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Bioinformatics Analysis of Key Genes and miRNAs Associated with Small-cell Lung Cancer

Imteyaz Ahmad Khan, Srikant Sharma*

Abstract

Small-cell lung cancer (SCLC) is a type of lung cancer, accounting for approximately 15% of lung cancers. SCLC is significantly associated with early recurrence, metastasis, and poor prognosis, with a 2-year survival rate after therapy, which is <5%. The molecular mechanisms underlying the development of SCLC are not clear. In this study, we identified SCLC-specific biomarkers by evaluating the differential expression of mRNA and microRNA (miRNA) profiles in SCLC tissue compared with normal lung tissue. A non-coding RNA sequence dataset (GSE19945) and transcriptome sequencing dataset (GSE6044) were downloaded from the GEO database. We identified 445 DEGs (differentially expressed genes) and 128 DE-miRNAs (differentially expressed miRNAs) using the GEO2R tool of the GEO and R limma software package. Furthermore, using the KEGG database, we identified 15 enrichment pathways, mostly associated with DNA replication, cell cycle, and oocyte meiosis mismatch repair, and the GO function was considerably enriched for 26 items. To investigate the molecular processes of key signaling pathways and cellular activity in SCLC, we used Cytoscape software to construct protein-protein interaction (PPI) networks. Using miRNAWalk, we identified 598 target genes of the 1380 miRNAs and constructed miRNA target networks. In addition, we identified eighteen overlapping genes that are regulated by 28 different miRNAs. The identified hub genes are important because they may be used as biomarkers for prognosis, diagnosis, and therapeutic target for SCLC.

Keywords: Biomarkers, Gene expression omnibus, miRNA, Protein-protein interaction, Small-cell lung cancer

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Introduction

Lung cancer is one of the most common cancers associated with increased morbidity and mortality rates worldwide.[1] Small cell lung cancer (SCLC) is a type of lung cancer that is extremely invasive and has a high risk of early metastasis, poor prognosis, and a high rate of recurrence and mortality. It accounts for approximately 25% of all cancer deaths and nearly 15% of bronchogenic carcinomas.[2,3] Although improvement has been achieved over the past few decades, the prognosis for SCLC patients remains poor. Recently, patients with SCLC have moderate benefit from the current targeted molecular therapy and immunotherapy; therefore, effective therapies are urgently needed for this disease.[4] Patients with SCLC have a bad prognosis, with a 5-year survival rate of ~ 2% for patients with unresectable locally advanced or metastatic disease.[5]

SCLC patients are resistant to conventional anticancer treatment including chemotherapy and radiotherapy. In SCLC patients, the increased recurrence risk is associated with a higher mutation rate.[6] Sundaresan *et al*. demonstrated that RB1 and TP53 were the most commonly mutated genes in SCLC.[7] Aberrant MYC family, SOX2 and fibroblast growth factor receptor 1 activity, resulting from chromosomal translocations, gene amplification, or increased mRNA/protein stability, is found in patients with SCLC.[8]

MicroRNAs (miRNAs) are single-stranded non-coding RNAs (~22–24 nucleotide long) that negatively regulate gene expression by binding to target mRNA to inhibit their translation.[9] Accumulating evidence suggests that miRNAs are involved in the regulation of various biological processes, such as tumor cell proliferation, differentiation, migration, and apoptosis.[10] Many studies have shown that miRNAs are aberrantly expressed in various types of cancer including SCLC and can function as oncogenes or tumor suppressor genes.[11,12] The expression levels of miR-25 were significantly high in SCLC and act as an oncogenic regulator.[13] Decreased expression of miR-126 promotes angiogenesis in lung cancer through activation of vascular endothelial growth factor A (VEGF).[14] By microarray analysis, Cao *et al*. reported the down-regulation of miR-886-3p and miR-150 in SCLC.[15] Accumulating evidence indicates that expression levels of various miRNAs, including miR-126, -450, -485-5p, and miR-195 are down-regulated in SCLC tumors, indicating that these miRNAs may act as tumor suppressors.[16-20] Yu *et al*. demonstrated that the expression level of miR-92a-2 in plasma is upregulated and differentiated SCLC from normal subjects with a sensitivity, specificity, and AUC of 100%, 56%, and 0.76, respectively; thus miR-92a-2 in plasma could serve as a potential biomarker for the diagnosis of SCLC.[21]

Using the GEO database and bioinformatics analysis, various studies identify differently expressed miRNAs in SCLC. For example, Li *et al*. identified 56 differentially expressed miRNAs from the GEO database (GSE19945). Using a similar dataset (GSE19945), Mao *et al*. identified 49 upregulated and 86 down-regulated miRNAs in SCLC.[22,23] MiRNAs have been proposed as prognostic biomarkers in SCLC. For instance, the expression of miR-886-3p, miR-195, miR-7, miR-495, and miR-450 was significantly lower in SCLC. Further analysis revealed that these miRNAs were associated with poor overall survival.[15,17,19,24-26] In this study, we used two microarray

Department of Biotechnology, Shri Venkateshwara University, Gajraula, Uttar Pradesh, India

Corresponding Author: Dr. Srikant Sharma, Department of Biotechnology, Shri Venkateshwara University, Gajraula, Uttar Pradesh, India. E-mail: shribioinfo@gmail.com

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gene expression datasets from the Gene Expression Omnibus (GEO) database and various bioinformatics analysis methods were applied to identify differentially expressed microRNAs (miRNAs) and genes in SCLC and to investigate the molecular processes associated with SCLC development.

Methods

Source of the Microarray Data

The gene expression profile datasets were retrieved from the public Gene Expression Omnibus (GEO) repository. To examine gene expression across human SCLC samples, the following two SCLC datasets were downloaded from GEO: GSE19945 and GSE6044. The miRNA expression profiles from the GSE19945 dataset included 43 SCLC surgical samples and eight normal lung tissue samples. The Agilent Human 0.6K miRNA Microarray G4471A platform was used to determine miRNA expression. The mRNA GSE6044 dataset includes samples from nine SCLC samples and five normal lung tissue, which were analyzed using the GPL201 platform.

Screening of Candidate DE-miRNAs and DE-genes

Normalization of raw data for GSE6044 was performed using the Affy package of Bioconductor in the R statistical software environment. The analysis of differential expression of miRNAs and genes was conducted using the LIMMA package in the Bioconductor project. To screen the DE-miRNAs and DE-genes between SCLC tumor tissue and non-tumor lung tissue, we used the GEO2R analytical tool from the GEO database. For the detection of DE-miRNAs and DE-genes, a *P*-value cutoff of <0.05 was used in combination with a $|\log2FC| > 1$.

Functional Annotation and Pathway Enrichment Analysis

In this study, ShinyGO v0.741 (http://bioinformatics.sdstate.edu/go/) bioinformatics software was used for the GO analysis of enriched genes. In addition, based on the results of the microarray dataset, DE-genes were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) molecular pathway analysis using ShinyGO v0.741.

PPI Network of the Differentially Expressed Genes

Using the Cytoscape program, a protein-protein interaction (PPI) network of DEGs was created (version: 3.6.0). An interaction score greater than 0.4 (indicating medium confidence) was considered to identify statistically significant PPIs. Then, the association between the genes was analyzed using the Network Analyzer plug-in of the Cytoscape package. Finally, the hub genes were confirmed by the Cytoscape plugin molecular complex detection (MCODE). The thresholds for screening were: Kappa score (K-core) = 2, degree cutoff = 2, node score cutoff fixed to 0.2, and maximum depth fixed to 100.

Prediction and Analysis of Target Genes of Differentially Expressed miRNAs

The candidate target genes of the DEmiRNAs were identified from online software including miRTarBase, TargetScan, miRDB, miRTarBase, and miRWalk and the miRNA target exists in the five databases concurrently were included in the study. FunRich is a stand-alone software tool that was used to find overlapping genes. The Cytoscape software was used to generate and visualize a regulatory network of miRNA-gene.

Results

Identification of DE-miRNAs and DE-genes

When comparing patients with SCLC versus normal lung tissue samples, we identified 451 DEGs in the GSE6044 dataset, of which 205 were down-regulated and 246 were upregulated. The *P*-value of 20 DEGs is shown in Table 1. In total, 134 DE-miRNAs were detected in SCLC tissue samples, of which the expression of 86 was high and 48 expression was low. The 20 DE-miRNAs are shown in Table 2.

Table 1: The 20 superior genes differentially expressed in SCLC.

Gene name	*Adjusted P-value*	*Log FC*
TMSB15	2.22×10^{-3}	4.6
MEST	3.10×10^{-3}	3.78
UCHL1	3.28×10^{-2}	3.08
TYMS	3.31×10^{-2}	2.88
TOP2A	3.51×10^{-3}	2.75
CDKN2A	1.65×10^{-4}	2.33
MCM6	3.12×10^{-4}	2.32
SOX4	3.13×10^{-4}	2.24
ACAA2	1.97×10^{-4}	2.12
MARCKSL1	2.26×10^{-3}	2.1
ID4	3.24×10^{-3}	1.99
IL17RB	1.23×10^{-4}	1.49
HDAC2	3.55×10^{-4}	1.39
CYP2J2	3.16×10^{-4}	-1.13
PDLIM4	3.17×10^{-4}	-1.41
FABP6	1.66×10^{-4}	-1.78
CES1	3.24×10^{-3}	-2.51
TSPAN8	3.34×10^{-4}	-2.94
CYP4B1	3.44×10^{-4}	-3.34
GSTA1	3.54×10^{-4}	-4.26

Table 2: Top 20 miRNAs identified as the most differentially expressed in SCLC

miRNAs	*Adjusted P-value*	*Log FC*
miR-7	4.88×10^{-9}	5.67
miR-183	7.78×10^{-13}	4.57
miR-130b	1.89×10^{-13}	4.22
miR-301b	2.78×10^{-11}	3.91
miR-96	1.55×10^{-13}	3.82
miR-182	2.45×10^{-9}	3.72
miR-18a	3.54×10^{-9}	3.35
miR-26a	7.67×10^{-8}	-1.82
miR-26b	2.67×10^{-7}	-1.92
miR-140-5p	3.55×10^{-7}	-1.99
miR-498	4.57×10^{-7}	-2.22
miR-140-3p	3.22×10^{-7}	-2.4
miR-638	3.65×10^{-9}	-2.93
miR-126	1.45×10^{-11}	-3.77
miR-338-3p	2.54×10^{-7}	-3.79
miR-145	6.35×10^{-7}	-3.88
miR-486-5p	2.25×10^{-7}	-4.04
miR-451	3.34×10^{-8}	-4.22
miR-144	5.56×10^{-7}	-4.61
miR-1	9.33×10^{-9}	-4.64

GO Analysis of DEGs

The identified DEGs were analyzed for enriched biological processes using the ShinyGO v0.741 bioinformatics software. As a result, a total of 418 DEGs were related to 390 GO terms. Thirty significant enriched functional clusters were identified at a 1% false discovery rate and *P*-value <0.05. Thirty GO terms were found to be considerably enriched, including 10 in a cellular component, 10 in a molecular function, and 10 in a biological process. Specifically, the top 10 GO terms in the biological process were cell cycle, cell cycle process, mitotic cell cycle, mitotic cell cycle process, cell division, mitotic nuclear division, chromosome segregation, nuclear chromosome segregation, sister chromatid segregation, and mitotic sister chromatid segregation [Figure 1a]; the top 10 significant GO terms in cellular component were spindle, nuclear lumen, cytoskeleton, chromosome, microtubule cytoskeleton, chromosomal region, centromeric region, nuclear chromosome, and mitotic spindle [Figure 1b]; and the top 10 GO terms in molecular function were adenyl nucleotide binding, ATP binding, adenyl ribonucleotide binding, anion binding, nucleotide binding, purine nucleotide binding, ribonucleotide binding, purine ribonucleotide binding, purine ribonucleotide triphosphate binding, and ATPase activity [Figure 1c].

Pathway Analysis of DEGs

We used the ShinyGO v0.741 online software to calculate the KEGG pathway on 440 DEGs. A total of 220 differentially expressed genes were mapped to KEGG software and pathways with $P < 0.05$ were considered as enriched KEGG pathways. A total of 20 functional clusters of the DEGs were identified, such as cell cycle (15 genes), DNA replication (10 genes), oocyte meiosis (10 genes), progesterone-mediated oocyte maturation (five genes), human T-cell leukemia virus one infection (five genes), pathways in cancer, pyrimidine metabolism, and nucleotide excision repair [Figure 2].

PPI Network Construction and Module Analysis

The STRING database and Cytoscape software were used to create a PPI network containing 440 differentially expressed genes. Next, MCODE was used to select key modules derived from the PPI network. We obtain a new network with a complex interaction after removing partially connected genes nodes. The resulting network consisted of 412 nodes and 29.38 edges. Of 412 total nodes, 20 differentially expressed genes with a larger degree of nodes were selected based on Cytoscape analysis [Figure 3]. We detected 85 hub genes with a degree value of >45 using the MCODE plugin in Cytoscape. Based on the highest degree

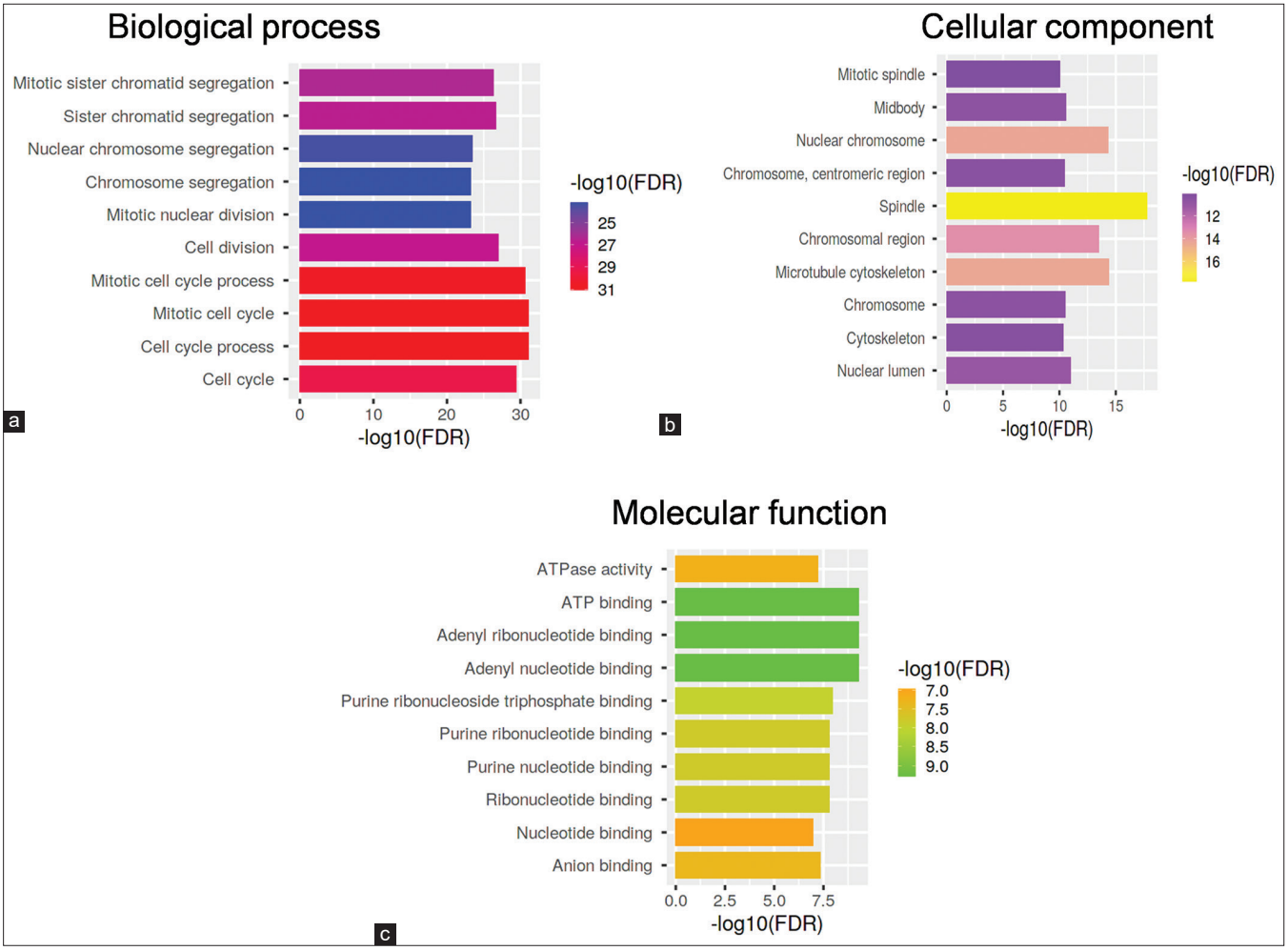

Figure 1: GO analysis of differentially expressed genes. (a) Biological process, (b) cellular component, and (c) molecular function

of nodes, we identified two key modules. In addition, these two key modules were further analyzed for functional and pathway enrichment using ShinyGO v0.741 online software. Module 1 was constructed with 54 nodes and 1.232 edges, and Module 2 was constructed with 17 nodes and 46 edges [Figure 4]. Module 1 consists of DNA replication, mismatch repair, cell cycle, nucleotide excision repair, and oocyte meiosis. Module 2 was associated with complement and coagulation cascades, pyrimidine metabolism, legionellosis, viral protein interaction, chemokine, and NF-kappa B signaling pathway.

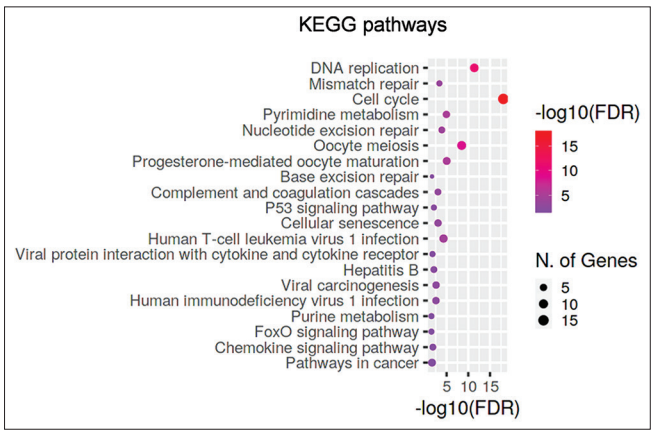

Figure 2: KEGG analysis of differentially expressed genes

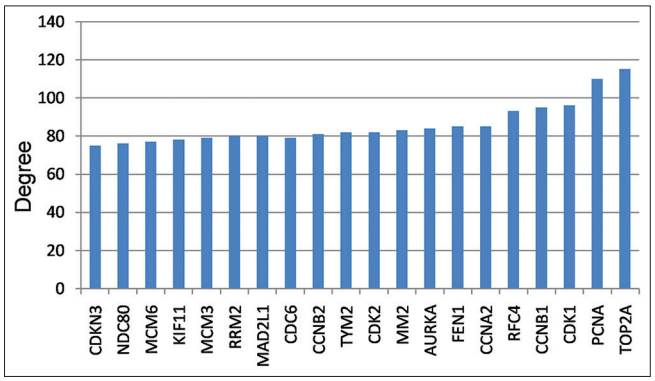

Figure 3: Top 20 hub genes in SCLC

Construction of the miRNA-Target Gene Regulatory Network

Using miRNA target prediction software, we identified 1387 regulatory modules containing 552 target genes of the DEmiRNAs. Furthermore, 14 genes were identified as overlapping genes between target genes of the DE-miRNAs and DE-genes. A total of 14 overlapping genes [SOX11, FGF9, polypyrimidine tract binding protein 2 (PTBP2), lamin B1 (LMNB1), nucleolar protein 4 (NOL 4), HLF transcription factor(HLF), RAD 21 cohesin complex component (RAD21), CELSR3, CDS1, EPB41L4B, SOX4, ALDH1A1, and fibulin 1 (FBLN1) were regulated by 22 different miRNAs [Figure 5a]. The 18 significantly deregulated genes and 28 DEmiRNAs are shown in a heat-map [Figure 6a and b]. In addition, three target genes (RAD 21, KIF11, and MSH2) of the 14 overlapping genes were regulated by five different miRNAs (miR-21, -25, -92a, -181a, and -101 [Figure 5b]. The expression levels of these three genes were upregulated in lung cancer patients as compared to normal control [Figure 7].

Discussion

Small cell lung carcinoma is characterized by highly invasive and metastatic capacity. Patients with advanced disease have a median survival time of only 10–15 months, and the 2-year survival rate is below 5%.[27] The high mortality rate is attributed to the fact that the majority of patients are diagnosed at a late stage.[28] The progression of SCLC is not well defined at the molecular level. Thus, there is an urgent need to identify potential biomarkers for the early detection of SCLC. Microarray is a well-established, cost-effective, and high-throughput technology for the identification of potential biomarkers. Recently, dysregulation of various miRNAs has been shown to have critical roles in the occurrence, metastasis, and recurrence of SCLC. In this study, bioinformatics tools were used to identify the differentially expressed genes and miRNAs of SCLC.

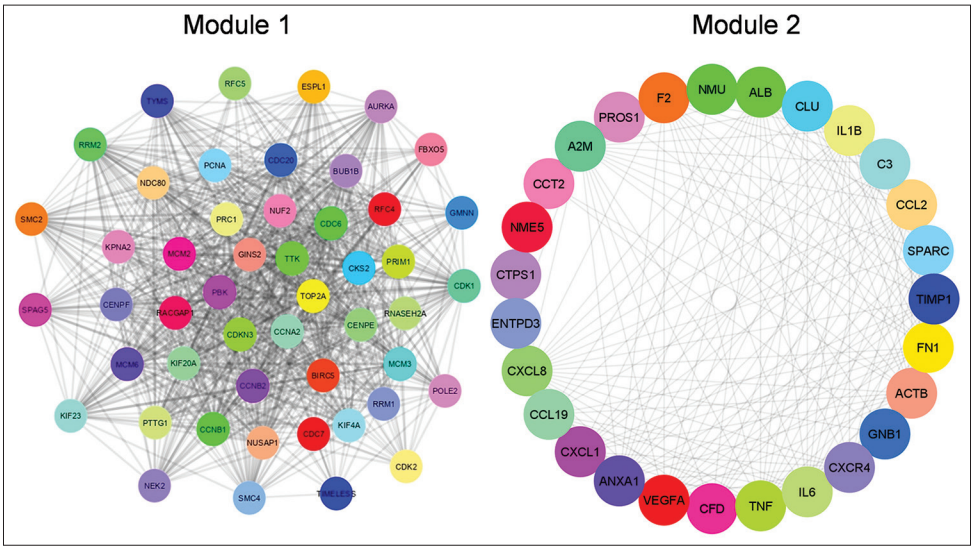

Figure 4: Modules 1 and 2 were derived using the MCODE plug-in in Cytoscape

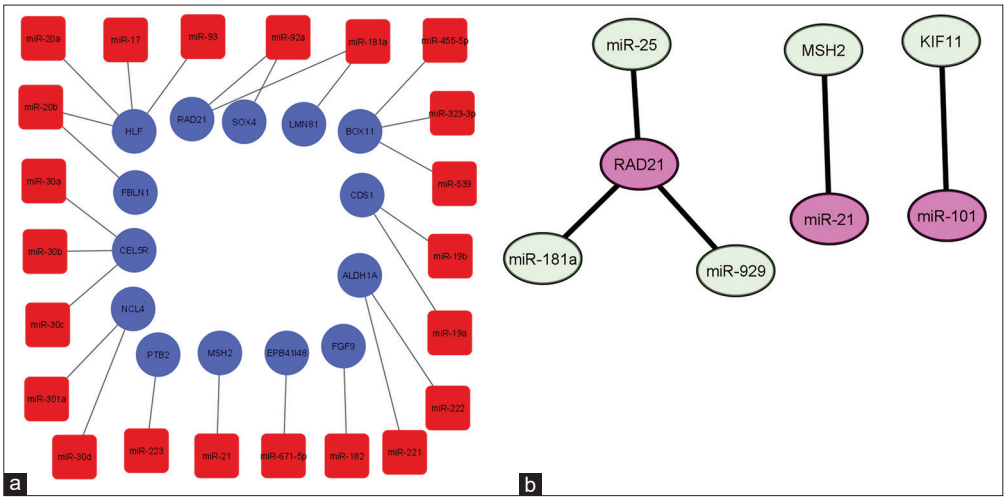

Figure 5: (a) Protein-protein interaction network of 18 target genes, regulated by 22 DE-miRNAs. (b) Three hub genes were regulated by five different miRNAs

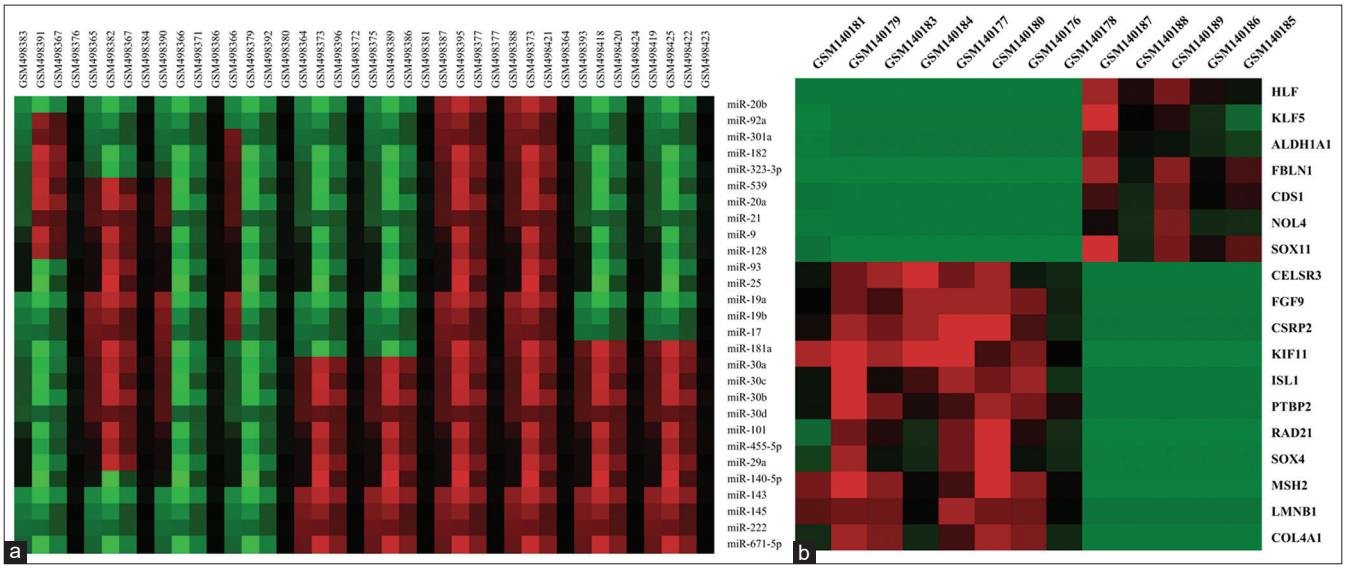

Figure 6: Heat-maps of differentially expressed miRNAs and genes. (a) Heat-map showing the expression intensity of 28 DEmiRNAs. (b) Heat-map showing the expression profiles of 18 DEGs. The red color represents a high value and the green color represents a low value

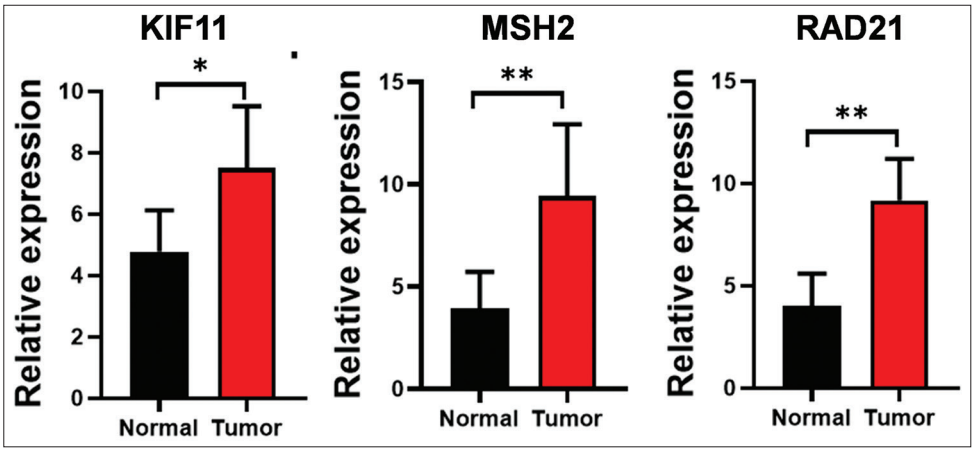

Figure 7: Expression levels of three genes are significantly different between SCLC tumor tissue and adjacent normal control lung tissues

In the GSE6044 dataset, we identified 440 differentially expressed genes. The KEGG and GO enrichment analysis of the DEGs showed that the genes enriched in many signaling pathways, such as p53 signaling pathway, DNA replication, mismatch repair, cell cycle, nucleotide excision repair, and oocyte meiosis were mostly upregulated; however, mismatch repair, nucleotide excision repair, and "base excision repair" were underexpressed. Recent research shows that p53 expression is altered in non-small cell lung cancer patients and involved in DNA damage response and apoptotic pathways.[7] During cell mitosis, the overexpression of kinases leads to carcinogenesis by promoting tumor cell proliferation.[29] Altered regulation of key genes such as TFIIH core complex helicase subunit, ERCC excision repair 3, and 8-oxoguanine DNA glycosylase involved in DNA damage repair pathways, such as DNA mismatch repair and nucleotide excision repair.[30,31]

Furthermore, the innermost 10 hub nodes are the core network identified from the PPI network, which is considered oncogenes and can promote the progression of SCLC. Recent research showed that TOP2A is overexpressed in SCLC.[32] In this study, microarray dataset analysis identified altered miRNA expression profiles in SCLC. In the dataset (GSE19945), 78 miRNAs were over-expressed and 45 miRNAs were under-expressed, in which two miRNAs (miR-1290 and miR-1) was the most significantly regulated in different types of cancer; miR-1290 inhibita MT1G and promotes the progression of NSCLC.[33] Furthermore, the integration of the miRNA-gene regulatory networks and differentially expressed genes disclosed that there are 14 overlapping genes regulated by 22 miRNAs. Gene ontology (GO) enrichment analysis of GSE6044 revealed that three target genes (RAD21, KIF11, and MSH2) were identified as the hub genes and are regulated by five microRNAs (miR-181a, -101, -21, -92a, and -25). Among them, three genes RAD21, KIF11, and MSH2 showed the highest degree, suggesting that these three genes might be involved in small cell lung cancer development. The expression of miR-101 was down-regulated in different types of cancer including lung, colon, and breast cancer.[34] MiR-21 was found to be upregulated in SCLC in the GSE6044 and GSE19945 datasets, and it was found to be strongly linked with the advanced stage of cancer.

In addition, it has been shown that three miRNAs (miR-181a, -92a, and -25) regulate the expression of RAD21. The RAD21 gene involved in DNA double-strand break repair and meiotic recombination and aberrant expression of RAD21 are shown in many types of cancer including lung, breast, and rectal cancer.[35-39] Consistent with the previous research, our results showed that miR-92a is upregulated in SCLC. Moreover, upregulation of miR-92a has been reported to be associated with chemotherapeutic resistance and poor survival of SCLC patients. Therefore, miR-92a could be considered a reliable biomarker for patient survival and chemotherapy resistance.[40] MiR-181a targets KLF6, thereby promoting macrophage-associated cell migration and invasion.[41] Mir-25 has been shown to be important for NSCLC cell growth, adhesion, and invasion *in vitro*.[13]

Conclusion

In the present work, we measured gene and miRNA expression in SCLC tissue samples and non-tumor lung tissue samples using the gene and miRNA expression datasets from the GEO database. We identified DEGs and miRNAs in SCLC patients. Furthermore, bioinformatics analysis on DEGs identified significant signaling pathways. In addition, three differentially expressed genes including RAD21, KIF11, and MSH2 identified as hub genes. Hence, these hub genes can be used as biomarkers for SCLC. However, further research is required to support our findings.

Acknowledgments

Not applicable.

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Ethics Approval and Consent to Participate

Not applicable.

Patient Consent for Publication

Not applicable.

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