Identification of Key Genes Between Lung Adenocarcinoma and Lung Squamous Cell Carcinoma by Bioinformatics Analysis

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Objective: To explore differentially expressed genes (DEGs) between lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC).

Methods: Based on GEO database, we used R software to identify the DEGs and conducted the bioinformatics analysis to explore the molecular mechanisms of DEGs and constructed PPI network to find the key DEGs. Then we assessed the effect of the eligible key DEGs on survival in LUAD by Kaplan-Meier plotter online tool.

Results: GSE10245 was downloaded from the GEO database, which contained a total of 58 tissue samples, including 18 LUSC and 40 LUAD. We identified 784 DEGs between LUAD and LUSC. DEGs were enriched in statistical significant GO annotation 201 items and KEGG pathways 17 items. By constructed PPI network, we obtained 10 hub genes. Of which, five genes were significantly correlated with the overall survival of LUAD.

Conclusions: P2RY1, CHRM3, LPAR3, NMU, and S1PR5 may be the potential prognostic markers and therapeutic targets for LUAD.

Keywords: Bioinformatics analysis; LUAD; Differentially expressed genes

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Non-small cell lung cancer (NSCLC) accounts for approximately 85% of LC [1], which the morbidity and mortality rank first in malignant tumors [2]. The incidence rates of lung adenocarcinoma (LUAD, approximately 50% of cases) and lung squamous cell carcinoma (LUSC, approximately 30% of cases) comprises 70% of NSCLC, which are the predominant histological subtypes of NSCLC [2-4]. Until recently, surgery is the recommended treatment for stage I - II NSCLC patients, 5-year survival of clinical stage I NSCLC and pathological stage I NSCLC after surgical resection can reach 68-92% and 73-90%, respectively [5,6]. However, due to lack of early effective

diagnostic methods, 70% of LC patients are diagnosed at advantage stage when the tumor lose the opportunity of surgical resection [7]. Experimental chemotherapy is the main treatment method for these patients, though the clinically outcomes are poor.

Attributed to the development of next-generation sequencing, the treatment strategies for advanced NSCLC patients have evolved to effective regimens that are targeted to specific molecular subtypes and specific genomic abnormalities of tumors [8,9]. According to statistics, up to 69% of advanced NSCLC patients have a potentially actionable molecular target [10]. Based on cell origin and different growth patterns, the specific

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targeted treatment produces modest improvements in survival for NSCLC patients [11-16]. However, Targeted treatment is limited to certain subsets of NSCLC patients, and patients with different histological subtypes and molecular abnormalities have different response to the same targeted therapy medications. For example, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) had great curative effect for patients with EGFR-mutated LUAD, but almost ineffective in EGFR-mutated LUSC [15-17]. Therefore, further progress is expected to discovery new and potentially great genotype-directed therapy targets for more subgroup's patients with NSCLC, which could be made a notable impact in outcomes for advanced NSCLC patients.

Since gene microarray and bioinformatics methods are broadly used in cancer researches, further identify the pathogenesis and search for potential specific diagnostic, prognostic markers and therapeutic targets have become possible. As a result, it is important to clarify the differentially expressed genes (DEGs), biological processes and molecular mechanisms of LUAD and LUSC from the perspective of biomedicine. In this study, we explored DEGs by comparison the genes expression profiles of tumor tissues between LUSC and LUAD patients from Gene Expression Omnibus (GEO) database (GEO database). The DEGs were conducted gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Furthermore, Protein-protein interaction (PPI) network, module analysis, and survival analysis were performed.

Materials and methods

Microarray data

GSE10245 was downloaded from the GEO DataSets, annotated by Human Genome U133 Plus 2.0 (Affymetrix, USA), and the chip platform was GPL570. A total of 58 tissue samples were collected, including 40 LUAD and 18 LUSC.

Data analysis and identification of DEGs

Software R x64 (version 3.4.3) was used to analyze the GSE10245 expression profile dataset, identify the DEGs compared LUAD with LUSC, plot heatmap and volcanic map. The DEGs screening criteria in this study were adjusted P value < 0.05 and $|\log FC| > 1$.

GO and KEGG analysis of DEGs

GO and KEGG enrichment analysis were applied by Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>) program. $P < 0.05$ was regarded statistically significant.

PPI network establishment and modules selection

STRING database (<https://string-db.org/>) was used to infer the PPI information. Entering all of DEGs names in STRING for a search, the combined score > 0.9 was considered significant. Molecular Complex Detection (MCODE), a plug-in in Cytoscape version 3.6.1, was used to screen the modules from PPI network. Degree Cutoff = 3, Node Score Cutoff = 0.2, K-Core = 2, Max.Depth = 100 were set as the cut-off criterion. Modules with MCODE score > 8 were presented. Eligible hub genes were selected as potential key genes.

Survival analysis

The Kaplan-Meier plotter online tool (<http://kmplot.com/>) was applied to assess the effect of the eligible hub genes on survival [18]. The Logrank test $P < 0.05$ was statistically significant.

Results

Identification of DEGs

A total of 784 DEGs were identified, 517 genes were upregulated and the remaining 267 genes were downregulated. Then we plotted heatmap of the top 100 DEGs (top 50 upregulated and downregulated genes), as described in Figure 1. All of the 20460 genes were represented on the volcano map, as presented in Figure 2. Red dots represented 784 DEGs and black dots represented the remaining genes with no different expression.

GO and KEGG analysis of DEGs

All the DEGs names were submitted to DAVID (<https://david.ncifcrf.gov/>) for GO and KEGG analysis, and we found statistically significant GO analysis 201 items and KEGG pathways 17 items. For the biological process (BP), DEGs were significantly aggregated in epidermis development, keratinocyte differentiation, negative regulation of endopeptidase activity, establishment of skin barrier, keratinization and peptide cross-linking, as shown in table 1. For the cellular component (CC), DEGs were significantly assembled in the extracellular space, cornified envelope, extracellular exosome, extracellular region, apical plasma membrane and apicolateral plasma membrane, as shown in table 1. For the molecular function (MF), DEGs were markedly enriched in serine type endopeptidase inhibitor activity and structural molecule activity, as shown in table 1. KEGG analysis showed that DEGs were significantly enriched in cell cycle, as shown in table 1.

PPI network construction and modules selection

All DEGs names were submitted to STRING and

PPI network was constructed. As shown in Figure 3, 344 nodes and 562 edges were identified, PPI enrichment p value $< 1.0 \times 10^{-16}$. Used Cytoscape plugin to identify the hub gene modules, and the cut-off criterion was set as degrees > 8 . Ten genes constituted one significant module, they were PMCH (pro-melanin concentrating hormone), NTS (neurotensin), P2RY1 (purinergic receptor P2RY1), CHRM3 (cholinergic

receptor muscarinic 3), NMU (neuromedin U), AGT (angiotensinogen), CXCL6 (C-X-C motif chemokine ligand 6), LPAR3 (lysophosphatidic acid receptor 3), HCAR3 (hydroxycarboxylic acid receptor 3) and S1PR5 (sphingosine-1-phosphate receptor 5), as shown in Figure 4. These ten hub genes were considered to be the major DEGs between LUAD and LUSC.

Table 1 GO and KEGG pathways analysis of differentially expressed genes (DEGs) with $P < 0.05$

Category	Term	Count	%	P Value	FDR
GOTERM_BP_DIRECT	GO:0008544~epidermis development	25	3.27654	8.44E-15	1.51E-11
GOTERM_BP_DIRECT	GO:0030216~keratinocyte differentiation	22	2.883355	6.51E-13	1.16E-09
GOTERM_BP_DIRECT	GO:0010951~negative regulation of endopeptidase activity	19	2.49017	9.47E-07	0.00169
GOTERM_BP_DIRECT	GO:0061436~establishment of skin barrier	8	1.048493	2.79E-06	0.004984
GOTERM_BP_DIRECT	GO:0031424~keratinization	11	1.441678	1.23E-05	0.022017
GOTERM_BP_DIRECT	GO:0018149~peptide cross-linking	11	1.441678	1.81E-05	0.032242
GOTERM_CC_DIRECT	GO:0005615~extracellular space	100	13.10616	7.97E-11	1.10E-07
GOTERM_CC_DIRECT	GO:0001533~cornified envelope	14	1.834862	8.95E-09	1.24E-05
GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	162	21.23198	1.35E-08	1.88E-05
GOTERM_CC_DIRECT	GO:0005576~extracellular region	100	13.10616	6.99E-07	9.69E-04
GOTERM_CC_DIRECT	GO:0016324~apical plasma membrane	30	3.931848	1.96E-06	0.002714
GOTERM_CC_DIRECT	GO:0016327~apicolateral plasma membrane	7	0.917431	2.43E-05	0.033725
GOTERM_MF_DIRECT	GO:0005198~structural molecule activity	32	4.193971	4.72E-09	7.20E-06
GOTERM_MF_DIRECT	GO:0004867~serine-type endopeptidase inhibitor activity	17	2.228047	6.75E-07	0.00103
KEGG_PATHWAY	hsa04110:Cell cycle	18	2.359108781	4.11E-06	0.005266549

BP, Biological process; CC, Cellular component; MF, Molecular function; GO, Gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; FDR, false discovery rate. E represents base of 10 to the power.

Survival analysis

Ten hub genes underwent survival analysis using Kaplan-Merier online tool (<http://kmplot.com/analysis/>), including 675 LUSC and 866 LUAD. The results showed that the LUAD patients with high expression of P2RY1 (HR=0.7 (0.55-0.89), logrank $P = 0.0031$), CHRM3 (HR=0.72 (0.56-0.92), logrank $P = 0.0085$) and LPAR3 (HR=0.54 (0.42-0.69), logrank $P = 6.8 \times 10^{-7}$) had significantly longer overall survival time than those with low expression groups, as shown in Figure 5. And the LUAD patients with low expression of NMU (HR=1.69(1.33-2.14), logrank $P = 1.3 \times 10^{-5}$) and S1PR5 (HR=1.93(1.52-2.45), logrank $P = 5.2 \times 10^{-8}$) had significantly longer overall survival time than those with high expression group, as shown in Figure 5.

Discussion

NSCLC has particularly high intratumoral heterogeneity due to distinct molecular features. Understanding the molecular mechanisms of NSCLC is critical for diagnosis, treatment and prognosis. In this study, we identified 784 DEGs either upregulated or downregulated in all compared LUAD with LUSC. Function annotation revealed statistically significant GO analysis 201 items and KEGG pathways 17 items. Through constructing PPI network, ten hub genes were considered to be the key DEGs. Three downregulated (P2RY1, CHRM3, and LPAR3) genes and two upregulated (NMU and S1PR5) genes were significantly related to worse overall survival in LUAD patients.

Figure 1 Heatmap of top 50 upregulated and downregulated genes differentially expressed genes (DEGs), red ones represented upregulation and green represented downregulation.

Figure 2 Volcano plot of the 20460 genes. Red dots represented the 784 differentially expressed genes (DEGs) and black dots represented the remaining genes with no different expression.

For cellular component (CP), DEGs were markedly located in the extracellular exosomes, extracellular space, cornified envelope, and extracellular region, as shown in table 1. Exosomes, which contain proteins, nucleic acids, lipids and the encapsulated contents, play an vital role in cell communication, tumor progression, drug resistance, and recent years have also play an important role in liquid biopsy of NSCLC [19-21]. It provides a new basis for early diagnosis, precision medicine and prognosis of NSCLC. However, the study of exosomes still is in their infancy, whether it can be applied in clinic remains need to be confirmed by further research. For molecular function (MF), DEGs were mainly promoted serine type endopeptidase inhibitor activity and structural molecule activity. Serine type endopeptidase inhibitor is a subtype of serine protease inhibitor, which is termed serpins. Serpins sit at intersections of normal physiology and pathology, both ensuring normal biological function

Figure 3 Protein-protein interaction (PPI) network of the 784 differentially expressed genes (DEGs).

but also, when dysfunctional, leading to disease [22-25]. It is involved in a variety of biological processes, such as inflammation, immune response, cell invasion, tissue remodeling and cancer development [22,26-28]. With cancers, altered levels of serpins and related proteases are correlated with invasion and metastasis, even can serve as markers for aggression [29]. For biological process (BP) enrichment, DEGs were mainly involved in epidermis development, keratinocyte differentiation, keratinization and peptide cross-linking. It mainly refers to the process

of epidermal cells from formation to maturity over time. In vivo, epithelial cells and mesenchymal cells can be converted under certain conditions during the epithelia cells development. The epithelial-mesenchymal transition (EMT) is critical for cancer progression [30-33]. EMT is driven by transcriptional factors, results in enhancer migration and invasive potential of epithelial cells, and is critical for the metastatic spread of epithelial tumors [30, 34, 35].

Figure 4 Significant module inferred from protein-protein interaction network.

Figure 5 Prognostic values of the five key DEGs in LUAD patients.

P2RY1 encodes a G-protein couples receptors, adenine di-phosphate (ADP) is a physiological agonist for P2RY1 [36]. P2RY1 couples to phospholipase C, which triggers Ca^{2+} release from the intracellular reservoir, leading to reversible platelet aggregation [37]. The P2RY1 polymorphism might participate in the control of the various physiological functions, and negative regulation of P2RY1 in the multidrug – chemoresistance of bladder cancer cells was reported [38,39]. The copy number variation (CNV) frequencies for pharmacogenes using The Cancer Genome Atlas dataset showed that P2RY1 may play a role in determining individual variations in drug responses in NSCLC patients [40]. CHRM3 is one of the muscarinic receptors and plays an important role in many kinds of cancer, such as breast cancer, colon cancer, and lung cancer [41]. Experienced study revealed that CHRM3 highly expressed in NSCLC and enhanced the expression and activity of matrix metalloproteinase 9 (MMP9) through PI3K/Akt, which regulated the transcription of MMPs and EMT-related genes in NSCLC [42,43]. It has demonstrated overexpression of CHRM3 in endometrial carcinoma patients, as an prognostic factor, had a shorter overall survival time [44]. NMU encodes a member of

gene, so its role in NSCLC is less studied. S1PR5 is a constitutively G protein-coupled receptor with the ability to inhibit cell proliferation and induce cell migration in cancer by $\text{G}\alpha_{12/13}$ -evoked stimulation of Rho, suggesting an inhibitory role of S1PR5 [52-54]. S1PR5 has also been shown to have pro-survival effect by inducing autophagy and S1PR5 activation was found to be involved by applying small interfering RNA and dihydro-S1P in cancer [55, 56].

Conclusion

In conclusion, our study discovered a series of key targets for further research on molecular mechanisms of LUAD by a comprehensive bioinformatics analysis, which provides new therapeutic and prognostic clues for LUAD patients. Other than bioinformatics exploration,

the neuromedin family of neuropeptides and is associated with cell motility, invasion, and resistance to anoikis [45]. In NSCLC pathogenesis, NMU might be a target of the lung metastasis suppressor effect of RhoGD12 and RhoGD12 reconstitution in NSCLC cells without expression resulted in lung metastasis suppression [46]. In Shuangjie You's study, it showed that NMU was the candidate promoting the tumorigenesis and alectinib resistance in NSCLC [47]. Sweta Rani et al reported that NMU was a candidate prognostic indicator and therapeutic target for HER2- overexpressing tumors, which associated with poor outcomes and effected sensitivity to lapatinib, trastuzumab, neratinib and afatinib in patients with HER2 overexpressing tumors [45]. LPAR3 indicates several biological functions, such as cell proliferation, migration, and tumor progression [48]. LPAR3 abnormally expressed in many cancer cells, such as ovarian cancer, colorectal cancer, and LC [49-51]. The developmental study found that aberrant expression of LPAR3 due to aberrant DNA methylation induced inhibition of lung cancer cell migration in rat, which suggested that LPAR3 may be promising chemotherapeutic target gene and chemoprevention for LC [51]. S1PR5 is the most recently deorphanized

functional researches that further delineate the effects of the key DEGs are needed to validate their specific roles in the pathogenesis of LUAD, even and in the diagnosis, individualized treatment, and prognosis of LUAD.

Conflict of Interest

The authors declare no conflict of interests.

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