Identification of miRNAs and differentially expressed genes in early phase non-small cell lung cancer

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Abstract. To explore the potential therapeutic targets of early-stage non-small cell lung cancer (NSCLC), gene microarray analysis was conducted. The microarray data of NSCLC in stage IA, IB, IIA, and IIB (GSE50081), were downloaded from the Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) in IB vs. IA, IIA vs. IB, IIB vs. IIA were screened out via R. ToppGene Suite was used to get the enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the DEGs. The GeneCoDis3 database and Cytoscape software were used to construct the transcriptional regulatory network. In total, 25, 17 and 14 DEGs were identified in IB vs. IA, IIA vs. IB, IIB vs. IIA of NSCLC, respectively. Some GO terms and pathways (e.g., extracellular space, alveolar lamellar body, bioactivation via cytochrome P450 pathway) were found significantly enriched in DEGs. Genes *S100P*, *ALOX15B*, *CCL11*, *NLRP2*, *SERPINA3*, *FoxO4* and *hsa-miR-491* may play important roles in the development of early-stage NSCLC. Thus, by bioinformatics analysis the key genes and biological processes involving in the development of early-stage NSCLC could be established, providing more potential references for the therapeutic targets.

Introduction

Lung cancer is the second most common cancer with high mortality in both men and women (1). More than 80% of lung cancer are non-small cell lung cancer (NSCLC) (2,3). In China, the incidence of lung cancer in the past few decades has doubled due to the aging population, smoking and decline in air quality (4). Although some treatments have achieved certain therapeutic effect, the overall 5-year survival rates of NSCLC patients is still below 15% (5). Most NSCLC patients are in mid-term and advanced stage when diagnosed, which could contribute its poor prognosis. The progression of NSCLC is related to many genetic factors, such as abnormal gene expression (6). Gu *et al* found that NSCLC, comparing to other cancers, had a significantly higher ratio of VEGF-A protein/mRNA and a significantly lower level of miR-497, suggesting the presence of the post-transcriptional control of VEGF-A in NSCLC which was obviously different from other cancers (7). It demonstrated that miR-497 played an important role in suppression of VEGF-A-mediated NSCLC by inhibiting the proliferation and invasion of cancer cells (7). Therefore, it is very important to identify potential biomarkers of NSCLC for its prevention and therapy.

With the rapid development of molecular biology and bioinformatics, we can explore the mechanisms of the development and progression of cancers at the molecular level. It also provides some significant references for the diagnosis, prevention and treatment of cancer (8). However, the mechanism and development of new target-based therapies related to lung carcinogens are still unknown. Gene microarray, as an efficient technology, has been used to detect the expression levels of some genes in cells and tissues at different stages of cancer. It is also widely used for the genome-scale detections, which may help discover the key genes associated with tumorigenesis (9).

In this study, we aimed to explore the key genes in the early and mid-stages of NSCLC via gene microarray data analysis. Differentially expressed genes (DEGs), Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways which were associated with NSCLC were identified. A transcription factor (TF) network was also constructed. Our study aimed to provide a new idea and method for a better understanding of NSCLC, so as to improve the diagnosis and treatment of early stage NSCLC.

Materials and methods

Affymetrix microarray data. Microarray data set GSE50081, submitted by Der *et al* (10), was obtained from the Gene

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Table I. The three groups of differentially expressed genes.

IB/IA	logFC	P-value	IIA/IB	logFC	P-value	IIB/IIA	logFC	P-value
CPB2	-1.46	3.83E-05	CYP1A1	1.06	0.000686	NOG	-1.03	0.007331
IL8	1.13	0.000285	SERPINA3	-2.03	0.000757	SERPINA3	1.59	0.010848
LRRK2	-1.03	0.000594	TSPAN1	-1.65	0.00116	SPRY2	-1.09	0.012028
AGR3	-1.38	0.000674	ALOX15B	1.31	0.001853	RPL39L	1.07	0.012364
PEBP4	-1.26	0.000772	ID1	-1.23	0.002166	NLRP2	1.63	0.012685
CYP4B1	-1.35	0.000864	MB	-1.18	0.005791	TMEM163	-1.05	0.013372
LY6D	1.13	0.000998	S100P	-2.49	0.006031	ALOX15B	-1.24	0.016598
ZNF385B	-1.06	0.001022	AGR2	-1.33	0.013493	TRIP13	1.04	0.023701
CYP24A1	1.08	0.00145	MFAP5	1.32	0.017172	MALL	-1.18	0.026681
HSD17B6	-1.03	0.001823	PLEKHS1	-1.02	0.017271	BAMBI	-1.19	0.027515
SFTPC	-1.39	0.003012	ANXA1	1.08	0.023946	EYA2	1.17	0.030051
C16orf89	-1.26	0.003714	NLRP2	-1.36	0.032368	SLAIN1	-1.04	0.033329
S100P	1.30	0.004488	DNER	-1.11	0.034532	PRSS21	1.23	0.035066
MMP10	1.17	0.005196	CA9	-1.02	0.036787	CCL11	-1.07	0.043612
SFTPD	-1.21	0.006222	SLPI	-1.18	0.041679			
C4BPA	-1.27	0.006294	FXYD3	-1.07	0.044774			
SFTA3	-1.30	0.0096	CCL11	1.16	0.045338			
SCGB2A1	-1.02	0.009966						
SFTPB	-1.01	0.010427						
KRT6A	1.74	0.01062						
SCGB3A2	-1.32	0.012366						
MMP1	1.14	0.013572						
BPIFA1	1.25	0.014045						
SCGB3A1	-1.19	0.019787						
NAPSA	-1.04	0.022362						

Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database. A total of 181 gene chips were available, including 48 samples in IA stage, 79 samples in IB stage, 9 samples in IIA stage and 45 samples in IIB stage of NSCLC, respectively. Microarray data were analyzed using GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array), which included 54,675 probes to detect the expression of gene transcription levels.

Data preprocessing and screening of DEGs. The raw data were preprocessed. CEL files were normalized and converted to expression profiles using Affy package of R (11). If multiple probes corresponded to one given gene, the mean expression value was defined as the expression value. Limma package of R was used to analyze the DEGs of stage IA samples, IB samples, IIA samples and IIB samples (12). The samples were divided into three groups: IA vs. IB, IIA vs. IB and IIA vs. IIB. The gene expression values were normalized followed by the t-test of the non-paired samples (13). The multiple testing corrections were performed through Beniamini-Hochberg. FDR <0.05 and |logFC (fold change)| >2 were used as the threshold for identifying the DEGs.

Functional enrichment analysis. GO and KEGG pathway analysis of DEGs were conducted via ToppGene Suite (http://toppgene.cchmc.org) (14). ToppGene Suite is a one-stop portal for gene list functional enrichment, candidate gene prioritization using either functional annotations or network analysis and identification and prioritization of novel disease candidate genes in the interactome (14). Adjusted P≤0.05 was used as cut-off criterion.

Construction of transcriptional regulatory network. With the cut-offs of adjusted P<0.05, the TFs and miRNAs which interacted with target DEGs were identified by the online database GeneCoDis (http://genecodis.cnb.csic.es/) (15). These pairs were used to construct transcriptional regulatory network. Visualization of transcriptional regulatory network was performed by Cytoscape software (16).

Results

Identification of DEGs. A total of 19,851 genes were obtained after the processes of background calibration, standardization and the transformation from probe symbols to the gene symbol, which were used for the further analysis. The total 56 DEGs, consisted of 25 in IB vs. with IA, 17 in IIA vs. IB and 14 in IIB vs. IIA, as detailed in Table I. The variation in trends of the DEGs expression values in the three groups are shown in Fig. 1.

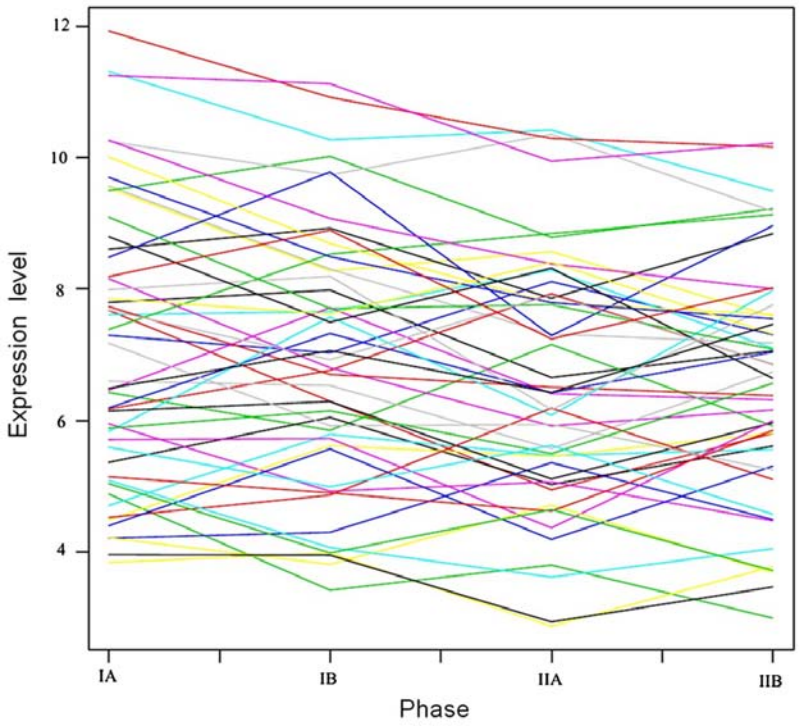

Figure 1. The trend of differentially expressed genes changes in the different phases of the non-small cell lung cancer.

Table II. The top 10 enriched GO terms for DEGs.

Category	GO ID	GO name	Gene number	P-value
CC	GO:0005615	Extracellular space	16	5.44E-08
CC	GO:0097208	Alveolar lamellar body	3	1.29E-06
CC	GO:0042599	Lamellar body	3	5.52E-06
BP	GO:0050828	Regulation of liquid surface tension	2	6.90E-06
BP	GO:0016477	Cell migration	12	1.72E-05
MF	GO:0070576	Vitamin D 24-hydroxylase activity	2	1.93E-05
BP	GO:0048870	Cell motility	12	4.05E-05
BP	GO:0051674	Localization of cell	12	4.05E-05
MF	GO:0005506	Iron ion binding	5	7.45E-05
CC	GO:0005771	Multivesicular body	3	8.75E-05

GO, Gene Ontology; DEGs, differentially expressed genes; CC, cellular component; BP, biological process; MF, molecular function.

Functional enrichment analysis of DEGs. A total of 110 enriched GO terms and 8 KEGG pathways for DEGs were identified. The top 10 enriched GO terms are listed in Table II, and 8 enriched KEGG pathways are shown in Table III. The top three pathways were bioactivation via cytochrome P450 (P=1.01E-04), Arachidonic acid metabolism (P=3.19E-04), Cytochrome P450, arranged by substrate type (P=3.57E-04).

Analysis of regulatory network between TFs and miRNAs. Following the screening of TFs, we constructed a regulatory network between TFs and miRNAs including 13 TFs, 120 miRNAs and 563 edges (Fig. 2). Based on this regulatory network, TFs (e.g., *hsa-miR-653*, *hsa-miR-548a-5p*, *hsa-miR-491-3p*, *hsa-miR-518d-3p*, *hsa-miR-518c*, *hsa-miR-330-3p* and *hsa-miR-374a*) were obtained which closely connected with others. Each of them interacted with seven genes, respectively. *ID1* was regulated by the most number of the genes including 35 TFs and miRNA. The top 10 genes which closely connected with others in the NSCLC samples of these four stages are shown in Fig. 3.

Discussion

Substantial research efforts has been made in exploring the mechanisms of NSCLC, however, the current understanding of the genetic alterations associated with the progressing NSCLC has not been elucidated. In our present study, 52 DEGs of NSCLC were identified using gene microarray analysis. These

Table III. The enriched KEGG pathways for DEGs.

Category	Pathway name	Gene number	P-value
KEGG_PATHWAY	Bioactivation via cytochrome P450	2	1.01E-04
KEGG_PATHWAY	Arachidonic acid metabolism	3	3.19E-04
KEGG_PATHWAY	Cytochrome P450 - arranged by substrate type	3	3.57E-04
KEGG_PATHWAY	Cytochrome P450	3	5.68E-04
KEGG_PATHWAY	Phase 1 - functionalization of compounds	3	8.44E-04
KEGG_PATHWAY	Plasminogen activating cascade	2	8.99E-04
KEGG_PATHWAY	TGF-β signaling pathway	3	1.19E-03
KEGG_PATHWAY	Fatty acid metabolic	2	1.66E-03

DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes.

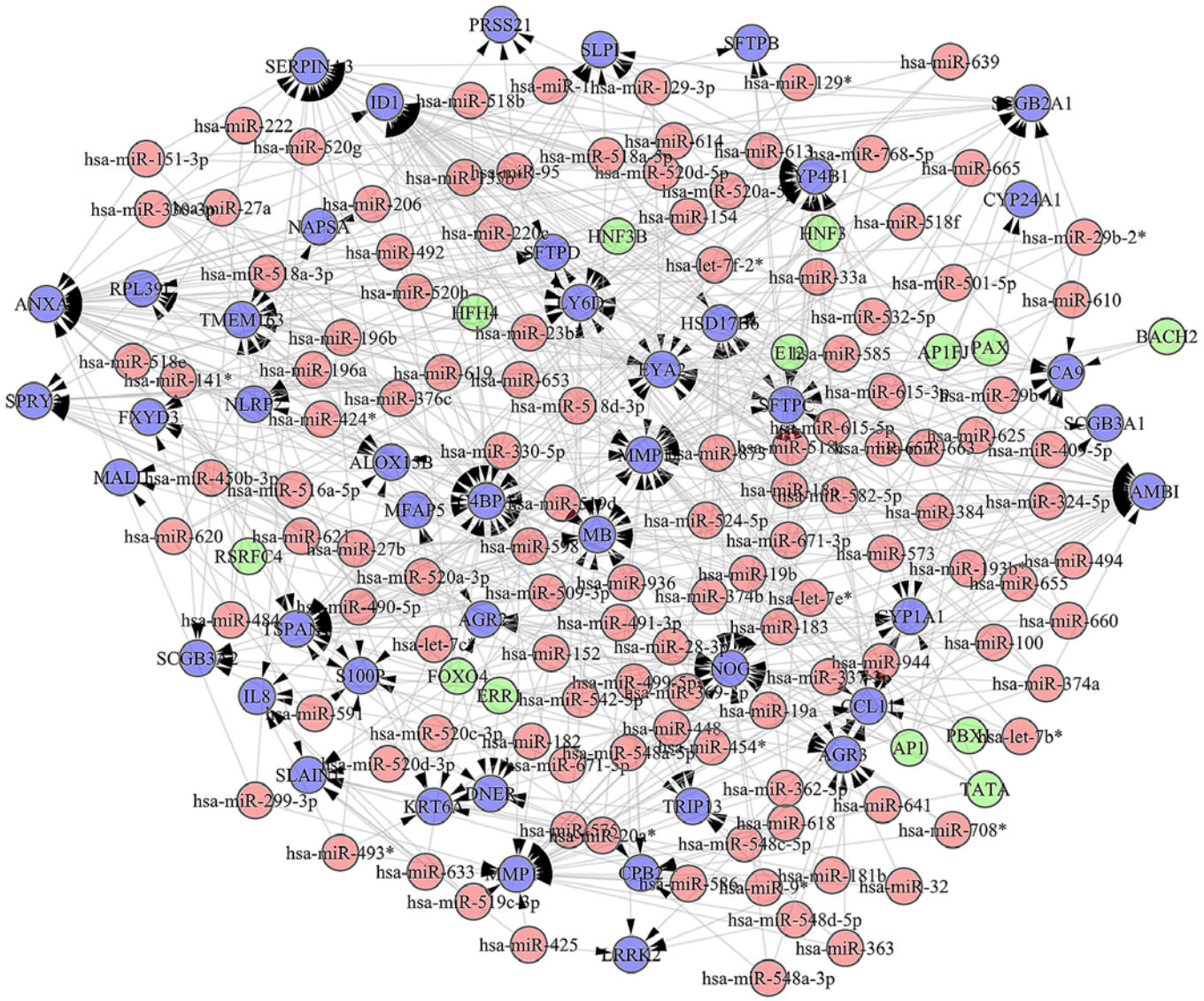

Figure 2. Regulatory network between transcription factors (TFs) and miRNAs. Red nodes, miRNAs; green nodes, differentially expressed genes; purple nodes, transcription factors.

DEGs may influence the occurrence and development of NSCLC, thus, they will provide important references for the diagnosis and treatment of NSCLC.

Gene microarray data in the early and mid-stage NSCLC were obtained from the GEO database. An important coinciding gene (*S100P*) was found differentially expressed in

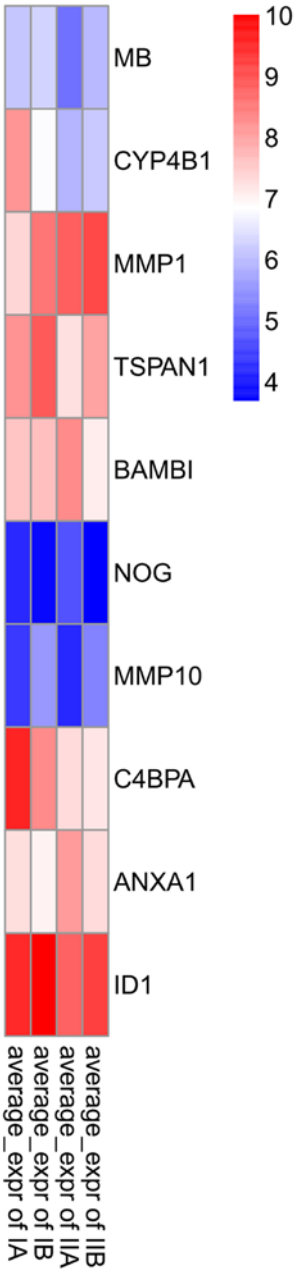

Figure 3. The mean expression of the top 10 genes with the largest regulatory degree in the samples of the four stages of NSCLC.

IA vs. IB and IIA vs. IB, respectively. *S100P* is a 95-amino acid member of *S100* protein family. It contains 2 EF-hand calcium-binding motifs (17) and involves in a series of cellular regulation processes, such as cell cycle progressions and differentiation (18). *S100P* was found to be one of the target genes studied in many cancers including NSCLC (19). The expression of *S100P* was associated with drug resistance, metastasis, and poor clinical outcome. According to our results, *S100P* was upregulated in IB vs. IA and downregulated in IIA vs. IB. It could be speculated that the expression of *S100P* increased in the processes of cancer migration and invasion in the early stage of NSCLC, and then its expression was inhibited by certain factors, such as the immune system.

Besides, 4 overlapped genes (*ALOX15B*, *CCL11*, *NLRP2* and *SERPINA3*) were identified in IIA vs. IB and IIA vs. IIB. The knockdown of the human arachidonate 15-lipoxygenase type B (*ALOX15B*) was reported to reduce the inflammation and lipid accumulation, suggesting its active pro-inflammatory and proatherogenic role (20). Pyrin domain-containing protein 2 (*NALP2*) was characterized by an N-terminal pyrin domain (PYD). It was involved in the activation of caspase-1 by Toll-like receptors (21). The expression of *NALP2* was regulated by inflammatory mediators which were closely related to cancer (22,23). As a member of the CC chemokine family, eotaxin-1 (*CCL11*) was initially regarded as an eosinophil chemoattractant, and involved in the recruitment of inflammatory cells, such as eosinophils and neutrophils (24). Overexpression *CCL11* was found in various inflammatory diseases, such as allergic asthma. *CCL11* was also reported as diagnostic marker for some cancers, such as prostate cancer (25,26). Collectively, the dysregulation of the 4 overlapped genes in different paired comparisons (Table I) revealed that the key genes may play different roles at different stages of NSCLC.

The regulatory network revealed that *FoxO4* was closely connected with other nodes. It participated in the processes of cell cycle, aging, apoptosis, stress response, tumorigenesis and metabolisms. The expression of *FoxO4* was confirmed in the studies of 8 cases of NSCLC patients. The immunohistochemistry method was used to characterize the expression of *FoxO4* (27). Many studies have demonstrated the relationships between *FoxO4* and a variety of cancers. According to Su *et al*, the transcription factor *FoxO4* was downregulated and inhibited tumor proliferation and metastasis in gastric cancer (28). The expression of *FoxO4* was significantly decreased in colorectal cancer, indicating that *FoxO4* acted as a tumor suppressor in the development and progression of colorectal cancer (29).

miRNA is a non-coding RNA of about 20-25 nt in length, which can affect the phase of post-transcriptional process to regulate the expression levels of genes (30). The development of cancer is associated with the reduction of tumor suppressor genes which are regulated by miRNAs. In this study, *hsa-miR-491* was regulated by the most number of TFs serving as modulators and biomarkers for the invasion and metastasis of oral squamous cell carcinoma (31). According to other reports, *hsa-miR-491* was found to be involved in metastasis of hepatocellular carcinoma by inhibition of matrix metalloproteinase and epithelial to mesenchymal transition based on the the methods of miRNA microarray analysis, RT-PCR, western blotting, Transwell invasion and immunohistochemistry (32), and it may even play a role in stemness of stem cells and cancer stem cells in lung tumors (33). Moreover, the roles of other target genes of *hsa-miR-491* have also been reported in many studies, so they may be the novel biomarkers of NSCLC.

In conclusion, a set of DEGs were screened and considered to be related to the development of NSCLC. *S100P*, *ALOX15B*, *CCL11*, *NLRP2*, *SERPINA3*, *FoxO4* and *hsa-miR-491* may act as candidate diagnostic markers for NSCLC patients. However, further experiments are still needed to confirm the results.

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