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ORIGINAL RESEARCH PAPER

Identification of key pathways and differentially expressed genes in bronchopulmonary dysplasia using bioinformatics analysis

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Abstract
Objectives The objective of this study was to discover unknown differentially expressed genes (DEGs) associated with bronchopulmonary dysplasia (BPD), analyze their functions and enriched signaling pathways, and identify hub genes correlating with BPD incidence and evolvement.
Results Of 1289 DEGs identified, 568 were downregulated and 721 were upregulated. The DEGs were mainly associated with oxidative stress, angiogenesis, extracellular matrix, inflammation, cell cycle, and protein binding. Eight DEGs were identified as hub genes, including C-X-C motif chemokine ligand 5 (*Cxcl5*), connective tissue growth factor (*Ctgf*), interleukin 6 (*IL6*), matrix metallopeptidase 9 (*Mmp9*), mitogen-activated protein kinase 14 (*Mapk14*), platelet and endothelial cell adhesion molecule 1 (*Pecam1*), TIMP metallopeptidase inhibitor 1 (*Timp1*), and TIMP metallopeptidase inhibitor 2 (*Timp2*). IL6 mRNA and protein expression levels were significantly increased in the peripheral blood of neonates with BPD.
Conclusions Hence, BPD involves complex biological changes. Our findings indicate that inflammation and angiogenesis may play major roles in BPD pathogenesis and that IL6 has the potential to serve as a biomarker for early BPD diagnosis.

Keywords Bioinformatics analysis · Bronchopulmonary dysplasia · IL6 · Neonatal · Transcriptome

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic respiratory disease that frequently occurs in premature infants, especially in very premature or very-low-birth-weight infants (VLBWIs) (Pasha et al. 2018). BPD was first described by Northway et al. in 1967 (Northway et al. 1967). With advances in perinatal medicine, the rate of successful treatment of BPD in premature infants has increased. Nevertheless, premature infants have extremely immature organ development, and respiratory insufficiency remains the main factor that contributes to perinatal morbidity and mortality (Jung et al. 2017). Since most VLBWIs are born during the saccular stage of lung development,

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which is critical for lung development, BPD can cause abnormal lung development and affect lifelong lung function (Principi et al. 2018). In addition, BPD can cause damage to nervous system and other systemic organs (Abman et al. 2017). However, the underlying pathogenesis of BPD is not well understood, and there are few early diagnostic tests and specific prevention or treatment strategies for this disease.

Oxygen supplementation can improve the survival rate of premature infants. However, oxidative stress caused by high-concentration oxygen therapy can lead to neonatal reactive oxygen species (ROS)-related diseases including necrotizing enterocolitis, retinopathy of prematurity, and BPD (Fukuhara et al. 2017). Because almost no protection against antioxidants occurs in premature infants, they are particularly sensitive to ROS (Pisoschi and Pop 2015). ROS induce the production of inflammatory factors, thus causing respiratory epithelial damage and decreased lung-surfactant activity (Ozsurekci and Aykac 2016). Although BPD pathogenesis has been continuously studied, the underlying molecular mechanisms remain inconclusive. Here, we aimed to screen for differentially expressed genes (DEGs) in BPD, analyze their biological functions and enriched signaling pathways, and identify hub genes that correlate with the incidence and evolvement of BPD through bioinformatics analyses.

Materials and methods

Microarray data

Four datasets (GSE25286, GSE32472, GSE51039, and GSE121097) were acquired from the GEO database (https://www.ncbi.nlm.nih.gov/geo; Barrett et al. 2013). GSE25286 was used to screen for DEGs by performing mRNA profiling at postnatal days 14 and day 29 in BPD and control groups. For verification, we used GSE51039, which includes expression data obtained from the lungs of 8–10-week-old male and female WT C57BL/6 J mice exposed to hyperoxia or room air (as a control) for 48 h. Two additional datasets (GSE32472 and GSE121097) were used to verify the expression of human hub genes in the peripheral blood of newborns with BPD. Peripheral blood microarray assessments of gene expression profiles from 294 VLBWIs around the 5th, 14th, and 28th days of life were included in GSE32472. GSE121097 included the protein expression profiles of blood samples obtained from infants with a gestational age of < 34 weeks, during their first week following delivery. The basic information obtained from each dataset is shown in Table 1.

Identification of DEGs

DEGs between BPD and non-BPD samples were filtered using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r), an online tool that compares two sets of samples in a GEO dataset. The limma packet was used to calculate P values and log fold-changes (LogFCs). Genes with a logFC of ≥ 1.0 or ≤ -1.0 and a P value of < 0.01 were considered were considered DEGs.

Gene Ontology (GO) pathway, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and Reactome pathway enrichment analyses of DEGs

WebGestalt is an interactive web tool (https://www.webgestalt.org) that employs multiple algorithms for enrichment analysis of various functions (Liao et al. 2019). GO is a database that aids in gene annotation and gene function analysis. The GO database contains three sub-databases, namely the biological process (BP), cellular component (CC), and molecular function (MF) databases (The Gene Ontology Consortium 2017). The KEGG database provides gene annotation data, which can be used to identify the signal transduction pathways in which genes function (Kanehisa et al. 2017). The Reactome database integrates a large amount of peer-reviewed information pertaining to pathophysiological response patterns and biological pathways in the human body (Croft et al. 2011). Gene function enrichment was captured using the WebGestalt tool. The Benjamini–Hochberg false-discovery rate (FDR) was applied to screen statistically significant functional modules and pathways and reduce the FDR. Functional terms with an FDR of < 0.05 were considered biologically meaningful.

Protein–protein-interaction (PPI) network construction and key module filtration

The PPI network was structured using the Search Tool for the Retrieval of Interacting Genes (STRING),

Table 1 Descriptions of the datasets analyzed in this study

Dataset	GSE25286	GSE51039	GSE32472	GSE121097
Species	*Mus musculus*	*Mus musculus*	*Homo sapiens*	*Homo sapiens*
Age	Neonatal	8–10 weeks	Neonatal	Neonatal
Organization	Whole lung	Whole lung	Whole lung	Peripheral blood
Source	mRNA	mRNA	mRNA	Protein
BPD group	6	6	182	82
Control group	4	6	112	20

version 11.0 (https://string-db.org), and interaction scores were generated using the setting 'highest confidence (0.900)'(Szklarczyk et al. 2019).The most central module in the PPI network was ascertained using the Cytoscape plug-in 'Molecular Complex Detection' (MCODE) as the key module (Bader and Hogue 2003). The key module was analyzed using WebGestalt to clarify its functionality. FDR values < 0.05 were considered to reflect statistically significant differences.

Selection and verification of hub genes

The top 10 genes with the highest key module scores were identified as latent hub genes, using the Cytoscape plug-in "cytoHubba." The maximal-clique centrality (MCC) method was used as an algorithm to calculate the score (Chin et al. 2014). GSE51039 was used to validate the accuracy of the selected hub genes. GSE32472 and GSE121097 were used to assess the expression levels of these genes in the peripheral blood of neonates with BPD.

Text mining

Pubmed2ensembl was used as a text-mining tool to extract genomic data from published research. (https://pubmed2ensembl.ls.manchester.ac.uk/; Baran et al. 2011). Metascape (https://www.metascape.org/) was used as an analytical approach to obtain functional annotations for the genes obtained from text mining (Zhou et al. 2019).

Visualization

The R package ggplot2, which visually combines expression data with functional analysis data, was used to generate visual representations of the enrichment analyses (Ito and Murphy 2013). Visual representations of the PPI network were prepared using Cytoscape, version 3.7.2 (Cline et al. 2007).

Results

Identification of DEGs in BPD

In total, 1289 DEGs were confirmed between the BPD and control groups, including 568 downregulated genes and 721 upregulated genes. Genes with a $|\log FC| > 1.0$ and a P value < 0.01 were DEGs (Fig. 1).

GO, KEGG pathway, and reactome pathway enrichment analyses of DEGs

The WebGestalt tool was used to analyze functional and pathway enrichment. GO analysis indicated that DEGs were highly enriched for GO terms such as angiogenesis, cell chemotaxis, response to oxidative stress, leukocyte migration, and extracellular matrix. KEGG pathway analysis suggested that DEGs were mostly involved in focal adhesion, cell-cycle progression, and the Rap1, PI3K–Akt, HIF-1, and MAPK signaling pathways. Changes in Reactome pathways were mainly related to cell-cycle progression, neutrophil degranulation, the VEGFA–VEGFR2 pathway, the apoptosis-execution phase, and the innate immune system. The results and further details are shown in Fig. 2 (FDR < 0.05).

PPI network construction and key module filtration

The PPI network is shown in Fig. 3. The key module was selected using the MCODE plug-in (Fig. 4a). As shown in Fig. 4b, the key module was mainly enriched in terms of cell migration, tissue remodeling, cell proliferation, cell chemotaxis, angiogenesis, and the tumor-necrosis factor (TNF)-signaling pathway.

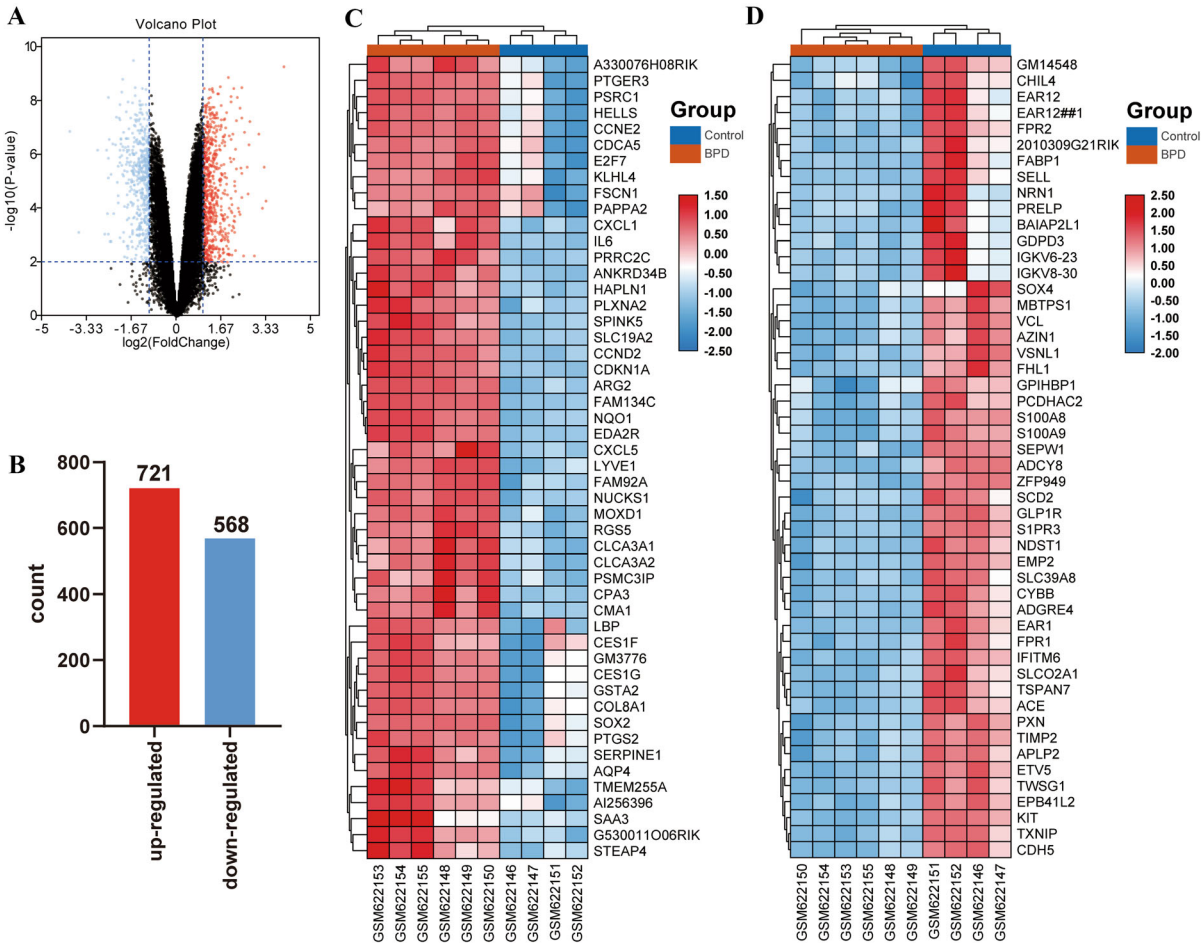

Fig. 1 DEGs between the BPD and control groups. **a** Volcano plot of the gene-expression levels observed in the BPD and control groups. Red dots represent upregulated genes in the BPD group, blue dots represent downregulated genes in the BPD group, and black dots represent no significant difference in terms of gene expression between the groups. **b** Histogram of the number of genes upregulated and downregulated in the BPD group. **c** The top 50 upregulated genes in the BPD group, based on a heat map. **d** The top 50 downregulated genes in the BPD group, based on a het map. BPD, bronchopulmonary dysplasia

Hub gene selection and functional-enrichment analysis

The top 10 hub genes were revealed using the MCC method with cytoHubba and included matrix metallopeptidase 9 (*Mmp9*), TIMP metallopeptidase inhibitor 1 (*Timp1*), mitogen-activated protein kinase 14 (*Mapk14*), transformation-related protein 53 (*Trp53*), platelet and endothelial cell adhesion molecule 1 (*Pecam1*), vascular cell adhesion molecule 1 (*Vcam1*), C-X-C motif chemokine ligand 5 (*Cxcl5*), connective tissue growth factor (*Ctgf*), TIMP metallopeptidase inhibitor 2 (*Timp2*), and interleukin 6 (*Il6*) (Fig. 5a). The expression of *Il6, Vcam1, Cxcl5, Ctgf,* and *Timp1* was significantly upregulated ($P < 0.001$), whereas that of *Mmp9, Mapk14, Trp53, Pecam1,* and *Timp2* was significantly downregulated ($P < 0.001$). We verified the expression of the abovementioned core genes in the GSE51039 dataset. The results showed that VCAM1 expression did not differ significant between two groups (Fig. 5b). The differential expression of TRP53 in GSE51039 was opposite to that in GSE25286. Based on these findings, V*cam1 and Trp53* were removed from the list of hub genes (Table 2). By examining the expression of hub genes in datasets generated using peripheral blood samples from neonatal subjects with BPD (GSE32472 and GSE121097), we found that the mRNA (P = 1.8e-11, Fig. 5c) and protein (P = 0.0097, Fig. 5d) expression levels of IL6 were significantly increased. Moreover,

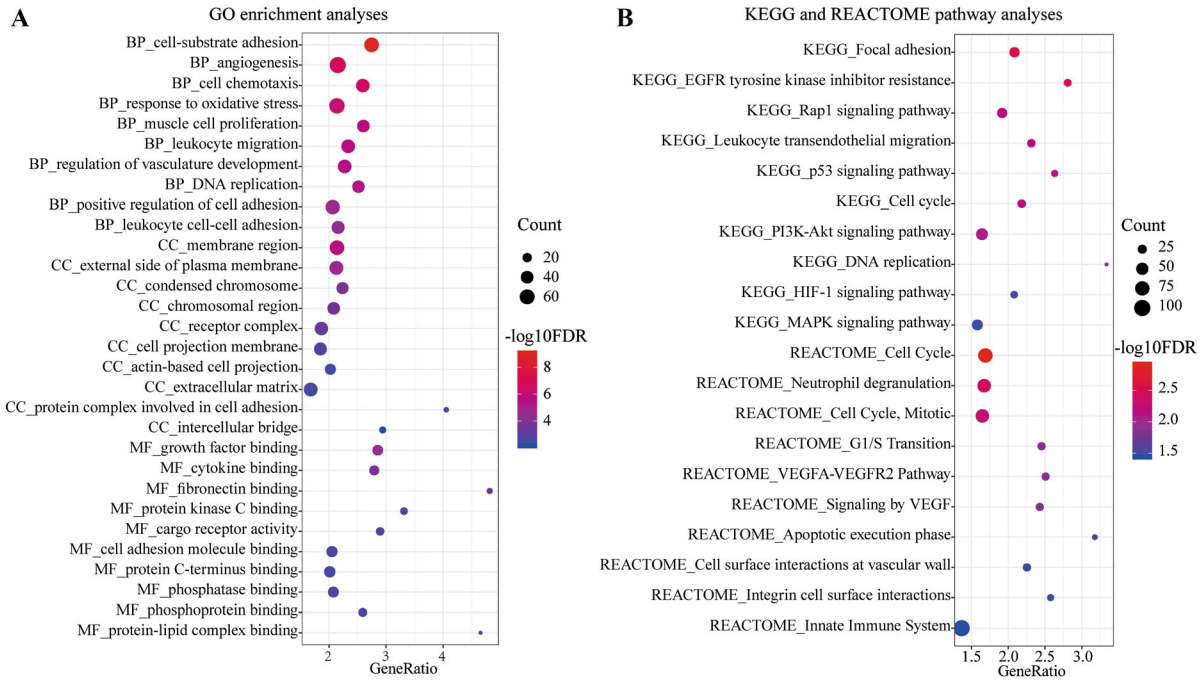

Fig. 2 Functional enrichment analysis of DEGs. **a** GO enrichment analysis. **b** KEGG and Reactome pathway analysis. The abscissa shows the gene-enrichment scores. The size of each dot represents the number of enriched genes. The color of each dot represents the FDR value. BP, biological process; CC, cellular components; MF, molecular function

the expression levels of IL6 increased with the severity of BPD.

Text mining

Using the text-mining tool, a total of 283 genes related to BPD were extracted from 7930 previous reports. The frequency of these genes in the gene set was determined. The top 10 genes with the highest number of reports are presented in Table 3. Enrichment analysis with Metascape showed that the above genes were dramatically enriched in interleukin signaling, regulation of exocytosis, response to toxic substances, leukocyte migration, blood vessel development, apoptotic signaling pathways, the IL-17-signaling pathway, the TNF-signaling pathway, the HIF-1-signaling pathway, aging, and extracellular structure organization (FDR < 0.05) (Fig. 6).

Discussion

BPD can impose a serious healthcare burden on society as a common complication in premature infants (Islam et al. 2015). Although great progress has been made in recent years, the pathological molecular mechanisms of BPD have not been fully elucidated because of the complexities of the interactions between its causative factors. Rapid progress of gene chip technology has enabled wide use of genomic analyses in disease research (Albertson and Pinkel 2003). Such technology provides a new way to extensively explore biomarkers and identify new therapeutic targets for BPD.

In this study, 1289 DEGs were identified, with 568 genes exhibiting downregulated expression and 721 genes exhibiting upregulated expression. GO enrichment analysis showed that the DEGs were mainly involved in oxidative stress, cell-cycle progression, angiogenesis, inflammation, the extracellular matrix, and protein binding. These results reflect, to some extent, the pathophysiological mechanism of BPD.

Inflammatory responses have been associated with BPD. A recent meta-analysis confirmed that chorioamnionitis exposure in premature infants increased the risk of BPD ($P < 0.001$) (Villamor-Martinez et al. 2019). Inflammatory activity in the lungs caused by mechanical injury and oxygen therapy

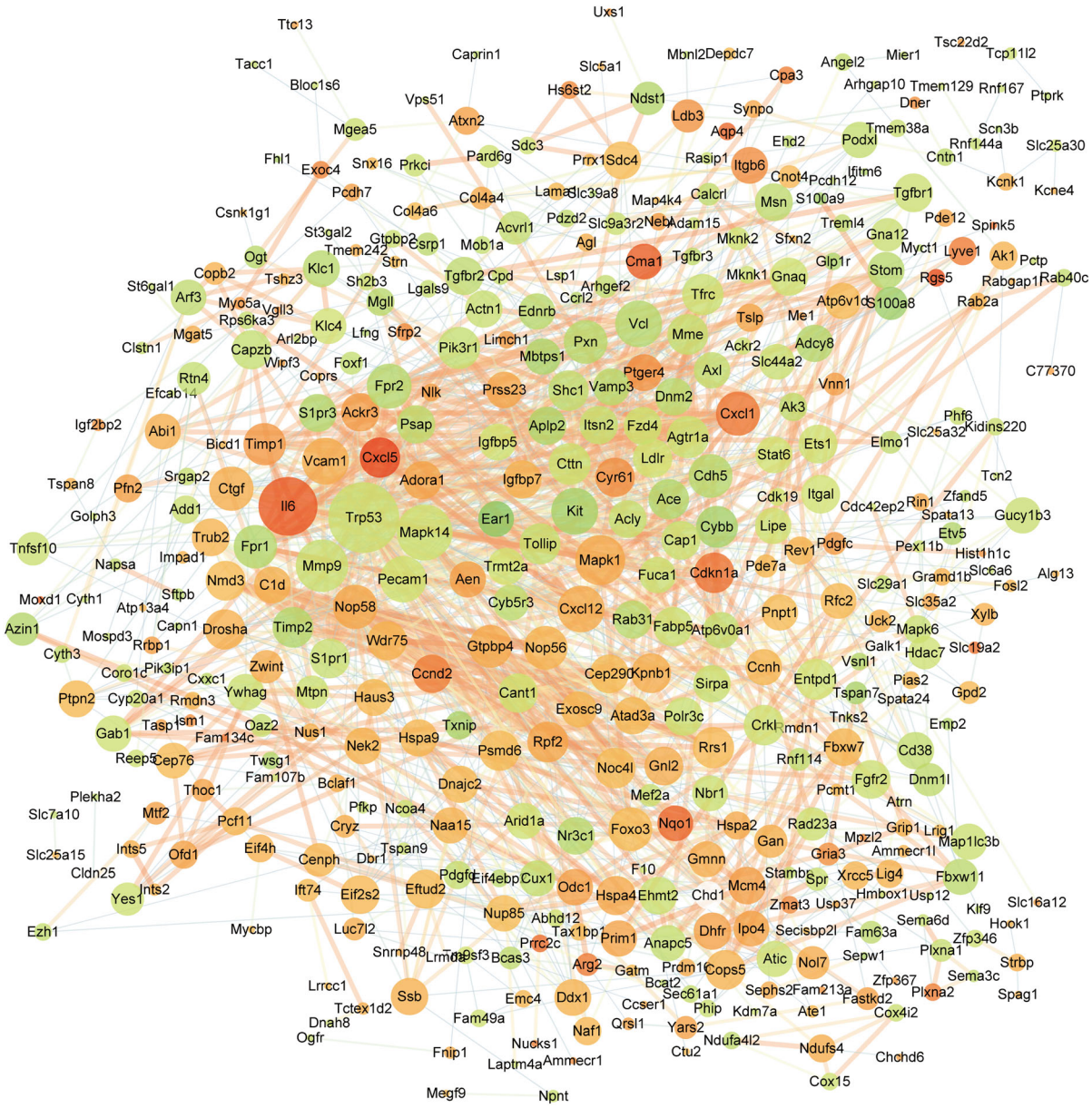

Fig. 3 PPI network. The larger the node diameter, the greater the connectivity. The color of a node represents the associated logFC value. Red shading represents upregulated genes in the BPD group. whereas green shading represents downregulated genes. The darker or thicker the link, the higher the connection score

leads to an imbalance of proinflammatory cytokines and growth factors; this causes inflammatory cells to migrate to the lungs (Rudloff et al. 2017). In this study, we found that multiple inflammation-related functional pathways were significantly associated with BPD. We screened IL6 as a possible key gene, as well as a recognized marker of inflammation, and found that both mRNA and protein expression levels of IL6 were increased in the peripheral blood of children with BPD. IL6 levels were significantly increased in the peripheral blood of children with BPD, even before the onset of clinical symptoms (Leroy et al. 2018). Moderate suppression of inflammation early in life may help prevent BPD (Nold et al. 2013; Liao et al. 2015).

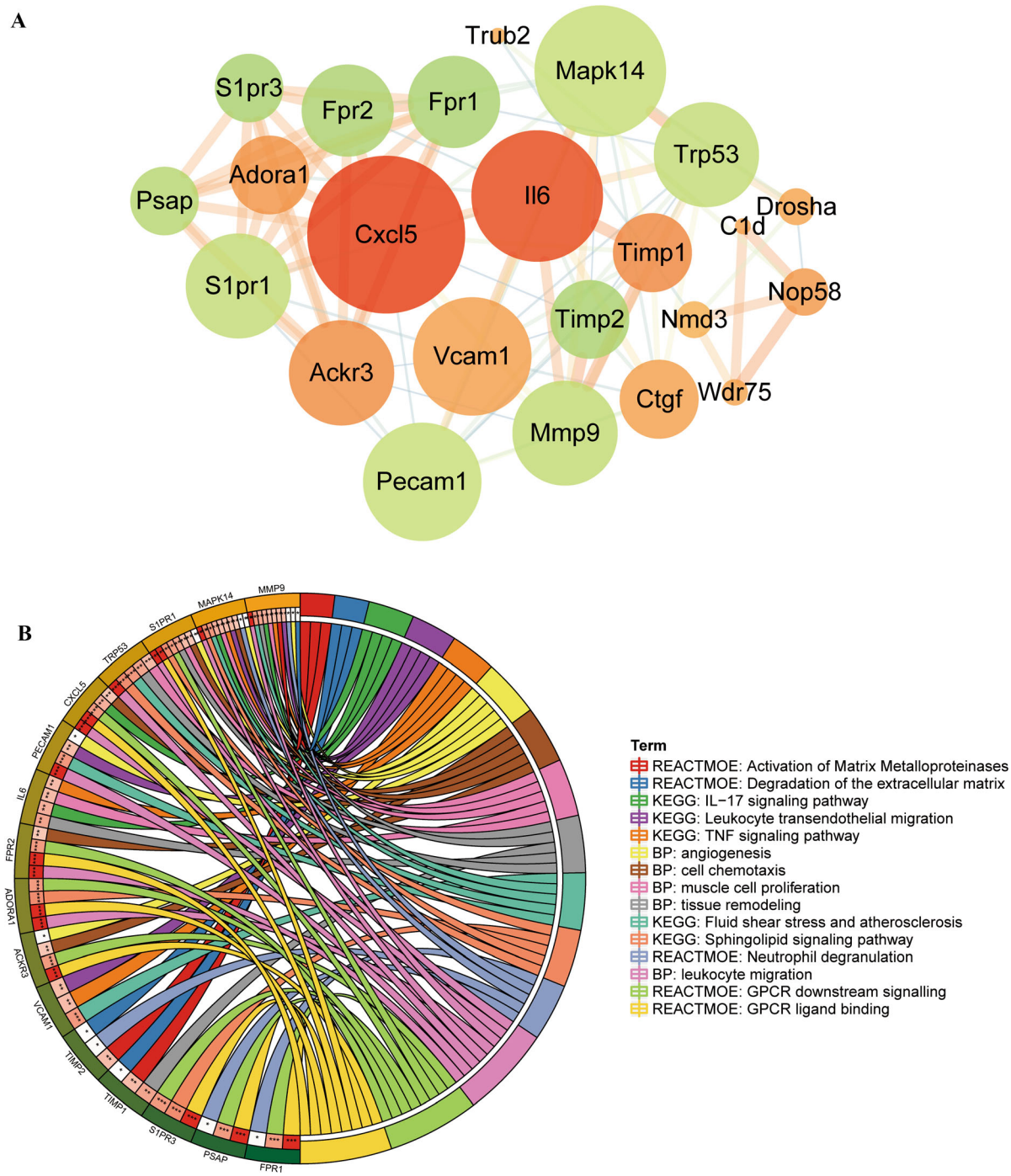

Fig. 4 Identification of the key module and enrichment analysis. **a** The key module network. The larger the node diameter, the greater the connectivity. The color of each node represents the associated logFC value. Red shading represents upregulated genes in the BPD group, whereas green shading represents downregulated genes. The darker or thicker the side, the higher the connection score. **b** Enrichment analysis of the key module. The semicircle on the left side of the circular diagram represents the gene names, and the module on the right side shows the enriched BP, KEGG pathway, and Reactome pathway. *FDR < 0.05; **FDR < 0.01; ***FDR < 0.001

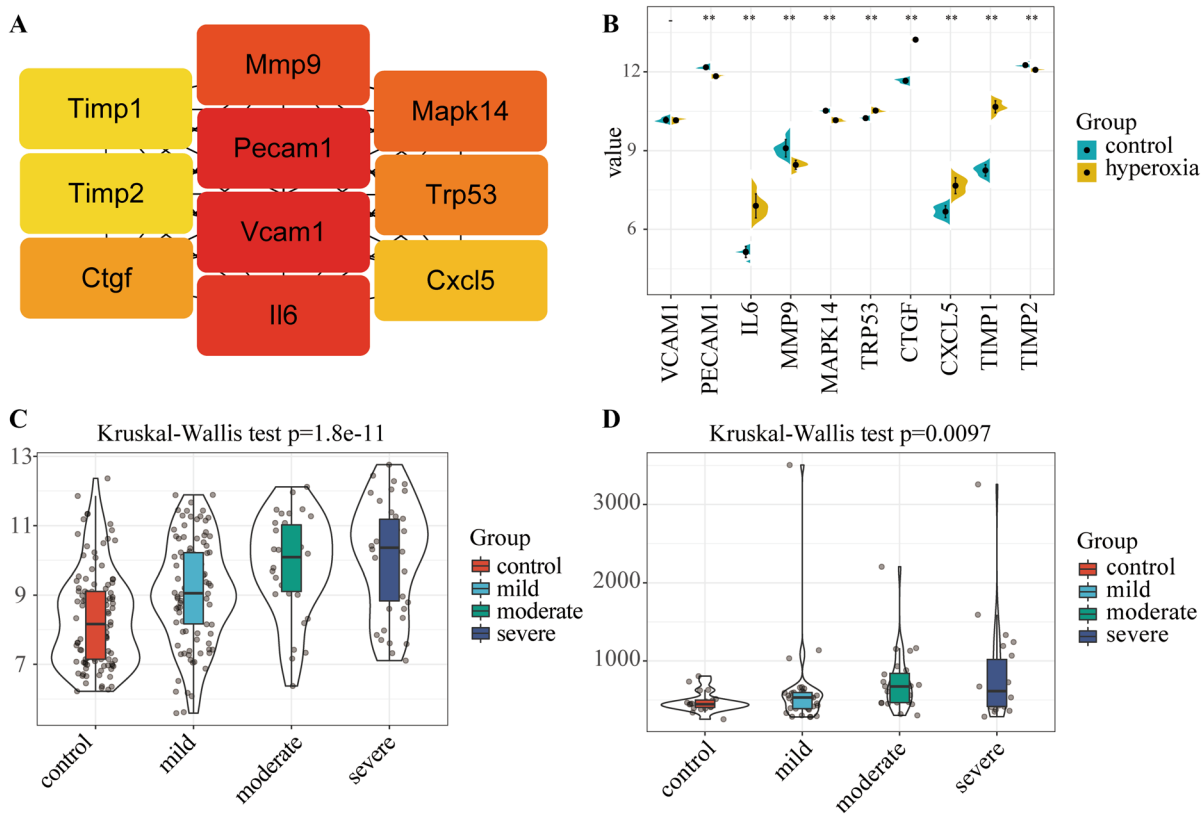

Fig. 5 Hub genes and their verification, **a** Potential hub gene network created using the MCC method. **b** The expression of potential hub genes in GSE51039. **P < 0.01. **c** Expression of IL6 in GSE32472. (D) Expression of IL6 in GSE121097

Table 2 List of the hub genes identified in this study

Gene symbol	Description
PECAM1	Platelet and endothelial cell adhesion molecule 1
IL6	Interleukin 6
MMP9	Matrix metallopeptidase 9
MAPK14	Mitogen-activated protein kinase 14
CTGF	Connective tissue growth factor
CXCL5	C-X-C motif chemokine ligand 5
TIMP1	TIMP metallopeptidase inhibitor 1
TIMP2	TIMP metallopeptidase inhibitor 2

Angiogenic disorders participate in BPD pathogenesis. In BPD, the numbers of pulmonary microvessels are decreased and distributed abnormally, or the numbers of capillaries are increased but are simplified and immature (Alvira and Morty 2017). In addition, angiogenesis can promote distal lung development (Jakkula et al. 2000). In our study, GO analysis of DEGs and the key module highlighted a close relationship between angiogenesis and BPD, thereby substantiating results from prior bioinformatics analyses of children with BPD (Yang et al. 2017). Angiogenic growth factors such as angiopoietin-1 and endostatin can be used to predict BPD (Mohamed et al. 2011). Vascular endothelial growth factor-based gene therapy can improve the survival rate, promote pulmonary angiogenesis, and prevent alveolar damage during lung injury caused by hyperoxia (Thébaud et al. 2005). Further study of pulmonary vascular

Table 3 Top 10 genes identified through text mining

Gene symbol	Description	Rank
VEGFA	Vascular endothelial growth factor A	400
SLC26A3	Solute carrier family 26 member 3	390
IL6	Interleukin 6	297
ALB	Albumin	220
C2	Complement C2	140
ELN	Elastin	140
CENPJ	Centromere protein J	110
NPRL3	NPR3-like, GATOR1 complex subunit	108
SFTPB	Surfactant protein B	90
CXCL8	C-X-C motif chemokine ligand 8	81

disturbances developing during BPD should be beneficial for identifying innovative treatments for BPD.

We found a correlation between BPD and the extracellular matrix (ECM), which involves a highly sophisticated meshwork with large molecules (Thébaud et al. 2005). Any interference with elastin expression and elastin fiber assembly usually leads to poor alveolar formation. This disturbance is caused by high oxygen levels and mechanical stress (Ke et al. 2019). Among the identified hub genes, multiple genes such as *MMP9*, *TIMP1*, and *TIMP2* are involved in ECM assembly and degradation (Table 4).

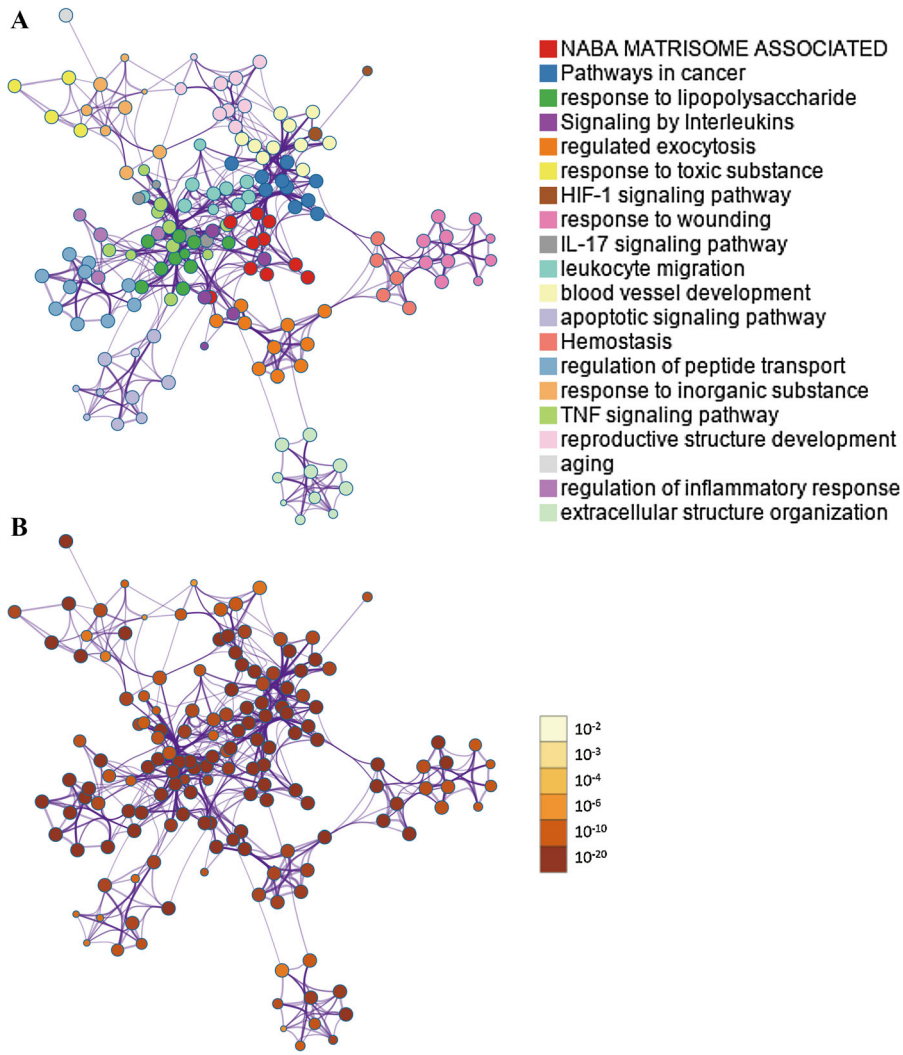

Fig. 6 Cluster plot generated by text mining gene set-enrichment analysis. **a** Coloring based on each enrichment cluster. **b** Coloring based on each P value

Table 4 Description of the hub genes identified in this study

Gene symbol	Description
PECAM1	High oxygen exposure can downregulate Pecam1 expression in the lungs of mice, with a more prominent effect observed in male mice (Firsova et al. 2014)
IL6	IL6 level was significantly increased in the peripheral blood of children with BPD, even before the onset of clinical symptoms (Leroy et al. 2018)
MMP9	Neonatal rats exposed to hyperoxia for 14 days had decreased MMP9 expression level but increased TIMP1 expression level in the lung tissues (Hosford et al. 2004)
MAPK14	MAPK14 (also known as p38alpha) regulated the proliferation and differentiation of lung stem cells or progenitor cells (Kim 2007). MAPK14 may be a key gene in ventilator-associated pneumonia, as identified by PPI network analysis (Xu et al. 2015)
CTGF	CTGF monoclonal antibodies improved alveolar formation and vascular development and reduced pulmonary vascular remodeling and pulmonary hypertension. CTGF may be a new target for BPD therapy in premature infants (Wang et al. 2019a)
CXCL5	The expression of CXCL5 and CXCR2 promoted the development of acute respiratory distress syndrome in mice (Wang et al. 2019b)
TIMP1	Neonatal rats exposed to hyperoxia for 14 days had increased TIMP1 expression levels in the lung tissues (Hosford et al. 2004)
TIMP2	A prospective study showed that low TIMP2 expression in the sera of preterm infants was related to the subsequent occurrence of BPD (Lee et al. 2015)

KEGG pathway analysis revealed a high correlation between BPD and some pathways such as the Rap1, PI3K–Akt, TNF, and IL-17 signaling pathways. Similar results were obtained previously. For example, Rap1-mediated signal transduction helped protect against pulmonary vascular-endothelial barrier injury induced by mechanical ventilation (Ke et al. 2019). Activation of the PI3K–Akt pathway reduced hypoxia-induced pulmonary epithelial cell apoptosis (Wu et al. 2018). TNF plays crucial roles in inflammation, cell proliferation, and cell death. Abnormal signal transduction of nuclear factor-kappa B induced by TNF receptor 1 can lead to chronic inflammation (Van Quickelberghe et al. 2018). Further investigation into these biological processes and pathways may provide a more thorough understanding of BPD pathogenesis. Our results also suggest that many other functional pathways participate in BPD pathogenesis. Although connections between these pathways with BPD remain unclear, they provide new insights for prospective studies of BPD.

Finally, we screened eight hub genes in this study (Table 4). Associations between BPD and *MAPK14* and *CXCL5* are not widely reported. We believe that the identification of these genes will provide new insights into the pathogenic mechanism of BPD.

In summary, we identified DEGs associated with BPD and that disturbance of the angiogenesis pathway and inflammation are key factors associated with the pathogenesis of BPD. We also identified a key module and hub genes, which may play crucial roles in BPD development. This study is the first to link MAPK14 and CXCL5 to BPD, and future studies may further reveal this link. In addition, our study systematically constructed the differentially expressed gene network in BPD. However, this study has some limitations. For example, relatively few samples were used to screen for DEGs in the GSE25286 dataset. Although the expression of each hub gene screened was verified, data from a larger sample size may yield more precise results. Despite these limitations, this study provides new ideas for further studies of the molecular mechanism of BPD and aids in screening of possible therapeutic targets for BPD. Future studies are necessary to characterize the roles of the hub genes in BPD and to determine whether these genes can be used as biomarkers for BPD diagnosis.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by WY and MJ. The first draft of the manuscript was written by WY and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

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