Bioinformatics-based study to detect chemical compounds that show potential as treatments for pulmonary thromboembolism

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Abstract. The objectives of the present study comprised the recognition of major genes related to pulmonary thromboembolism (PTE) and the evaluation of their functional enrichment levels, in addition to the identification of small chemical molecules that may offer potential for use in PTE treatment. The RNA expression profiling of GSE84738 was obtained from the Gene Expression Omnibus database. Following data preprocessing, the differently expressed genes (DEGs) between the PTE group and the control group were identified using the Linear Models for Microarray package. Subsequently, the protein-protein interaction (PPI) network of these DEGs was examined using the Search Tool for the Retrieval of Interacting Genes/Proteins database, visualized via Cytoscape. The most significantly clustered modules in the network were identified using Multi Contrast Delayed Enhancement, a plugin of Cytoscape. Subsequently, functional enrichment analysis of the DEGs was performed, using the Database for Annotation Visualization and Integrated Discovery tool. Furthermore, the chemical-target interaction networks were investigated using the Comparative Toxicogenomics Database as visualized via Cytoscape. A total of 548 DEGs (262 upregulated and 286 downregulated) were identified in the PTE group, compared with the control group. The upregulated and downregulated genes were enriched in Gene Ontology terms related to inflammation and eye sarcolemma, respectively. Tumor necrosis factor (*TNF*) and erb-b2 receptor tyrosine kinase 2 (*ERBB2*) were upregulated genes that ranked higher in the PPI network (47 and 40 degrees, respectively) whereas *C-JUN* was the most downregulated gene (46). Small chemical molecules ethinyl (135), cyclosporine (126), thrombomodulin precursor (113) and tretinoin (111) had >100 degrees in the DEG-chemical interaction network. In addition, ethinyl targeted to *TNF*, whereas *TNF* and *ERBB2* were targeted by cyclosporine, and tretinoin was a targeted chemical of *ERBB2*. Therefore, cyclosporine, ethinyl, and tretinoin may be potential targets for PTE treatment.

Introduction

Acute pulmonary thromboembolism (PTE), the obstruction by a thrombus in the pulmonary artery or its branches, is the most common form of pulmonary embolism (PE) (1). The common pathology of PTE comprises hemodynamic instability, hypoxia, and pulmonary hypertension, which may cause heart failure with hypotension (2,3). PTE is reported to be a complex disease caused by a variety of factors, including the vascular microenvironment and vascular cell dysfunction (4,5). During an episode of PTE, a thrombus trapped in the pulmonary blood vessels injures the vascular endothelium, causing the unregulated release of pro-inflammatory mediators (2,6). Pulmonary vascular remodeling caused by repeated pulmonary vascular injury may lead to secondary pulmonary hypertension (7), a major clinical consequence of PTE, which indicates that endothelial injury induced by PTE may be key in the pathophysiological consequences of PTE (8). PTE is a common disease, which is a contributing factor to the global non-communicable disease burden (8,9).

Previous studies have identified brain natriuretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP) in the blood as biomarkers for predicting echocardiographic right ventricular dysfunction in patients with acute PTE (10,11). Furthermore, it is reported that troponin I, D-dimer, and plasma tenascin-C are positively correlated with the occurrence of PTE, whereas the erythrocyte sedimentation rate is downregulated in patients with PTE (12). Tang *et al* identified interleukin 8 (*IL-8*),

tumor necrosis factor-α (*TNF-α*), and C-X-C motif chemokine ligand 5 as important factors in PTE in rabbits (13). In addition, it was revealed that these important genes are associated with cardiovascular disease, pulmonary disease, immune disease, and inflammation (13). However, small chemical molecules targeting differently expressed genes (DEGs) between the PTE group and the control group did not screen out. The use of small chemical molecules that target important disease-related genes has become an effective strategy for disease treatment (14,15).

In the present study, the *GSE84738* dataset was obtained from the Gene Expression Omnibus (GEO) database. Following data preprocessing, the DEGs between a PTE group and control group were identified. Subsequently, the protein-protein interaction (PPI) network of these DEGs was visualized using Cytoscape, and the most significantly clustered modules in the network were analyzed. Subsequently, functional enrichment analysis of the DEGs was performed. Furthermore, the chemical-target interaction networks were investigated using the Comparative Toxicogenomics Database (CTD), visualized via Cytoscape. The present study identified the important genes and their functional enrichment in relation to the occurrence of PTE, and identified small chemical molecules potentially useful for PTE treatment. These findings may provide an important basis to detect mechanisms underlying PTE and potential treatment methods for PTE.

Materials and methods

Data sources. The RNA expression profiling dataset of the European rabbit (*Oryctolagus cuniculus*) was obtained from the GEO database in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE84738) (13). The dataset included eight samples (four control samples and four PTE samples), collected from rabbit pulmonary artery tissue. All samples were assessed, based on the GPL13288 Agilent-020908 *Oryctolagus cuniculus* (rabbit) Oligo Microarray platform. The use of animals was approved by the Animal Ethics Committee of Affiliated Hospital of Nantong University (Nantong, China).

Screening of DEGs. The original file was downloaded and annotated. In cases where one gene was detected by multiple probes, the mean value of the probes was used to represent the expression level. The gene expression matrix was separated into a control group and a PTE group, and the DEGs between the two groups were screened according to the Linear Models for Microarray package (version 3.30.3, www.bioconductor. org/packages/release/bioc/html/limma.html) in R software. The P-values of these DEGs were calculated and adjusted using an unpaired t-test and Benjamini-Hochberg method, respectively, at a significance level of P<0.05. Subsequently, the heatmap in R package, which is used for drawing heatmaps of gene expression, was utilized to plot the heat map of DEGs (16). Clustering between the different samples and different genes was performed using Pearson's and Spearman's correlation coefficients, respectively.

Analysis of the PPI network. The PPIs of the DEGs between the control group and PTE group were extracted from the

Search Tool for the Retrieval of Interacting Genes/Proteins database (version 10.0, http://www.string-db.org/) with default parameters (species: *Oryctolagus cuniculus*) (17). Those PPIs meeting the combined score requirement of >0.4 were used to construct the PPI network, which was then visualized using Cytoscape (version 3.2.0, http://cytoscape.org/) (18). In the PPI network, each node represents a protein, and each edge between two nodes represents an interaction between these two proteins. The degree is defined as the number of proteins interacting with the node.

Furthermore, the degree centrality, a network topology index, was used to analyze the scores of nodes in the network. The nodes with higher degrees are considered to be important in the PPI network, and may be the key nodes.

Subnet module analysis. Genes often have a regulatory role by interacting with other cellular components. Proteins produced by genes in the same module tend to have the same function or similar functions, and where they act as a module they may have the same biological role. The most significantly clustered modules in the network were analyzed using the Multi Contrast Delayed Enhancement plugin of Cytoscape (19).

GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of regulatory network modules. In order to examine the gene function and pathways related to the DEGs, the Database for Annotation Visualization and Integrated Discovery (DAVID) online tool (version 6.8, https://david-d. ncifcrf.gov/) (20) was used to perform the Gene Ontology (GO) functional and pathway enrichment analyses of DEGs based on the GO database (20) and KEGG pathway database (21). Only function terms with an adjusted P-value of <0.05 and count ≥2 were considered as significant.

Analysis of disease-related chemistry small molecule-target gene interaction network. The CTD, a publicly available database (http://ctdbase.org/), provides manually curated information on chemical-disease, gene-disease associations and chemical-gene/protein interactions. Based on PE-related chemical-gene interaction data provided by the CTD (22), the chemical-gene interaction association pairs were visualized via Cytoscape. Chemicals interacting with a higher number of DEGs may be more important in this disease.

Results

Identification of DEGs. A total of 548 (262 upregulated and 286 downregulated DEGs) were identified in the PTE group, compared with the control group. The results of the hierarchical clustering heat map of DEGs showed that these DEGs were clearly distinguishable between the different sample types (Fig. 1).

Functional enrichment analysis. The upregulated DEGs were significantly enriched in 27 KEGG pathways, 51 GO biological processes (BPs), 27 GO cellular components (CCs) and 10 GO molecular functions (MFs). The downregulated genes were significantly enriched in 11 KEGG pathways, 15 GO BPs, 14 GO CCs, and 11 GO MFs. The enriched results are presented in Table I. The upregulated DEGs were

Table I. Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched by differentially expressed genes.

Pathway ID	Pathway name	Count	P-value
Upregulated			
ocu04145	Phagosome	15	5.51E-09
ocu04640	Hematopoietic cell lineage	10	4.39E-07
ocu05323	Rheumatoid arthritis	10	1.99E-06
ocu05140	Leishmaniasis	9	4.03E-06
ocu05162	Measles	9	1.80E-04
ocu05142	Chagas disease (American trypanosomiasis)	8	3.94E-04
ocu04620	Toll-like receptor signaling pathway	7	8.54E-04
ocu05133	Pertussis	6	2.08E-03
ocu05146	Amoebiasis	7	2.30E-03
ocu05144	Malaria	5	3.61E-03
Downregulated			
ocu05412	Arrhythmogenic right ventricular cardiomyopathy	7	5.12E-05
ocu04020	Calcium signaling pathway	9	2.26E-04
ocu05410	Hypertrophic cardiomyopathy	6	1.24E-03
ocu05414	Dilated cardiomyopathy	6	1.66E-03
ocu04080	Neuroactive ligand-receptor interaction	9	2.90E-03
ocu04022	cGMP-PKG signaling pathway	7	4.86E-03
ocu04261	Adrenergic signaling in cardiomyocytes	6	1.11E-02
ocu04066	HIF-1 signaling pathway	5	1.20E-02
ocu05416	Viral myocarditis	4	2.74E-02
ocu04151	PI3K-Akt signaling pathway	8	2.75E-02

cGMP-PKG, cyclic guanosine monophosphate-dependent protein kinase G; HIF-1, hypoxia-inducible factor-1; PI3K, phosphoinositide-3-kinase.
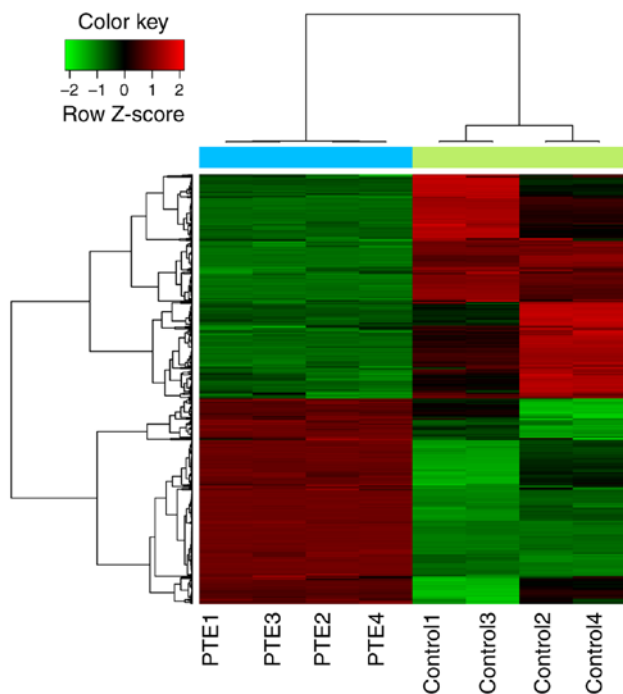

Figure 1. Clustered heat map of differentially expressed genes in the PTE group compared with the control group. The abscissa represents different samples, and the ordinate represents different genes. The red boxes indicate upregulated genes, and the green boxes indicate downregulated genes. Clustering between different samples and different genes were performed based on Pearson's and Spearman's correlation coefficients, respectively. PTE, pulmonary thromboembolism.

largely enriched in the phagosome (P=5.51E-09) and hematopoietic (P=4.39E-07, e.g. *TNF*) cell lineages in the KEGG pathways, and the downregulated DEGs were enriched in the arrhythmogenic right ventricular cardiomyopathy (P=5.12E-05) and calcium signaling (P=2.26E-04) pathways in the KEGG pathways (Table I). Interleukin-6 production (P=4.02E-07, e.g. *TNF*) in GO BP, external side of plasma membrane (P=1.46E-05, e.g. *TNF*), NADPH oxidase complex (P=2.08E-05) in GO CC, and low-density lipoprotein particle binding (P=6.06E-06) in MF were the upregulated genes, and were enriched among the abovementioned GO terms (Fig. 2A). The downregulated genes were largely enriched in lens development in camera-type eye (P=2.40E-05), sarcolemma (P=5.93E-05), and structural constituent of eye lens (P=1.01E-08) (Fig. 2B).

PPI network and module analysis. A total of 342 nodes and 1,128 interactional pairs were included in the PPI network. Among them, 10 proteins had >25 degrees (Fig. 3). *TNF* and *ERBB2* were the upregulated genes with the highest degree in the PPI network, with 47 and 40 degrees respectively. *C-JUN* was the downregulated gene with the highest degree of 46. Furthermore, *C-JUN* was an interacting protein of *TNF* and *ERBB2*.

In total, three subnet modules (A-C) were identified with a score >4. Module A (score=6.769) included 14 nodes (e.g. actin β) and 44 interactional pairs (Fig. 4A). Module B (score=6) had seven nodes (e.g. potassium voltage-gated
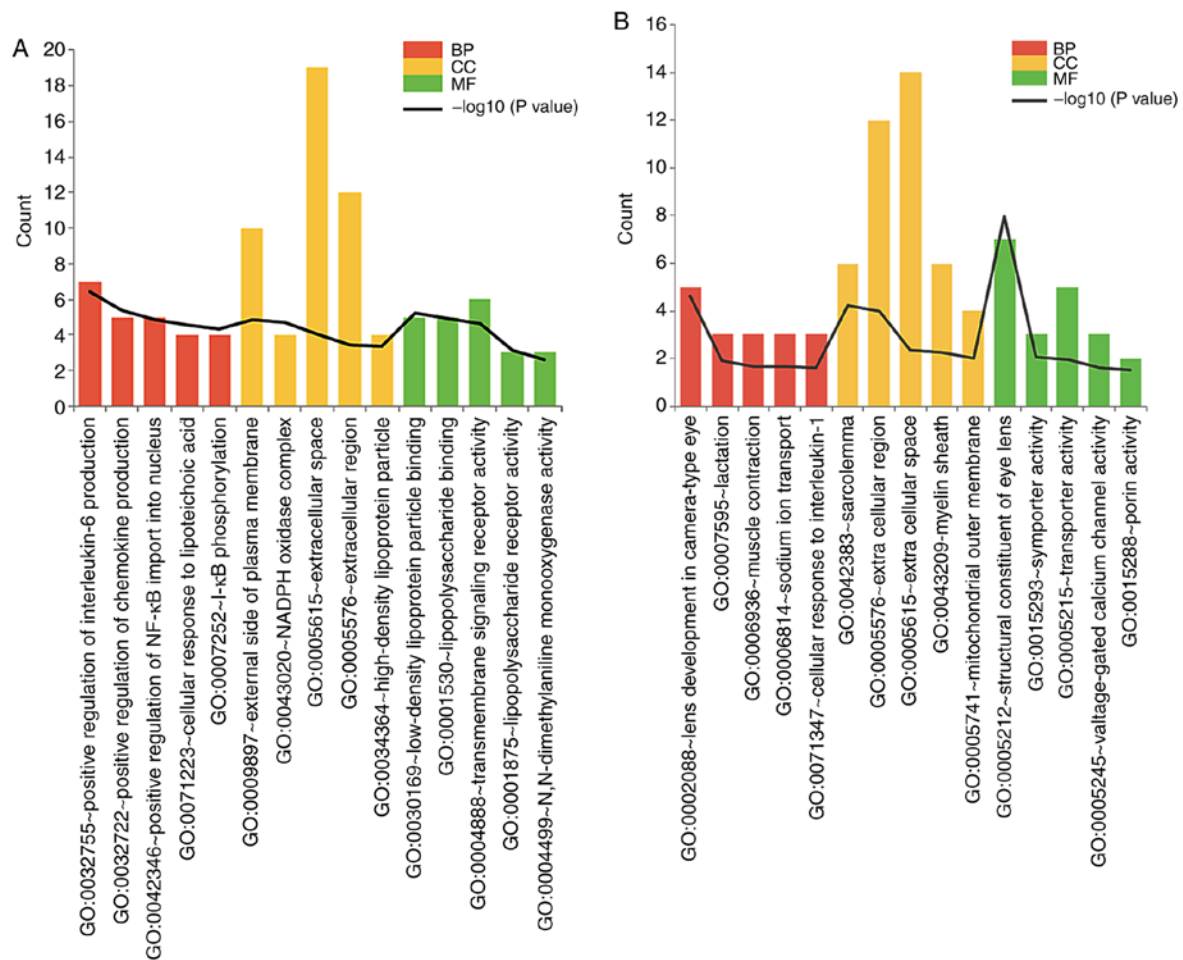
Figure 2. Top GO terms enriched in DEGs. Top 5 GO terms enriched in (A) upregulated and (B) downregulated DEGs. Red, yellow, and green boxes represent GO BP, CC, and MF, respectively. GO, gene ontology; DEGs, differentially expressed genes; BP, biological process; CC, cellular component; MF, molecular function.

channel subfamily B member 2) and 18 interactional pairs (Fig. 4B). Module C (score=4.435) included 24 nodes (e.g. *TNF* and *C-JUN*) and 51 interactional pairs (Fig. 4C). Genes in module A were most enriched in cardiac muscle contraction (P=3.97E-03), hypertrophic cardiomyopathy (P=4.79E-03) and dilated cardiomyopathy (P=5.45E-03). Genes in module B were most enriched in the oxytocin signaling pathway (P=1.33E-03), whereas biosynthesis of antibiotics in the KEGG pathways (P=1.61E-03) was enriched in module C genes.
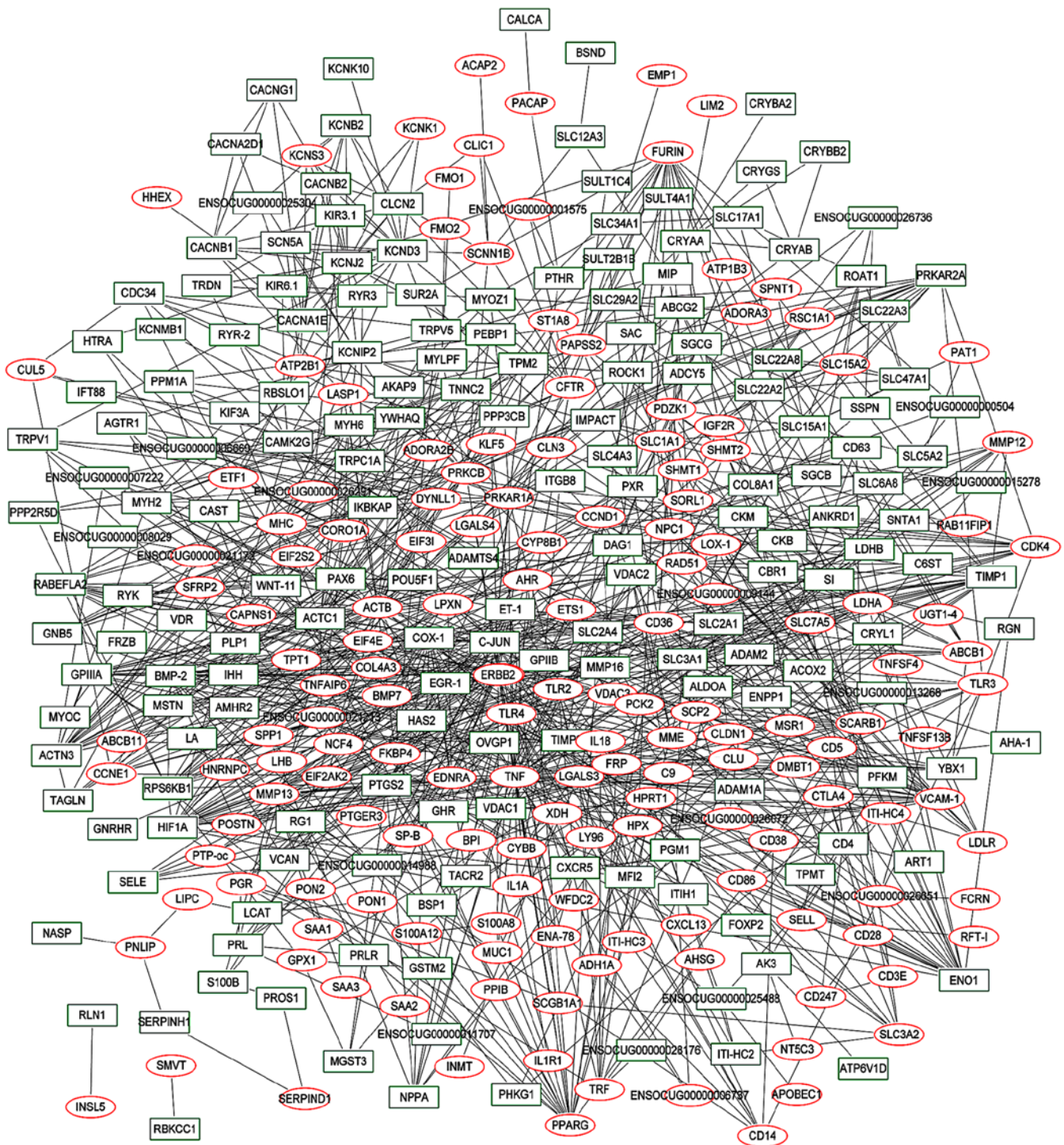
PTE-related chemical-small molecule-target gene inter-action network. In the CTD database, 50,719 PE-related chemical-target interactions, including 1,537 DEG-chemical interactions, were detected (Fig. 5). A total of 1,537 inter-actional pairs, 127 upregulated DEGs, 105 downregulated DEGs, and 117 chemicals, including four with a degree of >100 [ethinyl (135), cyclosporine (126), thrombomodulin precursor (THBD) (113) and tretinoin (111)], were found in the DEG-chemical interaction network. Among them, ethinyl targeted *TNF*, whereas *ERBB2* were targeted by cyclosporine. In addition to *THBD* being an upregulated gene, it was an important chemical interacting with ethinyl, cyclosporine and tretinoin. The chemical tretinoin targeted *ERRB2* (Table II).

Discussion

The findings of the present study demonstrated that *TNF* and *ERBB2* were upregulated, and *C-JUN* was the main downregulated gene in the PTE group. Additionally, ethinyl, cyclosporine, and tretinoin may have significant effects in the treatment of PTE. Among them, *TNF* was targeted by ethinyl and cyclosporine, *ERBB2* was targeted by cyclosporine and tretinoin, and *C-JUN* was targeted by *TNF* and *ERBB2*.

TNF is a multifunctional pro-inflammatory cytokine produced by macrophages. Previous studies have indicated that inflammation is associated with the occurrence of PTE (23). Due to stimulation caused by hypoxia and trauma during PTE, inflammation may trigger pulmonary platelet activation and endothelial dysfunction (24). Additionally, systemic inflam-matory and leukocytosis responses are prognostic factors for mortality following 30 days of PTE (25). In addition, pro-inflammatory factors, including *TNF* and *IL-6*, are signifi-cantly correlated with PTE mortality rate (26,27). Consistent with previous studies, the results of the present study suggested that, compared with the control group, *TNF* was upregulated in the PTE group, and that it was mainly enriched in the hematopoietic cell lineage, positive regulation of interleukin-6 production, and external side of the plasma membrane.

Figure 3. Protein-protein interaction network of differentially expressed genes. Red circles and green squares represent the upregulated and downregulated genes, respectively. Lines indicate protein-protein interaction.

Furthermore, it was targeted by small chemical molecules, including ethinyl and cyclosporine. Ethinyl is a contraceptive, although oral ethinyl increases the risk of venous thromboembolisms including PTE (28,29). Cyclosporine is a calcineurin inhibitor (CNI), which is beneficial for reducing the incidence of complications during homologous transplantation, including vascular toxicity (30). CNIs induce proinflammatory cytokine production and endothelial activation in the isolated mouse aorta and vascular smooth muscle cells (31). Cyclosporine also increases the mRNA levels of proinflamma-

tory cytokines, including *TNF-α*, *IL-6*, C-C motif chemokine ligand 5 (*CCL5*), and *CCL2*, which are involved in vascular injury (31). Consistent with previous studies, the results of the present study indicated that *TNF* was targeted by cyclosporine. Cyclosporine promotes inflammation-induced monocyte adhesion to human intestinal endothelial cells (32). Therefore, ethinyl and cyclosporine may increase the incidence of PTE via targeting *TNF* and promoting the onset of inflammation.

Cyclosporine is also targeted by *ERBB2*, which is a member of the epidermal growth factor receptor family.
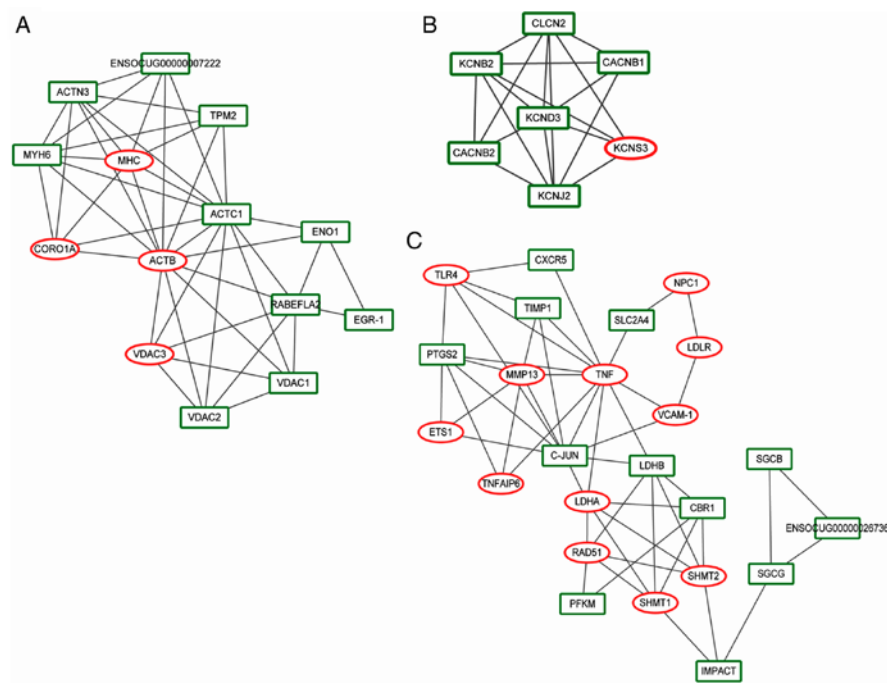
Figure 4. Results of subnet analysis of the PPI network. (A) Module A, (B) module B, and (C) module C of the PPI network are presented. Red circles and green squares represent the upregulated and downregulated genes, respectively. The lines indicate PPI. PPI, protein-protein interaction.

Table II. Top five Kyoto Encyclopedia of Genes and Genomes pathways enriched by differentially expressed genes in the top 10 degrees and modules.

| Pathway ID	Pathway name	Count	P-value	Genes
Degree top 10				
ocu05205	Proteoglycans in cancer	5	7.67E-06	ACTB, HIF1A, TNF, ERBB2, TLR4
ocu05140	Leishmaniasis	3	1.53E-03	TNF, PTGS2, TLR4
ocu04064	NF-κB signaling pathway	3	1.69E-03	TNF, PTGS2, TLR4
ocu05410	Hypertrophic cardiomyopathy	3	2.04E-03	ACTB, ACTC1, TNF
ocu05414	Dilated cardiomyopathy	3	2.33E-03	ACTB, ACTC1, TNF
Module A				
ocu04260	Cardiac muscle contraction	3	3.97E-03	ACTC1, MYH6, TPM2
ocu05410	Hypertrophic cardiomyopathy	3	4.79E-03	ACTB, ACTC1, TPM2
ocu05414	Dilated cardiomyopathy	3	5.45E-03	ACTB, ACTC1, TPM2
ocu04530	Tight junction	3	1.08E-02	ACTB, MYH6, ACTN3
ocu04261	Adrenergic signaling in cardiomyocytes	3	1.29E-02	ACTC1, MYH6, TPM2
Module B				
ocu04921	Oxytocin signaling pathway	3	1.33E-03	CACNB1, CACNB2, KCNJ2
ocu05412	Arrhythmogenic right ventricular cardiomyopathy	2	2.94E-02	CACNB1, CACNB2
ocu04260	Cardiac muscle contraction	2	3.22E-02	CACNB1, CACNB2
ocu05410	Hypertrophic cardiomyopathy	2	3.53E-02	CACNB1, CACNB2
ocu05414	Dilated cardiomyopathy	2	3.77E-02	CACNB1, CACNB2
Module C				
ocu01130	Biosynthesis of antibiotics	5	1.61E-03	SHMT1, LDHB, LDHA, SHMT2, PFKM
ocu00010	Glycolysis/gluconeogenesis	3	1.07E-02	LDHB, LDHA, PFKM
ocu01230	Biosynthesis of amino acids	3	1.25E-02	SHMT1, SHMT2, PFKM
ocu05140	Leishmaniasis	3	1.29E-02	TNF, PTGS2, TLR4
ocu04064	NF-κB signaling pathway	3	1.42E-02	TNF, PTGS2, TLR4

NF-κB, nuclear factor-κB.
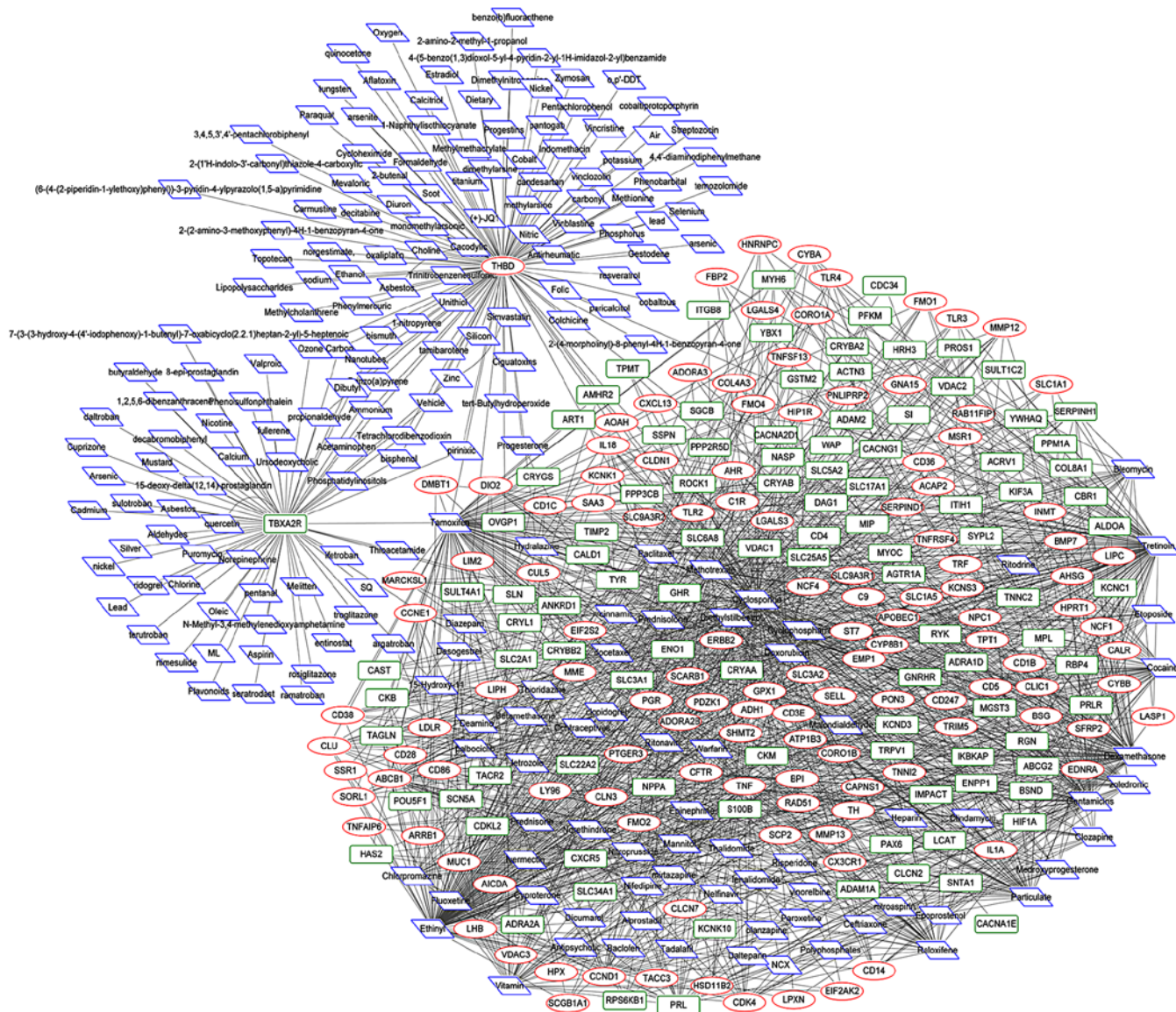
Figure 5. Chemical-target interactional network. The blue quadrilaterals, red circles, and green squares represent the chemical small molecules, the upregulated genes, and the downregulated genes, respectively. Lines indicate protein-protein interaction.

ERBB2 is increased in patients with cancer, including breast and ovarian cancer (33). In breast cancer, *ERBB2* is important in regulating oncogenic microRNA expression (34). *ERBB2* is also a potent independent predictor of the metastatic potential of breast cancer cells (35). However, few studies appear to have investigated the role of *ERBB2* in PTE. It is reported that breast cancer is closely associated with the development of vascular emboli (36). In the present study, *ERBB2* was the important upregulated gene in the PTE group, as it had a higher degree in the PPI network, and it was a target of the small chemical molecules, cyclosporine and tretinoin. Tretinoin, a metabolite of retinol, can repair lung tissue in pulmonary emphysema model rats (37). The role of tretinoin in promoting alveolar regeneration involves the regulation of vascular endothelial growth factor (*VEGF*), VEGF receptor 2, and matrix metallo-proteinase 1 (38). Therefore, tretinoin may reduce the degree of lung injury in PTE by downregulating *ERBB2*. Cyclosporine may contribute to the occurrence of PTE via targeting *ERBB2* and *TNF*, thereby regulating the expression of *C-JUN*.

In humans, C-JUN is a protein encoded by *JUN*. C-JUN in combination with C-FOS, forms the early response transcription factor, activator protein 1 (39). In addition, C-JUN cooperates with nuclear factor-κB to prevent apoptosis induced by TNF-α. C-JUN can also protect hepatocytes from apoptosis, as hepatocytes lacking C-JUN show increased sensitivity to TNF-α-induced apoptosis (40). However, the role of C-JUN in PTE has not been reported. As stated above, pro-inflammatory factors, including *TNF* and *IL-6*, show a significant correlation with PTE mortality rate. In addition, β4 integrin forms a complex with ERBB2 and enhances activation of the transcription factors signal transducer and activator of transcription 3 and C-JUN (41). C-JUN is also necessary for ERBB2-mediated hyperproliferation. Therefore, *C-JUN* may regulate *TNF* and *ERBB2*.

However, the results of the present study have not been experimentally validated, which is a limiting factor that requires resolution in the future. In addition, the regulation of small chemical molecules by PTE-related gene expression

was not investigated. The sample size used in the present study was also small (four control and four PTE pulmonary artery samples).

In conclusion, the findings of the present study indicate that small chemical molecules, cyclosporine and ethinyl, may trigger PTE by regulating the expression of *TNF* and *ERBB2*. Furthermore, tretinoin may delay the progression of PTE via targeting *ERBB2*. Taken together, cyclosporine, ethinyl, and tretinoin may be promising potential targets for PTE treatment.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

KS provided the conception and design of the study. ZX and CQ acquired the data. JW, YL and ML analyzed and interpreted the data and performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of animals was approved by the Animal Ethics Committee of Affiliated Hospital of Nantong University (Nantong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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