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Original Article

Identification of novel diagnosis biomarkers for lung adenocarcinoma from the cancer genome atlas

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Abstract: Lung adenocarcinoma is a type of non-small cell lung cancer (NSCLC) with poor prognosis in most cases. Early diagnosis followed with treatment can increase the survival rates significantly. We aim to identify differentially expressed miRNAs that could be potential biomarkers for early diagnosis of lung adenocarcinoma through an integrative framework. The miRNA-seq data extracted from TCGA (The Cancer Genome Atlas) database was preprocessed by hierarchy cluster analysis. Differentially expressed miRNAs were identified by the DESeq package in Bioconductor and TargetScan Human database version V6.2. After function enrichment analysis, the miRNA-gene network was constructed to identify symbolic miRNAs of lung adenocarcinoma by Cytoscape 3.2.0. Furthermore, CytoCluster plugin was used for identification of miRNA-gene clusters from the network. The miR-1298, miR-1, miR-206, miR-1269, miR-486, miR-503 and miR-3651 were found closely associated with NSCLC. More notably, miRNA-gene interaction network analysis showed that *GRB2, EGFR, SOS1* and *PIK3R1* served as the key channel of information circulation. The two miRNA-gene clusters in the network were associated with ErbB signaling pathway and *PI3K*-Akt signaling pathway, respectively. We firstly reported miR-1298, miR-206 and miR-1269 as biomarkers in lung adenocarcinoma and characterized a group of target genes closely associated with NSCLC, suggesting a potential application in early diagnosis of lung adenocarcinoma.

Keywords: Lung adenocarcinoma, microRNA, diagnostic marker, differential expression analysis, pathway and network analysis, cluster analysis

Introduction

Lung cancer is the most lethal cancer with 1.5 million new cases every year worldwide and the 5-year survival rates of patients is less than 15% [1, 2]. Lung cancers are mainly classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) and 40% of lung cancers are NSCLC. Among all types of NSCLC, lung adenocarcinoma is one of the most common types [3, 4]. Currently, lung adenocarcinoma diagnosis is mainly based on symptoms, which is not effective for early detection of lung adenocarcinoma. Early diagnosis of lung adenocarcinoma followed with treatments can increase the five-year survival rate to 60-80% [5]. Therefore, early diagnosis is critical for improving the prognosis of lung adenocarcinoma. Searching for early biomarkers of lung adenocarcinoma that could benefit clinical practice has been very active [6].

miRNA is a family of small non-coding RNAs, which regulates target gene expression by blocking the translation or degrading of target mRNAs [7]. miRNAs has been reported to associate with various types of cancers [8] and may act as oncogenes or tumor suppressors [9]. Previous studies reported that miRNAs play important roles in the oncogenesis pathway and miRNA expression patterns have been associated with the prognosis of lung cancer [10]. Moreover, it has been confirmed that miRNA expression profiles are more accurate to classify tumors than mRNA profiles. Thus, miRNA profiling might be a new tool for diagnosing lung adenocarcinoma at early stage. miRNAs, such as miR-148a [11], miR-21 [12], miR-218 [13] and miR-214 [14] has been reported to associate with different stages of NSCLC, which are considered as potential diagnosis markers. In lung adenocarcinoma studies, miR-31, miR-196b, miR-766, miR-519a-1, miR-375,

miR-187, miR-331 and miR-101-1 have been identified as biomarkers for predicting survival in lung adenocarcinoma [15]. However, none of them have been applied in in clinical practice yet. Therefore, searching for new differentially expressed miRNAs for early diagnosis of lung adenocarcinoma is still important.

With the development of emerging technology of miRNA-seq, it is now possible to screen for alterations of miRNAs and their target genes in cancers. As a rising high-throughput technology, miRNA-seq is based on deep sequencing and can provide a high dynamic range in detection with more precise and reproductive data [16]. Therefore, miRNA-seq has been widely used for detecting differentially expressed miRNAs in cancers.

In the present study, we aimed to identify differentially expressed miRNAs that may serve as biomarkers for diagnosis of lung adenocarcinoma by analyzing miRNA-seq data from TCGA (The Cancer Genome Atlas) database. We constructed a miRNA-gene network to identify differentially expressed miRNAs of lung adenocarcinoma, which may provide a basis for innovative diagnostic markers and therapeutic approaches for lung adenocarcinoma.

Materials and methods

miRNA-seq data

The miRNA-seq data Level3 was extracted from TCGA (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) database, which contained 46 normal samples and 458 lung adenocarcinoma samples with Platform of IlluminaHiSeq miRNASeq. The data was downloaded from the open-access database TCGA and there is no ethical and moral permission problem in our study.

Data preprocessing

Due to the variations in sample origin, cancer stage and subtype, we preprocessed the data by Hierarchical Clustering Algorithm analysis [17]. Samples were grouped based on the expression values (RPM values) of miRNAs and samples with great variation were excluded from further analysis. After integrated the tumor and normal data, we filtered out the miRNAs without detectable expressions to ensure that every miRNA corresponded to at least one count in average.

Differential expression analysis

The DESeq package in Bioconductor was used to identify differentially expressed miRNAs between normal and lung adenocarcinoma tissues [18]. Differential expression analysis was conducted after standardization and variance estimation. The $P<0.001$, $FDR<0.001$ and $|\log_2 FC|>3$ were used as the cut-off criterions.

Screening of target genes and NSCLC correlated genes

TargetScan Human database version V6.2 was used to predict target genes of differentially expressed miRNAs [19]. Target genes of differentially expressed miRNAs were mapped to the pathways related to NSCLC in KEGG database [20]. NSCLC correlated gene groups were further screened from these selected target genes.

Function enrichment analysis of differentially expressed miRNAs

All target genes and NSCLC correlated genes were taken as candidates for enrichment analysis. Enrichment degrees of NSCLC correlated genes to the target genes of each miRNA were analyzed by hypergeometric distribution test. miRNAs are closely associated with NSCLC when $P<0.05$ [20].

Construction of miRNA-gene interaction network

Interactions of NSCLC correlated genes were extracted from BIND database [21]. The regulations between differentially expressed miRNAs and corresponding target genes were integrated into an interaction network. Cytoscape 3.2.0 was utilized to generate network topological graph [22].

Basic analysis of interaction network

The basic properties of the network were analyzed by built-in tools of Cytoscape, including Edge Count In degree, out degree, Betweenness Centrality, Clustering Coefficient and Closeness Centrality parameters. Key nodes with different biological significance were selected from the network according to these parameters. Network clustering was used to establish the structure of network and for molecular function predictions. CytoCluster plugin (http://apps.cyto-

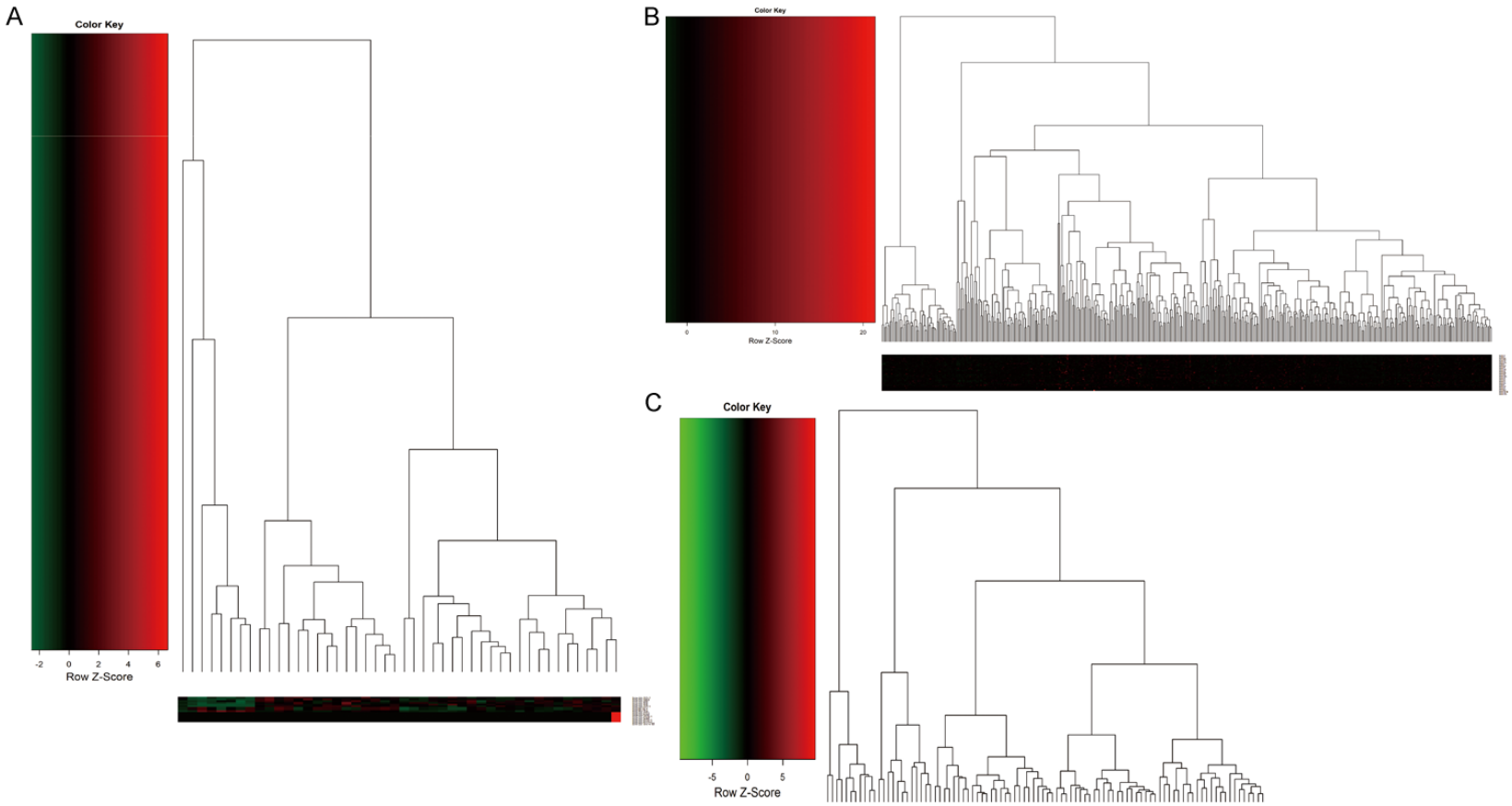

Figure 1. Hierarchical cluster dendrogram of lung cancer expression profiles. A. Clustering analysis to minimize the impact of variation. B. Subgroups of cancer samples. C. Samples after filtering out miRNAs without expressions. The horizontal axis represents miRNAs. The vertical axis represents samples. The clusters of samples are shown above the heat-map.

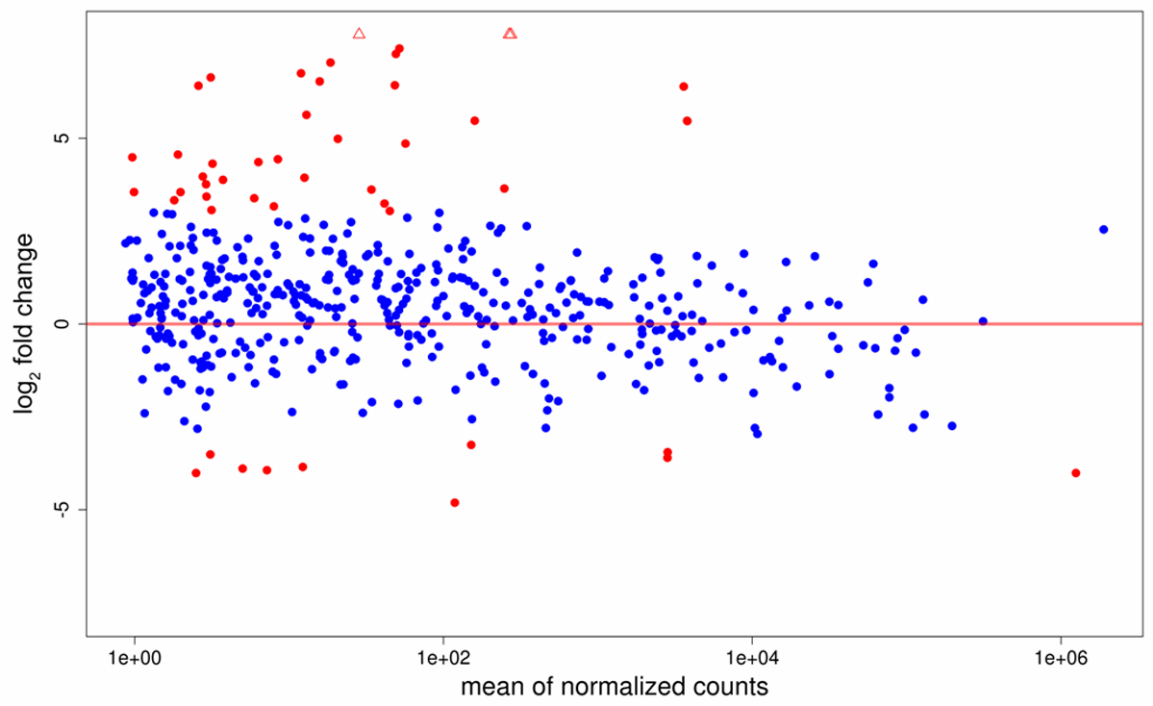

Figure 2. The relative expression levels of miRNAs. The red dot represents the tumor miRNAs and the blue dots represents the normal miRNAs.

scape.org/apps/cytocluster) was used for identifying gene clusters from the network.

Results

Data preprocessing

After clustering analysis, a cluster (8 samples in total) in 46 normal samples was found to differ from other samples (**Figure 1A**). To minimize the impact of variation in normal samples on further data analysis, this cluster was excluded in subsequent analysis.

To divide the samples into subgroups, hierarchy cluster analysis were applied to the miRNA profiling data of 458 lung cancer samples. Cancer samples were clustered into 5 main subgroups (**Figure 1B**). The first cluster with 56 cancer samples was selected for further analysis. After filtering out miRNAs without expressions, a total of 94 samples (56 cancer samples and 38 normal samples) and 481 miRNAs were selected for further analysis (**Figure 1C**).

Differential expression analysis

To identify differentially expressed miRNAs in lung adenocarcinoma, we analyzed the expressions of selected 481 miRNAs in cancer and normal samples. We found that the expression levels of 48 miRNAs (44 in total except multi-copy ones) were statistically significant different ($P<0.001$, $FDR<0.001$) between cancer and normal samples (**Figure 2**). Among these differentially expressed miRNAs, the expression levels of 38 miRNAs were up-regulated relative to that in normal samples while 10 miRNAs were down-regulated (**Table 1**).

To confirm that these identified miRNAs are differentially expressed in cancer and normal samples, hierarchy cluster analysis was applied to all samples selected for analysis. Tumor samples were distinguished from normal samples by clustering the differentially expressed miRNAs (**Figure 3**). Therefore, the miRNAs we identified do have different expression levels between lung adenocarcinoma and normal tissues.

Enrichment of miRNAs and their target genes that correlate with NSCLC

To understand the cellular functions of these identified differentially expressed miRNAs, we used TargetScan Human database to screen

Table 1. Differentially expressed miRNAs in lung adenocarcinoma

miRNA	log_2 Fold Change	p-val	p-adj
hsa-mir-105-1	6.426	0.000	0.000
hsa-mir-105-2	7.268	0.000	0.000
hsa-mir-122	4.560	0.000	0.000
hsa-mir-1229	3.430	0.000	0.000
hsa-mir-1269	8.593	0.000	0.000
hsa-mir-1293	6.637	0.000	0.000
hsa-mir-137	6.529	0.000	0.000
hsa-mir-147b	5.629	0.000	0.000
hsa-mir-196a-1	5.473	0.000	0.000
hsa-mir-196a-2	6.749	0.000	0.000
hsa-mir-196b	3.646	0.000	0.000
hsa-mir-210	6.392	0.000	0.000
hsa-mir-301b	4.358	0.000	0.000
hsa-mir-31	4.856	0.000	0.000
hsa-mir-3161	4.486	0.000	0.000
hsa-mir-323	3.165	0.000	0.000
hsa-mir-323b	4.435	0.000	0.000
hsa-mir-3607	3.043	0.000	0.000
hsa-mir-3617	3.761	0.000	0.000
hsa-mir-3651	3.330	0.000	0.000
hsa-mir-3662	3.551	0.000	0.000
hsa-mir-371	9.177	0.000	0.000
hsa-mir-372	9.626	0.000	0.000
hsa-mir-373	7.036	0.000	0.000
hsa-mir-489	3.385	0.000	0.000
hsa-mir-503	3.617	0.000	0.000
hsa-mir-513c	3.968	0.000	0.000
hsa-mir-514b	4.314	0.000	0.000
hsa-mir-548y	6.414	0.000	0.000
hsa-mir-577	4.984	0.000	0.000
hsa-mir-615	3.067	0.000	0.000
hsa-mir-616	3.937	0.000	0.000
hsa-mir-675	3.242	0.000	0.000
hsa-mir-767	7.415	0.000	0.000
hsa-mir-892a	3.549	0.000	0.000
hsa-mir-9-1	5.464	0.000	0.000
hsa-mir-9-2	5.470	0.000	0.000
hsa-mir-9-3	3.880	0.000	0.000
hsa-mir-1-2	-3.255	0.000	0.000
hsa-mir-1298	-4.012	0.000	0.000
hsa-mir-133a-2	-3.512	0.000	0.000
hsa-mir-133b	-3.848	0.000	0.000
hsa-mir-143	-4.011	0.000	0.000
hsa-mir-144	-3.601	0.000	0.000
hsa-mir-184	-4.810	0.000	0.000
hsa-mir-206	-3.935	0.000	0.000
hsa-mir-486	-3.450	0.000	0.000
hsa-mir-490	-3.892	0.000	0.000

their target genes and their interactions with other molecules. As a result, these 44 miRNAs (without multi-copy) regulate 7308 target genes and have 21768 interactions with other molecules.

Pathways like Wnt signaling [23] and EGFR induced signaling [24] contribute to the genesis of lung adenocarcinoma. Inhibitors targeting key components of these pathways have to be consider as potential clinical therapeutic drugs for lung adenocarcinoma patients [25, 26]. To screen the genes that are more relevant to lung adenocarcinoma, the selected target genes were mapped to NSCLC related pathways according to KEGG database. We found that 42 out of 7308 target genes were correlated with NSCLC related pathways and these 42 genes were considered as NSCLC related genes. These genes are targeted by 38 of differentially expressed miRNAs. Therefore, these 38 differentially expressed miRNAs may closely link to NSCLC. Based on hypergeometric distribution test ($P<0.05$), 7 miRNAs were closely associated with NSCLC related pathways and are potential biomarkers for early lung adenocarcinoma diagnosis. The target genes of these 7 miRNAs (**Table 2**) might be potential therapeutic targets and were selected for further analysis.

Construction and analysis of miRNA-gene interaction network that is associated with NSCLC

To characterize the roles of the identified differentially expressed miRNAs in molecular network of lung adenocarcinoma genesis, a miRNA-gene interaction network was constructed and analyzed. The genes served as nodes and interactions served as edges to construct miRNA-gene interaction network. Total 53 nodes and 89 edges were involved in the network (**Figure 4**). Among the NSCLC related 42 target genes, 20 genes had 23 pairs of interactions based on the BIND database (including self-interaction). These 20 target genes were regulated by 33 differentially expressed miRNAs, including 6 miRNAs enriched in NSCLC pathway (except miR-3651).

Hub node is the node which has the largest number of connections with other surrounding nodes in the network. In the constructed miRNA-gene interaction network, *CDK6, SOS1, STK4, EGFR, NRAS* and *PIK3R1* were the top

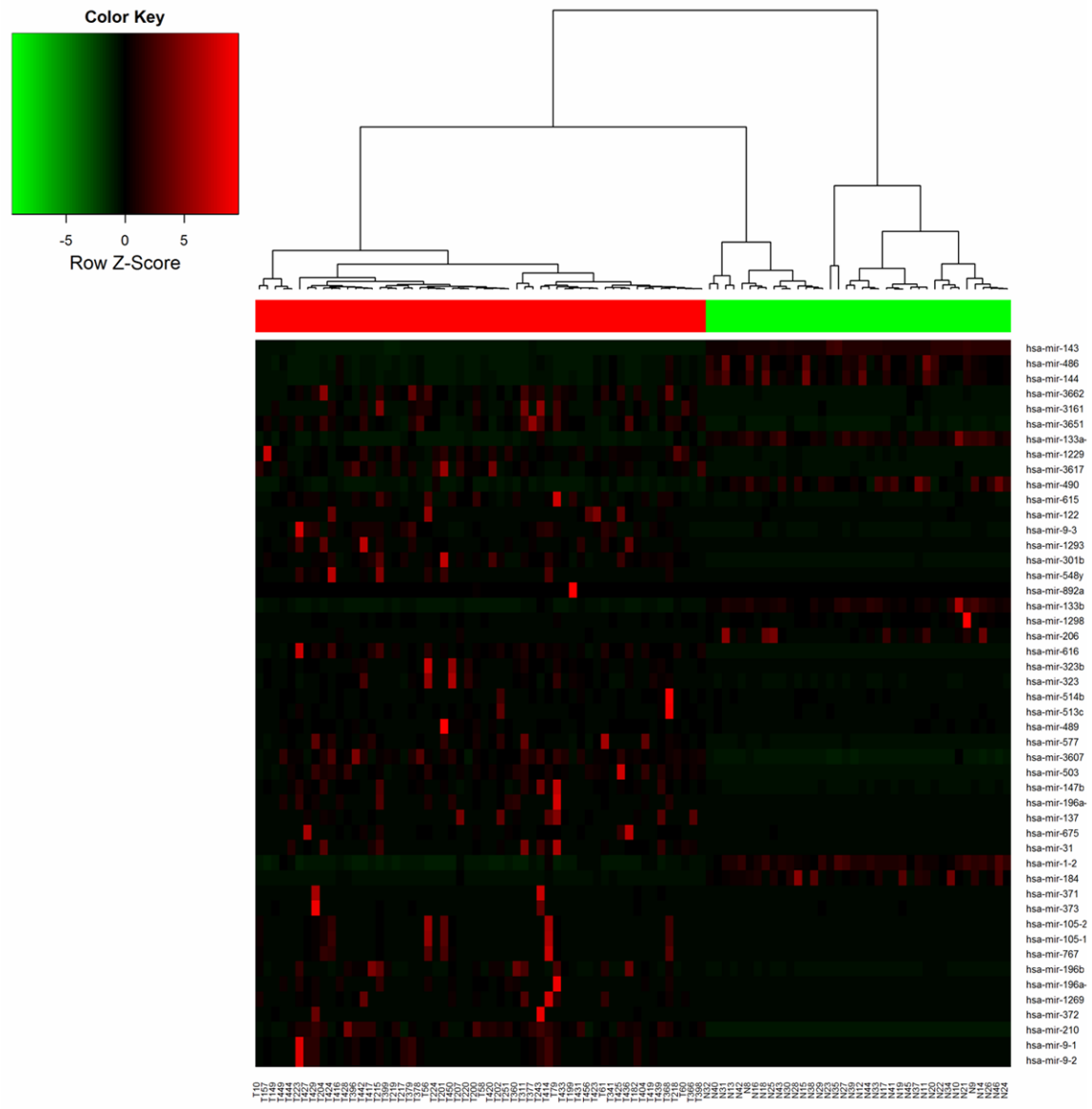

Figure 3. Hierarchical cluster dendrogram of differentially expressed miRNAs. The red edge represents tumor samples and the green edge stands for normal samples. The clustered structure of samples is shown above the expression hit-map of miRNAs.

10% nodes in the number of connections to other nodes. This result indicated that these genes may function as hub nodes in the network. We also evaluated the connections of a node using Betweenness Centrality. *GRB2, EGFR, SOS1 and PIK3R1* were connected more closely and may serve as the key channel of information circulation in the network. By using different methods, *SOS1, EGFR* and *PIK3R1* were picked up as hub nodes, indicating that SOS1, EGFR and PIK3R1 may have central roles in lung adenocarcinoma development.

To understand the association between the enriched differentially expressed miRNAs and lung adenocarcinoma, the differentially expressed miRNAs were ranked in descending order based on the numbers of NSCLC related target genes. As a result, miR-3607, miR-486, miR-1, miR-144, miR-206, miR-301-b, miR-372, miR-373, miR-503, miR-767 and miR-9 were the top 10% of miRNA that regulates the NSCLC related target genes. Moreover, miR-486, miR-1, miR-503 and miR-206 also stood out as differentially expressed miRNAs in lung adenocarcino-

Table 2. miRNAs associated with NSCLC

miRNA	Target genes	*P*-value
hsa-mir-1298	E2F1, EML4, RXRA, TGFA	0.0157
hsa-mir-1	CCND1, CDK6, EML4, KRAS, MAPK1, MAPK3, PIK3R3, PLCG1, RARB	0.0204
hsa-mir-206	CCND1, CDK6, EML4, KRAS, MAPK1, MAPK3, PIK3R3, PLCG1, RARB	0.0204
hsa-mir-1269	GRB2, MAPK1, STK4	0.0274
hsa-mir-486	CDK4, PIK3R1, PRKACA, RAF1, RASSF3, STK4	0.0295
hsa-mir-503	CCND1, E2F3, MAP2K1, PIK3R1, RAF1	0.0471
hsa-mir-3651	KRAS, PRKCA	0.0474

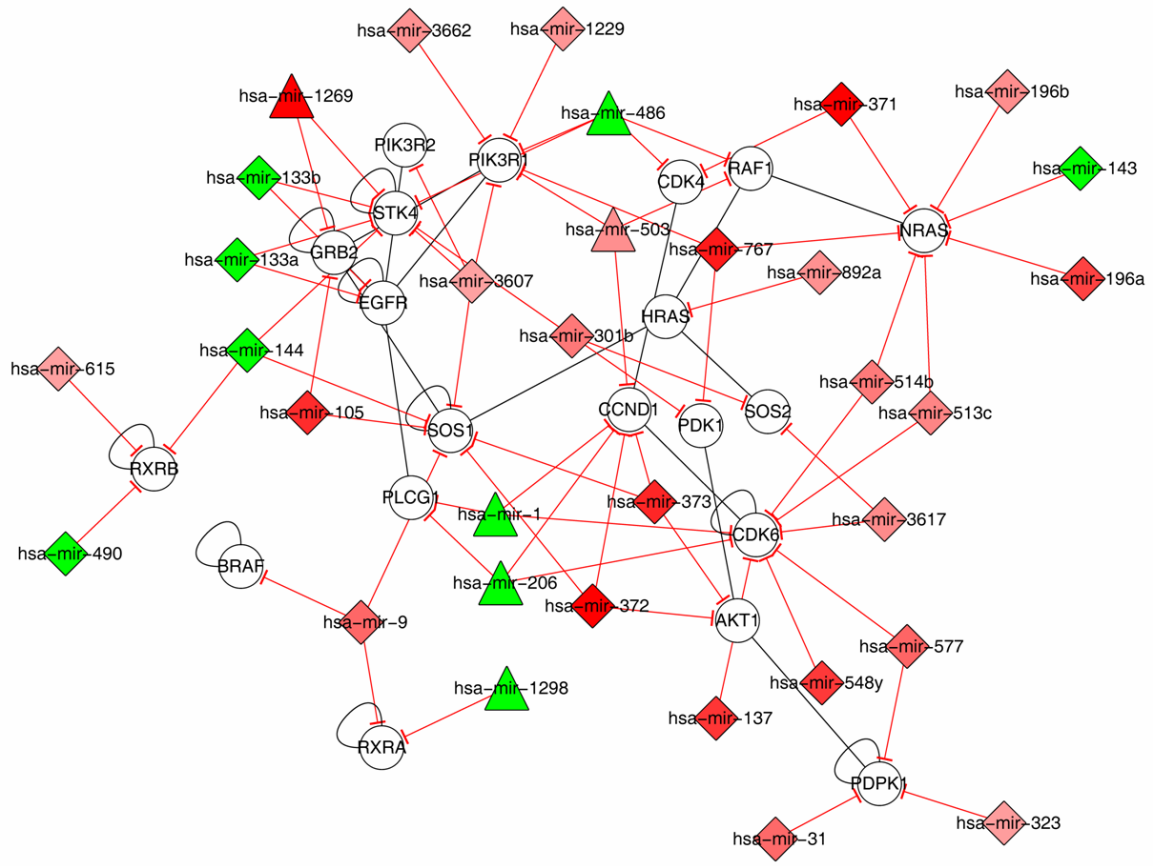

Figure 4. The miRNA-gene interaction network associated with lung adenocarcinoma. The triangle represents the miRNAs associated with NSCLC pathways, the diamond stands for other miRNAs and the circle represents target genes. The red line represents a regulation relation, and the black line represents an interaction relation. Red and green shows the change of expression profiles. Red is for up-regulation and green is down-regulation.

ma that are closely relative to NSCLC. Therefore, these miRNAs may have important roles in the progression of lung adenocarcinoma. To understand the roles of these miRNAs in the miRNA-gene interaction network, we performed clustering analysis. The miRNA-gene interaction network was clustered into two miRNA-gene groups without any overlaps. Cluster I included 25 genes with 40 interactions and it was mainly associated with ErbB signaling pathway (**Figure 5A**). Cluster II contained 14 genes with 17 interactions and it was mainly associated with PI3K-Akt signaling pathway and cell cycle (**Figure 5B**). These results suggest that ErbB and PI3K-Akt signaling play important roles in lung adenocarcinoma development.

Discussion

In this study, we identified 48 differentially expressed miRNAs and their target genes in

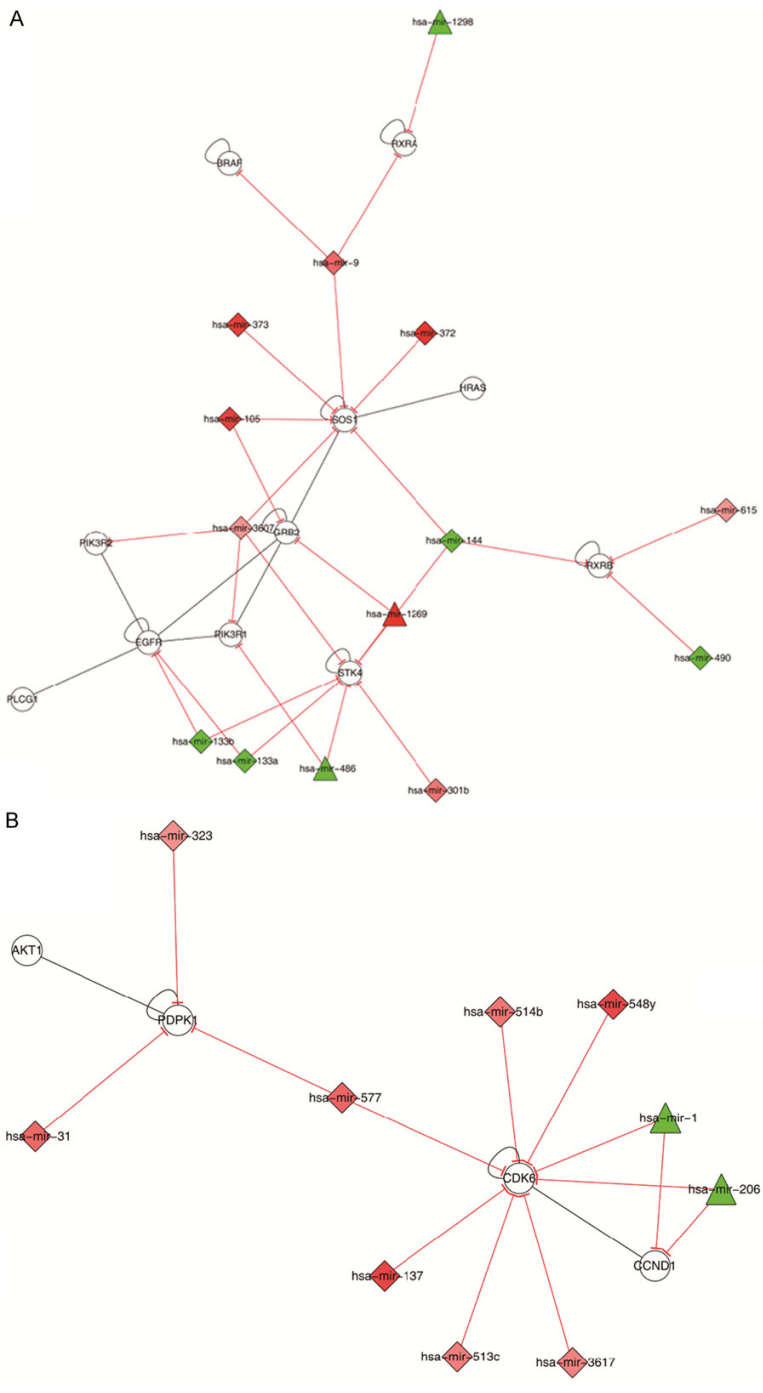

Figure 5. Gene clusters in the networks. A. Cluster I included 40 interactions among 25 genes which was mainly associated with ErbB signal pathway. B. Cluster II contained 17 interactions among 14 genes which was mainly associated with PI3K-Akt signal pathway and cell cycle. The triangle represents the miRNAs enriched in NSCLC pathways, the diamond stands for other miRNAs and circle represents target genes. The arrowed line represents regulation relations and the black line represents the interaction relations. Red stands for up-regulation in expression and green is for down-regulation.

lung adenocarcinoma. Seven of the identified differentially expressed miRNAs were closely associated with NSCLC pathway. Interaction network analysis showed that *GRB2, EGFR, SOS1* and *PIK3R1* served as the key channel of information circulation. The interaction network was clustered two main subnetworks which are associated with ErbB signaling and PI3K-Akt signaling respectively.

The miR-1298, miR-1, miR-206, miR-1269, miR-486 and miR-503 that identified in this study were closely associated with NSCLC. Most of NSCLC related target genes were regulated by miR-1, miR-503 and miR-486. It has been reported that miR-1 could reduce the expression levels of oncogenic targets like MET, Pim-1, FoxP1 and HDAC4 [27]. In our study, the expression level of miR-1 was statistical significantly down-regulated in lung adenocarcinoma. Interestingly, miR-1 has been considered as a therapeutic target for lung cancer [28] and drugs like doxorubicin that targets miR-1 could be used in clinical lung adenocarcinoma therapy potentially [27]. Therefore, miR-1 could be a potent biomarker for diagnosing lung adenocarcinoma. The miR-503 has been shown to suppress tumor cell proliferation [29] and it regulates cell apoptosis by targeting Bcl-2 [30]. We found that the expression level of miR-503 is up-regulated, indicating a strong self-defensive response to cancer cells. miR-486, down-regulated in our study, directly targets components of insulin growth factor (IGF) signaling including IGF1, IGF1R, and PIK3R1. Consistently, we also found that Hsa-mir-486 was down-regulated, indicating that it functions as a potent

tumor suppressor of lung adenocarcinoma cancer [31].

The miR-206 has been reported to suppress iMET and EGFR oncogenic signaling in lung squamous cell carcinoma [32], however, there is no reports elucidated its correlation with lung adenocarcinoma. Moreover, miR-1269 is only been reported to promote proliferation in human hepatocellular carcinoma via downregulation of FOXO1 [33]. Similarly, it is the first time that miR-1298 is reported to associate with lung adenocarcinoma. With further experimental support, these miRNAs might be biomarkers for early diagnosis or targets for treatment in lung adenocarcinoma.

The expression profiles of a group gene with relative functions also reflect the progress of cancers. Identifying gene clusters characterized in cancers may help picturing the development of cancer and it has been used in lung cancer study [34]. We found that GRB2, EGFR, SOS1 and PIK3R1 were connected more closely in the network and these genes may serve as the key nodes. GRB2 (growth factor receptor-bound protein 2) is an adaptor protein involved in signal transduction or cell communication [35]. ErbB2-induced GRB-2 overexpression could lead to lung cancer progression [36]. GRB-2 also interacts with SOS1 [37] and PIK3R1 [38], which are components of EGFR induced signaling pathways. EGFR (epidermal growth factor receptor) is a cell-surface receptor for extracellular protein ligands. Abnormal expression level or activity of EGFR has been reported in lung cancer [39, 40]. PIK3R1 functions as a tumor suppressor [41] and it also interacts with GRB2 [42]. Thus, the group profiling of *GRB2, EGFR, SOS1* and *PIK3R1* could be used for early lung adenocarcinoma diagnosis.

We also identified two clusters in miRNA-gene interaction network. One mainly associated with ErbB signal pathway and the other was mainly related to PI3K-Akt signal pathway and cell cycle. Overexpression of the ErbB family could lead to the genesis of lung cancer [40, 43]. The PI3K-Akt pathway is an intracellular signaling pathway important in regulating the cell cycle. PI3K could phosphorylate AKT to activate the AKT signaling [44]. A number of downstream genes are included in PI3K-Akt pathway such as CREB [44], p27, FOXO, PtdIns-3ps mTOR [45] and EGFR [46]. The group express profile of these two signaling may help to diagnose lung adenocarcinoma.

In conclusion, we identified 7 differentially expressed miRNAs in lung adenocarcinoma, among which miR-1298, miR-206 and miR-1269 are novel identified differentially expressed miRNAs. Among the genes regulated by the identified differentially expressed miRNAs, GRB2, EGFR, SOS1 and PIK3R1 were connected more closely in the network. These identified miRNAs and target genes may serve as early diagnosis markers or potential targets for the treatment of lung adenocarcinomas.

Disclosure of conflict of interest

None.

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