European Review for Medical and Pharmacological Sciences 2015; 19: 1364-1372

Network analysis of differentially expressed genes reveals key genes in small cell lung cancer

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Abstract. – OBJECTIVE: A combination of comparative analysis of gene expression profiles between normal tissue samples and small cell lung cancer (SCLC) samples and network analysis was performed to identify key genes in SCLC.

MATERIALS AND METHODS: Microarray data set GSE43346 was downloaded from Gene Expression Omnibus (GEO), including 42 normal tissue samples and 23 clinical SCLC samples. Differentially expressed genes (DEGs) were screened out with t-test. Coexpression network and gene regulatory network were then constructed for the DEGs. GO enrichment analysis as well as KEGG pathway were performed with DAVID online tools to reveal over-represented biological processes.

RESULTS: A total of 457 DEGs were obtained in SCLC, 259 up-regulated and 198 down-regulated. Some of them exhibited enzyme inhibitor activity and chemokine activity. A coexpression network including 457 nodes was constructed, from which a functional module was extracted. Genes in the modules were closely related with cell cycle. Top 10 nodes in the regulatory network were acquired and their sub-networks were extracted from the whole network. Genes in these sub-networks were related to cell cycle, apoptosis and transcription. A network comprising 43 microRNAs (miRNAs) and their target genes (also DEGs) were also constructed. Regulation of cell proliferation, cell cycle and regulation of programmed cell death were over-represented in these genes.

CONCLUSIONS: A range of DEGs were revealed in SCLC, which could enhance the understandings about the pathogenesis of this disease and provide potential molecular targets for diagnosis as well as treatment.

Key Words:

Small cell lung cancer, Microarray data, Differentially expressed genes, Functional enrichment analysis, Co-expression network, Gene regulatory network.

Introduction

Small cell lung cancer (SCLC) is an aggressive form of lung cancer that is strongly associated with cigarette smoking[1]. It grows quickly and often metastasizes to other parts of the body, including the brain, liver, and bone. Because of the high propensity of SCLC to metastasize early, surgery has a limited role as primary therapy. Although the disease is highly sensitive to chemotherapy and radiation, cure is difficult to achieve[2].

Microarray technology is a useful tool to globally investigate the alterations in gene expression during tumorigenesis. Bhattacharjee et al[3] report that integration of expression profile data with clinical parameters could aid in diagnosis of lung cancer patients. The study by Beer et al[4] shows that gene-expression profiles based on microarray analysis can be used to predict patient survival in early-stage lung adenocarcinomas. Lu et al[5] also report a gene expression signature that can predict survival of patients with stage I non-small cell lung cancer. Identification of deregulated pathways not only advances the understandings about the pathogenesis of cancer, but also serves as a guide to targeted therapies[6]. Using this technology, Kim et al[7] reveal the altered apoptotic balance in SCLC and suggest that MYC family genes might affect oncogenesis through distinct sets of targets. Radioresistance is a big challenge in treatment of cancers. Guo et al[8] carry out a microarray analysis to identify differentially expressed genes (DEGs) contributing to radioresistance in lung cancer cells. Bangur et al[9] adopt a combination of suppression subtractive hybridization and cDNA microarray to discover differentially over-expressed genes in SCLC.

Several potential diagnostic and therapeutic targets have been uncovered. Kijima et al[10] report that CXCR4 and c-Kit mediate the regulation of cellular proliferation, cytoskeletal function, and signal transduction in SCLC. Takamizawa et al[11] find that reduced expression of the let-7 microRNAs is associated with shortened postoperative survival. Tang et al[12] find that EPHB subgroup receptor kinases may modulate the biological be-

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havior of SCLC through autocrine and/or juxtacrine activation by ephrin-B ligands.

In order to better understand this disease and improve the outcomes of patients, more researches are necessary. Therefore, in this study, a comparative analysis of transcriptome between normal tissue samples and SCLC samples was performed, combined with network analysis and functional enrichment analysis, to identify important biological pathways as well as key genes disturbed in SCLC.

Materials and Methods

Gene Expression Profiles

Microarray data set GSE43346 was downloaded from Gene Expression Omnibus (GEO)[13], including 42 normal tissue samples [whole brain (cerebral cortex, hippocampus, diencephalon, pons, hypothalamus, cerebellum), skeletal muscle, heart, skin, tongue, esophagus, stomach, small intestine, colon, pancreas, liver, gallbladder, kidney, adrenal gland, bladder, salivary glands, tonsils, thyroid, thymus, trachea, lung, spleen, lymph nodes, adipose artery, vein, bone marrow, peripheral blood, monocytes, macrophages, testis, prostate, seminal vesicle, breast, uterus, ovary] and 23 clinical SCLC samples. The platform was Affymetrix Human Genome U133 Plus 2.0 Array. Annotation file was also acquired.

Raw Data Pretreatment and Screening of DEGs

Original CEL format was converted into expression matrix using function rma from package affy[14]. Probes were mapped to genes according to the annotation file with *R*. Average expression level was calculated for the probes corresponding to the same gene.

DEGs between normal tissue samples from non-SCLC individuals were removed through the goodness of fit test. If a gene was not differentially expressed, it should subject to the average distribution. We checked whether the statistic T subject to the chi-square distribution with degree of k-1 (k=42). Xi was the expression level of a gene in tissue i. p value < 0.05 was set as the cut-off.

Package limma was adopted for the differential analysis. |logFC| (fold change) >1.5 and p value < 0.05 were set as the criteria to screen out DEGs between normal tissue samples and SCLC samples.

Bioinformatic Analysis on the DEGs

GO (Gene Ontology) enrichment analysis and KEGG (kyoto encyclopedia of genes and genomes) pathway enrichment analysis were performed for the DEGs using the DAVID (database for annotation, visualization, and integrated discovery) online tools[15].

Previous study has indicated that genes sharing the same pathway or similar biological functions show similar gene expression pattern under same physical conditions[16]. Therefore, constructing the gene coexpression network could help to identify gene sets implicated in specific pathways or biological processes. In this study, Pearson correlation coefficient was used as a measure of gene coexpression. Coexpression with the coefficient > 0.85 and p value < 0.05 was retained.

With information from USUC (a database of transcription factors)[17] and miRBase (a database of miRNAs)[18], regulatory relationships between the DEGs and these factors were filtered out and then networks were constructed. Modules in the whole network were mined with MCODE from Cytoscape[19] and then functional enrichment analysis was applied on the genes in the modules.

Results

DEGs in SCLC

A total of 19944 gene expression values were obtained from 65 samples after raw data pretreatment. Gene expression data before and after normalization are shown in Figure 1 which presented a good performance of normalization (Figure 1).

No DEGs were detected among normal tissue samples from non-SCLC individuals according to the goodness of fit test. A total of 457 DEGs were revealed by comparing 42 gene expression profiles from normal tissue and 23 profiles from SCLC, 259 up-regulated and 198 down-regulated in SCLC. Heat map for expression of DEGs across samples is shown in Figure 2.

Functional Enrichment Analysis Results

GO enrichment analysis and KEGG pathway enrichment analysis were performed for the DEGs to reveal molecular functions and biological pathways (Tables I and II). Eight molecular functions and four KEGG pathways were over-represented in all the DEGs, such as enzyme inhibitor activity, chemokine activity, chemokine signaling pathway and cytokine-cytokine receptor interaction.

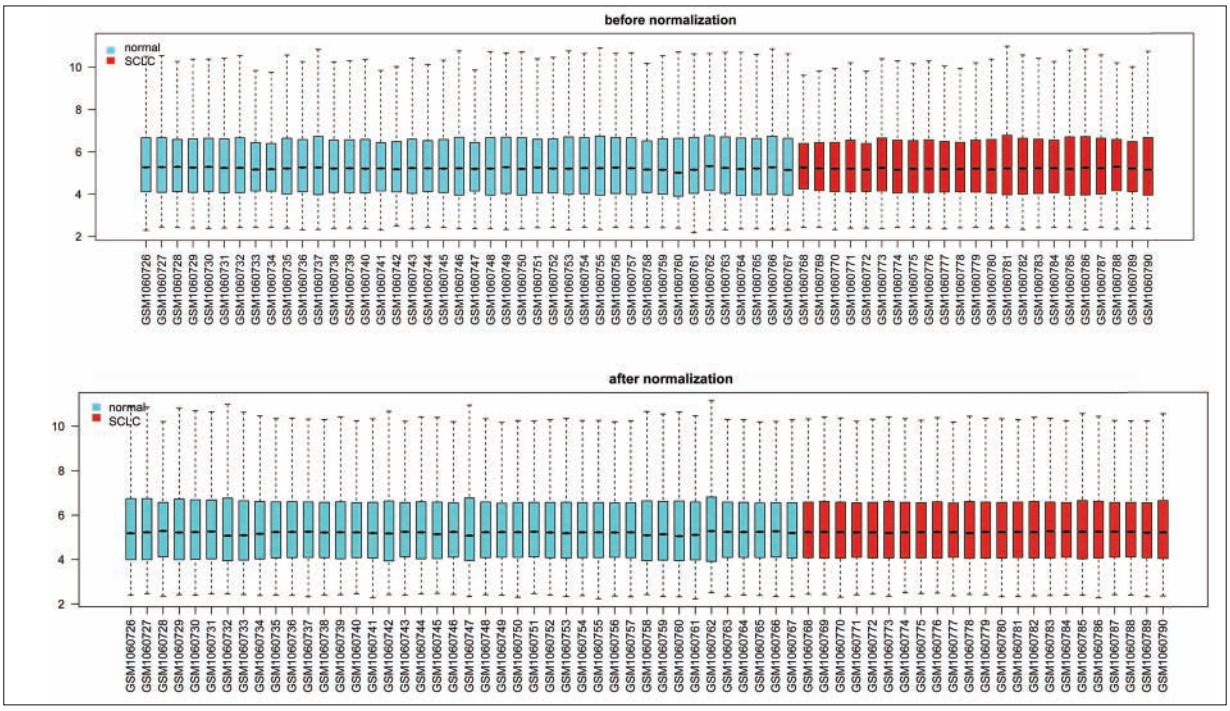

Figure 1. Box plots for gene expression data before (top) and after normalization (bottom). The medians (black lines) are almost at the same level, indicating a good performance of normalization.

Coexpression Network

A co-expression network was established in 1615 pairs of genes (Pearson correlation coefficient > 0.85, p value < 0.05). The network including 457 nodes was then visualized with Cytoscape, from which a functional module was identified with MCODE. According to the GO enrichment analysis using DAVID, genes in this functional module were enriched in cell cycle, mitosis as well as relevant biological processes (Table III).

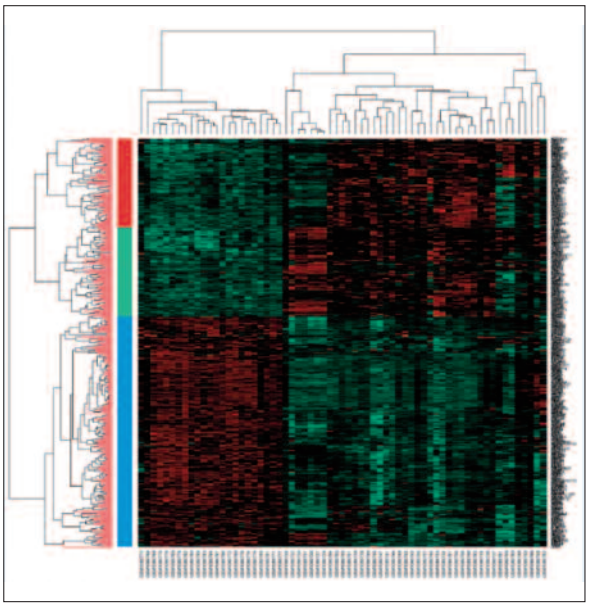

Figure 2. Heat map for expression of differentially expressed genes across the samples.

Gene Regulatory Network

The gene regulatory network was constructed with information from miRecords and UCSC. Degree was calculated for each node with package igraph of *R*. Top 10 nodes were selected out and then corresponding sub-networks were extracted from the whole network. Over-represented biological functions were revealed for each group of genes (Table IV). Similar to the above findings, these genes were enriched in cell cycle, cell proliferation and apoptosis (Figure 4).

A total of 43 miRNAs were included in the regulatory network and the corresponding subnetwork is in Figure 5. Top 5 biological processes over-represented in the target genes of these 43 miRNAs are shown in Table V, including regulation of cell proliferation, response to organic substance, regulation of RNA metabolic process, cell cycle and regulation of programmed cell death. Several processes were closely associated with tumorigenesis, suggesting important roles for these DEGs and miRNAs (Figure 5).

Table I. Significantly over-represented molecular functions in DEGs

GO term & molecular function	Count	*p* value
GO:0004857–enzyme inhibitor activity	11	4.57E-04
GO:0008009–chemokine activity	5	0.001230956
GO:0042379–chemokine receptor binding	5	0.001560885
GO:0019207–kinase regulator activity	6	0.002645966
GO:0004866–endopeptidase inhibitor activity	7	0.00375787
GO:0030414–peptidase inhibitor activity	7	0.004881871
GO:0019887–protein kinase regulator activity	5	0.009536542

Table II. Significantly over-represented KEGG pathways in DEGs.

KEGG term & pathway	Count	*p* value
hsa04062:Chemokine signaling pathway	8	0.010952703
hsa04270:Vascular smooth muscle contraction	6	0.015486143
hsa04060:Cytokine-cytokine receptor interaction	8	0.055432053
hsa04610:Complement and coagulation cascades	4	0.061912514

Table III. Top 10 biological processes over-represented in the genes from the module.

Go terms	Count	*p* value	FDR
GO:0007049–cell cycle	20	3.61E-15	4.93E-12
GO:0007067–mitosis	14	4.21E-15	5.68E-12
GO:0000280–nuclear division	14	4.21E-15	5.68E-12
GO:0000087–M phase of mitotic cell cycle	14	5.29E-15	7.17E-12
GO:0048285–organelle fission	14	7.12E-15	9.57E-12
GO:0022403–cell cycle phase	16	2.80E-14	3.77E-11
GO:0000279–M phase	15	2.81E-14	3.78E-11
GO:0022402–cell cycle process	17	1.35E-13	1.82E-10
GO:0000278–mitotic cell cycle	15	1.42E-13	1.91E-10
GO:0051301–cell division	14	1.86E-13	2.51E-10

Table IV. Top 10 nodes, sub-networks and biological functions.

Rank	Node	Degree	Main biological functions
1	MIA3	104	Cell cycle, DNA repair, cell division, DNA metabolic process
2	ARNT	104	Cell cycle, cell division, DNA damage stimulus, cell proliferation, cell death
3	SP1	97	Cell cycle, cytoskeleton organization, organelle fission
4	PSG1	97	Cell cycle, cell proliferation, cytoskeleton organization, organelle fission
5	DAND5	97	Cell cycle, cytoskeleton organization, cell proliferation, mitotic cell cycle, organelle fission
6	AHR	92	DNA metabolic process, cell proliferation, cell cycle, DNA replication
7	E2F3	86	Regulation of transcription, DNA-dependent, regulation of RNA metabolic process, regulation of transcription
8	PAX5	81	Regulation of programmed cell death, regulation of cell death, cell proliferation, cell cycle process, regulation of apoptosis
9	EGR3	80	Regulation of transcription from RNA polymerase II promoter, regulation of RNA metabolic process, regulation of transcription
10	EGR1	78	Regulation of RNA metabolic process, regulation of transcription, DNA-dependent

Count: the number of differentially expressed genes; FDR: false discovery rate obtained by Benjamini-Hochberg multiple correction; node, protein; degree, the number of interactions.

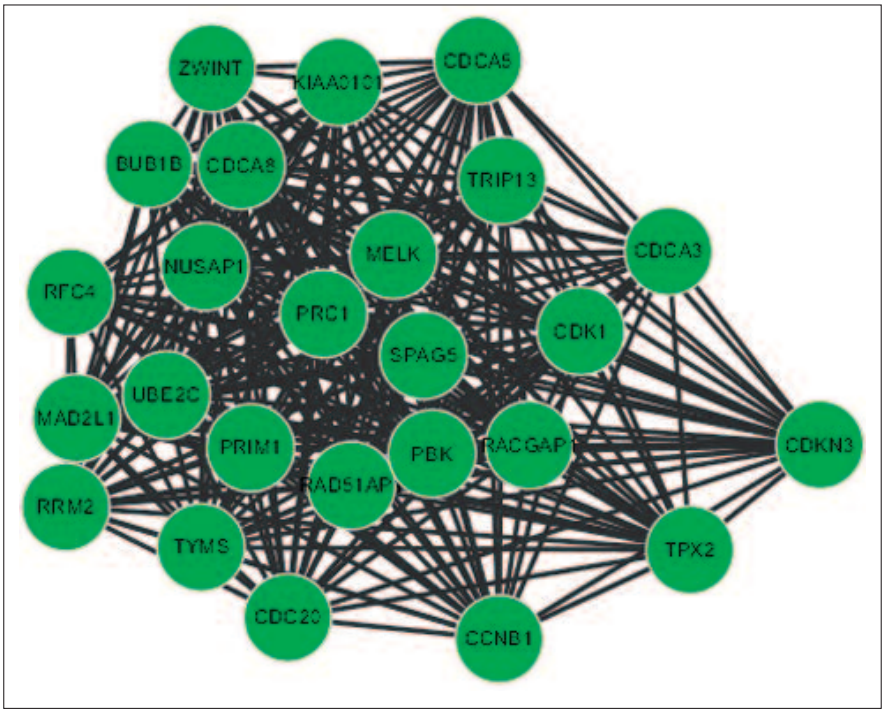

Figure 3. The functional module extracted from the whole co-expression network.

Discussion

In this work, a total of 457 DEGs were obtained in SCLC. Genes from the functional module of coexpression network were enriched in cell cycle and relevant pathways. In the sub-networks of gene regulatory network, cell cycle, cell proliferation and apoptosis were over-represented in these genes. Given the close relationships between these pathways and cancers, we considered our method was effective in mining key genes. Meanwhile, our findings offered a good guideline for future researches.

Most genes in the functional module were enriched in cell cycle and some have been linked to cancers. Cell division cycle associated 3 (CDCA3), part of the Skp1-cullin-F-box (SCF) ubiquitin ligase, refers to a trigger of mitotic entry and mediates destruction of the mitosis inhibitory kinase. Uchida et al[20] report that overexpression of CDCA3 promotes oral cancer progression by enhancing cell proliferation with prevention of G1 phase arrest. In prostate cancer, CDCA3 can be up-regulated by HoxB3 and, thus, promotes cancer cell progression[21]. The upregulation of maternal embryonic leucine zipper kinase (MELK) has been observed in breast cancer[22,23] and prostate cancer[24]. In breast cancer, dysregulated expression of MELK is associated with poor prognosis[22]. Cyclin-dependent kinase inhibitor 3 (CDKN3) can dephosphorylate CDK2 kinase and, thus, prevent the activation of CDK2 kinase. It's found to be up-regulated in breast cancer[25]. Overexpression of cell division cycle 20 (CDC20) is reported in several cancers, such as oral cancer[26]. Kidokoro et al[27] indicate it may be a good potential therapeutic target for a broad spectrum of human cancer.

In the gene regulatory network, most of the top 10 nodes were also related to cell cycle. Sp1 transcription factor (SP1) is a zinc finger transcription factor that involved in many cellular processes, including cell differentiation, cell growth and apoptosis. Dysregulation of p53/sp1 control leads to DNA methyltransferase-1 overexpression in lung cancer, which subsequently results in epigenetic alteration of multiple tumor suppressor genes and ultimately leads to lung tumorigenesis and poor prognosis[28]. It's also found to regulate expression of cancer-associated molecule CD147, which plays an important role in the invasion and metastasis of human lung cancer[29]. In accordance with its role in the development of cancer, Wang et al[30] suggest that it's a significant predictor of survival in human gastric cancer. Paired box 5 (PAX5) is a member of the PAX family of transcription factors. Kanteti et al[31] find that it's expressed in SCLC and positively regulates c-Met transcription. Loss of endogenous PAX5 significantly decreases the viability of

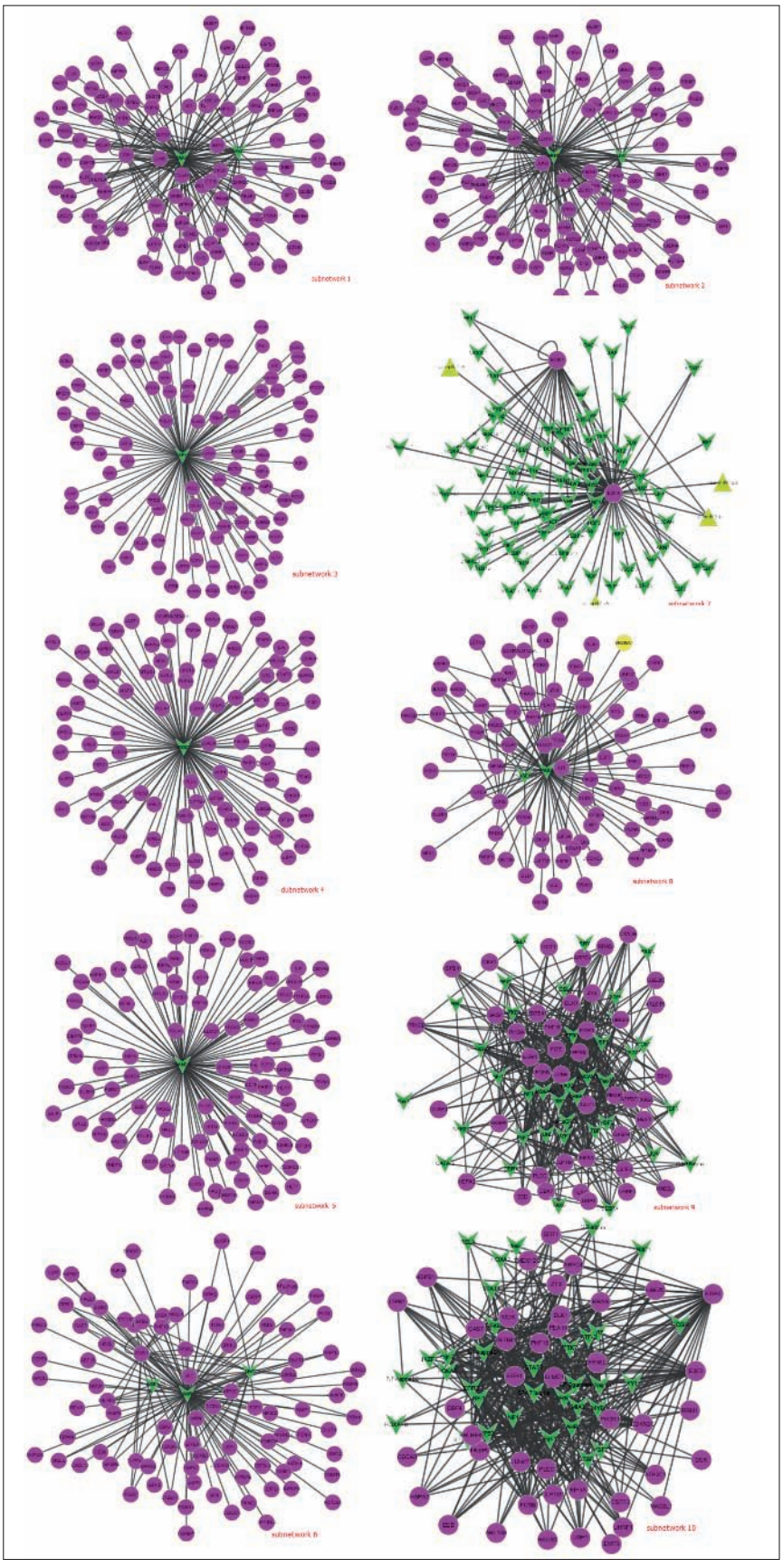

Figure 4. Ten sub-networks extracted from the whole regulatory network. Circles represent for differentially expressed genes, arrows for transcription factors and triangles for miRNAs. The lines indicate the interaction relationship.

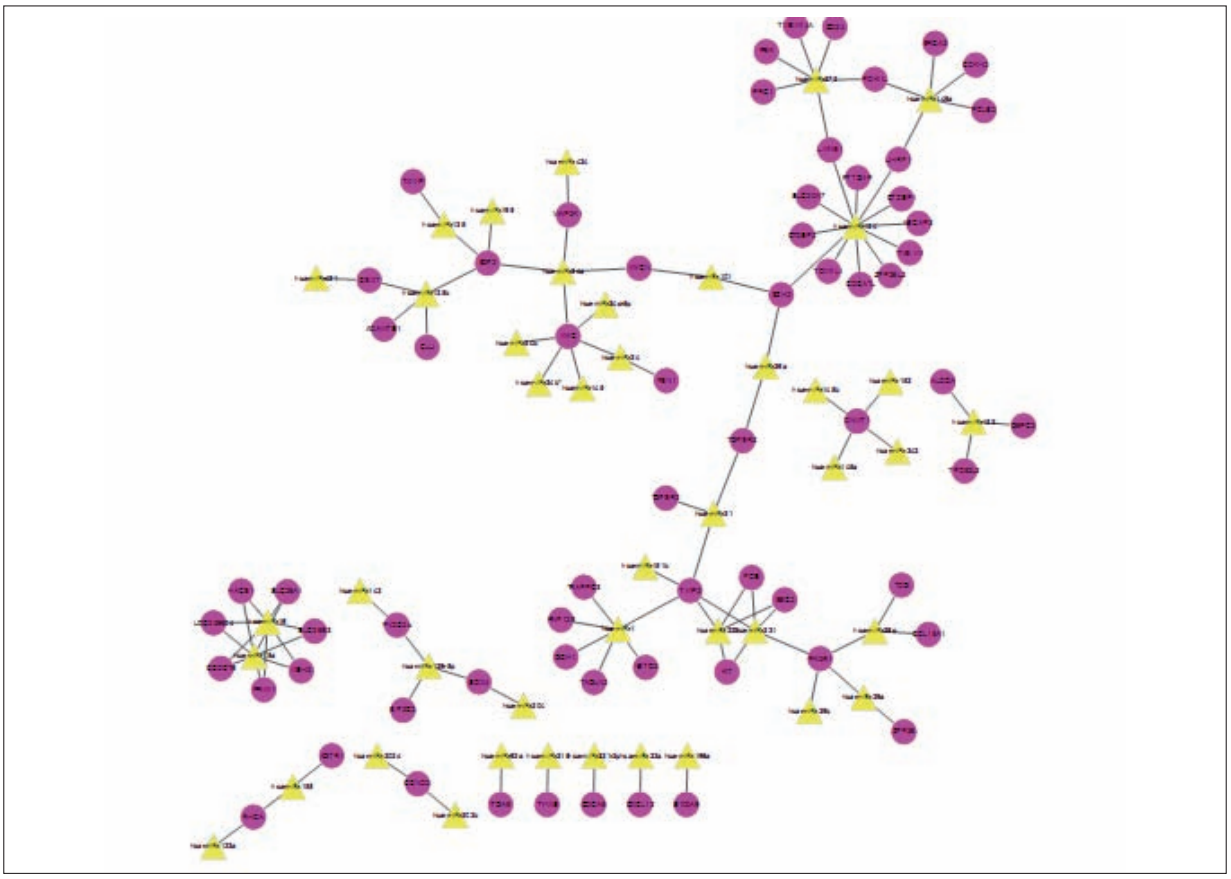

Figure 5. The sub-network comprised of miRNAs and target genes. Circles represent for differentially expressed genes and triangles for miRNAs.

SCLC cells, so it may be a potential target for therapy. E2F transcription factor 3 (E2F3) is a member of a small family of transcription factors that function through binding of DP interaction partner proteins[32]. Cooper et al[33] report that high expression level of nuclear E2F3 is found in almost all SCLCs. Besides, early growth response 1 (EGR1) and EGR3 have also been linked to lung cancer[34] and breast cancer[35].

MiRNAs are key regulators in the development of cancers. In present study, we attempted to discover important miRNAs using the miRNA-target network. c-Myc (MYC) plays an important role in the phenotypic conversion and malignant behavior of human lung cancer[36]. Amplification and/or high levels of expression of c-myc are observed in variant type SCLC lines[37]. Zajac-Kaye[38] points out that the overexpression of Myc and the deregulation of the pRB/E2F pathway promotes the G1 to S transition in parallel by activating cyclinE/cdk2 complexes in lung cancer cells. According to the network, it's regu-

Table V. Top 5 biological processes over-represented in miRNA-target gene network.

Term	Count	*p* value	FDR
GO:0042127–regulation of cell proliferation	15	3.35E-06	0.005452577
GO:0010033–response to organic substance	13	6.35E-06	0.010342655
GO:0051252–regulation of RNA metabolic process	13	1.51E-05	0.024561035
GO:0007049–cell cycle	12	2.05E-05	0.033437953
GO:0043067–regulation of programmed cell death	12	3.70E-05	0.06018597

Count: the number of differentially expressed genes; FDR: false discovery rate obtained by Benjamini-Hochberg multiple correction.

lated by several miR-34 members, which can induce apoptosis, cell-cycle arrest or senescence. In many tumor types the promoters of the miR-34a and the miR-34b/c genes are subject to inactivation by CpG methylation[39]. Gallardo et al[40] indicate that it's a prognostic marker of relapse in surgically resected non-small-cell lung cancer. Wiggins et al[41] even develop a miR34-based therapy for lung cancer. We also found many DEGs were regulated by miR-124, which is reported to be an epigenetically silenced tumor-suppressive miRNA in hepatocellular carcinoma[42]. Moreover, miR-15 and miR-16 are included in the network, and they are implicated in chronic lymphocytic leukemia[43] and prostate cancer[44]. Therefore, we believed more works on these miRNAs and target genes might bring in valuable findings.

Conclusions

Overall, we combined comparative analysis of transcriptome between SCLC and normal tissue with network analysis to mine important genes and pathway in the development of SCLC. Our findings could promote the understanding about this disease and also disclose potential targets for diagnostic and therapeutic usage.

Conflict of Interest

We certify that regarding this paper, no actual or potential conflicts of interests exist.

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