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A Bioinformatics Approach to Identify Potential Biomarkers in Non-Small Cell Lung Cancer

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ABSTRACT

Non-small cell lung cancer (NSCLC) is responsible for about 85% of lung cancer types. The molecular mechanism of NSCLC has not been completely elucidated. The current study aims to explore the potential biomarkers and targets for NSCLC. The gene and miRNA expression profiles were downloaded from the Gene Expression Omnibus (GEO) database. The differentially expressed miRNAs (DEMs) and genes (DEGs) were determined and used for further analysis. Functional enrichment analyses were applied using the DAVID program. Moreover, the miRNA targets were predicted based on the miRWalk. The STRING software was constructed protein-protein interaction (PPI) and miRNA-mRNA networks and Cytoscape software was used to visualize PPI and miRNA-mRNA networks and to identify hub genes. As a result of bioinformatic analysis, a total of 159 DEGs and 22 DEMs were identified and DEGs were mostly enriched in the terms like ECM receptor interaction, signal transduction and leukocyte transendothelial migration. The identified hub genes were IL6, COL1A1, CLDN5, CAV1, CDH5, SPP1, GNG11, PPBP, CXCL2 and CXCR2. A total of 239 target genes were identified as potential mRNAs. The most significantly identified genes and miRNAs could serve as potential biomarkers for NSCLC.

Keywords: Non-small cell lung cancer, miRNA, mRNA, Bioinformatics analysis.

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Introduction

Lung cancer is the crucial cause of cancer-related deaths in worldwide. Diagnosis and treatment of lung cancer are of critical importance to improve survival of cancer patients, especially earlier stages [1]. In particular, smokers are more likely to develop lung cancer and therefore smoking is one of the most important causes in lung cancer. However, several researchers have been reported that non-smokers also get lung cancer. The incidence of lung cancer in men is higher than in women [2]. Lung cancer is histopathologically divided into two groups as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) and NSCLC is accounting for 85 % of lung cancers with two major subtypes: adenocarcinoma (AD) and squamous cell carcinoma (SCC) [3]. Despite significant improvements in NSCLC treatments, the overall survival rate (20%) and the 5-year survival rate (16%) is still very low for NSCLC patients [4]. The lack of effective tools and methods are major problems in early detection of lung cancer. Therefore, it is urgent to screen new potential molecular markers and methods for understanding of the molecular mechanisms underlying lung cancer.

MicroRNAs (miRNAs) are small (19–25 nt long), single stranded and highly conserved non-coding RNA molecules that are a critical regulator in the gene expression. miRNAs regulate posttranscriptionally gene expression through binding to complementary sequences in target mRNAs. They suppress gene expression by translation inhibition or degradation of mRNA. MicroRNAs recognize with 3' region (3'-UTR) of their target mRNAs which are generally complementary nucleotides by 5' region of miRNAs. These small RNAs are involved in several types of cellular activities like apoptosis, cell proliferation or differentiation, development and progression [6]. MicroRNAs have been discovered to be connected with different cancer types. Especially, the changes in miRNA expression profiles play an important role in cancer pathogenicity. Comparison of miRNA expression profiles between tumor and healthy tissues is a crucial approach for an early detection, diagnosis, prognostic and therapy.

Microarray data analysis and bioinformatics methods have been widely applied to identify new molecular targets using gene expression data in many cancer types. Cai et al. [7] identified 8 miRNAs and 211 common genes using gene expression datasets via bioinformatic analysis for NSCLC. As a result of the bioinformatic analysis, they reported that identified miRNAs and genes can be a major regulator in the occurrence and development of NSCLC. In a different study, the differential expression levels of mRNA and miRNA between cancer tissues and healthy tissues were compared and the key miRNA-gene pairs were identified. The related genes were presented as potential biomarkers and as potential drug targets [8].

It was determined to differentially expressed genes (DEGs) and miRNAs (DEMs) between NSCLC tissues and healthy lung tissues using bioinformatic analyses in the current study. Subsequently, functional enrichment analysis, PPI network, prediction of miRNA targets and

survival analysis were applied and miRNA–mRNA regulatory network was finally constructed to identified genes in NSCLC. The aim of this study was to identified key miRNAs and genes as potential biomarkers in NSCLC and to contribute to the clarification of the molecular mechanism in NSCLC.

Materials and Methods

Microarray Data

Two genes (GSE18842 and GSE19804) and two miRNAs (GSE19945 and GSE102286) expression profiles were obtained from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) database. The GSE18842 datasets contain 46 NSCLC and 45 healthy lung tissues. GSE19804 datasets include 60 NSCLC and 60 healthy lung tissues. In the present study, it was randomly selected 9 tumor and 8 healthy tissues for the GSE19945 datasets and 40 tumor and 40 healthy tissues for the GSE102286 datasets.

Data Processing

In the microarray datasets, GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r) was used to identify differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) between NSCLC and healthy lung tissues [9]. GEO2R includes a huge number of experimental datasets and an adjusted P-value (adj. P) is applied to correct false-positive rates. The adjusted p value cutoff was set as P<0.05 and llogFCl>2 for DEGs selection and llogFCl>1 for DEMs selection. Following, the overlapping DEGs and DEMs in these datasets were analyzed and drawn Venn diagrams using Venny online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/)

Gene Ontology (GO) and Pathway Analysis

Database for Annotation Visualization and Integrated Discovery (DAVID) software (https://david.ncifcrf.gov/ Version 6.8) was used to explore the potential functions of the overlapping DEGs and DEMs for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The cut-off value was set as P<0.05.

Establishment of PPI Network

A PPI network was constructed for DEGs using The Search Tool of the Retrieval of Interacting Genes (STRING) database (https://string-db.org/cgi/) and was visualized by Cytoscape tool (www.cytoscape.org) with cut-off criterion of confidience score >0.4. Moreover, DEGs were analyzed to determine hub genes with the significant degree of nodes (> 5% degree) by Cytohubba algoritm.

Survival Analysis

Differentially expressed genes were entered into the Kaplan Meier (KM) plotter in order to assess the effect of these genes on survival in the lung cancer (http://kmplot.com/analysis/index.php?p=background). A 95% confidence interval for the hazard ratio and log-rank P value were determined and showed on the KM plotter tool.

Prediction of miRNA targets

Target genes of the overlapped DEMs among miRNA datasets were predicted from the miRWalk database (http://mirwalk.umm.uni-heidelberg.de/search_mirnas Version 3.0) [10]. TargetScan, miRDB and miRTarBase algorithms in MiRWalk database were performed to increase the accuracy in miRNA target prediction. Putative mRNAs were selected from target genes only recognized by all three databases.

Construction of miRNA–mRNA Regulatory Network

The regulatory network was constructed using a combination of overlapped miRNAs and putative mRNAs and visualized using by Cytoscape software (version 3.8.2).

Results

Identification of DEGs and DEMs

A total of 1022 and 265 genes showed significantly deregulation in the GSE18842 and GSE19804 microarray datasets, respectively. Among them, 607 and 213 upregulated genes for GSE18842 and 415 and 52 downregulated genes for GSE19804 were identified in NSCLC tissues. In addition, a total of 130 upregulated and 29 downregulated genes were determined common in both microarray datasets (Figure 1).

Figure 1. Venn Diagrams of identified DEGs in NSCLC. (a) upregulated (b) downregulated DEGs.

According to the results of GEO2R analysis, it was determined 118 DEMs from GSE19945 (65 upregulated and 53 downregulated DEMs) and 51 DEMs from GSE102286 (19 upregulated and 32 downregulated DEMs). A total of 12 upregulated DEMs (hsa-miR-30d-5p, hsa-miR-30a-5p, hsa-miR-1-3p, hsa-miR-145-5p, hsa-miR-144-5p, hsa-miR-30b-5p, hsa-miR-126-5p, hsa-miR-218-5p, hsa-miR-551b, hsa-miR-223-5p, hsa-miR-451 and hsa-miR-195-5p) and 10 downregulated DEMs (hsa-miR-93-5p, hsa-miR-130b-5p, hsa-miR-193b-5p, hsa-miR-183-5p, hsa-miR-196b, hsa-miR-9-5p, hsa-miR-21-5p, hsa-miR-96-5p, hsa-miR-18a and hsa-miR-135b) were found to overlap in both miRNA datasets (Figure 2).

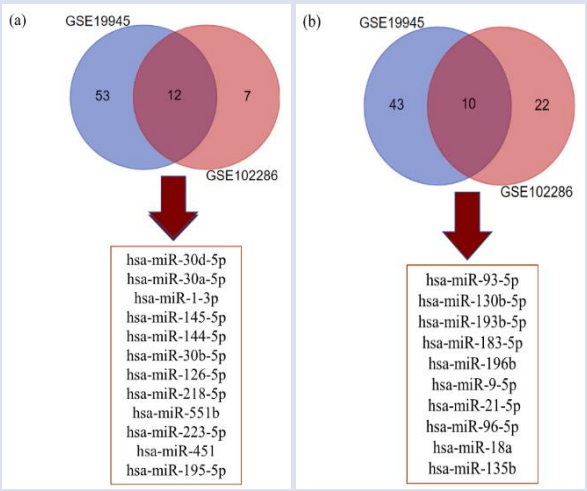

Figure 2. Venn Diagrams of identified DEMs in NSCLC. (a) upregulated (b) downregulated DEMs.

Functional Enrichment Analysis

The GO ontology contains three terms as biological process (BP), cellular component (CC) and molecular function (MF) [8]. GO classification and KEGG pathway enrichment analysis were conducted to investigate the function of the DEGs with the DAVID software. According the results of the GO analysis, 88 % (110/179) of the upregulated DEGs were mainly enriched in signal transduction, inflammatory response and negative regulation of transcription from RNA polymerase II promoter. In total, 94.4 % (118) and 81.6 % (102) upregulated DEGs were importantly enriched in cellular components and molecular functions, respectively. In addition, 96.6 % (28) involved genes for biological processes, 96.6 % (28) involved genes for cellular components and 93.1 % (27) involved genes for molecular functions were enriched in total of 36 downregulated DEGs. The KEGG pathway analysis showed that several disease-related pathways including leukocyte transendothelial migration, adrenergic signaling in cardiomyocytes, focal adhesion and ECM receptor interaction played an essential role in NSCLC pathogenesis. The some of the most significant GO categories and the KEGG pathways analysis are showed in Table 1.

Table 1. Some of the most significant DEGs in NSCLC

Category	Term	Description	Gene Counts	P-value
Up regulated gene				
GO:0007165	GOTERM_BP_DIRECT	signal transduction	10	3,40E-01
GO:0000122	GOTERM_BP_DIRECT	negative regulation of transcription from RNA polymerase II promoter	9	9,60E-02
GO:0006954	GOTERM_BP_DIRECT	inflammatory response	8	1,20E-02
GO:0043547	GOTERM_BP_DIRECT	positive regulation of GTPase activity	8	1,20E-02
GO:0045944	GOTERM_BP_DIRECT	positive regulation of transcription from RNA polymerase II promoter	8	7,50E-02
GO:0001525	GOTERM_BP_DIRECT	angiogenesis	8	4,50E-01
GO:0016021	GOTERM_CC_DIRECT	integral component of membrane	44	2,90E-02
GO:0005886	GOTERM_CC_DIRECT	plasma membrane	40	5,10E-03
GO:0005576	GOTERM_CC_DIRECT	extracellular region	31	6,60E-08
GO:0070062	GOTERM_CC_DIRECT	extracellular exosome	26	5,30E-02
GO:0005515	GOTERM_MF_DIRECT	protein binding	64	2,30E-02
GO:0008201	GOTERM_MF_DIRECT	heparin binding	9	5,20E-06
GO:0038023	GOTERM_MF_DIRECT	signaling receptor activity	6	9,80E-03
hsa05144	KEGG_PATHWAY	Malaria	5	5,50E-04
hsa05143	KEGG_PATHWAY	Leukocyte transendothelial migration	4	6,10E-02
hsa04670	KEGG_PATHWAY	Adrenergic signaling in cardiomyocytes	4	9,40E-02
hsa04261	KEGG_PATHWAY	Cell adhesion molecules (CAMs)	4	1,00E-01
Down regulated gene				
GO:0030574	GOTERM_BP_DIRECT	collagen catabolic process	6	5,2E-8
GO:0030199	GOTERM_BP_DIRECT	collagen fibril organization	4	3,3E-5
GO:0022617	GOTERM_BP_DIRECT	extracellular matrix disassembly	4	2,4E-4
GO:0005576	GOTERM_CC_DIRECT	extracellular region	11	5,8E-5
GO:0005615	GOTERM_CC_DIRECT	extracellular space	9	5,4E-4
GO:0031012	GOTERM_CC_DIRECT	proteinaceous extracellular matrix	6	4,1E-5
GO:0005581	GOTERM_CC_DIRECT	collagen trimer	5	9,8E-6
GO:0005509	GOTERM_MF_DIRECT	calcium ion binding	5	2,3E-2
GO:0004252	GOTERM_MF_DIRECT	serine type endopeptidase activity	4	6,9E-3
GO:0004222	GOTERM_MF_DIRECT	metalloendopeptidase activity	3	1,3E-2
hsa04512	KEGG_PATHWAY	ECM receptor interaction	4	5,1E-4
hsa04510	KEGG_PATHWAY	Focal adhesion	4	6,1E-3
hsa04151	KEGG_PATHWAY	PI3K Akt signaling pathway	4	2,5E-2

PPI Network

The two PPI networks were constructed for significantly up regulated DEGs (Fig ure 3a) and down regulated DEGs (Figure 3b). Nine DEGs with the significant degree of nodes (> 5% degree) were determined by Cytohubba algoritm with ranked method “degree”. These DEGs were identified as hub genes including IL6, COL1A1, CLDN5, CAV1, CDH5, SPP1, GNG11, PPBP, CXCL2 and CXCR2.

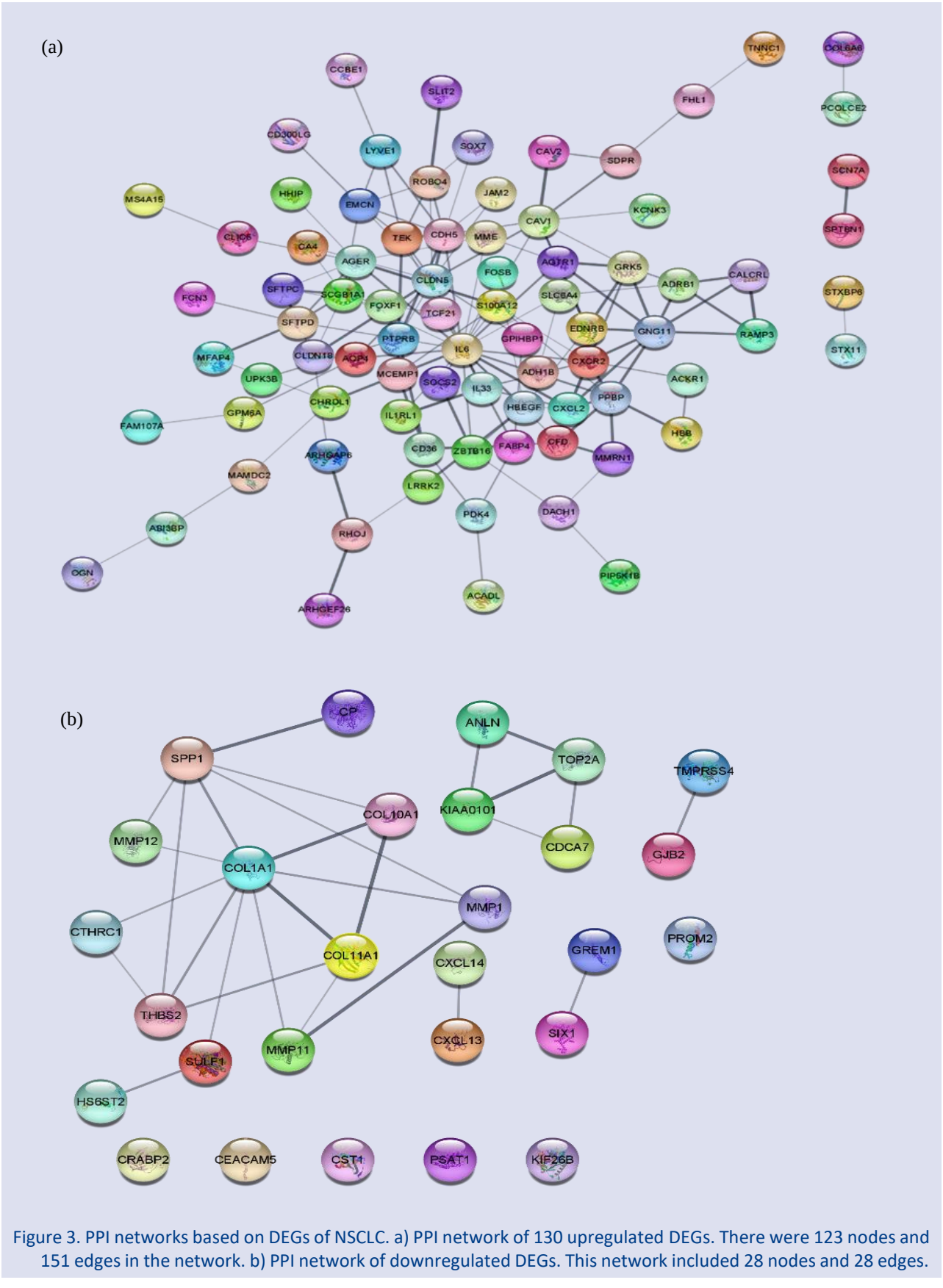

Figure 3. PPI networks based on DEGs of NSCLC. a) PPI network of 130 upregulated DEGs. There were 123 nodes and 151 edges in the network. b) PPI network of downregulated DEGs. This network included 28 nodes and 28 edges.

Survival Analysis

The identified hub genes were analyzed to evaluate the prognostic value by Kaplan-Meier plotter. The low and high expression level of the hub genes were used to define features of overall survival in NSCLC patients. Survival curves were plotted for the NSCLC patients (n=1,925) with adenocarcinoma (n=865) and squamous cell carcinoma (675). According to results of KM analysis, the high expression levels of IL6, PPBP, CXCR2, SPP1 and COL1A1 hub genes predicted worse prognosis of patients with NSCLC (P<0,005). In addition, low expression levels of CLDN5, CAV1, CDH5, GNG11 and CXCL2 were associated with poor overall survival (P<0,005) (Figure 4).

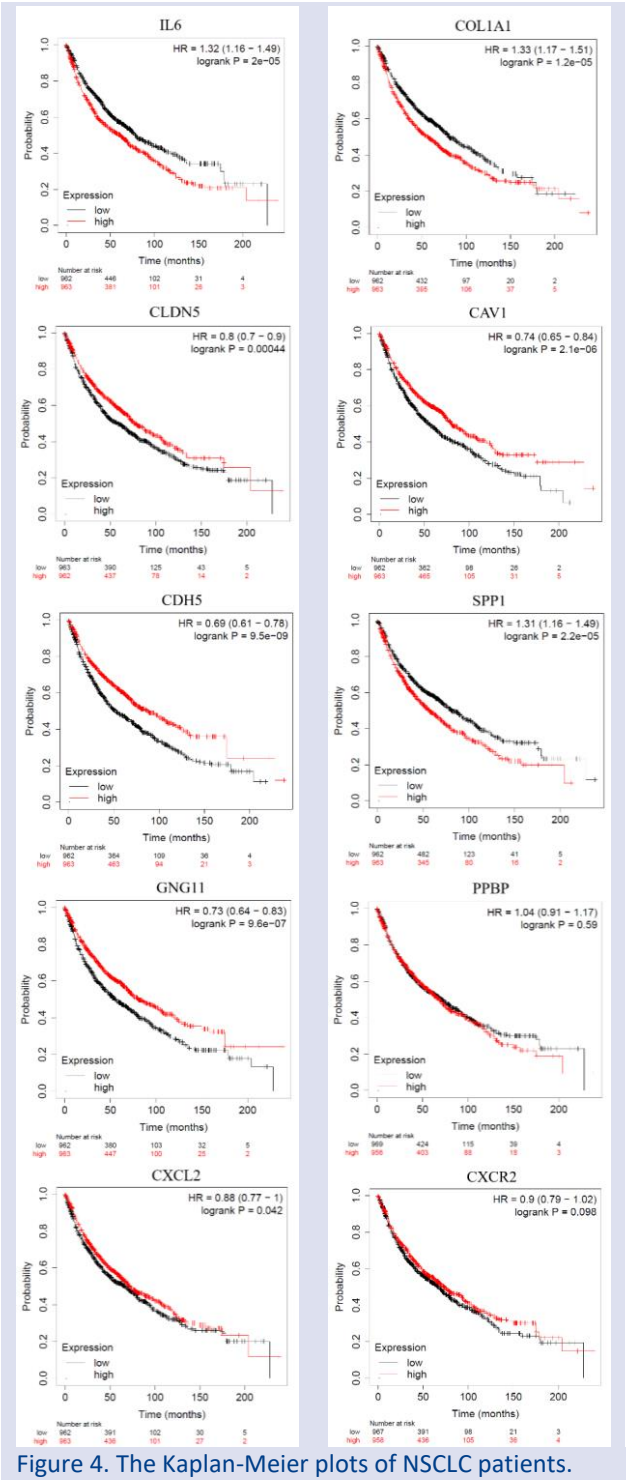

Figure 4. The Kaplan-Meier plots of NSCLC patients.

Prediction of miRNA Target

Target genes of the overlapped DEMs were predicted using TargetScan, miRDB and miRTarBase databases in MiRWalk. A total of 239 target genes were determined as potential mRNAs. When compared target genes with DEGs, hsa-miR-1-3p and hsa-miR-145-5p upregulated DEMs presented the same expression trend compared to their predicted targets in NSCLC. In this study, hsa-miR-1-3p was the most upregulated miRNAs and predicted to target the upregulated ANKRD29 gene. Similarly, hsa-miR-145-5p was upregulated and predicted to target the upregulated two genes including MYRF and ERG genes. However, hsa-miR-195-5p (an upregulated DEMs) and hsa-miR-93-5p (a downregulated DEMs) showed the opposite expression trend compared to their predicted targets in NSCLC. For example, hsa-miR-195-5p was predicted to target one upregulated (TGFBR3 gene) and two downregulated genes (CHEK1 and RASEF genes). Also, hsa-miR-93-5p was predicted to target one upregulated (RAB11FIP1 gene) and one downregulated gene (RACGAP1 gene).

miRNA-mRNA Regulatory Network

miRNA-mRNA Regulatory Network showed that a mRNA may be targeted by two or more miRNA. PPP1R12A gene had interactions with hsa-miR-30b-5p and hsa-miR-96-5p (Supplementary Figure 1). In addition, REST (hsa-miR-93-5p and hsa-miR-9-5p), YOD1 (hsa-miR-93-5p and hsa-miR-30b-5p), ATXN1 (hsa-miR-96-5p and hsa-miR-93-5p), GID4 (hsa-miR-21-5p and hsa-miR-93-5p) and PURA genes (hsa-miR-195-5p and hsa-miR-93-5p) had interactions with two different miRNAs.

Discussions

With the introduction to molecular targeted therapy of cancer, miRNAs and genes have emerged as important molecules used in the diagnosis and treatment for cancer patients. These molecules have widely used to find new approaches and treatments for NSCLC. The proliferation of NSCLC is slower than SCLC, however, it is generally diagnosed at the later stages of disease [4]. The 5-year survival rate of NSCLC patients is reported to be less than 20% [11]. Therefore, understanding the molecular mechanisms of NSCLC progression is very important. Also, identifying the potential biomarkers and therapeutic targets is a critical factor for early diagnosis and treatment of NSCLC.

Considering many studies conducted in recent years, it is seen that bioinformatics approaches are a new trend especially in cancer research. Large biological data obtained through experimental analyzes are used in bioinformatics approaches. These data sets are analyzed and transformed into meaningful information with computational methods. Large amounts of biological data stored in public databases such as Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) are invaluable resources for researchers to use in bioinformatics analysis. By comparing the gene expression

profiles between normal and tumor tissues with bioinformatics approaches, a lot of information can be obtained about tumor progression and development. Moreover, with these applications, new potential biomarkers can be discovered for the diagnosis and treatment of the diseases. These potential biomarkers can be guiding especially in clinical applications. In many recent studies on lung cancer, new molecular markers have been identified among miRNAs, mRNAs and circRNAs using bioinformatics approaches and their interactions with each other have been investigated. In a recent study, differentially expressed circRNAs (DECs), miRNAs (DEMs), and genes (DEGs) between NSCLC tumor and healthy lung tissues are examined via bioinformatics analysis. A circRNA–miRNA–mRNA network was conducted using DECs, DEMs, and DEGs for NSCLC. Furthermore, the researchers reported that identified molecule networks (hsa_circ_0001947/hsa-miR-637/RRM2 and hsa_circ_0072305/hsa-miR-127-5p/DTL) have a critical role in the occurrence and development of NSCLC [7]. Bioinformatic approaches have been used to explore the genes involved in the development of SCLC and their molecular mechanisms. Gene and miRNA expression profiles were compared between lung cancer tissues and normal lung tissues using transcription sequencing data and non-coding RNA data stored in geodatabase and differentially expressed miRNAs and genes were determined. In addition, some hub genes such as KIF11, MSH2 and RAD21, which were predicted to be regulated by miRNAs, were identified and it was estimated that these hub genes could be a potential biomarker in the diagnosis and treatment of SCLC [8]. The findings are very promising and guide for further research. Similarly, in the present study, it was aimed to identify new molecular markers in lung cancer using bioinformatics approaches. The obtained data have a promising potential for the diagnosis and treatment of NSCLC.

Functional enrichment analysis of the DEGs showed that these genes have an important relationship with lung cancer and these findings are validated with literature researches. For example, caveolin 1, CAV1 gene product, interferes cell growth of lung cancer by interacting with phospho-ERK1/2, estrogen receptor and progestin receptor [12]. Similarly, many studies have been showed that this protein is an important factor for the metastasis, proliferation, cell migration and invasion of lung cancer [13-15]. Interleukin-6 (IL-6) has a strong positive association with C-reactive protein and is a prognostic factor for NSCLC patients [16]. As a result of GO enrichment analysis for DEGs, it is determined to many significant terms including integral component of membrane, extracellular region, cell adhesion, protein binding and integral component of membrane. GNG11, FABP4 and IL33 genes act as tumor suppressors in lung adenocarcinoma similar to the current study. GNG11 is a lipid-anchored protein and FABP4 have a role in fatty acids metabolism. IL33 is responsible for many biological processes as a cytokine and is known to be an important factor in cancer progression [17].

In the present study, the results of KEGG pathways analysis indicate that the many DEGs were frequently clustered in cell adhesion molecules, leukocyte transendothelial migration, protein digestion and absorption, focal adhesion and ECM-receptor interaction pathway. Similar to the current study, Lu et al. [18] developed and validated a novel gene expression signature in NSCLC patients. They have showed that many pathways (e.g. leukocyte transendothelial migration and cell adhesion) were associated with recurrence free survival. In addition, determination of CAMs expression was proposed as biomarker for cancer therapy.

In survival analysis applied for identified hub genes, the high expression of IL6, PBP, IL8RB, SPP1 and COL1A1 genes was correlated with worse overall survival and low mRNA expression of CLDN5, CAV1, CDH5, GNG11 and CXCL2 were correlated with poor overall survival for NSCLC patients. Consistent with these results, the low expression of CDH5 associates with poor prognosis in NSCLC [19]. In addition, the key genes were recognized by constructing the PPI network for NSCLC patients. IL6, COL1A1, CLDN5, CAV1, CDH5, SPP1, GNG11, PPBP, CXCL2 and CXCR2 genes were identified as hub genes.

The 22 DEMs and target genes of them were recognized for NSCLC in the current study. Among the miRNAs, hsa-miR-1-3p found to be one of the most miRNAs and predicted to target the ANKRD29 gene. miR-1-3p play a role as a remarkable tumor suppressor in different types of cancers such as lung cancer [20], prostate cancer [21] and colorectal cancer [22]. Among the miRNAs, the expression of miR-9 was considerably lower than other downregulated miRNAs, while miR-451a was markedly upregulated in NSCLC tissues. In a different a study, it is reported that upregulation of miR-451a increases the sensitivity to radiotherapy in A549 cells by enhancing of apoptosis [23]. Additionally, Kim et al. [24] showed that downregulation of miR-9 associates with poor prognosis in colorectal cancer. These findings are consistent with our findings. However, Piao et al. [19] reported that the miR-451a was identified as tumor suppressors, while miR-9 was oncogenes.

According to the present study, hsa-miR-195-5p targets TGFBR3, CHEK1 and RASEF genes, while EGLN3, RACGAP1 and RAB11FIP1 genes are identified as target genes of hsa-miR-93-5p in NSCLC tissues. TGFBR3 (Transforming growth factor beta receptor type III) has shown to be a key molecule in progression and metastasis of cancer as a suppressor for breast, prostate, ovarian, pancreatic, renal and NSCLC [25]. Overexpression of CHEK1 gene (Cell cycle checkpoint kinase 1) has the potential to be an important factor in the development of human malignant tumors [26]. Oshita et al. [27] have reported that the higher expression of RASEF was associated with the poorer prognosis and suggested that RASEF is a new molecular marker and target for lung cancer patients.

In the current study, the bioinformatic analyses are only performed by comparison of NSCLC and health lung tissues. The 10 most significantly hub genes and 22 miRNAs could be considered as potential prognostic biomarkers and therapeutic targets. Moreover, bioinformatic analysis may present different ways to explore function of miRNAs and mRNAs and to clarify the underlying mechanisms of NSCLC. Therefore, the results of the current study may considerably provide benefit to NSCLC patients in clinical studies.

Conclusions

In conclusions, mRNA and miRNA expression profiles between NSCLC and healthy lung tissues were analyzed using microarray datasets downloaded from the GEO database and differentially expressed genes and miRNAs were identified in NSCLC. Pathogenesis and therapeutic targets of NSCLC were explored using bioinformatics approaches. The identified genes, miRNAs and miRNA targets have significant potential for the occurrence and development of NSCLC. These target molecules could serve as prognostic biomarkers and therapeutic targets. However, these findings need to be supported by further studies.

Conflicts of interest

The author declare that they have no conflict of interests

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