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Investigation of key miRNAs and potential mechanisms in non-small cell lung cancer development from chronic obstructive pulmonary disease

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Abstract. Lung cancer (LC) is the prominent cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) represents approximately 85% of all diagnosed LC cases. It is stated that LC and chronic obstructive pulmonary disease (COPD) are directly linked at a molecular genetics level. Early diagnosis of LC is important for individuals affected by COPD. This study aims to construct a molecular network to discover molecules in NSCLC development from COPD. We downloaded the expression profiles of COPD patients from Gene Expression Omnibus database. The Database Annotation for Visualization and Integrated Discovery tool was utilized for enrichment analysis; STRING and Cytoscape were used for network construction. 15 hub genes were detected among 1517 differentially expressed genes (DEGs). Additionally, 20 differentially expressed miRNAswere identified from five datasets. We constructed miRNA-mRNA regulatory network between the groups of overlapping predicted target genes/DEGs and miRNAs that contained miRNA-mRNA pairs. UALCAN and OncomiR web-portals were used to validate hub genes and miRNAs in NSCLC. JUN, IL6, CD4 and hsa-miR-497-5p, hsa-miR-130b-5p were verified in both lung adenocarcinomas and lung squamous cell carcinomas. This study presents potential biomarkers and mechanisms underlying NSCLC development from COPD that would be targeted for early intervention.

Key words: COPD — NSCLC — Functional enrichment analysis — Protein-protein interaction — miRNA-mRNA regulatory network

Abbreviations: BP, biological processes; COPD, chronic obstructive pulmonary disease; DAVID, Database annotation for visualization and integrated discovery; DEGs, differentially expressed genes; DEMs, differentially expressed miRNAs; GEO, Gene Expression Omnibus; GO, gene ontology; IRGs, immune-response related genes; KEGG, Kyoto encyclopedia of genes and genomes; LC, lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinomas; MCODE, molecular complex detection; NCBI, National center for biotechnology information; NSCLC, non-small cell lung cancer; PPI, protein-protein interaction; TCGA, The cancer genome atlas.

Introduction

Lung cancer (LC) is one of the leading causes of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all diagnosed LCs and there are two main histological subtypes: lung adenocarcinoma (LUAD) and lung squamous cell carcinomas (LUSC) (Team NLSTR 2011). It is provided that LC and chronic obstructive pulmonary disease (COPD) are directly associated with molecular genetics level (Young and Hopkins 2011) and it was shown that 40–70% of patients with LC have COPD (Anthonisen et al. 2005). Therefore, early diagnosis of LC is important for COPD patients and there is a need for clinical biomarkers that reveal the risk of increased cancer development.

MicroRNAs (miRNAs) are small non-coding oligonucleotides capable of negatively regulating expression of mRNAs by inhibiting protein translation (Ma and Weinberg

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2008). miRNAs participate in various biological processes and depending on their miRNA profiles; tumor cells can be distinguished from normal cells (Calin and Croce 2006). In addition, tumor cells can release the miRNAs in circulation in such a way that they can be detected in body fluids (Mitchell et al. 2008). Increased studies confirm the potential role of miRNAs as disease-specific biomarkers, which is promising for diagnostic, preventive, or therapeutic targets (Arroyo et al. 2011; Chan et al. 2013). Due to miRNAs high stability, strong specificity, high sensitivity, and detection easily in blood, they have been implicated in a variety of lung diseases (Tzortzaki et al. 2013).

Numerous public resources have been installed such as Gene Expression Omnibus (GEO) of National Center for Biotechnology Information (NCBI) with the improvement of high-throughput microarray and sequencing technology. Bioinformatics analyses based on the GEO present valuable data for searching biomarkers in several diseases (Wang et al. 2016; Manchia et al. 2017). However, to the best of our knowledge, there is no study available, that have been reported on the bioinformatics-based identification of potential biomarkers concerning NSCLC development from COPD.

In our study, we aimed to find key genes and miRNAs from GEO datasets that could play an important role in the development of NSCLC from COPD patients by establishing gene ontology (GO), pathway enrichment, protein-protein interaction (PPI) network and miRNA-gene network. UALCAN and OncomiR web-portals were utilized to validate the determined hub genes and miRNAs in NSCLC.

Materials and Methods

Selection and inclusion criteria of studies

We examined the GEO database (https://www.ncbi.nlm.nih.gov/geo/) by using the following keywords: "chronic obstructive pulmonary disease OR COPD" (study keyword), "Homo sapiens" (organism), "Expression profiling by array" (study type). Besides, available datasets for related miRNAs were searched using the following keywords; "chronic obstructive pulmonary disease OR COPD", "miRNA", "Homo sapiens". The inclusion criteria were peripheral blood samples of COPD patients compared with control, and sufficient information to perform the analysis. Then, six datasets were collected for analysis. The bioinformatics workflow with the followed steps is depicted in Fig. 1.

Microarray data and data processing

One mRNA and five miRNA expression profiles were downloaded from the GEO database. The included miRNA expression profiles were GSE31568, GSE61741, GSE70080, GSE24709 and GSE102915, which consist of 24 COPD/70 control, 47 COPD/94 control, 16 COPD/16 control, 24 COPD/19 control, and 6 COPD/6 control samples respectively and one included mRNA expression profile GSE94916 dataset consists of six COPD and six control samples. We compared two groups of samples in every dataset to determine differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs). The comparison was performed by limma Rpackage based online program, GEO2R (http://

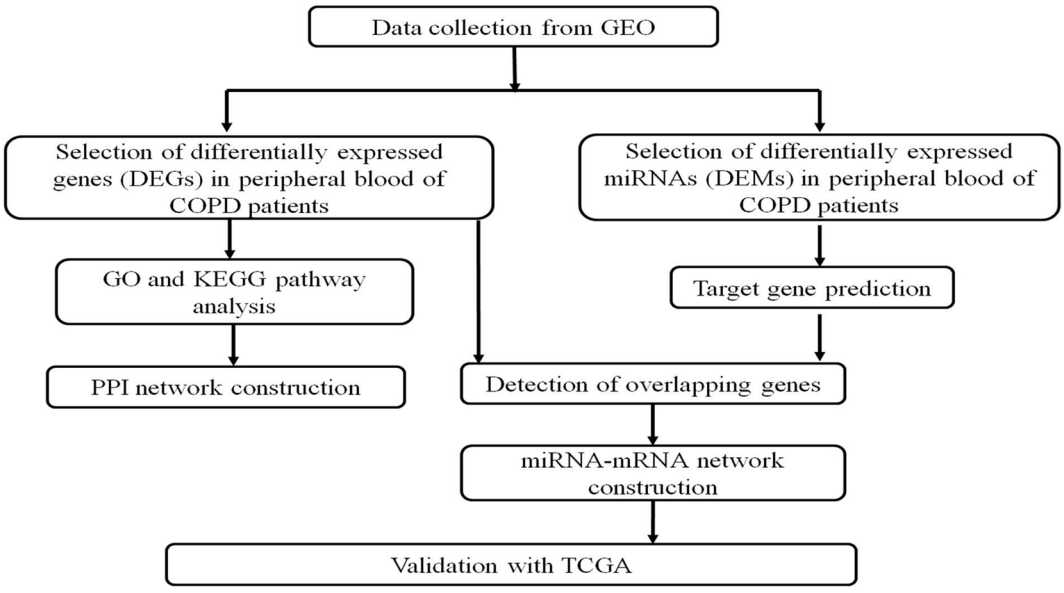

Figure 1. Flow chart of the data processing and analysis.

www.ncbi.nlm.nih.gov/geo/geo2r/) according to the cut-off criteria $p < 0.05$ and fold change > 2. According to these criteria, DEMs detected in two or more of the five datasets were considered as significant.

Functional enrichment analysis

The Database Annotation for Visualization and Integrated Discovery (DAVID) is a program that exhibits functional annotation of the huge amount of genes obtained from several genomic resources (Huang et al. 2008). We used the DAVID database to implement GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on significant DEGs. The species was limited to "Homo sapiens" and the $p < 0.05$ cut-off was considered as significant.

PPI network construction and analysis of modules

The STRING database (http://string-db.org/) is online software that aims to present a crucial estimation and combination of protein-protein interactions, including physical and functional relationships (Szklarczyk et al. 2019). Cytoscape is open-source software, used for the visual investigation of biomedical networks comprised of protein, gene, and other types of interactions (Shannon et al. 2003). The DEGs were plotted to STRING with a confidence score > 0.7 as a cut-off criterion to estimate the PPI information, and then interactions were visualized with Cytoscape. The genes with a node degree ≥ 25 were considered as hub genes. Next, the Molecular Complex Detection (MCODE) plug-in was used to screen modules of hub genes (Bader and Hogue 2003). Modules with MCODE scores > 5 and number of nodes > 10 were selected as significant. Moreover, the functional and pathway enrichment analyses of DEGs in mostly significant module were conducted by DAVID.

miRNA-gene network construction

All miRNA names were standardized according to miRBase v22 by using miRNAme Converter available in Bioconductor R package (Haunsberger et al. 2016). Then, MultiMiR package (http://multimir.ucdenver.edu/) includes 14 databases which were used to predict targets of miRNAs with the criterion of primary score listed in top 35 (Ru et al. 2014). Genes obtained by minimum three predicted algorithms were chosen for the following analysis. We subsequently selected the overlapping genes of significant DEMs-mRNA and the DEGs data. The miRNA-mRNA networks were visualized by Cytoscape. The combination of miRNAs and genes with degree ≥ 3 in miRNA-gene network and hub genes detected by PPI&Cytoscape were considered as potential key genes.

Validation analysis

The identified potential key genes from GEO datasets were searched and verified in LUSC and LUAD based on The Cancer Genome Atlas (TCGA) datasets. UALCAN is an interactive web resource used for analyzing cancer transcriptome data which allows users to define biomarkers and provides publication-quality graphs and plots illustrating gene expression (Chandrashekar et al. 2017). $p < 0.05$ cut-off was considered as significance criterion. Also, OncomiR WashU Pan-Cancer miRNome Atlas was used for miRNA validation which is freely available to all users in which aligned and normalized miRNA-seq and RNA-seq data were obtained from TCGA. It enables the statistical analysis of DEMs for each cancer type (Wong et al. 2018).

Results

Identification of DEGs and DEMs

Gene and miRNA expression data were obtained from the GEO database. Following the GEO2R analysis, 1517 DEGs were extracted from the expression profile dataset GSE94916 of which were 31 upregulated and 1486 downregulated ($p < 0.05$ and $|\log FC| > 2.0$). Besides, the miRNA profile datasets were analyzed to screen DEMs in COPD using the GEO2R tool. Totally 20 DEMs (seven upregulated and 13 downregulated) were identified which appeared in at least two datasets and matched the cut-off criteria ($p < 0.05$ and $|\log FC| > 2.0$). They all showed consistent expression patterns in different datasets.

Functional enrichment analysis

For the DEGs, we listed top five statistically significant enriched GO terms on biological processes (BP), and KEGG pathways ($p < 0.05$) (Fig. 2).

PPI network and identification of hub genes

The DEGs were used to set the PPI network by STRING, which composed of 1158 nodes and 1789 edges. Subsequently, we analyzed the STRING results using Cytoscape and 15 genes in the PPI network were identified as hub genes (degree ≥ 25). These hub genes included *TP53, JUN, IL6, LCK, PLCG1, CD3G, CD3D, IL4, CD4, CCR7, CD3E, ZAP70, CTLA4, GNB5,* and *CD28*. To further understand the interaction of 15 hub genes, the PPI network of them was constructed by STRING, which composed of 15 nodes and 48 edges (Fig. 3). We identified four clusters from the PPI network using MCODE. According to their degree of importance, the most important cluster that consists of

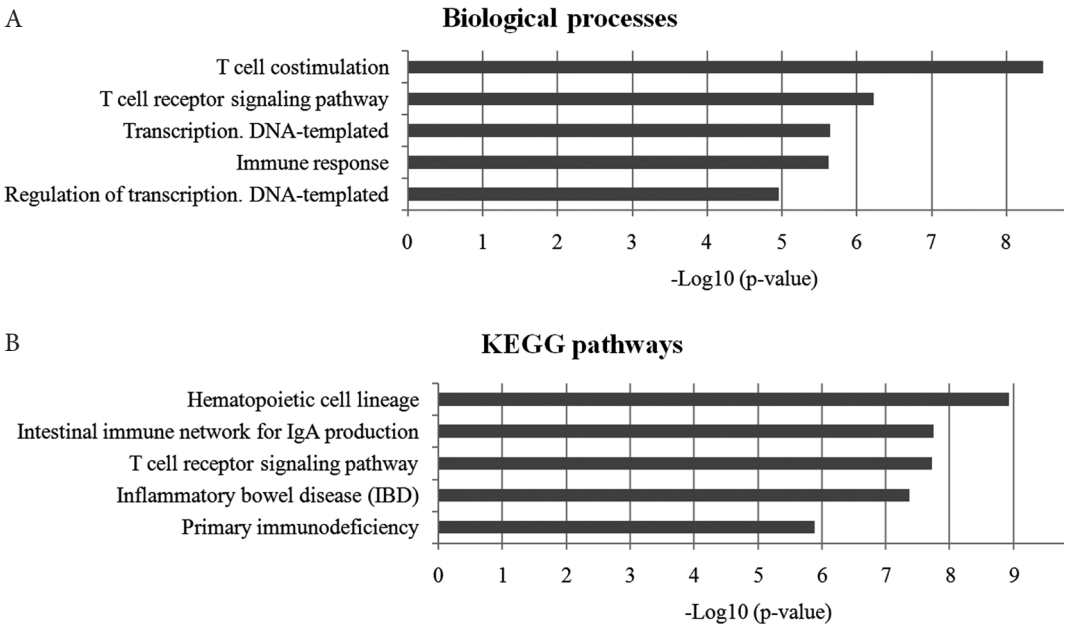

Figure 2. Enriched gene ontology terms of top five differentially expressed genes obtained from the DAVID of biological processes (**A**) and KEGG pathway (**B**).

41 nodes and 269 edges was selected for further analysis. KEGG pathway enrichment analysis of the genes involved in this cluster was performed by DAVID (Fig.4). The pathway enrichment analysis showed that the genes were mostly enriched in T cell receptor signaling pathway, primary immunodeficiency, intestinal immune network for IgA production, Staphylococcus aureus infection and HTLV-I infection.

Construction of the miRNA-gene regulatory network

The overlapping mRNAs of the miRNA-target gene predictions and DEGs in GSE94916 were determined and these overlapped 255 DEGs were used to construct the regulatory network. The miRNAs with no targets were excluded and inversely correlated miRNA-target gene regulatory network was constructed. The remaining nine

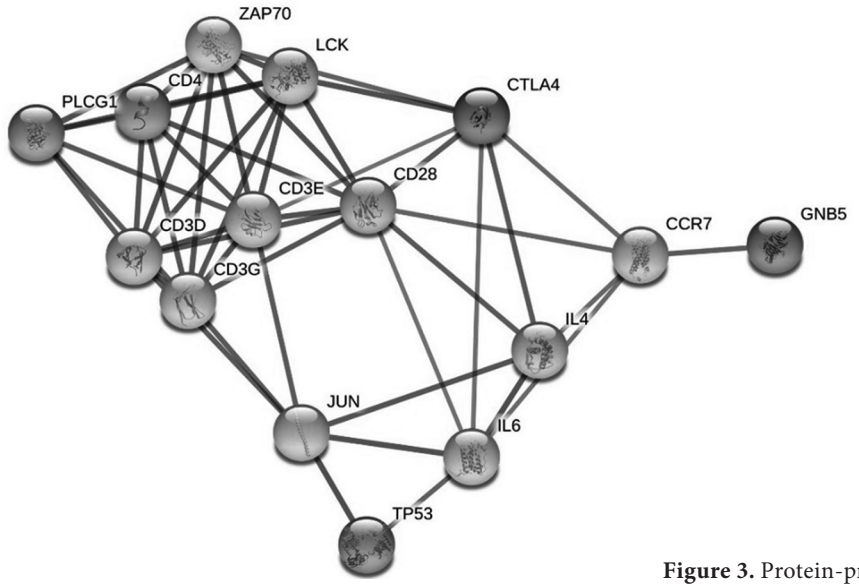

Figure 3. Protein-protein interaction network of 15 hub genes.

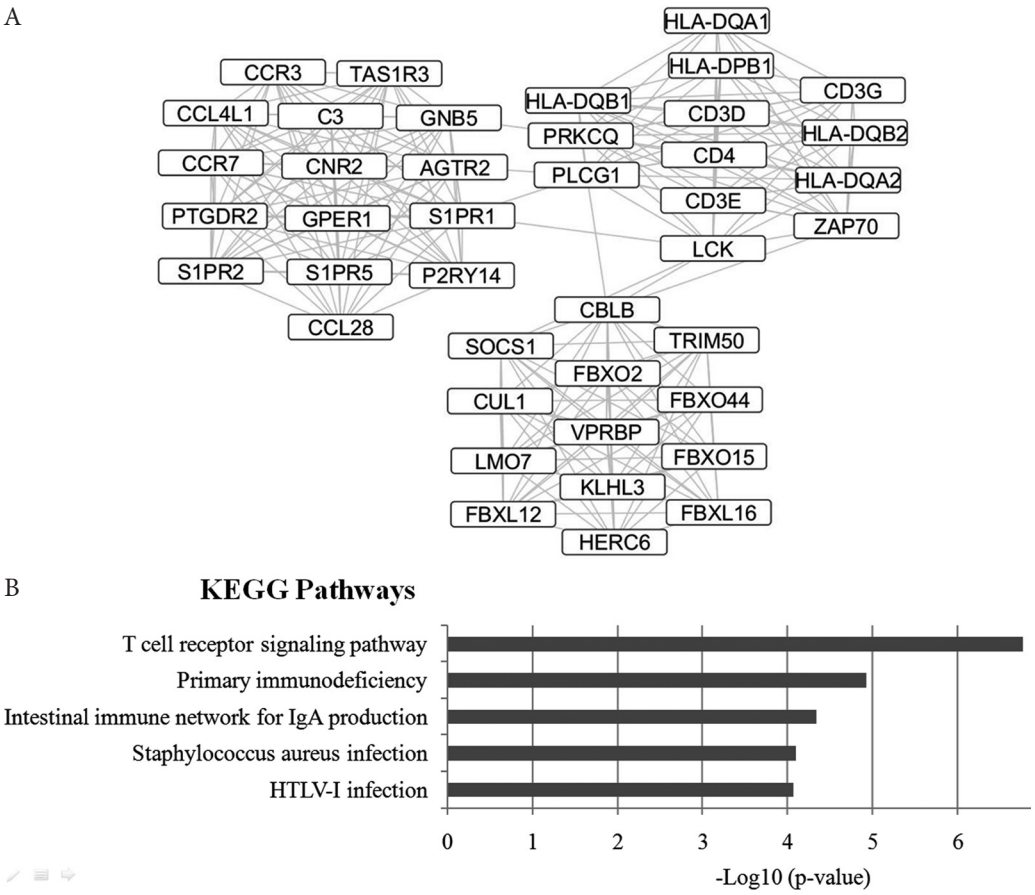

Figure 4. A. The most important module generated by MCODE. **B.** KEGG pathway in the module of A.

miRNA and 82 mRNA made 93 miRNA-mRNA pairs. The relationship between miRNAs and mRNAs is shown in Fig. 5. miRNAs; hsa-miR-1299, hsa-miR-556-3p, hsa-miR-1246, hsa-miR-1258, hsa-miR-130b-5p, hsa-miR-497-5p, and FLT3 showed degree ≥ 3 in the miRNA-gene network. These results were combined with the hub genes and considered to be potential key genes in developing NSCLC from COPD.

TCGA verification of potential key genes

TCGA data of LUAD and LUSC patients were used *via* the UALCAN data portal and OncomiRWashU Pan-Cancer miRNome Atlas to demonstrate the aberrant expression of potential key genes. Considering the cut-off criterion of $p < 0.05$ and the fact that our genes and miRNAs show the same expression pattern in all GEO, UALCAN and OncomiR WashU Pan-Cancer miRNome Atlas datasets; *JUN, IL6, CD4* genes (Fig. 6) and hsa-miR-497-5p, hsa-miR-130b-5p (Table 1) miRNAs were found to be significant in LUAD and LUSC.

Discussion

The morbidity and mortality of LC are both relatively high among the cancers (Shen et al. 2016) and various epidemiological studies, including LC screening trials, have determined 2–4 fold increase in LC risk in COPD patients when compared to control (Gonzalez et al. 2016). With well-developed microarray technology, it is easier to identify the genetic changes underlying the development of NSCLC from COPD patients. Also, by using bioinformatics tools, it is possible to identify new biomarkers and establish networks

Table 1. The *p*-values of the detected miRNAs in both cancer type LUAD and LUSC obtained from OncomiR web-portal

	Cancer type	*p*-value
hsa-miR-497-5p	LUAD	1.33×10^{-2}
	LUSC	8.79×10^{-10}
hsa-miR-130b-5p	LUAD	8.71×10^{-10}
	LUSC	2.54×10^{-17}

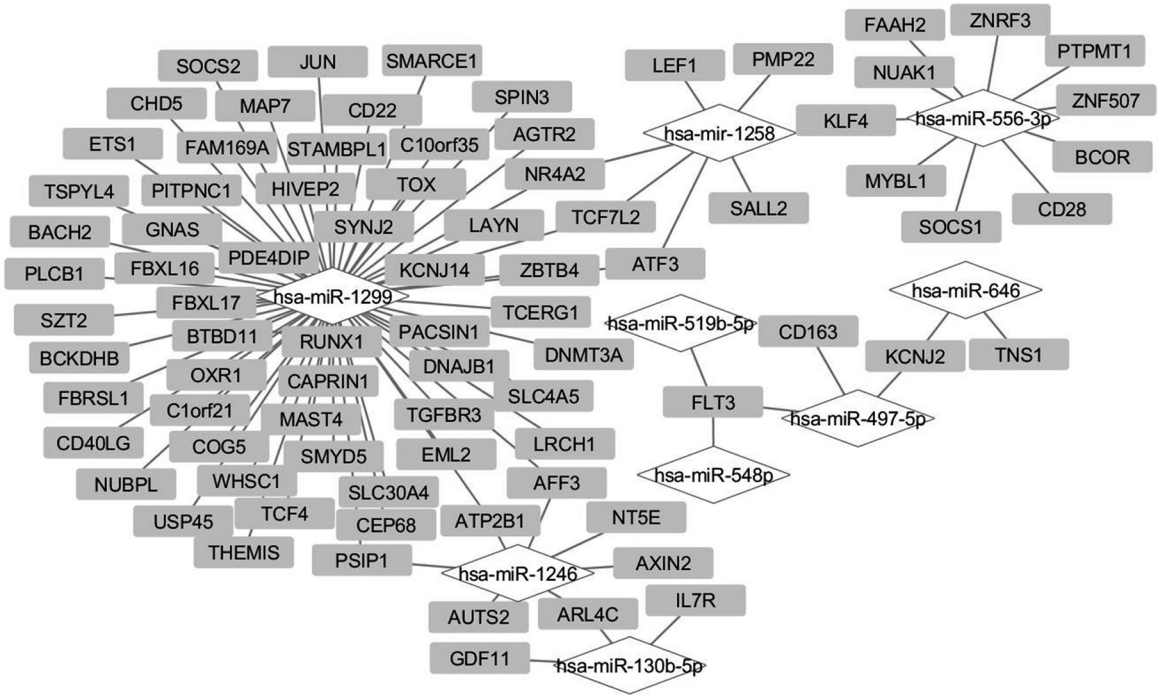

Figure 5. miRNA-mRNA regulatory network of COPD. Rectangle corresponds to the differentially expressed genes and diamond to the differentially expressed miRNAs screened.

that could be helpful to determine the relationship between these two diseases.

In our study, pathway enrichment analysis revealed that the hematopoietic cell lineage pathway was mostly enriched. It was demonstrated that this pathway is one of the key pathways in occurrence and migration in NSCLC (Li et al. 2016) also in COPD (Bi et al. 2015). Additionally, four clusters were acquired from the PPI network using MCODE. T cell receptor signaling pathway is the most enriched pathway in the highest significant cluster. It was also associated with COPD (Cruickshank-Quinn et al. 2018) and NSCLC (Chen et al. 2017) in some other studies. By constructing PPI, among 1517 of DEGs, 15 genes were identified as hub genes in COPD according to their high degrees in the network. *JUN* (degree = 41), *IL-6* (degree = 41) and *CD4* (degree = 29) were validated in LUAD and LUSC by using UALCAN. Jun proto-oncogene, activator protein-1 transcription factor subunit (JUN), is important for cell proliferation, survival, and apoptosis, and was reported to be a crucial contributing factor for tumorigenesis due to its downregulation in numerous types of human cancer (Fan and Ye 2018). IL-6 is one of the most important regulators of the cytokine-related tumor biology (Łukaszewicz et al. 2007). CD4 is a membrane glycoprotein and associated with the T-cell receptor signaling pathway (Kohm et al. 2002). CD4 T cells and macrophages are the crucial immune cells that mediate senescence surveillance of pre-malignant cells. Cells become malignant when they escape from senescence surveillance and progress further during tumor development, and then go through cancer surveillance.CD4 and CD8 T cell responses play a pivotal role in mediating the elimination of malignant cells (Ostroumov et al. 2018). Chen et al. (2017) searched the roles of immune-response related genes (IRGs) in lung cancer progression and found different expression profiles of IRGs in LUAD and LUSC but it is still unclear the precision mechanism of development of cancer in COPD. Evolving evidence has shown that the dysregulation of miRNAs is an important component of the pathogenesis of different cancers, including NSCLC. miRNAs regulate the expression of most genes and create a complex expression regulation network that interacts tightly with known gene regulatory networks. In this study, 20 DEMs were identified from five microarray datasets due to our cut-off criterion, of which seven were upregulated and 13 were downregulated. miRNA-gene regulatory network was constructed between targets of these miRNAs that overlap to DEGs which made 93 miRNA-mRNA pairs. hsa-miR-1299, hsa-miR-556-3p, hsa-miR-1246, hsa-miR-1258, hsa-miR-130b-5p, hsa-miR-497-5p and FLT3 were considered to be significant (degree ≥ 3) and hsa-miR-497-5p (degree = 3) and hsa-miR-130b-5p (degree = 3) were statistically significant according to the OncomiR WashU Pan-Cancer miRNome Atlas in both

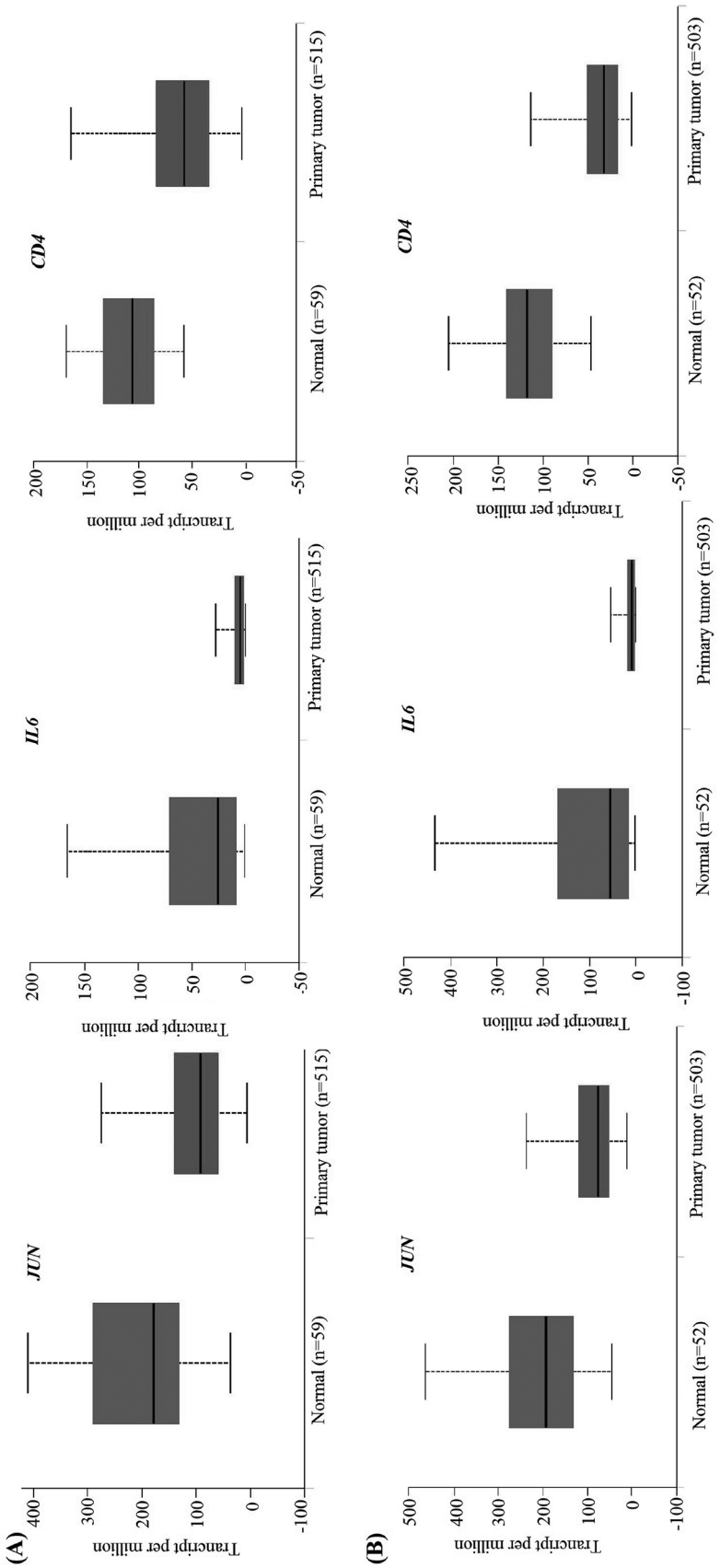

Figure 6. TCGA dataset analysis *JUN*, *IL6* and *CD4* expression in LUAD (lung adenocarcinoma; **A**) and LUSC (lung squamous cell carcinoma; **B**).

LUAD and LUSC. Abnormal expression and function of miR-497 have been presented in different types of cancer (Hu et al. 2016; Pengcheng et al. 2017). Besides, there are some reports concerning NSCLC and miR-497-5p (Huang et al. 2019; Li et al. 2019). These two studies concluded that miR-497-5p is a tumor suppressor miRNA and exhibit its potential use in the treatment of human NSCLC in the future. miR-130b was downregulated in cancer tissues, and they acted as anti-tumor miRNA in different types of cancer (Wang et al. 2014; Ramalho-Carvalho et al. 2017). Furthermore, the importance of miR-130b was also determined in NSCLC (Mitra et al. 2014).

In this study, we applied bioinformatics analysis to identify key genes and miRNAs that may be used as prognostic biomarkers in NSCLC development from COPD patients. In conclusion; *JUN, IL6*, and *CD4* hub genes and additionally hsa-miR-497-5p and hsa-miR-130b-5p were determined in COPD were validated in both LUAD and LUSC. This bioinformatics analysis contributed a comprehensive view to understand the mechanism underlying NSCLC development from COPD patients.

Disclosures. There is no a potential conflict of interest between the authors.

Author contributions. Tuba Denkçeken and Elif Pala designed the study, extracted corresponding data, prepared and approved the manuscript for submission.

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