Expression profile analysis of microRNAs and downregulated miR-486-5p and miR-30a-5p in non-small cell lung cancer

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Abstract. Lung cancer is the leading cause of cancer-related mortality worldwide and although there have been improvements in treatment there is a low survival rate. The aim of the present study was to investigate the effect of microRNA (miRNA) on cell pathways. A miRNA microarray was used to profile miRNAs of lung cancer tissues. It was identified that 33 miRNAs with >2.0-fold change and FDR <0.05 were differentially expressed between the adjacent non-cancerous lung tissues and non-small cell lung cancers NSCLCs (P<0.005). The data were optimized in combination with physical interaction analysis to obtain crucial miRNAs. The results showed that differentially expressed miRNAs were associated with biological processes such as cell migration, protein phosphorylation and neuron differentiation, and signaling pathways such as MAPK, TGF-β and PI3K/Akt signaling pathways. Validation of significant miRNAs in independent 40 paired NSCLC tissues demonstrated that the expression level of miR-486-5p and miR-30a-5p was significantly downregulated in another 40 paired lung cancer tissues. Taken together, the results provided strong evidence of the possible involvement of miRNAs in the development and progression of NSCLC. Thus, the results are of importance for clinical investigators and for those who design miRNA-based novel cancer therapeutics.

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Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1), with non-small cell lung cancer (NSCLC) accounting for 80% of all cases of lung cancer. Despite improvement in cancer treatment, the 5-year survival rate remains at <10%. However, diagnosis followed by treatment in the form of surgery at early stage may lead to a 5-year survival rate of 55-80% (2). Therefore, preferential understanding of the mechanisms of tumor development and progression are of great importance in early detection and prevention as well as the targeted treatment of NSCLC.

MicroRNA (miRNA) are a class of small non-coding RNAs between 19 and 24 nt that can repress translation or promote the degradation of target mRNAs (3,4). It has been confirmed that approximately 30% of human protein-coding genes are regulated by miRNAs (5). Thus, miRNAs play an important role in regulating various biological processes, such as cell proliferation (6), differentiation (7) and apoptosis (8). Previous findings showed that an altered expression of miRNAs in cancer relative to corresponding normal tissues, and specific expression signatures or panels can classify human cancers (9), distinguish tumor subtype (10), and are closely associated with prognosis (11). In a recent study, it was shown that altered miRNAs can regulate epithelial-to-mesenchymal transition (EMT) in tumor progression (12). Moreover, increasing evidence suggested that dysregulation of miRNAs contributed to stem cell fate (13).

hsa-miR-486-5p is located on chromosome 8p11.21, which is one of the most frequent genomic deletion regions that contain potential tumor-suppressor genes in various types of tumors, such as NSCLC (14,15). hsa-miR-486-5p was first cloned from the fetal liver. Additionally, a reduced miR-486-5p expression is a frequent molecular event in human malignances (16-19).

hsa-miR-30a-5p is a member of the microRNA-30 family, and is located on chromosome 6q13, which has been reported to be a genomic fragile region due to loss of heterozygosity in breast and lung cancer (20,21). miR-30a-5p has received more attention due to its important role in various biological and pathological processes, including development, differentiation, autophage and apoptosis (22,23).

In the present study, the microRNA microarray was employted to profile miRNA of lung cancer tissues from Chinese subjects. The data were optimized in combination with physical interaction analysis to obtain crucial miRNAs. The results showed the expression level of miR-486-5p and miR-30a-5p was significantly downregulated in another 40 paired lung cancer tissues.

Materials and methods

Patients and tissue samples. Forty-four paired NSCLC and adjacent non-cancerous lung tissues were collected at the time of surgery and before chemotherapy. The samples were obtained from patients at the First Affiliated Hospital of the Soochow University between 2007 and 2013. Informed consent was provided the patients participating in the study. Histological and pathological diagnostics of tissue samples from the patients were obtained from the Department of Pathology according to the Revised International System for Staging Lung Cancer. Tissue samples were snap-frozen and stored in an cryofreezer at -80˚C. Study approval was obtained by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Analysis of miRNA microarray. miRNA microarray assays were performed using the Agilent Human miRNA microarray platform (Agilent Technologies, Santa Clara, CA, USA) at Shanghai Biochip Co., Ltd. (Shanghai, China). The microarray containing probes for 2006 human miRNAs from the Sanger database V19.0. Total RNA (100 ng) derived from cells was labeled with Cy3. Microarray slides were scanned using an Agilent microarray scanner. Labeling and hybridization were performed according to the instructions in the Agilent microRNAs microarray system. The microarray image information was converted into spot intensity values using Feature Extraction software 10.7 (Agilent Technologies). The signal after background subtraction was exported directly into the GeneSpring software 11.0 (Agilent Technologies) for quantile normalization. Log transformation with base 2 was then performed. The limma algorithm (24) was then applied to filter the differentially expressed genes, after the significant analysis and FDR analysis (25) under the specified criteria: i), fold change >2 or <0.5; and ii), FDR <0.05. Targetscan 6.1 was utilized as the tool for predicting miRNA target on the differentially expressed miRNA.

Gene ontology (GO) analysis. GO analysis was performed to facilitate elucidating the biological implications of unique genes in the significant or representative profiles of the target gene of the differentially expressed miRNA in the experiment (26). The GO annotations were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/) and the Gene Ontology (http://www.geneontology.org/). Fisher's exact test was applied to identify the significant GO categories and FDR was used to correct the p-values.

GO-Tree. The Gene Ontology is structured as a directed acyclic graph, and each term has defined relationships to one or more other terms. GO-Tree is constructed based on the Gene Ontology Directed Acyclic Graph to provide user friendly data navigation and visualization. The significant GO-Term (P<0.01) in GO analysis was selected based on the up and down differentially expressed genes to construct the GO-Tree to summarize the function affected in the experiment (27).

Pathway analysis. Pathway analysis was used to identify the significant pathway of the differential genes according to the KEGG database. The Fisher's exact test was used to select the significant pathway, and the threshold of significance was defined by p-value and FDR (28).

Path-act-network. KEGG included metabolism, membrane transport, signal transduction and cell cycle pathways. Genes in the enriched biological pathway were selected (P<0.05) and Cytoscape (29) was used for the graphical representations of pathways.

RNA isolation, cDNA synthesis, and reverse transcriptase-quantitative polymerase chain reaction (qRT-PCR). Total RNA was extracted from tissues using an HP Total RNA kit (Omega Biotech, Stamford, CT, USA) according to the manufacturer's instructions. The amount of RNA was measured on a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Synthesis of cDNA with reverse transcriptase (RT) was performed with a M-MLV First Strand kit (Life Technologies, Gaithersburg, MD, USA). Primer sequences for miR-486-5p, miR-30a-5p and U6 detection were obtained from Riobobio (Guangzhou, China). The RT primer for mature miRNAs and U6 was designed according to the concept of a stem-loop RT primer (30). RT-qPCR analysis was carried out using Platinum SYBR-Green qPCR SuperMix-UDG kits (Life Technologies) according to the manufacturer's instructions. Real-time PCR was performed on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Ct values of miRNAs were equilibrated to U6, which were used as internal controls. Relative expression was calculated using the $\Delta\Delta C_t$ method.

Statistical analysis. Differences in miR-486-5p and miR-30a-5p expression between NSCLC (T) and adjacent paired non-cancerous lung tissues (N) were analyzed using a paired t-test (two-tailed) by GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). The Fisher's exact was applied to identify the significant GO categories and FDR was used to correct the p-values. The Chi-square test was used with SPSS16.0 software to test independence between two variables (SPSS, Chicago, IL, USA).

Results

Differentially expressed miRNAs in NSCLC. We applied the limma algorithm to filter the differentially expressed genes, followed by the significant analysis and FDR analysis under the specified criteria: i), fold change >2 or <0.5; and ii), FDR <0.05. The data from miRNA expression profiling indicated that 33 miRNAs with >2.0-fold change and FDR <0.05 were differentially expressed between the lung normal tissue and NSCLCs (P<0.005), with a higher expression in NSCLC for 15 miRNAs and a lower expression for 18 miRNAs (Fig. 1). Among the miRNAs with a decreased expression, miR-486-5p and miR-30a-5p were identified as the most significantly downregulated, respectively, by bioinformatics.

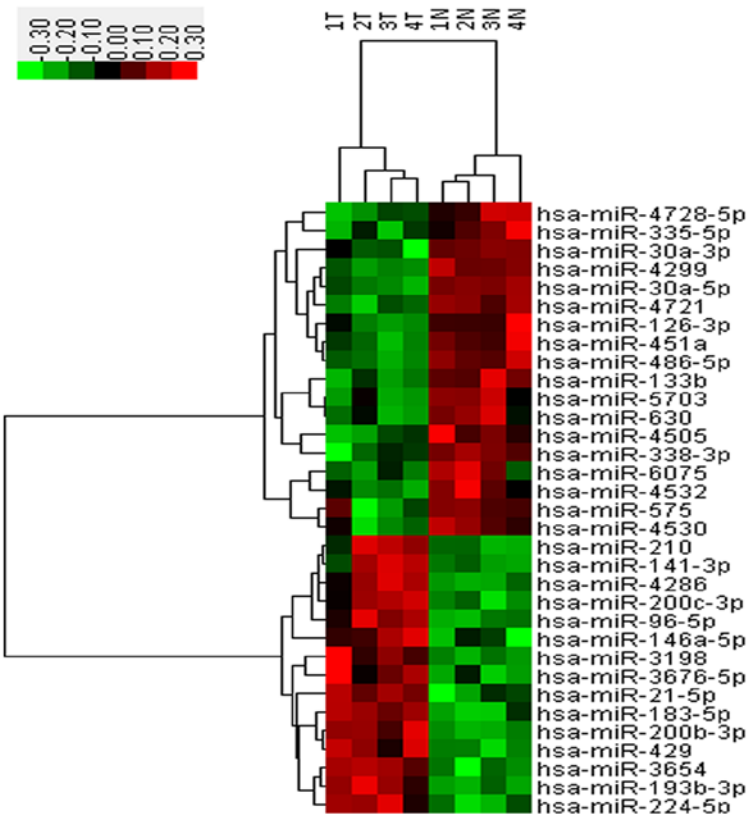

Figure 1. The miRNAs differentially expressed in lung cell carcinoma vs. paired normal tissues. Hierarchical clustering of 33 miRNA genes with a significantly different expression, i.e., i) fold change >2 or <0.5 and ii) FDR <0.05 and P<0.005. Rows are individual microRNA; columns are tumor and normal tissue samples. The color scale is the relative expression ratio of a miRNA following normalization (red, high expression level; green, low expression level).

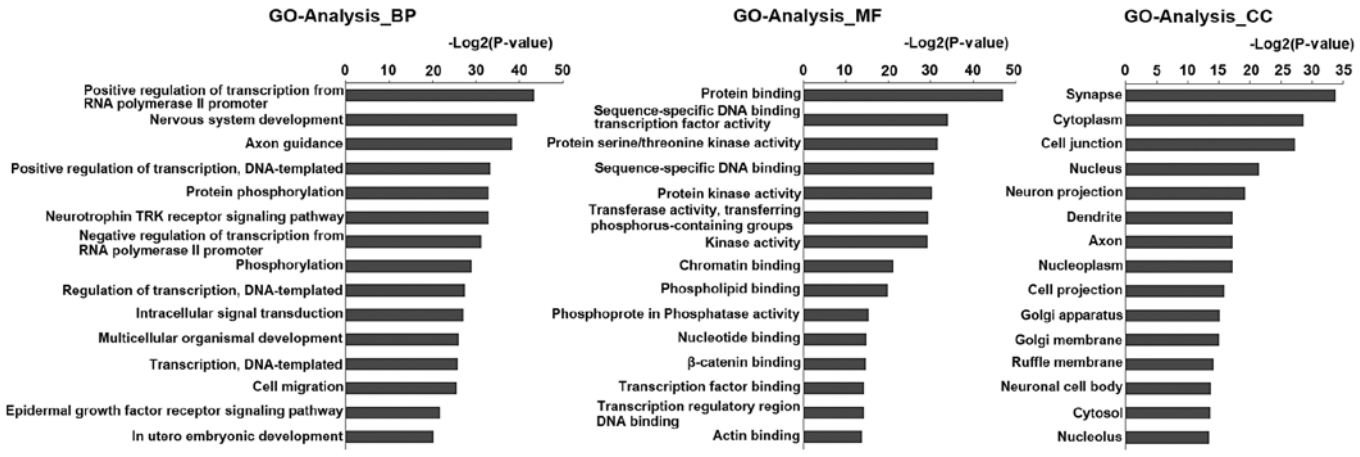

Figure 2. Fisher's exact test was applied to identify the significant GO categories and FDR was used to correct the p-value. GO, Gene Ontology; BP, biological process ; MF, molecular function; CC, cellular component.

Physical interaction analysis. Based on the miRNA array data, to identify the effect of the differentially expressed miRNA towards the biological process and signal transduction on the lung cancer, GO analysis was performed to facilitate elucidating the biological implications of unique genes in the significant or representative profiles of the target gene of the the differentially expressed miRNA in the experiment (Fig. 2), The Gene Ontology is structured as a directed acyclic graph, and each term has defined relationships to one or more other terms. The GO-Tree is constructed based on the Gene Ontology Directed Acyclic Graph to provide user friendly data navigation and visualization. The significant GO-Term (P<0.01) in GO analysis was selected based on the up and down differentially expressed genes to construct the GO-Tree to summarize the function affected in the experiment (Fig. 3).

It has been widely accepted that in solid tumors miRNAs are deregulated, suggesting their involvement in crucial cell pathways including cell-cell adhesion and signaling, cell cycle regulation and apoptosis, which play a significant role in the pathogenesis of cancer. Enriched KEGG and pathway

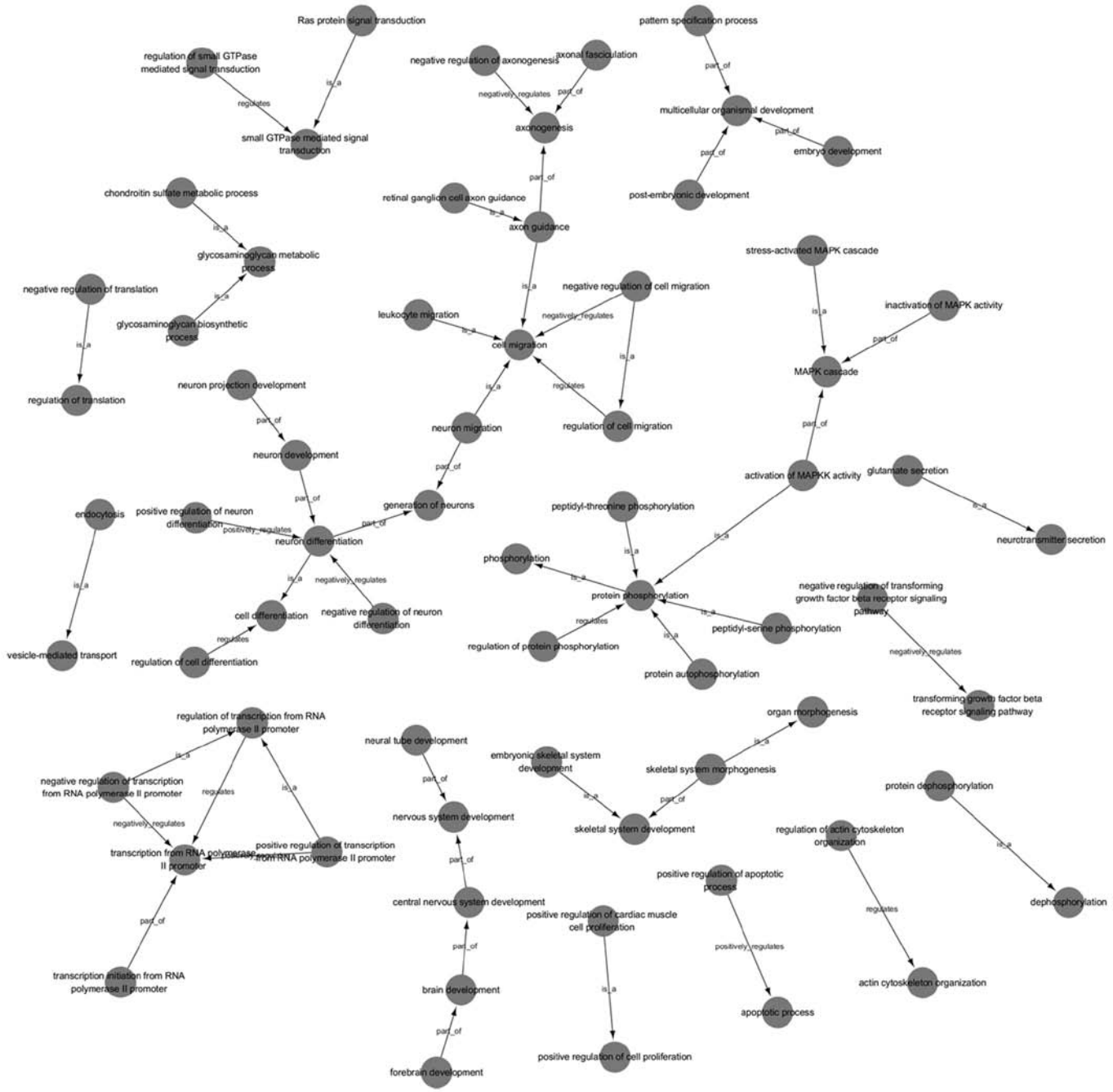

Figure 3. GO-Tree is constructed based on the Gene Ontology Directed Acyclic Graph to provide user friendly data navigation and visualization. We selected the significant GO-Term (P<0.01) in GO analysis based on the up and down differentially expressed genes to construct the GO-Tree to summarize the function affected in the experiment. GO, Gene Ontology.

act networks for several miRNAs target sets were frequently associated with cell signaling pathways, such as the MAPK, Wnt and PI3K-AKT signaling pathways, but also with cell migration, cytoskeleton. Thus, the significant pathway of the differential genes according to the KEGG database was idntified (Fig. 4), and the genes in the enriched biological pathway were selected (P<0.05). Cytoscape was used for the graphical representations of pathways (Fig. 5).

Validation of miRNA microarray results by RT-qPCR in NSCLC. To validate the results of miRNA microarray, miR-486-5p and miR-30a-5p were selected and assessed on the paired tissues of 40 NSCLC patients using RT-qPCR. The RT-qPCR results indicated that miR-486-5p and miR-30a-5p were significantly downregulated in NSCLCs samples used for validation, which was consistent with the results of miRNA microarray (Fig. 6).

Expression of miR-486-5p and miR-30a-5p in relation to clinicopathological characteristics. Patient characteristics with respect to the decreased expression of miR-486-5p and miR-30a-5p are shown in Table I. miR-486-5p and miR-30a-5p exhibited no association with patient age, gender, histology, lymph node status, smoking history and distant

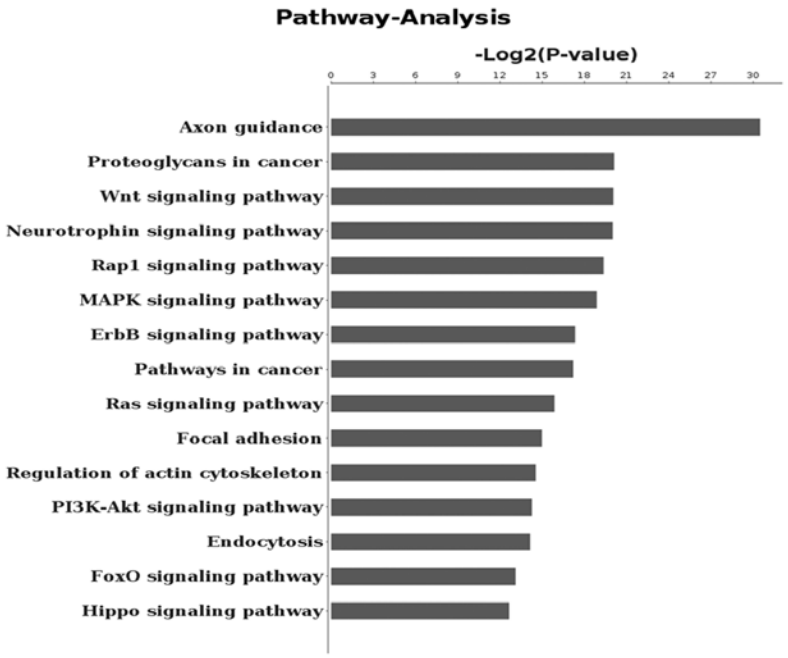

Figure 4. Pathway analysis was used to identify the significant pathway of the differential genes according to the KEGG database. We used the Fisher's exact test to select the significant pathway, and the threshold of significance was defined by p-value and FDR.

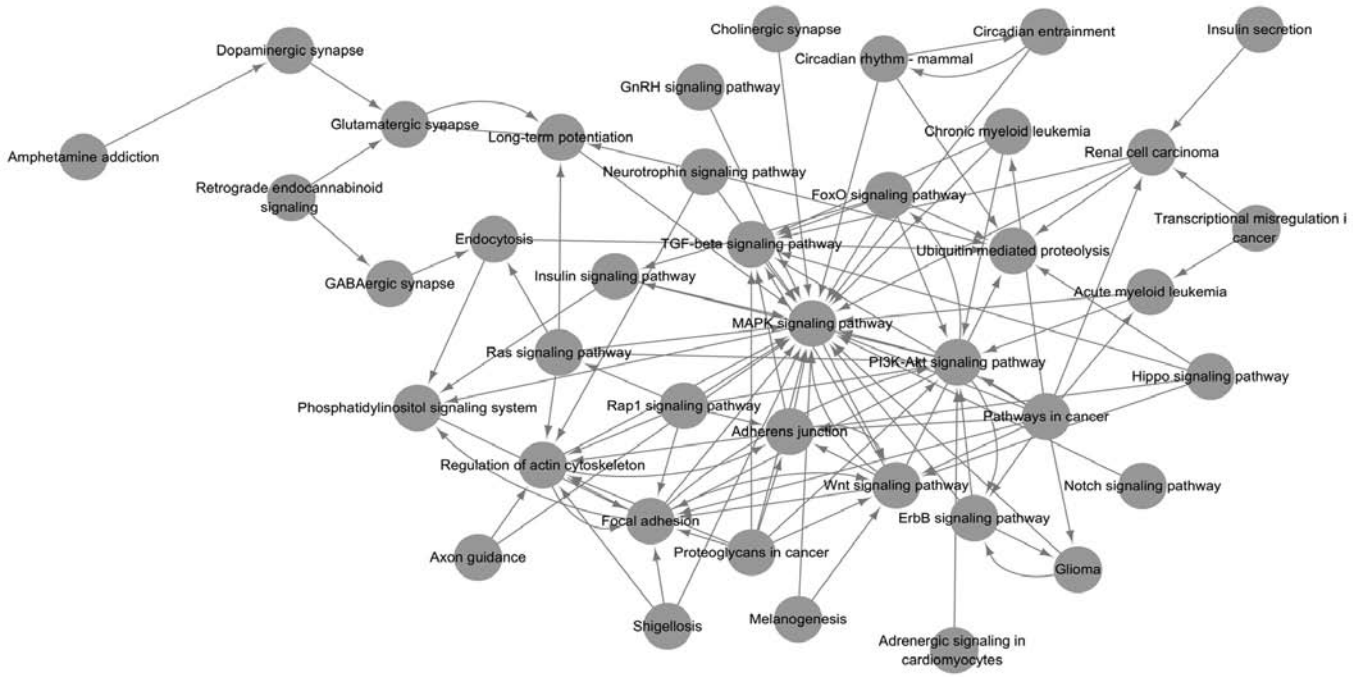

Figure 5. KEGG included metabolism, membrane transport, signal transduction and cell cycle pathways. We picked the genes in enriched biological pathway (P<0.05) and using Cytoscape for graphical representations of pathways.

metastases. To a certain degree, these results suggeseted that the changes in miR-486-5p or miR-30a-5p may occur at the early stage of tumorigenesis, rather than during cancer progression or metastasis.

Discussion

An incremental improvement in the survival rate of patients with cancer has been observed over the last several decades. However, survival advances identified in other common malignancies have not been realized in lung cancer, which remains the leading cause of cancer mortality worldwide (1). The current 5-year survival rate for lung cancer is only 15%.

Up to 25% of all lung cancer cases are not associated with smoking and this proportion is expected to increase as the number of smoking-associated cancer types decreases. The molecular genetic alterations including genetic and epigenetic ones occur prior to morphological changes that can be

Table I. Demographic and clinical characteristics of NSCLC patients and the association with miR-486-5p and miR-30a-5p expression in tumor tissue specimens.

	All patients		Patients with lower miR-30a-5p expression			Patients with lower miR-486-5p expression		
Characteristics	n	%	n	%	P-value[a]	n	%	P-value[a]
All patients	40		34	85.0		35		
Age (years)								
≥60	29	72.5	25	73.5	0.921	26	74.3	0.862
<60	11	27.5	9	26.5		9	25.7	
Gender								
Male	28	70.0	26	76.5	0.532	25	71.4	0.892
Female	12	30.0	8	23.5		10	28.6	
Histology								
Adenocarcinomas	20	50.0	14	41.2	0.448	16	45.7	0.711
Squamous cell carcinomas	20	50.0	20	58.8		19	54.3	
Smokers								
Yes	26	65.0	24	70.6	0.609	23	65.7	0.948
No	14	35.0	10	29.4		12	34.3	
Degree of differentiation								
Low	17	42.5	17	50.0	0.519	17	48.6	0.598
Middle	23	57.5	17	50.0		18	51.4	
Lymph node metastasis								
No	23	52.5	19	55.9	0.889	19	54.3	0.780
Yes	17	42.5	15	44.1		16	45.7	
Distant metastasis								
No	33	82.5	29	85.3	0.745	30	85.7	0.705
Yes	7	17.5	5	14.7		5	14.3	
Histology grade								
I/II	21	52.5	16	47.1	0.641	17	48.6	0.734
III/IV	19	47.5	18	52.9		18	51.4	

[a]Chi-square test; NSCLC, non-small cell lung cancer.

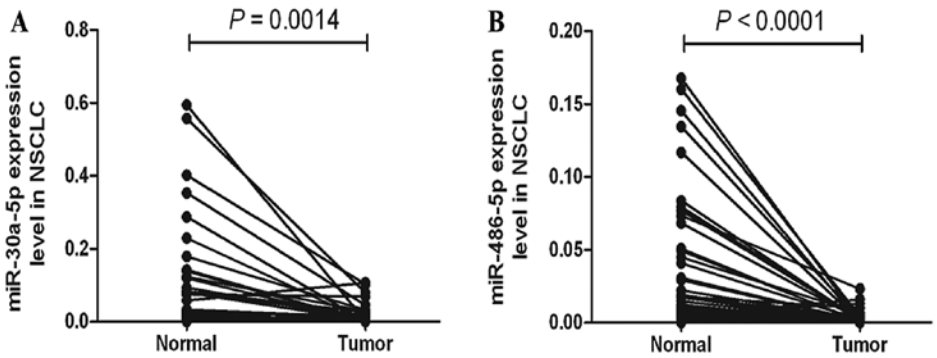

Figure 6. RT-qPCR detection of miR-30a-5p and miR-486-5p expression in lung cancer. (A) miR-30a-5p and (B) miR-486-5p were significantly downregulated in NSCLC samples used for validation,. N, adjacent non-cancerous lung tissues; T, cancerous tissues prepared form the same patients.

identified by a cytological test (31-34). miRNA expression has been directly profiled in lung cancer, and unique groups of miRNAs were identified to characterize the neoplastic tissues or identify patients with poor prognosis (10,35-37).

In this study, we used miRNA expression arrays to determine the miRNA profiles for NSCLC and paired adjacent non-cancerous lung tissues. The miRNA expression profiles distinguished NSCLC from normal lung tissue, and the

samples were classified into two clusters: normal and NSCLC. The expression levels of miR-486-5p and miR-30a-5p were determined by RT-qPCR and consistent with the results of miRNA microarray in 80 samples.

Among miRNAs identified (P<0.005), a few of the miRNAs, including miR-21 and miR-200b were found to be upregulated in NSCLCs. These results were consistent with those of Tan *et al* and Yu *et al* (17,38). miR-21 is one of the best-studied miRNAs, and more extensive efforts have been taken to identify the downstream genes and gene networks regulated by miR-21 and the upstream factors that can regulate dysfunction of miR-21. miR-21 can serve as an important biomarker for the early detection of lung cancer (38), and a high level of miR-21 is associated with worse prognosis of lung cancer patients. miR-200b, located on chromosome 1p36.33, is one of the most common regions with genomic amplications in solid tumors including lung cancer (39). Although the biological mechanism of miR-200b dysfunction in lung tumorigenesis is unclear, miR-200b has been recently identified as one of a set of miRNAs whose aberrant expression was associated with recurrence of stage I NSCLC after surgical resection (40). Consistently, we found that miR-200b overexpression existed in microRNA expression profiles data.

By contrast, a few of the miRNAs, including miR-486-5p and miR-30a-5p were found to be significantly downregulated in NSCLCs. miR-486 is located on one of the most frequent genomic rearrangement regions, chromosome 8p11.21, which contains potential tumor-suppressor genes in lung tumorigenesis. Recent findings have shown miR-486-5p plays a critical role in breast and lung cancer by targeted PIM-1 (41,42). The results from the present study are consistent with those of previous findings, suggesting that miR-486-5p is a potential tumor suppressor in carcinogenesis. miR-30a-5p is a member of the microRNA-30 family, located on chromosome 6q13, which has been reported to be a genomic fragile region due to loss of heterozygosity in breast and lung cancer (20,21). Zhang *et al* showed that miR-30a-5p can suppress breast tumor growth and metastasis by targeting metadherin (43). In lung cancer, Kumarswamy *et al* showed that miR-30a is downregulated in NSCLC and inhibits EMT by targeting Snail (44).

It has been widely accepted the tumorigenesis is a heterogeneous disease and develops from complex and multistep processes, including lung cancer. Evidence suggests that multiple signaling pathways involve the development of lung cancer. A meta-analysis of microRNA expression in lung cancer suggested that significant miRNAs are regulatory drivers of the oncogenic process, and may be good candidates for the development of tests for monitoring remission during postoperative follow-up, this is supported by the gene enrichment analysis (45). In our study, based on the miRNA profiles in lung cancer, an enrichment analysis was carried out, and the results showed differentially expressed miRNAs associated with the biological process and signaling pathway in lung cancer.

In conclusion, based on the miRNA array combined with the physical interaction analysis, our results have shown the impact of significant miRNAs on cell pathways and biological processes. Further validation of miRNA microarray results by RT-qPCR in NSCLC show that miR-486-5p and miR-30a-5p were significantly downregulated in NSCLC tissues. Therefore, the results provide strong evidence of the possible involvement of miRNAs in the development and progression of NSCLC, The findings are of importance for both clinical investigators and for those who design miRNA-based novel cancer therapeutics.

Acknowledgements

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