics of miRNA expression based on the Kaplan-Meier survival curves. The test level was set as α=0.05. P<0.05 was considered to indicate a statistically significant difference.

Results

Difference in miRNA expression profile of cancer tissues. The heatmap of gene expression in each group, obtained using the Cluster software, showed the difference in the expression of each gene in the different groups. Cluster analysis was performed for the miRNA expression in 4 cases in lung cancer group and 5 cases in normal group using the Cluster chip technique, and the differential genes were screened by the system according to the parameter setting and grouping, thus obtaining the distribution diagram (Fig. 2A) and list of differential genes. In the diagram with abscissa of log_2 (fold change) and ordinate of $-log_{10}$ (P-value), the data closer to the left and right bottom corresponded to the lower P-value, larger fold change and more significant difference (Fig. 2B). A total of 76 differentially expressed genes were screened, and no gene was upregulated (Table I).

Prediction results of target genes of differentially expressed miRNAs. There were 140,405 target genes predicted by at least 6 out of 12 commonly used prediction methods of miRNA target

Table I. Differentially expressed miRNAs.

Gene ID	Score(d)	P-value (%)	Fold change	Transcript ID (array design)
20503875	-2.023293031	4.960182025	0.4813	hsa-miR-500a-5p
20517816	-2.640103593	2.254628193	0.4595	hsa-miR-3609
20506867	-2.236471814	3.600132115	0.4578	hsa-miR-1270
20505746	-2.693594792	2.254628193	0.4553	hsa-miR-874-3p
20534325	-2.080624824	4.650170648	0.4378	HBII-85-2
20534221	-2.110884506	4.021769209	0.4247	HBII-13
20515540	-2.093015723	4.021769209	0.4245	hsa-miR-3136-5p
20500735	-2.000720498	4.960182025	0.4214	hsa-miR-130a-3p
20500713	-2.539879295	2.254628193	0.4207	hsa-let-7g-5p
20501201	-2.201987788	3.600132115	0.4169	hsa-miR-362-5p
20538284	-3.541567197	0	0.4097	mgU12-22-U4-8
20538271	-3.541567197	0	0.4097	U91
20500152	-4.070180503	0	0.4022	hsa-miR-26a-5p
20532691	-2.339013412	2.861643476	0.3802	ACA54
20501280	-2.324838379	2.861643476	0.3718	hsa-miR-342-3p
20503908	-3.378079284	0	0.3658	hsa-miR-532-3p
20500490	-2.234977738	3.600132115	0.354	hsa-miR-224-3p
20500385	-2.711696402	2.254628193	0.3511	hsa-miR-192-5p
20534237	-2.165774907	4.021769209	0.347	HBII-289
20500720	-2.014983155	4.960182025	0.3424	hsa-miR-23b-5p
20502122	-3.132721492	0	0.3257	hsa-miR-422a
20500400	-2.507082441	2.254628193	0.3141	hsa-miR-199a-3p
20500458	-2.507082441	2.254628193	0.3141	hsa-miR-199b-3p
20534233	-3.222214722	0	0.311	HBII-239
20500444	-2.425279357	2.861643476	0.3092	hsa-miR-181a-5p
20500755	-2.570514383	2.254628193	0.3083	hsa-miR-145-5p
20500752	-2.538495565	2.254628193	0.3037	hsa-miR-143-3p
20501299	-2.759869484	2.254628193	0.2934	hsa-miR-339-3p
20511549	-2.513431114	2.254628193	0.2843	hsa-miR-2110
20504584	-2.06130082	4.650170648	0.2833	hsa-miR-378d
20534249	-4.614743071	0	0.2831	HBII-436
20500149	-2.092452784	4.021769209	0.2805	hsa-miR-24-2-5p
20500746	-2.266082512	3.600132115	0.2794	hsa-miR-140-3p
20500179	-2.385781433	2.861643476	0.2772	hsa-miR-98-5p
20500399	-2.157881528	4.021769209	0.2706	hsa-miR-199a-5p
20500796	-2.646379389	2.254628193	0.256	hsa-miR-193a-3p
20500786	-2.165642544	4.021769209	0.2491	hsa-miR-184
20501183	-2.681288323	2.254628193	0.2485	hsa-miR-30e-3p
20532631	-2.389108936	2.861643476	0.2431	ACA20
20534505	-3.419080689	0	0.2411	hsa-mir-139
20500724	-4.572998345	0	0.2405	hsa-miR-30b-5p
20500470	-1.995623568	4.960182025	0.2404	hsa-miR-181a-3p
20501242	-4.40904384	0	0.223	hsa-miR-378a-5p
20500777	-3.304790153	0	0.222	hsa-miR-138-1-3p
20500457	-2.798683433	0	0.2213	hsa-miR-199b-5p
20500472	-2.479406791	2.254628193	0.2133	hsa-miR-214-3p
20500725	-3.281305219	0	0.2105	hsa-miR-30b-3p
20500798	-2.266005058	3.600132115	0.2084	hsa-miR-195-5p
20500455	-2.443075633	2.861643476	0.2025	hsa-miR-187-3p
20504378	-2.961664196	0	0.1999	hsa-miR-628-3p
20500769	-2.748124213	2.254628193	0.1907	hsa-miR-126-3p
20500421	-3.140677949	0	0.1907	hsa-miR-148a-3p

Table I. Continued.

Gene ID	Score(d)	P-value (%)	Fold change	Transcript ID (array design)
20500751	-3.615143592	0	0.1878	hsa-miR-143-5p
20500181	-2.252023278	3.600132115	0.1792	hsa-miR-99a-5p
20501159	-3.522911239	0	0.1785	hsa-miR-29c-5p
20501160	-2.004342033	4.960182025	0.1766	hsa-miR-29c-3p
20500433	-4.0848626	0	0.1765	hsa-miR-139-3p
20503809	-2.106652473	4.021769209	0.1749	hsa-miR-497-5p
20503793	-2.877805839	0	0.1612	hsa-miR-146b-5p
20500745	-4.285173248	0	0.1605	hsa-miR-140-5p
20500448	-3.262063359	0	0.1563	hsa-miR-181c-5p
20501278	-2.530236435	2.254628193	0.153	hsa-miR-328-3p
20532991	-2.436811645	2.861643476	0.1529	ENSG00000207118
20518919	-2.947617982	0	0.1481	hsa-miR-4521
20500189	-2.469462691	2.254628193	0.1474	hsa-miR-29b-2-5p
20500767	-3.049601189	0	0.147	hsa-miR-125b-2-3p
20500471	-4.243698759	0	0.1436	hsa-miR-214-5p
20501279	-4.160054969	0	0.139	hsa-miR-342-5p
20503786	-3.059282787	0	0.137	hsa-miR-489-3p
20500163	-2.907652913	0	0.1189	hsa-miR-30a-3p
20500154	-4.609102845	0	0.1011	hsa-miR-26b-5p
20501237	-2.009850173	4.960182025	0.0991	hsa-miR-375
20500743	-2.406363169	2.861643476	0.096	hsa-miR-138-5p
20500423	-6.212996193	0	0.0923	hsa-miR-30c-2-3p
20500782	-3.711337554	0	0.0907	hsa-miR-150-5p
20500432	-2.542450843	2.254628193	0.0639	hsa-miR-139-5p

miRNA, micro ribonucleic acid.

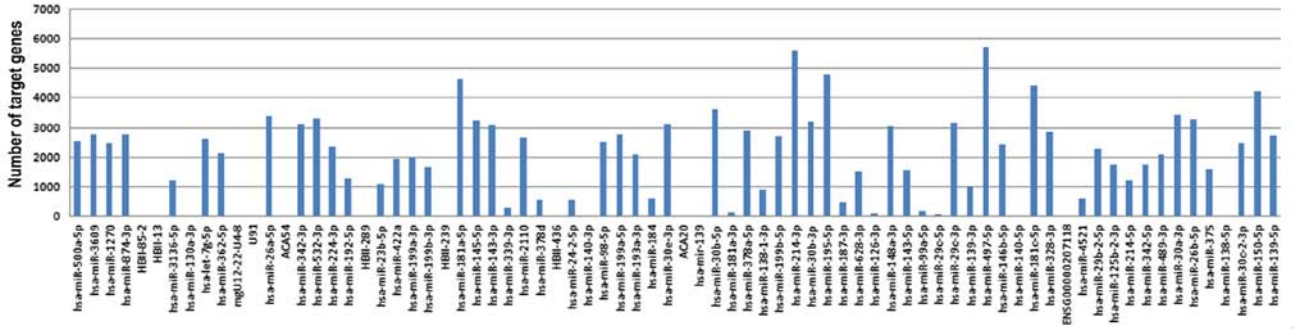

Figure 3. Number of target genes of differentially expressed miRNAs. Target genes were predicted by at least 6 out of 12 commonly used prediction methods of miRNA target genes. The maximum number of target genes predicted was 5,715 for miRNA-497-5p. The second highest number of target genes was 5,588 for miRNA-214-3p, and the third highest was 4,796 for miRNA-195-5p. miRNA, micro ribonucleic acid.

genes (miRWalk, DIANA-microTv4.0, miRanda-rel2010, mirBridge, miRDB4.0, miRmap, miRNAMap, PicTar2, PITA, RNA22v2, RNAhybrid2 and Targetscan6.2) based on miRWalk2.0 (Fig. 3).

Enrichment analysis of predicted target genes of differentially expressed miRNAs. GO enrichment analysis and KEGG pathway enrichment analysis were performed for the target genes obtained.

GO enrichment analysis. The analysis of the functions of target genes of differentially expressed miRNAs via GO enrichment revealed that they mainly influenced the binding process of intracellular components to protein, the positive regulation of biological process and the regulation of metabolic process (Table II and Fig. 4).

KEGG enrichment analysis. KEGG pathway enrichment analysis showed that these target genes were mainly enriched in the immunity, gene expression, metabolism and signal

Table II. Top 30 functions of target genes predicted by Gene Ontology enrichment analysis.

Term	ID	Input no.	P-value
Intracellular part	GO:0044424	10,337	4.88E-86
Intracellular	GO:0005622	10,575	8.05E-85
Binding	GO:0005488	10,658	5.45E-82
Protein binding	GO:0005515	8,315	7.84E-77
Organelle	GO:0043226	9,722	3.48E-61
Membrane-bounded organelle	GO:0043227	9,087	4.35E-60
Intracellular organelle	GO:0043229	9,017	1.16E-59
Cytoplasm	GO:0005737	8,121	1.14E-57
Positive regulation of biological process	GO:0048518	4,488	1.7E-57
Regulation of metabolic process	GO:0019222	5,396	2.95E-56
Intracellular membrane-bounded organelle	GO:0043231	8,249	2.35E-55
Positive regulation of cellular process	GO:0048522	3,863	6.5E-55
System development	GO:0048731	3,452	9.28E-54
Anatomical structure development	GO:0048856	4,310	1.25E-50
Regulation of cell communication	GO:0010646	2,730	3.08E-50
Multicellular organismal development	GO:0007275	3,869	5.33E-50
Developmental process	GO:0032502	4,556	2.3E-48
Regulation of signaling	GO:0023051	2,705	8.05E-48
Single-organism developmental process	GO:0044767	4,484	7.71E-47
Metabolic process	GO:0008152	8,668	1.17E-46
Ion binding	GO:0043167	4,768	3.55E-45
Regulation of cellular metabolic process	GO:0031323	4,694	2.12E-43
Nervous system development	GO:0007399	1,794	3.64E-43
Intracellular signal transduction	GO:0035556	2,336	9.01E-43
Cellular metabolic process	GO:0044237	7,818	1.84E-42
Positive regulation of metabolic process	GO:0009893	3,020	2.44E-42
Anatomical structure morphogenesis	GO:0009653	2,103	1.42E-40
Regulation of macromolecule metabolic process	GO:0060255	4,502	2.42E-40
Cytoplasmic part	GO:0044444	6,197	3.54E-40
Cellular macromolecule metabolic process	GO:0044260	6,384	9.37E-40

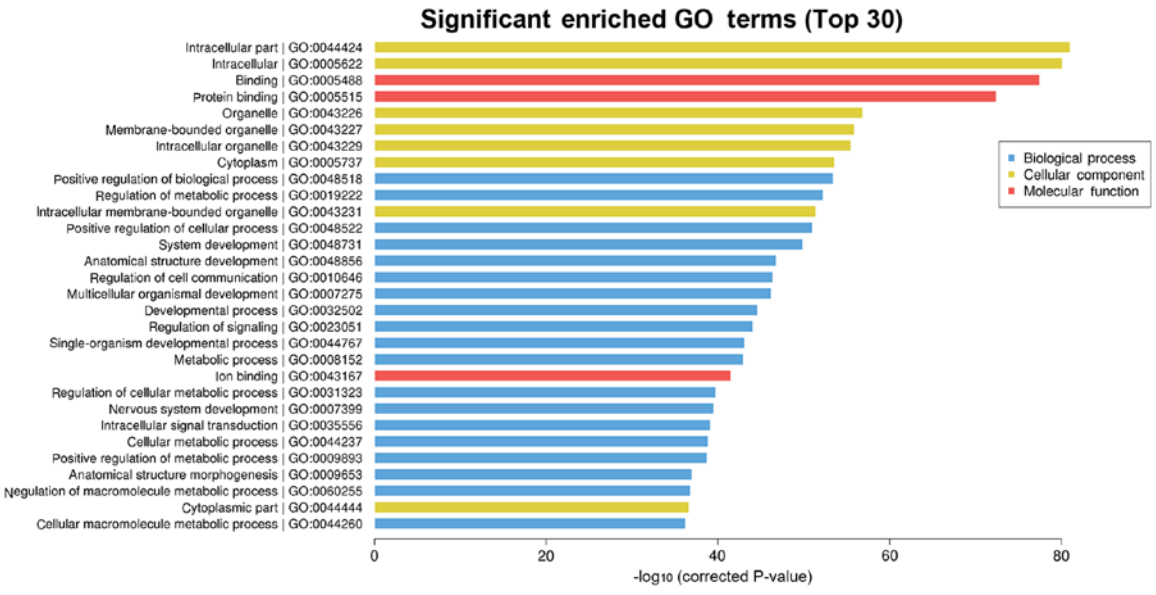

Figure 4. Top 30 functions of target genes predicted by GO enrichment analysis. The top 30 functions are classified into three categories, i.e., biological processes, cellular components and molecular functions. Among them, 19 functions are related to biological processes which mainly influence the positive regulation of biological processes, metabolic processes and cellular processes. Eight functions are related to cellular components which mainly influence intracellular components, and 3 functions are related to molecular functions which mainly influence protein binding and ion binding. GO, Gene Ontology.

Table III. Top 30 signaling pathways by Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis.

Term	ID	Input no.	P-value
Immune system	R-HSA-168256	1,223	1.86E-14
Developmental biology	R-HSA-1266738	687	3.37E-11
Gene expression	R-HSA-74160	1,266	5.31E-11
Generic transcription pathway	R-HSA-212436	668	5.66E-11
Axon guidance	R-HSA-422475	475	2.44E-10
Membrane trafficking	R-HSA-199991	459	1.69E-09
Vesicle-mediated transport	R-HSA-5653656	481	3.53E-09
Adaptive immune system	R-HSA-1280218	623	4.57E-09
Metabolism	R-HSA-1430728	1,388	1.7E-08
Transmembrane transport of small molecules	R-HSA-382551	530	2.38E-08
Pathways in cancer	hsa05200	345	5.64E-08
Signaling by NGF	R-HSA-166520	374	9.21E-08
Hemostasis	R-HSA-109582	486	1.11E-07
Metabolic pathways	hsa01100	896	2.36E-07
Disease	R-HSA-1643685	676	5.16E-07
Innate immune system	R-HSA-168249	582	9.72E-07
Cytokine signaling in immune system	R-HSA-1280215	486	1.14E-06
Metabolism of lipids and lipoproteins	R-HSA-556833	541	1.62E-06
PI3K-Akt signaling pathway	hsa04151	289	2.22E-06
Endocytosis	hsa04144	232	2.25E-06
Neuronal system	R-HSA-112316	288	2.38E-06
NGF signaling via TRKA from the plasma membrane	R-HSA-187037	304	2.86E-06
Signaling by PDGF	R-HSA-186797	299	2.93E-06
Post-translational protein modification	R-HSA-597592	625	3.14E-06
Wnt signaling pathway	P00057	254	3.36E-06
Fc epsilon receptor signaling	R-HSA-2454202	293	4.37E-06
Extracellular matrix organization	R-HSA-1474244	250	5.24E-06
Proteoglycans in cancer	hsa05205	188	6.53E-06
Signal transduction	R-HSA-162582	1,634	6.87E-06
Metabolism of proteins	R-HSA-392499	965	7.64E-06

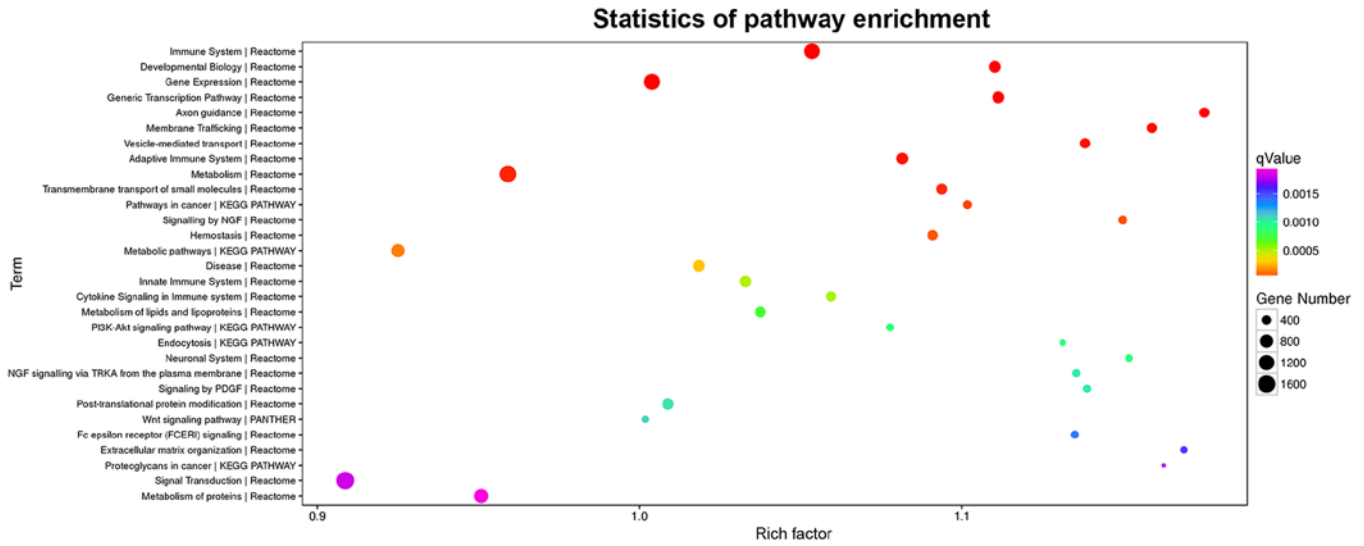

Figure 5. Top 30 signaling pathways by KEGG pathway enrichment analysis. The top 30 signaling pathways of the differentially expressed miRNAs were obtained by KEGG enrichment analysis. The size of each dot represents the amount of gene enrichment. The larger the dot, the more genes are enriched. The smaller the dot, the smaller the number of the genes enriched. The color of each dot represents the P-value. The more red the dot, the smaller the P-value. KEGG pathway enrichment analysis showed that these target genes are mainly enriched in the immunity, gene expression, metabolism and signal transduction, among which signal transduction was enriched with the most genes. KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, micro ribonucleic acid.

Table IV. Top 30 related diseases by disease enrichment analysis.

Term	Database ID	Input no.	P-value
Obesity-related traits	NHGRI GWAS Catalog	553	9.65E-09
Nervous system diseases	KEGG DISEASE	469	4.65E-08
Other nervous and sensory system diseases	KEGG DISEASE	364	1.11E-06
Other congenital disorders	KEGG DISEASE	370	4.66E-06
Congenital disorders of metabolism	KEGG DISEASE	442	6.5E-06
Height	NHGRI GWAS Catalog	381	7.71E-06
Schizophrenia	NHGRI GWAS Catalog	341	2.43E-05
Musculoskeletal diseases	KEGG DISEASE	226	3.18E-05
Body mass index	NHGRI GWAS Catalog	266	8.04E-05
Cancers	KEGG DISEASE	182	9.03E-05
Congenital disorders of development	KEGG DISEASE	250	0.000146
Inflammatory bowel disease	NHGRI GWAS Catalog	221	0.00015
Skeletal diseases	KEGG DISEASE	169	0.000159
Others	KEGG DISEASE	128	0.000344
Skin diseases	KEGG DISEASE	155	0.000494
Skin and soft tissue diseases	KEGG DISEASE	155	0.000494
Amyotrophic lateral sclerosis (sporadic)	NHGRI GWAS Catalog	137	0.000534
Cardiovascular diseases	KEGG DISEASE129	223	0.000678
Menarche (age at onset)	NHGRI GWAS Catalog	145	0.000978
Crohn's disease	NHGRI GWAS Catalog	200	0.00128
Type 2 diabetes	NHGRI GWAS Catalog	150	0.001504
Neurodegenerative diseases	KEGG DISEASE	132	0.002359
Immune system diseases	KEGG DISEASE	186	0.003778
Diisocyanate-induced asthma	NHGRI GWAS Catalog	135	0.004021
Congenital disorders of amino acid metabolism	KEGG DISEASE	102	0.004472
Kidney diseases	KEGG DISEASE	72	0.004562
Urinary system diseases	KEGG DISEASE	72	0.004562
HDL cholesterol	NHGRI GWAS Catalog	112	0.004949
Rheumatoid arthritis	NHGRI GWAS Catalog	114	0.005032
Breast cancer	NHGRI GWAS Catalog	100	0.005145

transduction, among which signal transduction was enriched with the most genes (Table III and Fig. 5).

Disease enrichment analysis. The tenth disease enriched was cancer (Table IV and Fig. 6).

Comparison of miRNA-139-5p and miRNA-150-5p relative expression levels between the lung cancer and control group and its influence on prognosis. According to RT-qPCR verification with an expanded sample size, the expression levels of miRNA-139-5p and miRNA-150-5p in lung cancer group were lower than those in control group, in accordance to the GeneChip results, displaying statistically significant differences ($P<0.05$) (Fig. 2C). The use of online resources (http://www.oncomir.org/) predicted that low-expression miRNA-139-5p in lung squamous cell carcinoma and high-expression miRNA-150-5p in lung adenocarcinoma have a good prognosis (Tables V and VI, Fig. 2D and E).

Discussion

NSCLC is a kind of solid tumor that is often diagnosed in the late stage, due to the lack of specific early symptoms, resulting in fewer opportunities for operation. Moreover, most of the samples in clinical survey are obtained via bronchoscopic small biopsy or cytology, so the number of tumor cells available is small with poor quality. miRNAs, as a kind of biomarkers, have expression dysregulation in the resected primary NSCLC tissues (17), which plays important roles in the classification of lung cancer subtypes, prognosis of patient, and sensitivity to chemotherapy (18,19). In the present study, a comprehensive miRNA expression profile was identified, and abnormally expressed miRNAs in tumor tissues were confirmed, providing references for the in-depth research on the role of miRNAs in occurrence and development of tumors.

According to the systematic analysis of miRNA in various human cancers and 217 types of mammals, the miRNA profile has a surprising amount of information, reflecting the developmental lineage and differentiation status of tumors. miRNAs in tumors are generally downregulated, compared with those in normal tissues, and the miRNA expression profile is valuable for the classification of poorly differentiated tumors (20). The results of this study are consistent with those in the above studies: All of the 76 differentially expressed miRNAs screened were downregulated, and no differentially expressed miRNAs were upregulated. miRNAs are a kind of non-coding regulatory RNAs involved in the occurrence of

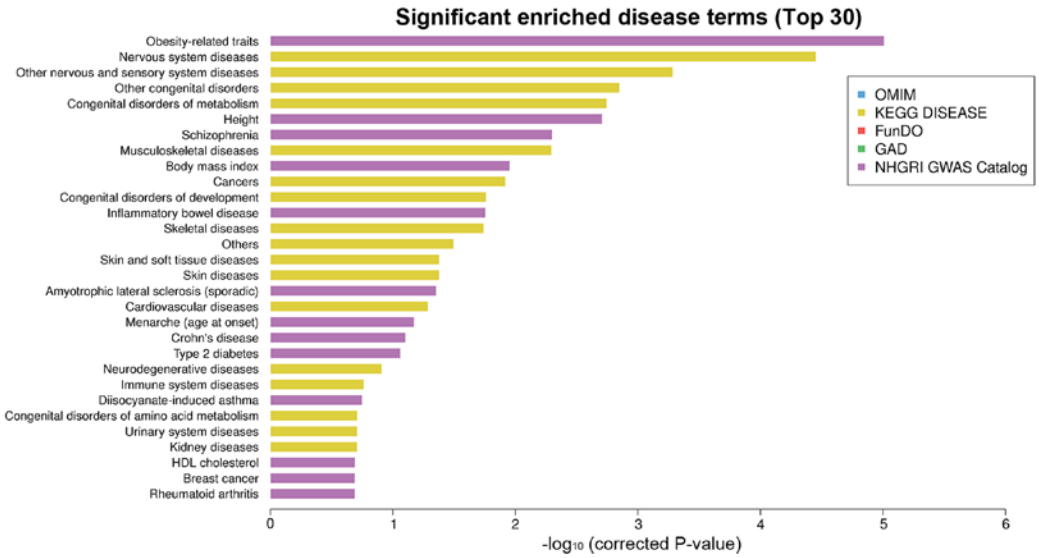

Figure 6. Top 30 related diseases by disease enrichment analysis. The top 30 related diseases of the differentially expressed miRNAs obtained by OMIM, KEGG, FunDO, GAD and NHGRI GWAS Catalog enrichment analyses. The length of each bar represents the P-value. The longer the bar, the smaller the P-value. Among the top 30 related diseases mainly enriched by KEGG and NHGRI GWAS, the 10th disease enriched was cancer. miRNA, micro ribonucleic acid; OMIM, Online Mendelian Inheritance in Man; KEGG, Kyoto Encyclopedia of Genes and Genomes; FunDO, Functional Disease Ontology; GAD, Genetic Association Database; NHGRI, National Human Genome Research Institute; GWAS, genome-wide association study.

Table V. Cancer types where survival is significantly associated with miR-139-5p and miR-150-5p.

		Log-rank				Deceased $\log_2$ mean	Living $\log_2$ mean	t-test	
miRNA	Cancer	P-value	FDR	Z-score	Upregulated in	expression	expression	P-value	FDR
miR-139-5p	LUSC	1.55e-02	9.92e-01	2.452	Deceased	5.00	4.82	2.16e-01	7.43e-01
miR-150-5p	LUAD	7.52e-03	9.56e-02	2.693	Living	9.94	10.18	4.22e-02	4.85e-01

LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma.

Table VI. Value of miR-139-5p and miR-150-5p for survival prognosis.

Cancer	Total no. of patients	Living patients	Deceased patients	Low risk	High risk	Value of miRNA for survival prognosis
LUSC	472	272	200	236	236	$S = 2.452 \times E_{miR\text{-}139\text{-}5p} + 0.173 \times E_{miR\text{-}150\text{-}5p}$
LUAD	500	319	181	250	250	$S = 1.196 \times E_{miR\text{-}139\text{-}5p} + 2.693 \times E_{miR\text{-}150\text{-}5p}$

LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; miRNA, micro ribonucleic acid; S, survival; E, expression.

tumors and they display significant tissue specificity, which can serve as effective biomarkers for tracking the cancer of unknown origin (21). Previous studies have demonstrated that the miRNA expression profile is correlated with the survival of lung adenocarcinoma. Univariate analysis has shown that the high expression of hsa-mir-155 and low expression of hsa-let-7a-2 are correlated with the low survival rate, while multivariate analysis have revealed that hsa-mir-155 is still related to the survival rate, and the miRNA expression profile is a diagnostic and prognostic marker for lung cancer (22). The above results also suggest that the expression of miRNA in NSCLC is different from that of mRNA (23,24). The role of miRNA in the overall survival of patients with NSCLC has been analyzed currently, which may provide valuable information for the treatment of NSCLC (25). However, its expression in lung cancer tissues was less consistent in the previous studies. Whether the lung cancer stage, case type and smoking status affect the results is worthy of further exploration and analysis. The low expression of cell adhesion molecule 1 (CADM1) is closely related to the short survival of NSCLC (26), and it is essential to search for new markers.

In the present study, five miRNAs were selected and verified via RT-qPCR with an expanded sample size, and it was found that both miR-139-5p and miRNA-150-5p were downregulated,

which is consistent with the GeneChip results. It is also reported that miR-139-5p is significantly downregulated in primary NSCLC tissues and cell lines. On the one hand, the ectopic expression of miR-139-5p significantly inhibits cell growth through suppressing the upregulation of cyclin D1 and p57 (Kip2), on the other hand, miR-139-5p induces apoptosis through upregulating cleaved caspase-3, a key apoptotic gene, and downregulating Bcl-2, an anti-apoptotic gene. In addition, miR-139-5p inhibits cell migration through inhibiting matrix metalloproteinase (MMP)-7 and MMP-9, and it was also found that miR-139-5p inhibits cell proliferation and metastasis promoting apoptosis through targeting oncogenic c-Met, thus playing a key role in lung cancer (27). In primary NSCLC, the miR-139 silencing mediated by histone H3 lysine 27 trimethylation (H3K27me3) enhances the distant lymph node metastasis and histological invasion (lymphatic invasion and vascular invasion) of NSCLC (28). Moreover, miR-139-5p inhibits *in vitro* proliferation, migration and invasion of lung cancer cells through targeting the insulin-like growth factor 1 receptor (IGF1R) (29). miRNA-150-5p has not been reported in NSCLC in detail, however it has been reported that miRNA-150-5p and miRNA-34c-3p are closely related to skeletal muscle mitochondrial function in human body (30), and the downregulation of miR-150-5p, a new circulating biomarker for acute heart failure (AHF), is correlated with the pathophysiology of AHF (31). In multiple myeloma, miR-150-5p arouses the specific effect of glucocorticoid receptor (GR) to increase the therapeutic response of glucocorticoids through indirectly regulating the interaction of GR with transcription factors and GR chaperones and motivating mRNA of various effectors, protein stress and chemokines of GR (32). In future research, the role of downregulation of miR-139-5p and miR-150-5p in NSCLC needs to be further clarified, and their specific values in target genes and functions should also be determined.

In the present study, the enrichment analysis of the functions of target genes of differentially expressed miRNAs revealed that they mainly influence the binding process of intracellular components to protein, the positive regulation of biological process and the regulation of the metabolic process. Moreover, KEGG pathway enrichment analysis showed that these target genes are mainly enriched in the immunity, gene expression, metabolism and signal transduction, among which signal transduction is enriched with the most genes. It was confirmed by previous studies that inhibiting miR-192 and miR-662 reduces the clonality and motility of early squamous cell carcinoma cells and increases the sensitivity to etoposide (33). Downregulation of miR-30a-5p and upregulation of miR-210-3p in NSCLC have excellent sensitivity and acceptable specificity, which can assist in distinguishing cancer and non-cancer tissues (34). The chronic treatment of NSCLC with gefitinib will alter the miRNA expression, including the significant decline in miR-155 and miR-200c accompanied by epidermal growth factor receptor (EGFR) mutation, and the decline in miR-155 and miR-200c may be correlated with epithelial-mesenchymal transition and histone modification, which may reduce the sensitivity to gefitinib, independent of the secondary EGFR mutation (35). miRNA may play a role in immune escape, resistance to chemotherapeutic drugs and biological functions of lung cancer cells. Research on the roles of miR-139-5p and miR-150-5p in diagnosis, metabolism, signal transduction, biological behaviors and drug resistance in NSCLC will benefit the diagnosis and treatment of lung cancer patients, and may even bring an unexpected breakthrough in immunotherapy.

In conclusion, in the present investigation through GeneChip, it was revealed that miRNAs are mainly downregulated in the miRNA expression profile of lung cancer tissues in the middle-altitude area, which is not completely consistent with the miRNA expression difference in NSCLC tissues as reported by previous studies, indicating the individual difference and complexity of miRNA in pathogenesis. According to the verification with an expanded sample size, both miR-139-5p and miR-150-5p are downregulated, thus, deeply exploring their important roles in the occurrence and development of disease and regulation of tumor cell functions has great clinical value in the diagnosis, treatment and evaluation of lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YG wrote the manuscript and was responsible for the immunohistochemistry. YG and XS performed PCR. XW and XL were responsible for GeneChip scanning of lung cancer tissues. YG and YX assisted with statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Qinghai Provincial People's Hospital (Xining, China) (approval no. 2015-07); and informed consents were signed by the patients and/or their guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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