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Communication

Identification of Key Biomarkers in Pulmonary Arterial Hypertension Based on Weighted Gene Co-expression Network Analysis

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Abstract: Pulmonary arterial hypertension (PAH) is a complex and devastating disease that the underlying cellular and molecular mechanisms are largely remains unknown. This study aimed to elucidate the key hub genes and pathways in PAH by bioinformatics analysis. In the current study, we performed WGCNA analysis to systematically identify the hub genes for PAH using transcriptome microarray data. From the Gene Expression Omnibus (GEO) database, one microarray dataset (GSE113439) was downloaded for this study. There were 26 samples in this data set, including 15 PHA samples and 11 normal controls. Based on weighted correlation network analysis, 11 modules were identified and the MEgreenyellow module showed a significantly positive correlation with PAH ($r=0.93$, $P=1e-06$). The genes in greenyellow module were mainly significantly enriched in mitophagy related pathways by KEGG analyses. Combined with the protein-protein interaction (PPI) and co-expression networks, ten hub genes were identified as candidate biomarkers for PAH in the greenyellow module, including CTNNB1, NIPBL, ROCK2, ROCK1, SCAF11, JAK1, BIRC6, KIF5B, PPP1R12A and XRN1. These hub genes might be potential targets for clinical therapy against PAH. The molecular mechanisms involved in these genes that affected the prognosis of PAH should be further validated through biological and basic studies.

Keywords: WGCNA; Pulmonary arterial hypertension; hub genes; microarray; modularization

1. Introduction

Pulmonary arterial hypertension (PAH) is a devastating and complex disease in the worldwide. According to the World Health Organization (WHO) classification system, a resting mean pulmonary artery pressure (mPAP) of PAH is more than 25 mm Hg, pulmonary capillary wedge pressure (PCWP) of PAH is less than 15 mm Hg, and a pulmonary vascular resistance (PVR) of PAH is more than three Wood units.[1]-[3] PAH affects the pulmonary vasculature by remodeling of the pulmonary vasculature primarily, which can eventually cause vascular dysfunction and structural remodeling of the small pulmonary arteries, then ultimately leading to right ventricular (RV) failure.[4,5] Median survival is only 5–7 years for PAH patients.[6] The pathophysiology of PAH is complex, for this condition can be influenced by multiple processes including DNA damage, genetic modifying factors, environmental factors, microRNAs (miRs), oxidative stress, sex hormones and altered cell metabolism.[7]-[10] Even though great efforts have been made in this field during the past few years, the underlying mechanism of PAH is not entirely clear.

Weighted gene co-expression network analysis (WGCNA), which is a widely used technique to detect modules and hub genes that are based on comparison between two groups and complex network analysis.[11] WGCNA method is based on some Network Medicine hypothesis.[12] To gain further insight into the mechanisms and factors leading to the development of PAH, we performed WGCNA to identify co-expression modules and hub genes for PAH using transcriptome microarray data. One microarray dataset (GSE113439) was obtained from the Gene Expression Omnibus (GEO) database for WGCNA analysis. The results of WGCNA found that 11 modules were identified and

the MEgreenyellow module showed a significantly positive correlation with PAH ($r = 0.93$, $P = 1e-06$). In this module, ten hub genes were identified as potential biomarkers for PAH, including CTNNB1, NIPBL, ROCK2, ROCK1, SCAF11, JAK1, BIRC6, KIF5B, PPP1R12A and XRN1. These hub genes might be potential targets for clinical therapy against PAH.

2. Material and Methods

2.1. Microarray Data Sources

The GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) is a public database containing a large number of gene profiles in various diseases. The gene expression profile was obtained from the GEO database. For the present study, the selection criteria were: (1) a total sample size was more than 15; (2) data accessibility (raw data or processed data); (3) studies were conducted on human samples of normal control and PAH patients. The GSE113439 dataset was included in the end, for its highest quality and appropriate sample size. Ethical approval was not necessary in this study, for the data we used was extracted from public GEO database.

2.2. Data and Statistical Analysis

Statistical analyses were performed by the R software (v.4.0.3) and the aforementioned packages. The limma (linear models for microarray data) package was used for the quality control.[13] Since the data were already normalized through Robust Multi-array Average (RMA) method,[14] we conducted the WGCNA analysis on the entire GSE113439 dataset.

2.3. Identification Key Gene Modules by WGCNA and Functional Enrichment Analysis

The WGCNA package in R was used to build the co-expression network with the GSE113439 dataset. The samples were clustered by Pearson's correlation coefficient to filter the obvious outliers first. Second, using the automatic network construction function, the gene co-expression network was obtained. Third, the soft thresholding power β was used to calculate the WGCNA adjacency. Modules were determined through hierarchical clustering dendrogram and dynamic tree cut function. Then, we calculated the module membership (MM), the gene significance (GS) and the correlation between the modules and the clinical data. We set a threshold of 0.25 to merge the similar modules to obtain the correct module number and clarify gene interaction. Genes in the most significant module were exported for further analysis. The linear regression relationship between the clinical features and gene expression was based on the P -value. To explore the potential biological themes and pathways of genes from the key module, the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment terms were analyzed via the clusterProfiler package[15] in R. We also used DAVID 6.8 (<https://david-d.ncifcrf.gov/>) online tools to perform the Gene Ontology (GO) analysis. The "ggplot2" R package was used to display the GO and KEGG enrichment results. A P value < 0.05 was considered significant pathways.

2.4. Identification of Hub Genes

After the identification of the key modules, the genes in the key module with high connectivity were screened. Next, to perform protein-protein interaction (PPI) network analysis, we uploaded these genes to STRING (v11.0) database (<https://string-db.org/>).[16] The combined score was set to more than 0.4 and other parameters were set to default. The "TSV: tab separated values" file was downloaded and imported into the Cytoscape software (version 3.8.2). The top 10 key genes were identified as "hub genes" by Maximal Clique Centrality (MCC) using the "cytoHubba" app. [17]

3. Results

3.1. Identification Key Gene Modules by WGCNA

In this study, we screened gene expression profiles from the GEO dataset for WGCNA analysis. The GSE113439 dataset was obtained from the GEO website. GSE113439, which was established on the platform of the Human Gene 1.0 set array (Affymetrix Inc., Santa Clara, CA, USA), was submitted by Mura. There were 26 samples in this data set, including 15 PAH samples and 11 normal controls. In our study, we comprehensively analyzed the expression data of GSE113439. The limma package was used for data normalization and quality control. Then, the Pearson’s correlation coefficient was used to cluster the samples in GSE113439. 45 was set as the cut-off height value. The samples that did not meet this criterion were removed. To construct a scale-free network, power of $\beta = 17$ (scale-free $R^2=0.9$) was selected as the soft-thresholding power. We then constructed the gene co-expression network by WGCNA package in R software, and 11 modules were detected and constructed (Figure.1). The sizes ranges of the 11 modules were from 75 to 551 genes. The correlations between each module and clinical trait (PAH or normal) were also calculated to determine significant associations (Figure.2). The results revealed that the greenyellow module was the most significantly correlated with PAH.

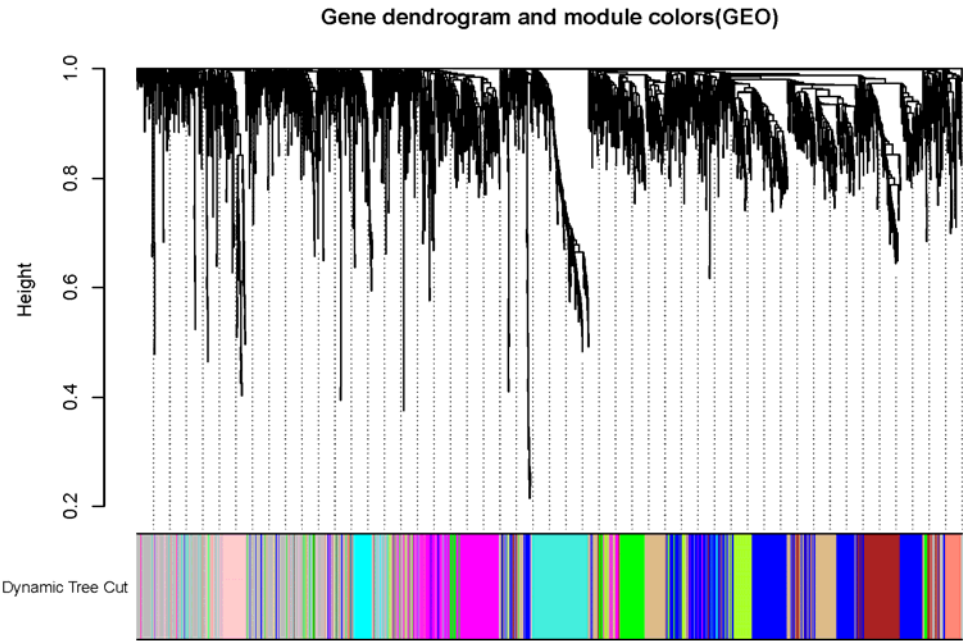


Figure 1. Clustering dendrogram of genes.

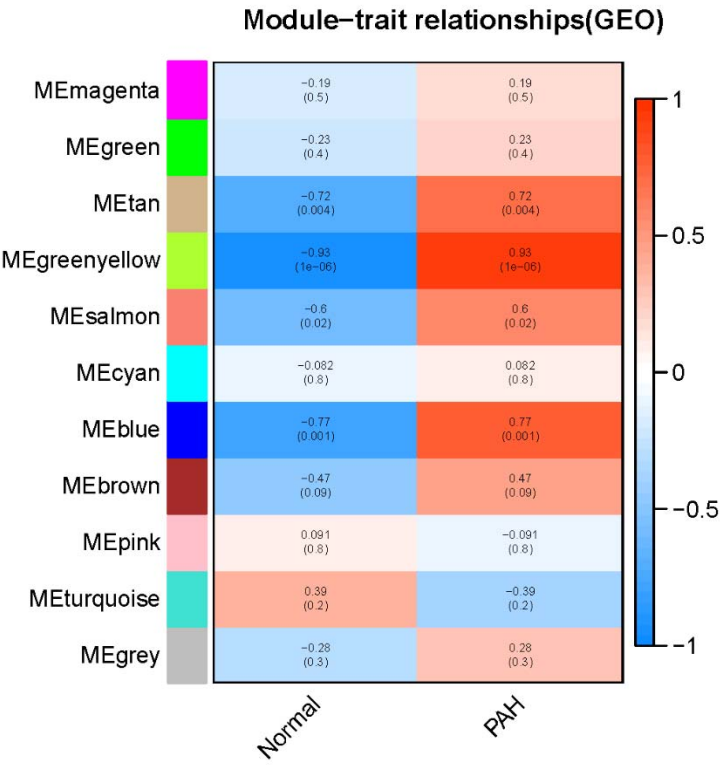


Figure 2. Heatmap of the correlation between the genes module and clinical features of PAH. Red represents high adjacency (positive correlation). Blue represents low adjacency (negative correlation).

3.2. Functional GO and KEGG Pathway Enrichment Analyses of the Key Module

To study the function of the greenyellow module, we conducted a GO analysis of all genes in this module. A total of 13 significantly enriched biological process (BP) were observed in the current study, such as regulation of establishment of endothelial barrier, regulation of stress fiber assembly, ubiquitin-dependent protein catabolic process, protein phosphorylation, resolution of recombination intermediates, regulation of gene expression, negative regulation of bicellular tight junction assembly, regulation of cell adhesion, negative regulation of myosin-light-chain-phosphatase activity, actin cytoskeleton reorganization, regulation of actin cytoskeleton organization, cellular response to DNA damage stimulus, positive regulation of DNA-templated transcription, initiation (GO IDs: 1903140, 0051492, 0006511, 0006468, 0071139, 0010468, 1903347, 0030155, 0035509, 0031532, 0032956, 0006974, 2000144, respectively). A total of 8 significantly enriched cellular component (CC) were observed in the current study, such as nucleoplasm, nucleus, membrane, intracellular, cytoplasm, cell cortex, centrosome, transcriptional repressor complex (GO IDs: 0005654, 0005634, 0016020, 0005622, 0005737, 0005938, 0005813, 0017053, respectively). A total of 8 significantly enriched molecular function (MF) were observed in the current study, such as protein binding, ATP binding, cysteine-type endopeptidase activity, thiol-dependent ubiquitin-specific protease activity, nucleic acid binding, SMAD binding, poly(A) RNA binding, DNA binding (GO IDs: 0005515, 0005524, 0004197, 0004843, :0003676, 0046332, 0044822, 0003677, respectively).

We also conducted a KEGG analysis of all genes in the greenyellow module. Figure 3 shows that the most significantly enriched KEGG pathway was mitophagy (pathway ID: 04137), which is directly associated with PAH. Moreover, the present study detected the others significantly enriched KEGG pathways, including signaling pathways regulating pluripotency of stem cells, necroptosis, salmonella infection, cGMP-PKG signaling pathway, leukocyte transendothelial migration, focal

adhesion, proteoglycans in cancer, platelet activation, notch signaling pathway, cAMP signaling pathway and regulation of actin cytoskeleton (Figure 3).

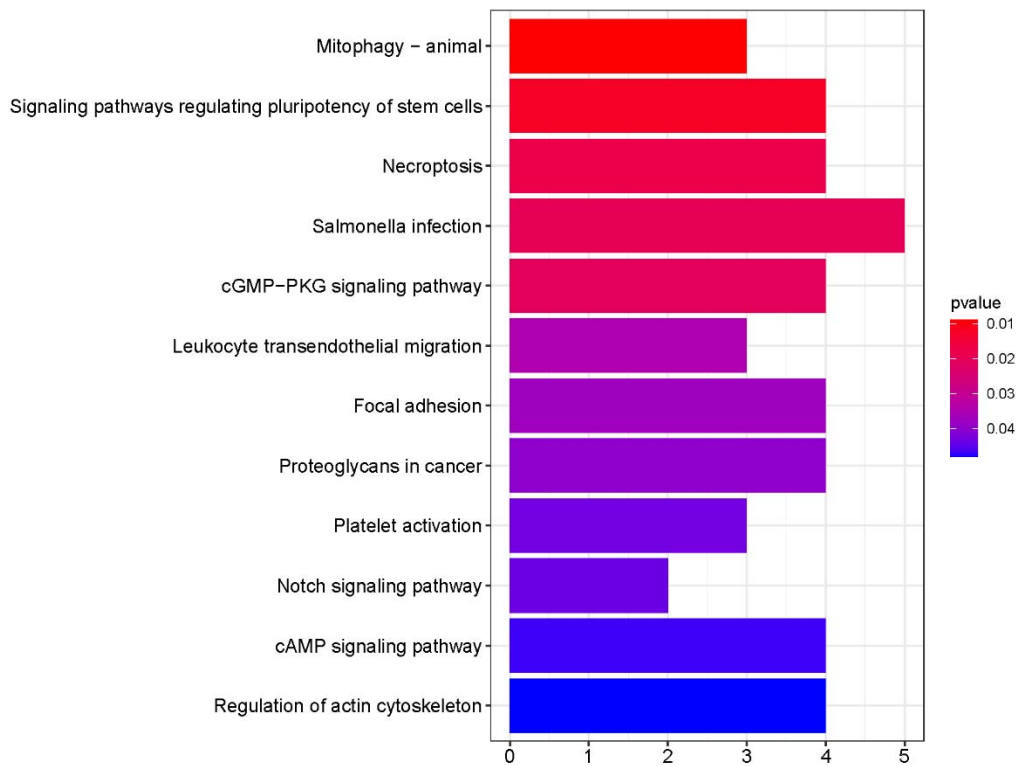


Figure 3. The significant KEGG pathways of genes involved in greenyellow module. $P<0.05$ was defined as the thresholds in selecting significant KEGG pathways.

3.3. PPI Network Construction

To define the protein interactions, STRING was used to create PPI network. With the cytoHubba plugin, the top 10 hub genes were identified as CTNNB1, NIPBL, rho-associated coiled-coil-containing protein kinase (ROCK)2, ROCK1, SCAF11, JAK1, BIRC6, KIF5B, PPP1R12A and XRN1(Figure.4). KEGG analysis revealed that the mainly enriched pathways for the 10 hub genes were proteoglycans in cancer, focal adhesion and vascular smooth muscle contraction.

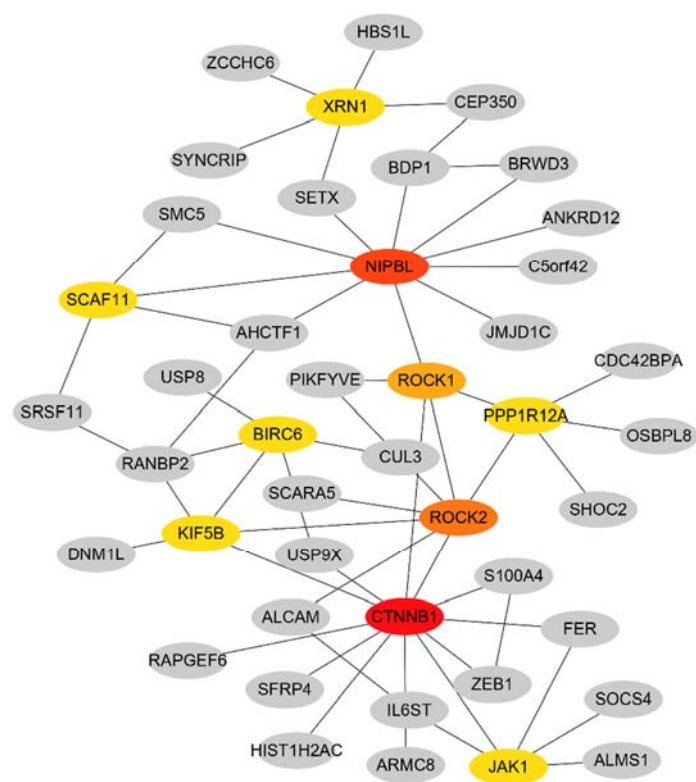


Figure 4. The PPI network of the genes from the greenyellow module. The nodes other than the grey nodes represent the hub genes with the highest prediction scores. The top 10 identified hub genes are shown from red (high degree value) to yellow (low degree value).

4. Discussion

PAH is a complex and devastating disorder. Histopathological examination of PAH reveals that proliferation of pulmonary artery endothelial cells (PAECs), fibroblasts and pulmonary artery smooth muscle cells (PASMCs) might occlude pulmonary arterioles, then eventually leads to right heart hypertrophy and heart failure.[18,19] The exact pathophysiology and mechanisms for PAH are largely unknown. Therefore, it is of great important to identify the underlying cellular and molecular mechanism of PAH, and might be the potential targets for clinical treatment of PAH.

In this study, we screened key gene modules and hub genes from the GSE113439 dataset through WGCNA analysis, and 11 modules were detected and constructed. The greenyellow module was highly correlated with PAH. We then conducted GO and KEGG analysis of all genes in the greenyellow module. The most significantly enriched KEGG pathway was mitophagy, which is directly associated with PAH. Mitophagy is a process referred to mitochondria degradation via autophagy. A number of studies have demonstrated that enhanced autophagic pathway promotes the development of PAH through several mechanisms, such as the removal of misfolded proteins and the preservation of mitochondrial homeostasis.[20]-[22] The present study also detected the others significantly hub pathways, including signaling pathways regulating pluripotency of stem cells, necroptosis, salmonella infection, cGMP-PKG signaling pathway, leukocyte transendothelial migration, focal adhesion, proteoglycans in cancer, platelet activation, notch signaling pathway, cAMP signaling pathway and regulation of actin cytoskeleton. Recently, Hashimoto et al.[3] demonstrated an unexpected function for bone marrow-derived hematopoietic stem cells (HSCs) in the pathogenesis of PAH, which is consist with our findings. In a recent animal study, necroptosis pathway was found associated with the pathogenesis of PAH through the RNA-seq data set and bioinformatics approach.[23] In this study, we also identify the necroptosis pathway was significantly enriched in the greenyellow module. Previous studies have shown that the platelets of PAH patients

are activated.[24,25] Similarly, in the current study, we have also found that platelet activation pathway was significantly enriched. These finding suggests that the hub pathways may be involved in modulation of PAH and have a therapeutic potential.

STRING was used to create PPI network of the genes in the greenyellow module to define the protein interactions. With the cytoHubba plugin, the top 10 hub genes were screened as CTNNB1, NIPBL, ROCK2, ROCK1, SCAF11, JAK1, BIRC6, KIF5B, PPP1R12A and XRN1. Previous studies have analyzed the same public microarray dataset (GSE113439), and have indicated different results. Luo et al. found that SMC2, CDK1, SMC4, CENPE, and KIF23 might be closely associated with the pathogenesis of PAH.[26] Li et al. identified nine hub genes related to PAH, including the PLK4 and SMC2 genes.[27] In another study, Ma et al. suggested that SMC4, TOP2A, SMC2, ANLN, KIF11, KIF23, SMC3, ARHGAP11A, RAD50 and SMC6 may involve in the pathogenesis of PAH.[28] In a recent study, Yao et al. revealed several hub genes associated with PAH, including HSP90AA1, ANGPT2, HSPD1, HSPH1, TTN, SPP1, SMC4, EEA1, and DKC1.[29] Another recent study found that 5 hub genes (CCL5, CXCL12, VCAM1, CXCR1, and SPP1) might play an important role in the development of PAH.[30] All of the above studies compared the differentially expressed genes (DEGs) between control samples and PAH samples. The methodology is different from our study. According to the tutorials of WGCNA (<https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/>), the input data should be the unfiltered expression matrix. Different approaches often give different results. To our knowledge, this is the first time using WGCNA methods to comprehensively reveal the biological processes of PAH in patients.

Among the top 10 hub genes in this study, CTNNB1, also known as catenin beta 1 or β -catenin, has an important role in adherens junctions. Yu et al.[31] have found that the mRNA and protein levels of β -catenin were remarkably elevated in the lung tissues of monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) rats group than control group. A case report showed that the variation of CTNNB1 is associated with abnormal lung growth and pulmonary hypertension.[32] NIPBL is a multifunctional protein and functions as a loading factor for cohesion.[33] Publications have reported that loss of NIPBL promotes autophagy through impairs the DNA damage response and decrease lung cancer cells proliferation, migration and invasion.[34] Previous reports have shown that ROCK1 and ROCK2 were involved in autophagy and fibrosis of the heart.[35] Reduced expression of either ROCK1 or ROCK2 might be a protective factor of pulmonary fibrosis.[36] These findings suggest that identification of the hub genes will allow greater understanding of the mechanisms and prognosis of PAH, as well as promote discovery of novel diagnostic and therapeutic direction.

In this study, there are some limitations. Firstly, the sample size was still relatively small. Second, although this study was based on WGCNA approach, which is a useful systems biology method, it was still limited to a certain computer analysis. Finally, there is still lack of experimental verification.

In the future, large amounts of data will be needed to further verify the hub genes and co-expression networks relationships of PAH, and the results need to be verified by experiments in vivo and in vitro.

5. Conclusions

Taken together, our findings indicate that CTNNB1, NIPBL, ROCK2, ROCK1, SCAF11, JAK1, BIRC6, KIF5B, PPP1R12A and XRN1 are closely associated with PAH. The molecular mechanisms involved in these genes that affected the prognosis of PAH should be further validated through biological and basic studies.

Author Contributions: ZZ: MW, XY and PC conceived and designed the idea; MW and HJ extracted data, analyzed the data and drafted the manuscript. MW, HJ and HJ checked raw data. PC and ZZ revised the manuscript. PC and XY revised the final manuscript.

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Conflicts of Interest: No.

Abbreviations

PAH Pulmonary arterial hypertension
 GEO Gene Expression Omnibus
 PPI protein–protein interaction
 WHO World Health Organization
 mPAP mean pulmonary artery pressure
 PCWP pulmonary capillary wedge pressure
 PVR pulmonary vascular resistance
 RV right ventricular
 WGCNA Weighted gene co-expression network analysis
 miRs microRNAs
 RMA Robust Multi-array Average
 MM module membership
 GS gene significance
 KEGG Kyoto Encyclopedia of Genes and Genomes
 MCC Maximal Clique Centrality
 BP biological process
 CC cellular component
 MF molecular function
 PAECs pulmonary artery endothelial cells
 PASMCs pulmonary artery smooth muscle cells
 ROCK rho-associated coiled-coil–containing protein kinase
 DEGs differentially expressed genes

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