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Research Article

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Identification of key genes associated with epithelial barrier dysfunction for patients with asthma using comprehensive analysis and experimental validation

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Abstract: **Background:** Airway epithelial barrier dysfunction is a significant aspect of asthma, but the crucial regulatory mechanism behind epithelial barrier dysfunction remains unclear. Moreover, epithelial barrier dysfunction is also prevalent in other conditions such as atopic dermatitis and ulcerative colitis, and it is uncertain whether there are common regulatory mechanisms that contribute to epithelial functional dysregulation in these diseases. **Methods:** We obtained gene expression profiles of asthma, atopic dermatitis, and ulcerative colitis from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEG) were identified using the "limma" package, and a common DEG set was produced for further analysis. We conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) functional enrichment analysis on the common DEG and performed correlation analysis with junction molecules to identify key genes involved in epithelial barrier dysfunction. Interaction networks of the key genes were constructed, and their correlation with clinical features of asthmatic patients or immune infiltration was examined. The effects of key genes on CLDN1 expression were determined by qPCR in 16HBE. **Results:** Twenty common DEG were identified from the intersection of asthma, atopic dermatitis, and ulcerative colitis datasets. GO and KEGG enrichment analyses indicated their involvement in cell proliferation regulation, viral protein interaction with cytokines, and transmembrane receptor protein tyrosine kinase-related signaling pathways. Correlation analysis revealed cell division cycle 7 kinase (CDC7), peroxidasin (PXDN), transcobalamin I (TCN1), and tissue inhibitor of metalloproteinase 1 (TIMP1) as key genes, which showed upregulation in the epithelium in all three diseases and were negatively correlated with junction molecules. A chemical–gene interaction network suggested that benzo(a)pyrene could promote the expression of these key genes. Additionally, a correlation between the expression of key genes and the clinical features of asthmatic patients or immune infiltration was found. Finally, it was observed that knocking down key genes in 16HBE could promote CLDN1 expression. **Conclusion:** Our study reveals the pivotal genes implicated in epithelial barrier dysfunction, which may significantly aid in the treatment of asthma and other diseases associated with epithelial barrier dysfunction.

Key words: Epithelial barrier dysfunction; Asthma; Atopic dermatitis; Ulcerative colitis; CDC7; PXDN; TCN1; TIMP1

1 Introduction

Bronchial asthma is one of the most common chronic inflammatory diseases worldwide, affecting more

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than 300 million people (Porsbjerg, et al.,2023). While leucocytes have long been considered the key players in the development of airway inflammation (Huang, et al.,2019), there is increasing evidence that epithelial cells also play an important role in inflammation activation (Izquierdo, et al.,2022). Previous studies have demonstrated the presence of dysfunctional epithelial tight junctions and underlying adherens junctions in the airway epithelium of asthmatic patients (Georas and Rezaee,2014; Heijink, et al.,2020). Epithelial barrier dysfunction not only allows greater leakage of allergens into subepithelial tissue but also propagates pro-inflammatory cytokines, which activate subepithelial immune cells and promote airway inflammation (Cecchi, et al.,2022). Therefore, the disruption of the epithelial barrier plays a crucial role in initiating airway inflammation in asthma.

The first connection between the epithelial barrier and inflammatory diseases was established in the early 1990s with the discovery of a defective intestinal barrier in celiac disease and inflammatory bowel disease (Schulzke and Riecken,1989; More, et al.,1993). In addition, a defective epithelial barrier has been demonstrated in various other chronic inflammation conditions, including asthma, atopic dermatitis (AD), allergic rhinitis (AR), chronic rhinosinusitis, eosinophilic esophagitis, and chronic obstructive pulmonary disease (COPD) (Aghapour, et al.,2018; Simon, et al.,2018; Akdis,2021; Nur Husna, et al.,2021; Dong, et al.,2022; He, et al.,2023; van den Bogaard, et al.,2023). These diseases with epithelial barrier dysfunction can also influence each other (Vutcovici, et al.,2016; Durrani, et al.,2018; Hikichi, et al.,2018; Lee, et al.,2019; Matsumoto, et al.,2020; Raftery, et al.,2020; Sella, et al.,2020; Sugita and Kabashima,2020; Alenezy, et al.,2022). Therefore, preserving and maintaining a healthy epithelial barrier is a potential therapeutic approach for asthma and other epithelial barrier dysfunction diseases (Goleva, et al.,2019). However, whether the mechanisms regulating airway, skin, and intestinal epithelial barrier dysfunction are shared among these diseases is still uncertain. The purpose of our work is to illustrate the epithelium transcriptome crosstalk among asthma, AD, and ulcerative colitis (UC) to provide potential common pathways, key genes, and drugs that might be useful for the treatment of epithelial barrier dysfunction in asthma and other diseases.

In the present study, we utilized comprehensive bioinformatics and enrichment analysis to determine the common differentially expressed genes (DEGs) and their functions in asthma, atopic dermatitis (AD), and UC, based on data downloaded from the Gene Expression Omnibus (GEO). Furthermore, we identified four key genes associated with junctional molecule expressions and constructed interaction networks of these key genes, including transcription factor–gene, miRNA–gene, protein–protein, and chemical–gene interactions. We also validated the expression of key genes in other epithelial barrier dysfunction diseases such as COPD, eczema, Crohn's disease, psoriasis, and AR. Moreover, we found a definite correlation between key gene expressions and clinical features of asthmatic patients or immune infiltration. Finally, we found that knocking down key genes could promote CLDN1 expression in 16HBE. The identification of these key genes can provide new insights into the common pathogenesis of these epithelial barrier dysfunction diseases.

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2 Materials and methods

2.1 Data resource

The gene expression data used in this study were obtained from the GEO database. The GSE43696 dataset included 20 samples from healthy controls, 50 samples from mild-moderate asthmatic (MMA) patients, and 38 samples from severe asthmatic (SA) patients. Airway epithelial cells were collected through bronchoscopy using epithelial brushings. The GSE60709 dataset comprised 19 skin samples from patients with atopic dermatitis (AD) and 14 skin samples from control participants. The GSE36807 dataset contained 15 intestinal biopsy samples from patients with ulcerative colitis (UC) and 7 intestinal biopsy samples from healthy controls.

2.2 Identification of differentially expressed genes

The data extracted from the microarray were normalized and processed through log2 transformation.

Normalization of the microarray data was carried out using the Preprocess Core package available on the R software platform (version 3.4.1). The platform annotation information was utilized to convert the probes to gene symbols in accordance with the normalized data. The DEGs were analyzed using the "limma" package in R software. Cut-off values of adjusted P value < 0.05 and fold-change (FC) ≥ 1.3 or ≤ −1.3 were employed. The ggplot2 package in R was used to create volcano plots, displaying expression levels of all DEGs. Any grammatical errors have been corrected.

2.3 Enrichment analysis

The R package "Cluster Profiler" was utilized for carrying out Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. GO analysis was conducted to identify the potential targets of mRNAs based on Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). Enriched KEGG signaling pathways were chosen to depict the principal biological actions of the major mRNA targets. A P-value less than 0.05 was regarded as statistically significant, indicating a meaningful pathway.

2.4 Correlation heatmaps and boxplot graphs of gene expression

The R software package "pheatmap" was utilized to generate multi-gene correlation heatmaps. Spearman correlation was employed to evaluate the expression correlation between genes, and a $P < 0.05$ was considered statistically significant. Box plots were constructed using the R package 'boxplot'. The Wilcoxon test was utilized to compare the statistical difference between two groups, while the Kruskal–Wallis test was conducted to assess differences among the three groups. Statistical significance was determined at $P < 0.05$.

2.5 Construction of protein–protein, transcription factor–gene, miRNA–gene, and chemical–gene interaction networks

GeneMANIA is a valuable online biological resource database capable of constructing protein–protein interaction (PPI) networks, protein–DNA interactions, signal pathways, and physiological and biochemical responses, which is regularly updated. The PPI network of candidate genes was generated using the GeneMANIA website (https://genemania.org) (Warde-Farley, et al.,2010).

Transcription factor (TF)–gene and miRNA–gene interaction networks were constructed using NetworkAnalyst, a visual analytics platform for comprehensive gene expression profiling and meta-analysis (Zhou, et al.,2019).

The chemical–gene interaction network was constructed using the GenDoma database (https://ai.citexs.com/homePath). This database analyzes human genes from multiple dimensions, including their molecular mechanisms in disease transformation and treatment, by integrating over 70 database sources and information extracted by self-built models.

2.6 Analysis of the expression of key genes in other diseases related to epithelial barrier dysfunction

The mRNA expression of the identified key genes was analyzed in several datasets, namely GSE37147, GSE79704, GSE10616, GSE36807, and GSE19187. The GSE37147 dataset includes bronchial epithelial brushing samples from 87 patients with COPD and 151 controls. The GSE79704 dataset includes skin biopsy samples from 44 patients with psoriasis and 20 controls. The GSE10616 and GSE36807 datasets comprise intestinal biopsy samples from 45 patients with Crohn's disease and 18 controls. Lastly, the GSE19187 dataset consists of nasal epithelial brushing samples from 14 patients with allergic rhinitis (without asthma), 13 patients with allergic rhinitis combined with asthma, and 11 controls. Statistical differences between two groups were compared using the Wilcox test, while the Kruskal–Wallis test was used to test the significant differences between three groups. A P-value < 0.05 was considered significant.

2.7 The relation between key genes and clinical characteristics in patients with asthma

In the GSE89809 dataset, we analyzed the correlation between key genes and the clinical features of

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asthmatic patients. This dataset includes bronchial epithelial brushing samples and clinical information from 38 asthmatic patients. The clinical features consisted of asthma control questionnaire (ACQ) scores, inhaled corticosteroid dosage (ICS), and lung function. Correlations were analyzed using Spearman coefficients.

2.8 Immune infiltration analysis

CIBERSORTx is a unique analytical tool that implements a deconvolution algorithm to assess the abundance of immune cell subpopulations within tissue samples, utilizing gene expression data (Newman, et al.,2019). To evaluate the immune cell population in the bronchial epithelium of asthmatic patients, GSE43696 gene expression profile data from both asthmatic patients and healthy participants were uploaded onto CIBERSORTx. The LM22 Signature matrix file, which encompasses 22 different immune cell components, was applied to serve as a reference tool for effective cell quantification. Furthermore, a Spearman correlation analysis was conducted to establish the existing correlation between genes and immune cells.

2.9 Cell culture and RNA interference

Human bronchial epithelial cell line 16HBEo- (16HBE) was used in this study. 16HBE cells were cultured with high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (PAN, German), 100 μg/ml streptomycin, and 100 UI/ml penicillin (Gibco, USA). siRNA transfections were performed using KeygenMAX 3000 reagent (KGA9705-1.5, KeyGEN, China) according to the manufacturer's instructions. The siRNAs were constructed by KeyGEN (KGARNA, China) and the sequences information is as follows: siCDC7: 5'-CUGUCUAGAAAGUUAGCAA-3'; siTIMP1: 5'-CGUUAUGAGAUCAAGAUGA-3'; siTCN1: 5'-GCGUCUCUGCUUCAGGUAA-3'; siPXDN: 5'-GCGACACCAUCUACUAUGA-3'.

2.10 Quantitative PCR (qPCR)

RNA was isolated using the Trizol kit (TAKARA). The reverse transcription was carried out according to the reverse transcription kit protocol (Evo M-MLV RT Premix, AG11706). qPCR was carried out with SYBR Green (YEASEN 11202ES08, Shanghai, China) on a Real-Time PCR instrument (Bio-Rad). Data analysis was performed using the 2-ΔΔCt method. The primer sequences were obtained from the National Center for Biotechnology Information (NCBI) and are listed below: CDC7 forward: 5′-CAGCAGTCCACCACAAAAGC-3′; reverse: 5′-AGTTTCACTGCAGGGCTCTC-3′; TIMP1 forward: 5′-TGCGGATACTTCCACAGGTC-3′; reverse: 5′-TTCCAGGGAGCCACAAAACT-3′; TCN1 forward: 5′-TAGCACAGGAGAAGCCATGC-3′; reverse: 5′-GCAGCGTTTGGATTGCTGAA-3′; PXDN forward: 5′-ATGGCCAAGCGCTCCAG-3′; reverse: 5′-AATGCCCCAGGTTGGATCTC-3′; CLDN1 forward: 5′-CCTCCTGGGAGTGATAGCAAT-3′; reverse: 5′-GGCAACTAAAATAGCCAGACCT-3′; GAPDH forward: 5′-ATCAGCAATGCCTCCTGCAC-3′; reverse: 5′-TGGCATGGACTGTGGTCATG-3′.

2.11 Immunofluorescence

Cells were fixed in 4% PFA for 20 minutes, followed by incubation with the CLDN1 (28674-1-AP, Proteintech) primary antibody at 4 ℃ overnight. Subsequently, cells were incubated with either Alexa 488-labeled goat anti-rabbit or 596-labeled goat anti-mouse IgG antibody at 37 ℃ for 2 hours. Nuclei were counterstained with DAPI for 5 minutes, and images were acquired using a confocal fluorescence microscope.

2.12 Transepithelial electrical resistance (TER) and permeability to FITC-dextran

The integrity and permeability of the airway epithelial barrier were assessed by measuring transepithelial electrical resistance (TER) and permeability to 4-kDa FITC-dextran. Once the 16HBE cells reached full monolayer confluency, TEER measurements were conducted using a Millipore electric resistance system (ESR-2, Millipore). After TER experiments, the medium was replaced with 200 μL of phenol red-free RPMI 1640 containing 0.5 mg/ml 4-kDa fluorescent-dextran-FITC in the upper chamber, while the lower chamber was

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filled with 800 μL of phenol red-free RPMI 1640 without FITC-Dx. The cells were then incubated at 37° C for 120 minutes. Fluorescence intensity in both the upper and lower chamber media was measured using an excitation light of 492 nm and an emission light of 520 nm.

2.13 Statistical analysis

When comparing two groups, statistical analysis was performed using the unpaired two-tailed Student's t-test (normal distribution data). When comparing multiple groups, multiple comparisons were analyzed using one-way ANOVA followed by Bonferroni's test. Spearman's rank correlation analysis was used for correlation analysis. Data were analyzed with GraphPad Prism 6.0 (GraphPad software). The data were presented as mean ±SEM. Differences were considered statistically significant when the P-value < 0.05.

3 Results

3.1 Identification of DEG and enrichment analysis in the epithelium of asthma, AD, and UC patients compared to normal controls.

The workflow diagram of this research is presented in Fig. 1. We first conducted a differential expression analysis between patients and normal controls to identify DEGs. We identified 694 DEGs in the asthma dataset (GSE43696), 1135 DEGs in the AD dataset (GSE60709), and 2475 DEGs in the UC dataset (GSE36807) with |fold-change (FC)| ≥ 1.3 and |adj. P value| < 0.05 (Fig. 2a-2c). Subsequently, we performed GO/KEGG enrichment analysis for the DEGs. Bubble plots showing the top 10 enriched GO/KEGG terms are presented in Fig. 2d-2i. We found that the DEGs from asthma, AD, and UC could be enriched in common pathways such as extracellular space, extracellular region, and the collagen-containing extracellular matrix in the GO enrichment analysis. The KEGG enrichment analysis showed viral protein interaction with cytokine and cytokine receptor and amoebiasis. Our findings revealed that DEGs from asthma, AD, and UC are closely related and share a large number of common pathways.

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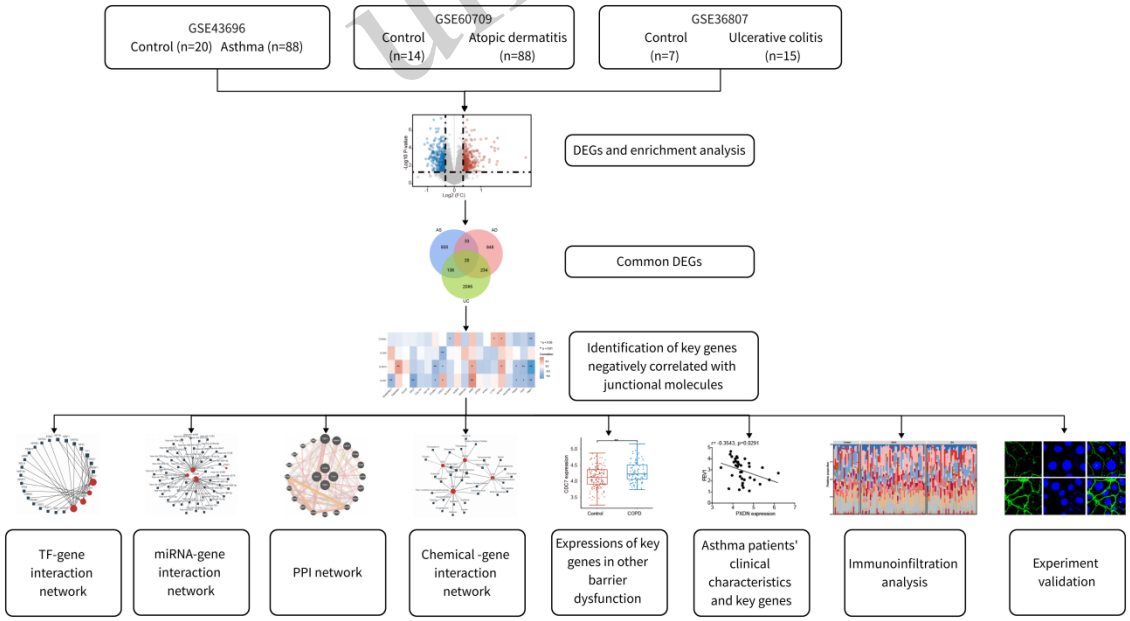

Fig. 1 The workflow diagram of this research.

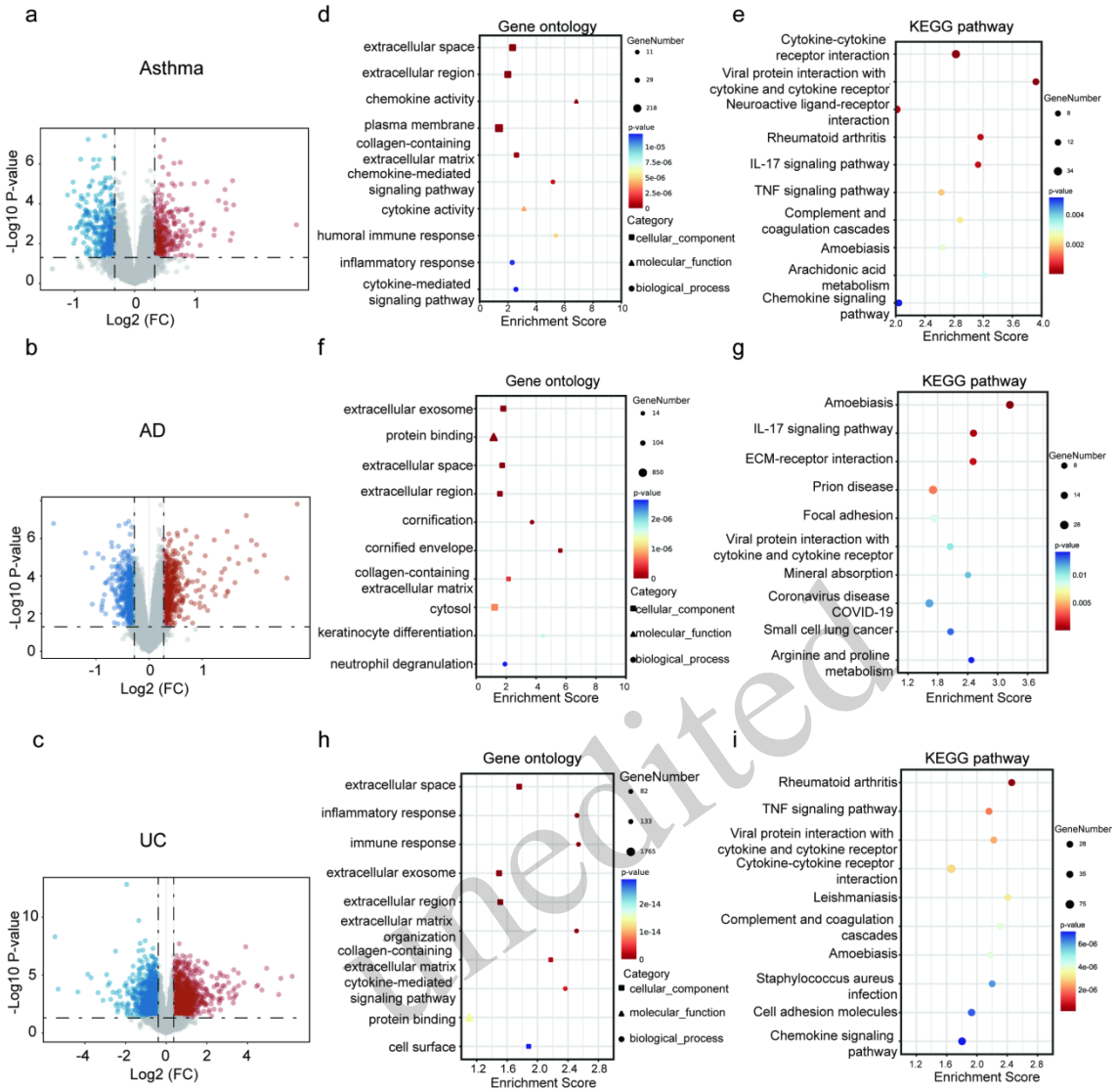

Fig. 2 Volcano map and GO/KEGG functional enrichment analysis of DEGs. (a-c) Volcano plots show the DEGs in asthma (a), AD (b), and UC (c) datasets. The red dots denote the upregulated genes; the blue dots indicate the downregulated genes. (d-f) GO/KEGG pathway enrichment analysis of DGEs in asthma (d and e), AD (f and g), and UC (h and i) datasets. The enriched pathways are displayed on the vertical axis, and their corresponding P-values are shown in various colors. The enrichment score is shown on the horizontal axis, and the size of the bubble represents the count of genes in each pathway. AS, asthma; AD, atopic dermatitis; UC, ulcerative colitis.

3.2 Identification of common DEG and analysis of the functional characteristics of common DEG from asthma, AD and UC

We identified common DEGs from asthma, AD, and UC epithelial expression datasets and obtained 20 common DEGs by taking the intersection of the DEGs from each dataset using a Venn diagram (Fig. 3a). Furthermore, the expression of 20 common DEGs in asthma, AD, and UC epithelial cells was depicted using a heatmap (Fig. 3b-3d). Interestingly, the expression signature of these 20 common DEGs was not the same in the three diseases. GO/KEGG pathway enrichment analysis was then performed to analyze the biological functions and pathways involved in the 20 common DEGs. The GO analysis revealed that the top five GO terms were the positive regulation of cell population proliferation, transmembrane receptor protein tyrosine kinase signaling pathway, positive regulation of kinase activity, platelet-derived growth factor binding, and response to hyperoxia (Fig. 3e). The top five pathways of KEGG were viral protein interaction with cytokine and cytokine

receptor, and the PI3K-Akt signaling pathway, Rap1 signaling pathway, Ras signaling pathway, and MAPK signaling pathway (Fig. 3f). These results suggest that positive regulation of cell population proliferation, viral protein interaction with cytokine and cytokine receptor, and transmembrane receptor protein tyrosine kinase-related signaling pathways (PI3K-Akt signaling pathway, Rap1 signaling pathway, Ras signaling pathway, and MAPK signaling pathway) are involved in these epithelial barrier dysfunction diseases.

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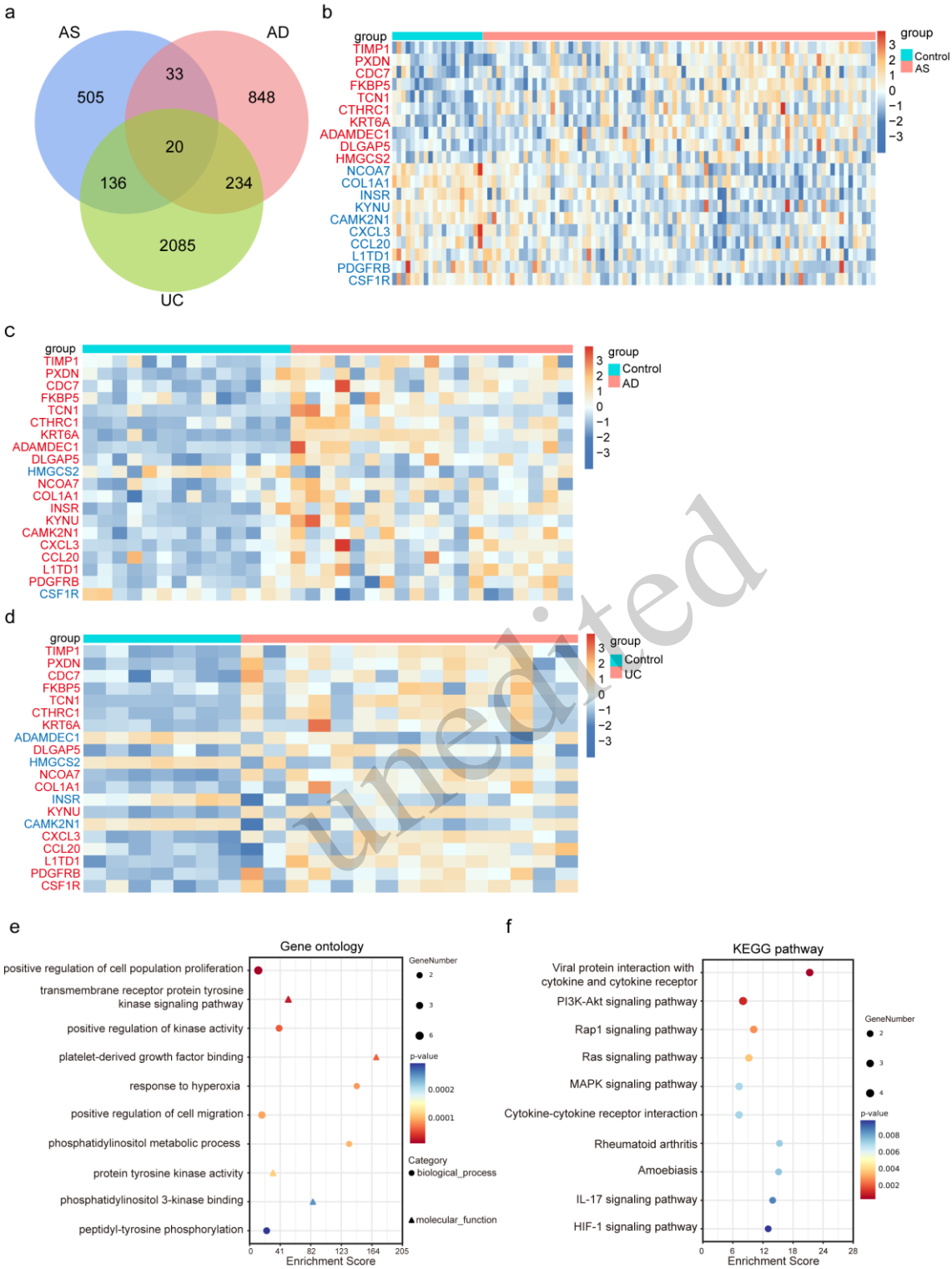

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Fig. 3 Identification and analysis of the functional characteristics of common DEGs. (a) The overlap of DEGs among AS, AD, and UC is displayed in the Venn diagram. (b-d) The expression of 20 common DEGs in asthma (b), AD (c), and UC (d) epithelial cells is displayed in the heatmap. Red color represents upregulation; blue color represents downregulation. (e and f) GO/KEGG pathway enrichment analysis of common DEGs. AS, asthma; AD, atopic dermatitis; UC, ulcerative colitis.

3.3 Expressions of junctional molecules in epithelium from asthma, AD, UC compared to normal control

The epithelial barrier plays a crucial role in preventing the entry of harmful substances into the underlying tissues by various intercellular junctions, especially tight junctions (TJs) and adherens junctions (AJs) (Izquierdo, et al.,2022). However, the expression signatures of junctional molecules in asthma, AD, and UC remain unclear. Next, we analyzed the expression levels of TJs and AJs genes in asthma, AD, and UC datasets. Our results showed that the expression of CLDN1, CLDN4, CLDN16, and CTNNB1 genes was significantly downregulated in the epithelium of individuals with asthma, compared to controls (Fig. 4a). The expression of CLDN7, CLDN8, CLDN16, CLDN22, and CLDN23 genes was significantly downregulated in the epithelium of individuals with AD (Fig. 4b), and the expression of CLDN3, CLDN4, CLDN8, TJP1, and CDH1 genes was significantly downregulated in the epithelium of individuals with UC (Fig. 4c). Interestingly, the expression of the CLDN10 gene was significantly upregulated in individuals with asthma (Fig. 4a). Furthermore, the expression of CLDN3, CLDN4, CLDN9, CLDN25, and TJP2 genes was significantly upregulated in individuals with AD (Fig. 4b), while the expression of CLDN1, CLDN2, and CLDN18 genes was significantly upregulated in individuals with UC (Fig. 4c). These results suggest that the expression signatures of junctional molecules varied among these diseases.

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Fig. 4 Expression signatures of junctional molecules in AS, AD, and UC. (a-c) Expression signatures of junctional molecules in AS (GSE43696), AD (GSE60709), and UC (GSE36807). The statistical difference between the two groups was assessed using the Wilcoxon test. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01, ***P < 0.001. AS, asthma; AD, atopic dermatitis; UC, ulcerative colitis.

3.4 Correlation analysis between common DEG and reduced junction molecules in epithelium from asthma, AD, UC

To explore the potential involvement of 20 common DEGs in the regulation of epithelial barrier dysfunction, a correlation analysis was conducted between these DEGs and reduced junction molecules in asthma, AD, and UC. The results revealed that four DEGs were consistently negatively associated with one or more reduced junction molecules in all three diseases (Fig. 5a-5c and Table 1). These genes are CDC7, PXDN, TCN1, and TIMP1. The expression analysis demonstrated upregulation of CDC7, PXDN, TCN1, and TIMP1 in the epithelium of asthma, AD, and UC compared to controls (Figure 6 a-c). These findings suggest that CDC7, PXDN, TCN1, and TIMP1 may play crucial roles in the development of epithelial barrier dysfunction.

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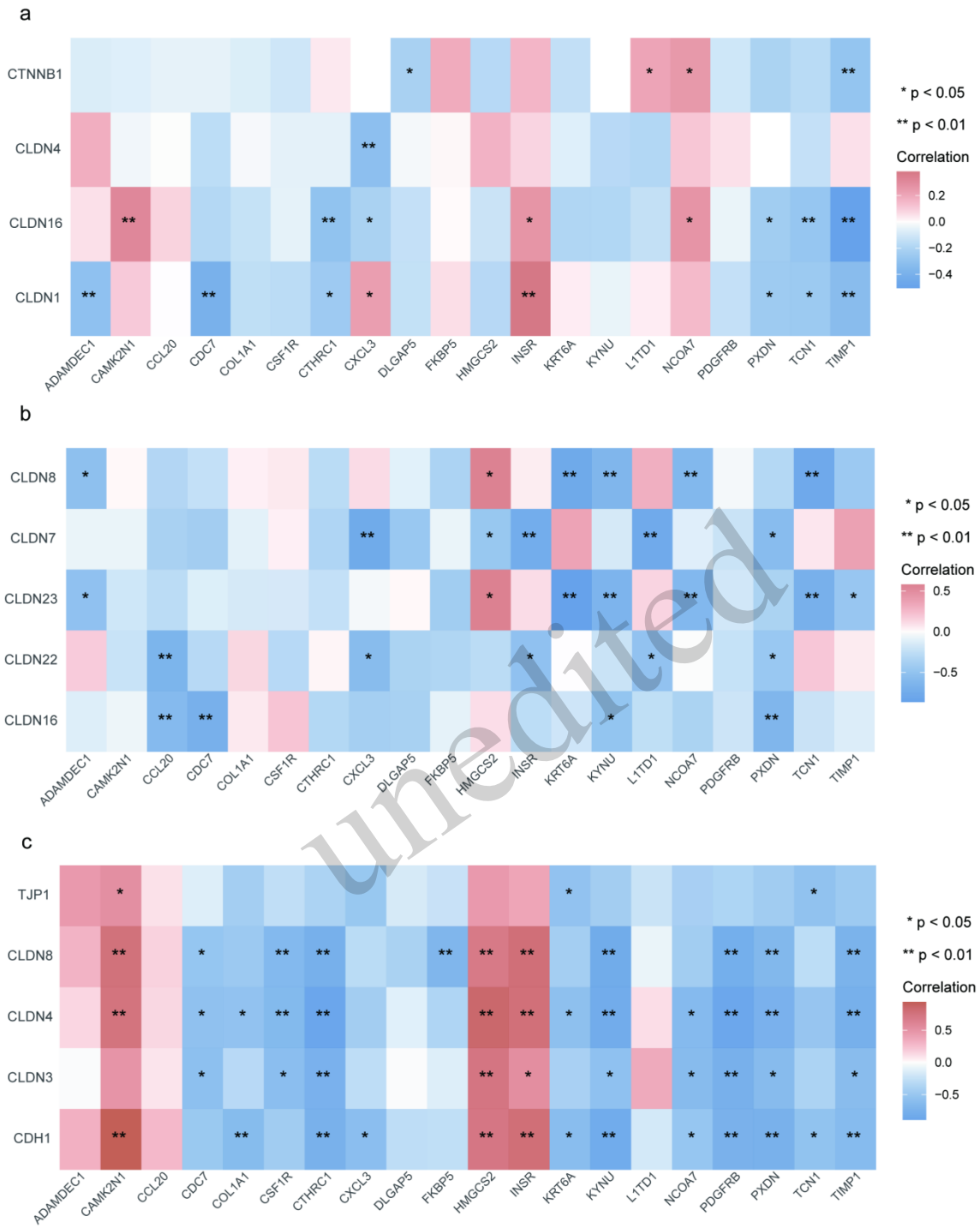

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Fig. 5 Correlation analysis between common DEGs and reduced junction molecules. (a-c) The heatmap depicts the correlation between common DEGs and reduced junction molecules in AS (GSE43696), AD (GSE60709), and UC (GSE36807). The Spearman correlation coefficient was used to analyze the expression correlation between two genes. Genes are represented on the x-axis and y-axis, and different colors indicate different correlation coefficients. Red indicates a positive correlation, while blue indicates a negative correlation; the intensity of the color represents the strength of the relationship. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01. AS, asthma; AD, atopic dermatitis; DEGs, differentially expressed genes; UC, ulcerative colitis.

Table 1 Details of key genes and their negatively correlated junction genes in AS, AD and UC

Gene symbol	Negatively correlated junction genes			Gene description
	AS	AD	UC	
CDC7	CLDN1	CLDN16	CLDN3, CLDN4, CLDN8	This gene encodes a cell division cycle protein with kinase activity that is critical for the G1/S transition.
PXDN	CLDN1, CLDN16	CLDN7, CLDN16, CLDN22	CLDN3, CLDN4, CLDN8, CDH1	This gene encodes a heme-containing peroxidase that is secreted into the extracellular matrix. It is involved in extracellular matrix formation.
TCN1	CLDN1, CLDN16	CLDN8, CLDN23	TJP1, CDH1	This gene encodes a member of the vitamin B12-binding protein family. This protein is a major constituent of secondary granules in neutrophils and facilitates the transport of cobalamin into cells.
TIMP1	CLDN1, CLDN16, CTNNB1	CLDN23	CLDN3, CLDN4, CLDN8, CDH1	This gene belongs to the TIMP gene family. The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases (MMPs).

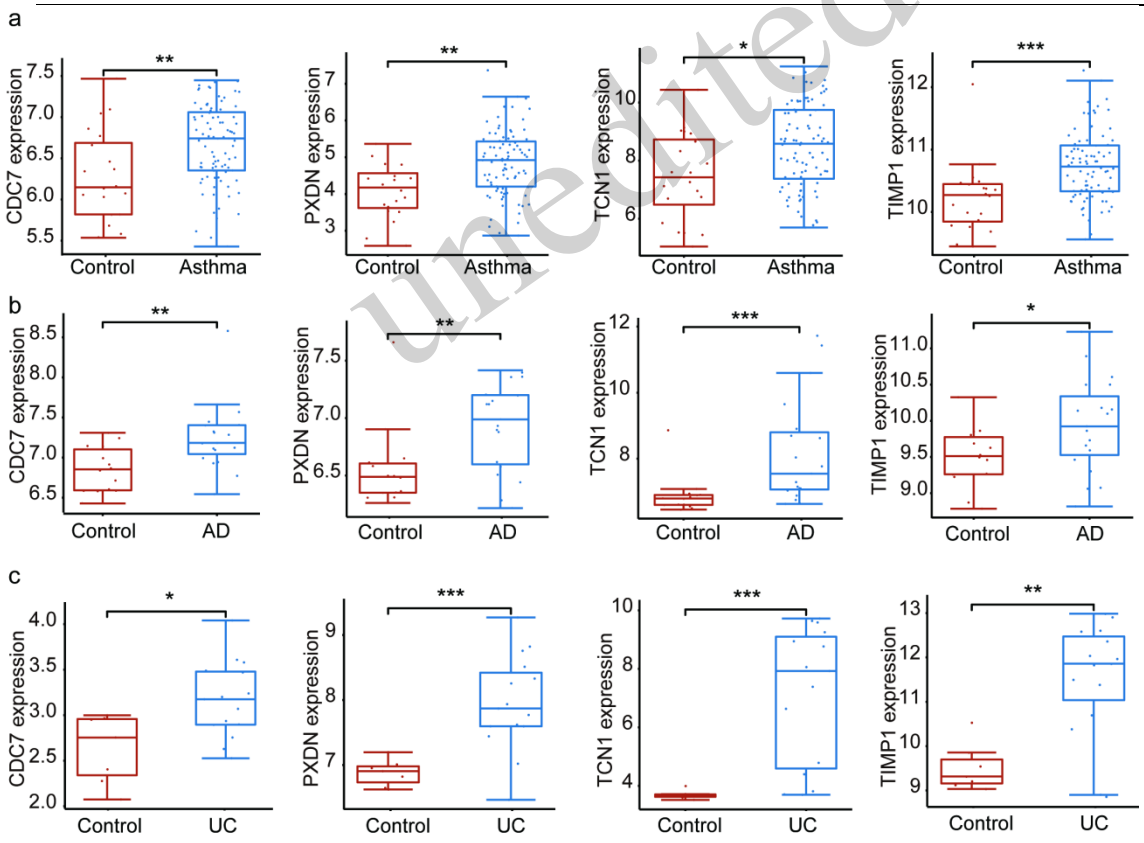

Fig. 6 Expressions of key genes in epithelial barrier dysfunction related diseases. (a) The expression levels of key genes in asthma were analyzed. (b) The expression levels of key genes in AD were examined. (c) The expression levels of key genes in UC were investigated. The Wilcox test was used to compare the statistical differences between two groups, while the Kruskal–Wallis test was used for three groups. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. AS, asthma; AD, atopic dermatitis; UC, ulcerative colitis.

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3.5 Construction of transcription factor–gene, miRNA–gene, and protein–protein interaction networks for key genes

Transcription factor, miRNA, and proteins regulate gene expression at the transcriptional, posttranscriptional, and posttranslational levels, respectively. To further investigate the regulatory mechanisms of key genes, we constructed TF–gene, miRNA–gene, and protein–protein interaction networks. Using NetworkAnalyst (Fig. 7a and 7b), a comprehensive gene expression profiling visual analytics platform, we predicted 22 TFs and 57 miRNAs capable of interacting with four key genes. The protein–protein interaction network was constructed using the GeneMANIA tool (Fig. 7c). Among these regulatory factors, the transcription factors HOXA5, SRF, E2F1, HNF4A, RELA, JUND, FOXC1, TEAD1, NFKB1, GATA3, EGR1, and CREB1, miRNAs hsa-mir-29a-3p and hsa-mir-1-3p, and interaction proteins MMP9, MMP1, MMP3, and TIMP3 have been reported to be involved in modulating epithelial barrier function (de Guzman Strong, et al.,2006; Chen, et al.,2008; Fréour, et al.,2009; Bin, et al.,2016; Fujino, et al.,2017; Lee, et al.,2018; Sumitomo, et al.,2019; Zhang, et al.,2019; Kunkl, et al.,2020; Ma, et al.,2020; Montenegro-Miranda, et al.,2020; Al-Sadi, et al.,2021; Herrnreiter, et al.,2021; Jin, et al.,2021; Mayangsari, et al.,2021; Muhammad, et al.,2021; Barroso, et al.,2022; Sun, et al.,2022). These results provide guidance for further mechanistic research, highlighting their relevance in the field.

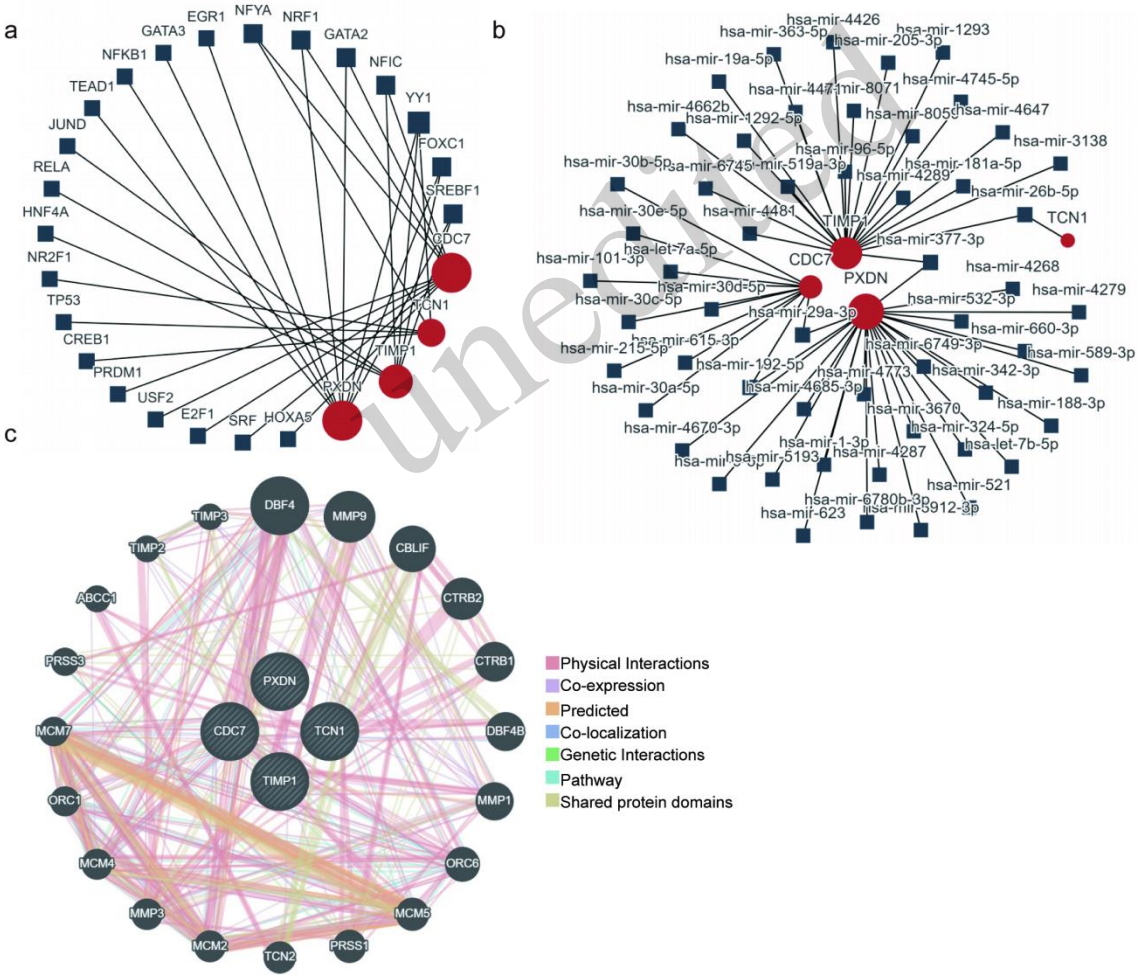

Fig. 7 The regulation network of key genes. (a) The prediction of the key genes-related transcription factors. (b) The prediction of the key genes-related miRNAs. (c) Protein–protein interaction network of the key genes.

3.6 Identification of potential chemicals that interact with key genes

Next, we constructed a chemical–gene interaction network using the Gendoma web server (Fig. 8a). The

network was made up of solid lines, which indicated that chemicals promoted target gene expression, while the dotted lines represented chemicals inhibited target gene expression. We discovered that smoke had the ability to promote TIMP1 expression, a finding that was validated through ex-smoker and current smoker epithelium gene expression analysis (GSE37147 dataset). Our results revealed that not only was TIMP1 expression up-regulated, but also TCN1 expression was increased in current smoker epithelia (Fig. 8b). Additionally, the network showed that dexamethasone, which is commonly used for the treatment of asthma exacerbation (Roddy, et al.,2023), could inhibit TIMP1 expression. In accordance with this finding, we discovered that TIMP1 expression was downregulated in the epithelium of asthmatics treated with maintenance inhaled corticosteroids (ICS+) as compared to asthmatics not treated with ICS (ICS-) (GSE179156 dataset) (Fig. 8c). Interestingly, among these chemicals, benzo(a)pyrene was found to promote the expression of four key genes. This suggests that it may be a harmful exposure in the context of epithelial barrier dysfunction diseases. These results will inspire us to explore potential drugs or environmental risk factors for epithelial barrier function in the near future.

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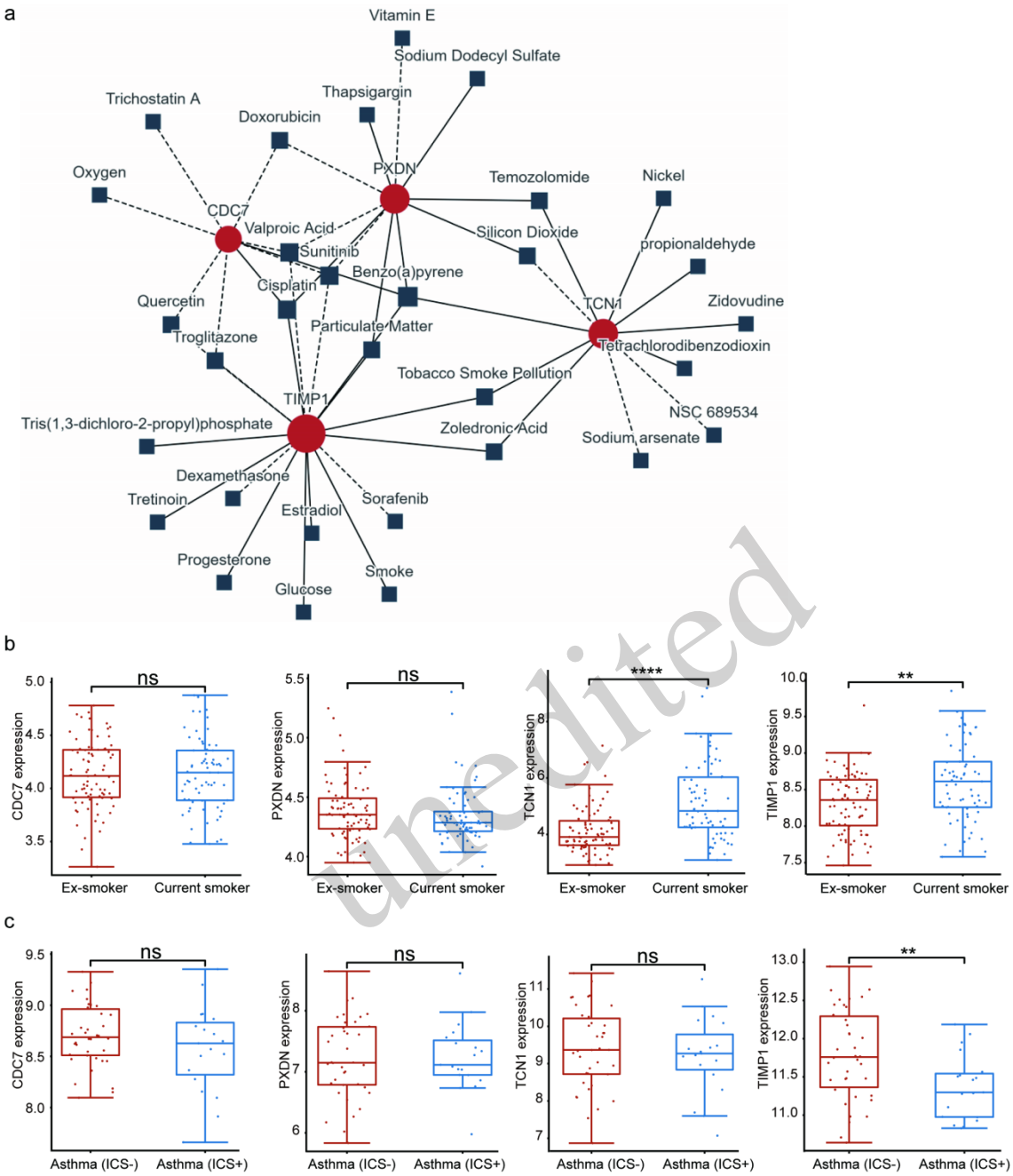

Fig. 8 Chemical–gene interaction network. (a) Potential chemicals that interact with key genes. (b) The effect of smoke on the expressions of key genes was validated in ex-smoker and current smoker epithelia from the GSE37147 dataset. (c) The effect of dexamethasone on the expressions of key genes was validated in the epithelium of asthmatics with or without inhaled corticosteroid (ICS) treatment from the GSE179156 dataset. The statistical difference between the two groups was assessed using the Wilcox test. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, not significant. ICS (-) refers to patients not treated with ICS, while ICS (+) represents patients treated with ICS. ICS, inhaled corticosteroids.

3.7 Analysis of the expressions of key genes in other epithelial barrier dysfunction related diseases

Next, we aimed to investigate the expressions of key genes in other epithelial barrier dysfunction-related diseases such as COPD, psoriasis, and Crohn's disease. We found that all four key genes were upregulated in the epithelium of COPD patients (Fig. 9a). Furthermore, in the case of Crohn's disease, we observed upregulation in

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the expressions of PXDN, TCN1, and TIMP1 (Fig. 9b). Similarly, in psoriasis patients, we detected upregulation in the expressions of TCN1 and TIMP1 (Fig. 9c). PXDN and TIMP1 were also found to be upregulated in allergic rhinitis patients (Fig. 9d). It is worth mentioning that allergic rhinitis often coexists with asthma, worsening the course of both diseases (Thomas,2006; Eriksson, et al., 2011). Therefore, we explored the expression of key genes in nasal epithelium from allergic rhinitis (AR) patients with or without asthma and found that those with asthma expressed higher levels of PXDN and TIMP1 (Fig. 9d). Based on these findings, it is evident that the key genes may play a role in multiple epithelial barrier dysfunction diseases.

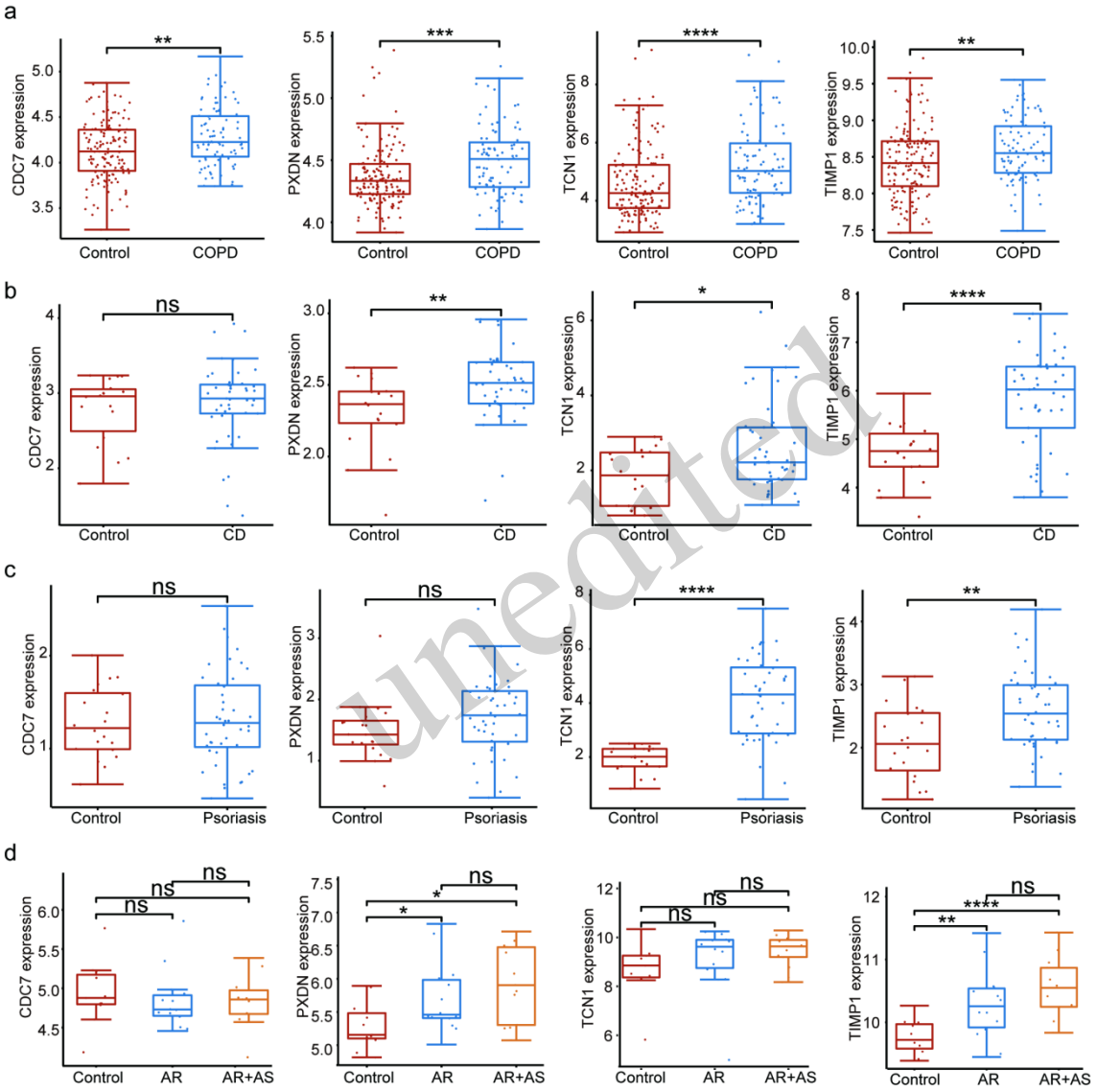

Fig. 9 Expressions of key genes in other epithelial barrier dysfunction-related diseases. (a) The expression levels of key genes in COPD were analyzed. (b) The expression levels of key genes in CD were examined. (c) The expression levels of key genes in psoriasis were investigated. (d) The expressions of key genes in allergic rhinitis (AR) with or without asthma (AS) were compared. The Wilcox test was used to compare the statistical differences between two groups, while the Kruskal–Wallis test was used for three groups. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, not significant. COPD, chronic obstructive pulmonary disease; CD, Crohn's disease; AR, allergic rhinitis; AS, asthma.

3.8 Association between clinical features in asthmatic patients and expression of key genes

Next, we analyzed the relationship between the clinical characteristics of asthmatic patients and the expressions

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of key genes. We found that the ACQ score was positively correlated with the expression of TCN1 (r=0.3214, P=0.0491) (Fig. 10a). The ACQ is a questionnaire used to measure the adequacy of asthma control, with higher scores indicating poor asthma control (Meltzer, et al.,2011). The ICS dose was negatively correlated with the expression of CDC7 (r=-0.3857, P=0.0168) but positively correlated with PXDN (r=0.3857, P=0.0564) or TCN1 (r=0.3096, P=0.0586), although the latter did not reach the threshold for statistical significance (Fig. 10b). Lung function, specifically the forced expiratory volume in the first second (FEV1), was negatively correlated with the expression of PXDN (r=-0.3543, P=0.0291) or TCN1 (r=-0.3594, P=0.0267) (Fig. 10c). The ratio of the forced expiratory volume in the first second to the forced vital capacity (FEV1/FVC) was negatively correlated with the expression of PXDN (r=-0.3528, P=0.0298) or TCN1 (r=-0.5295, P=0.0006) (Fig. 10d). To determine whether the expressions of the key genes are associated with asthma severity, we analyzed the expressions of the key genes in healthy controls, mild-moderate asthmatic (MMA) patients, and severe asthmatic (SA) patients. The results showed that the expressions of the key genes were higher in patients with severe asthma and were related to the degree of disease severity (Fig. 10e). In conclusion, there is a definitive correlation between the clinical features of asthmatic patients and the expression of key genes.

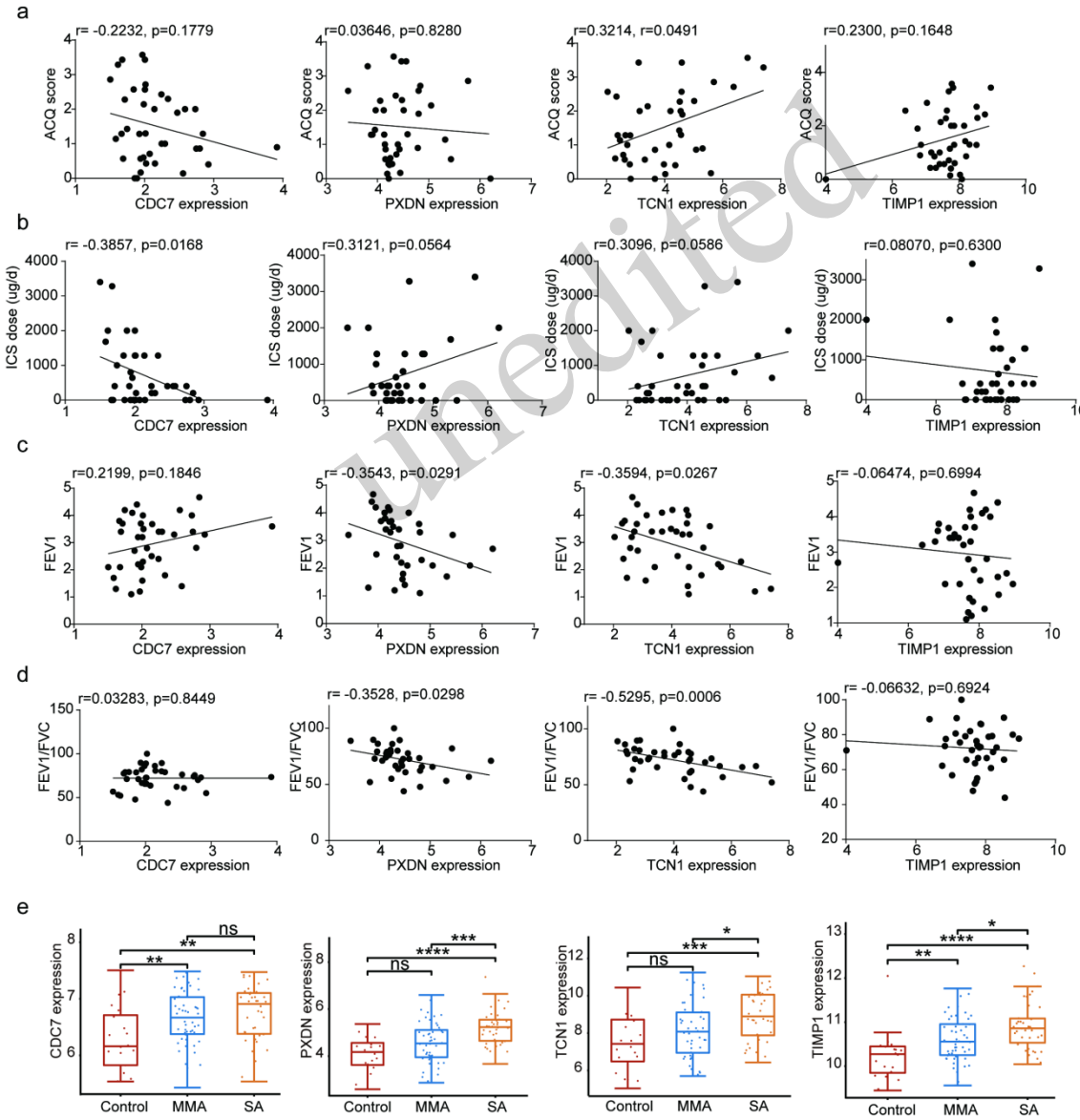

Fig. 10 The relationship between asthma patients' clinical characteristics and key genes. (a-d) The relationship between

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key genes and clinical characteristics (ACQ score, ICS dose, FEV1, and FEV1/FVC) of asthmatic patients was analyzed using the GSE89809 dataset. (e) The expression levels of key genes were compared among different asthma severities. Correlations were analyzed using Spearman coefficients. The significance of the differences among the three groups was determined using the Kruskal–Wallis test. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, not significant. ACQ, Asthma Control Questionnaire; ICS, inhaled corticosteroids; FEV1, forced expiratory volume in the first second; FEV1/FVC, the ratio of the forced expiratory volume in the first second to the forced vital capacity; MMA, mild-moderate asthma; SA, severe asthma.

3.9 Relationship between the expression of key genes and infiltration of immune cells in the epithelium of asthmatic patients.

Immunoinfiltration analysis is a commonly used technique to evaluate the presence and density of different immune cells in tissues or tumors. This involves analyzing gene expression data with specialized software algorithms to determine the abundance of various immune cell types, including T cells, B cells, macrophages, and dendritic cells (Newman, et al.,2019). Such analysis is frequently used in cancer research to identify immune-related biomarkers that predict patient outcomes and inform treatment decisions (Zhang, et al.,2020; Zhao, et al.,2022). In this study, an immune cell infiltration analysis of airway epithelium in asthmatic patients and control subjects was conducted, as presented in the cluster heatmap (Fig. 11a) with a histogram showing the proportion of each immune cell in each sample (Fig. 11b). The correlation between immune cell abundance and key gene expression levels was analyzed, and the results are presented using a correlation heatmap and a correlation matrix plot (Fig. 11c and 11d). The findings indicated that the expression of PXDN, CDC7, and TCN1 was negatively correlated with M2 macrophages. Additionally, PXDN and TIMP1 exhibited a negative correlation with T regulatory cells (Tregs), while PXDN, TIMP1, and TCN1 showed a positive correlation with plasma cells.

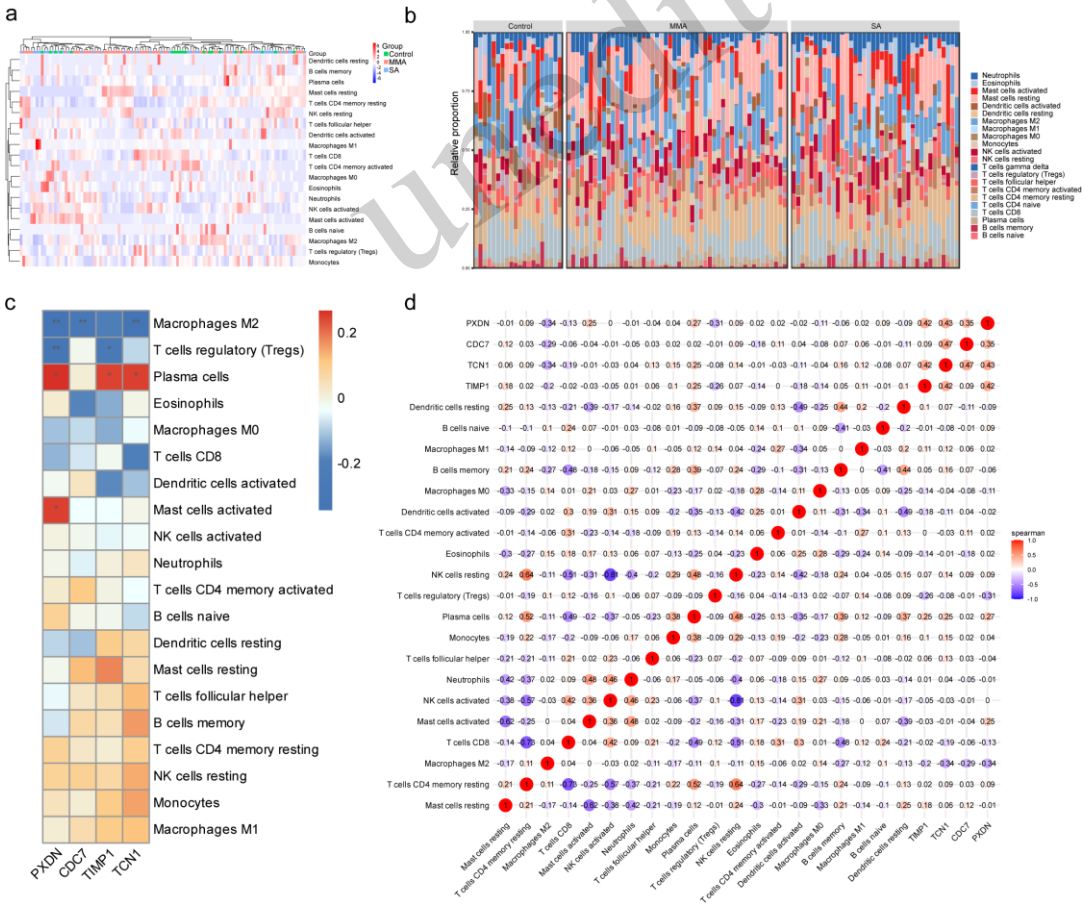

Fig. 11 The analysis of immune infiltration. (a) A clustering heatmap is used to display the relative fold change in the

abundance of 22 immune cells in control subjects, patients with mild-moderate asthma, and severe asthma. An increase in abundance is indicated by red; blue indicates a decrease. Sample clusters are distinguished by color, with control shown in green, mild-moderate asthma in pink, and severe asthma in blue. (b) Histograms are used to present the relative proportion of each immune cell in each sample, with each sample represented by a column and each cell type shown in a different color. (c-d) Correlation between immune cell abundance and key gene expression levels was assessed by developing a correlation heatmap (c) and a correlation matrix plot (e). Red represents a positive correlation; blue represents a negative correlation. A Spearman correlation analysis was carried out to determine the correlation between genes and immune cells. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01. MMA, mild-moderate asthma; SA, severe asthma.

3.10 The expression of CLDN1 was upregulated after knocking down key genes in human bronchial epithelial cells.

Our findings suggest that four key genes all exhibit a negative correlation with the expression of CLDN1 in asthmatic airway epithelium (Table 1). CLDN1 plays a vital role as an essential component of tight junctions in various epithelia. To further confirm whether these key genes can regulate the expression of CLDN1, we detected the expression of CLDN1 by qPCR in 16HBE cells after knocking down CDC7, PXDN, TCN1, and TIMP1, respectively. The results revealed that the expression of CLDN1 was upregulated following interference with the expression of these key genes (Fig. 12a-12d). Moreover, we found that cell membrane localization of CLDN1 was enhanced after interfering with the expression of these key genes in 16HBE cells (Fig. 12e-12h). Finally, we found that the knockdown of these key genes significantly enhanced the integrity and permeability of the airway epithelial barrier by improving transepithelial electrical resistance and reducing permeability to FITC-dextran (Fig. 12i-12j). These findings suggest that the key genes might be involved in epithelial barrier dysfunction by regulating CLDN1 expression.

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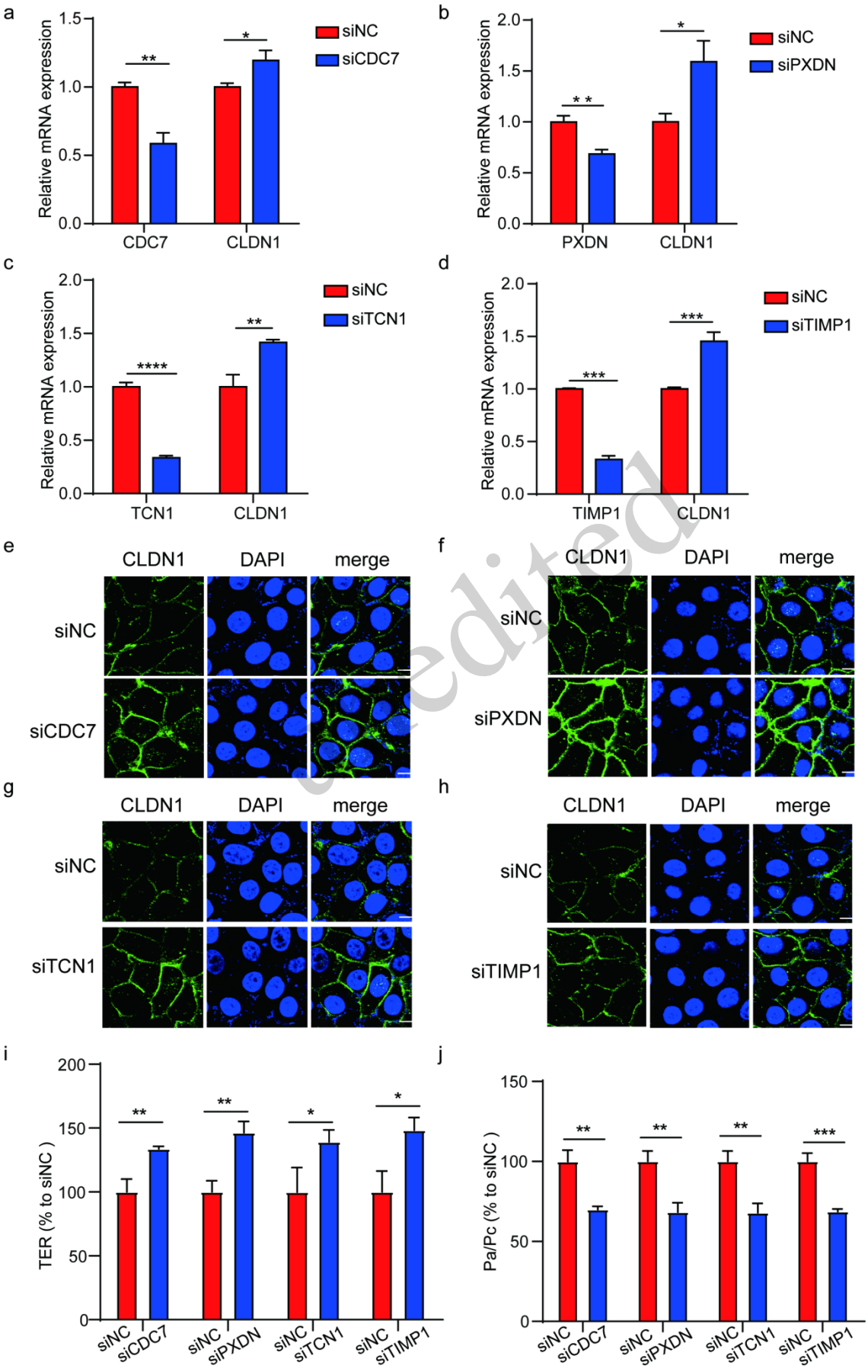

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Fig. 12 Knocking down key genes promotes CLDN1 expression. (a-d) The expression of CLDN1 was detected by qPCR in 16HBE transfected with negative control (NC) siRNA, CDC7 siRNA (a), PXDN siRNA (b), TCN1 siRNA (c), and TIMP1 siRNA (d). (e-h) The cellular distribution of CLDN1 was detected by immunofluorescence in 16HBE transfected with negative control siRNA, CDC7 siRNA (e), PXDN siRNA (f), TCN1 siRNA (g), and TIMP1 siRNA (h). (i and j) The integrity and permeability of airway epithelial barrier were assessed by transepithelial electrical resistance (i) and permeability to FITC-dextran (j). (a-d, i and j) Data are shown as the means ± SEM of three independent experiments and analyzed using the unpaired two-tailed Student's t-test. A P-value < 0.05 was considered statistically significant. *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

4 Discussion

Although epithelial barrier dysfunction has been demonstrated to occur in asthma, ulcerative colitis, and atopic dermatitis, our present study revealed that the expression signatures of junctional molecules varied among these diseases. This may be due to different epithelia or epidermis in the airway, intestine, and skin. The airway epithelium is a pseudostratified columnar epithelium. The intestinal epithelium is simple columnar, while the skin is a continuous keratinized stratified squamous epithelium. However, the exploration of common regulatory mechanisms leading to epithelial barrier dysfunction is an important yet neglected research subject. In this study, we conducted a comprehensive bioinformatics analysis of the transcriptome profiling data from asthma, atopic dermatitis, and ulcerative colitis epithelia, which enabled us to identify four key genes (CDC7, PXDN, TCN1, and TIMP1) that may regulate epithelial barrier function. Further, the expression of these key genes was also validated in COPD, Crohn's disease, psoriasis, and allergic rhinitis. Finally, we found that knocking down key genes in 16HBE could promote CLDN1 expression. These key genes may be potential therapeutic targets of epithelial barrier dysfunction diseases.

Among the key genes, TIMP1 has been demonstrated to be involved in asthma, COPD, and inflammatory bowel disease (IBD). TIMP1 is a natural inhibitor of matrix metalloproteinases (MMPs), a group of peptidases involved in extracellular matrix degradation. It is capable of promoting cell proliferation in a broad range of cell types and may also have an anti-apoptotic function (Grünwald, et al.,2019). TIMP-1 polymorphism has been reported to be associated with the development of asthma (Kumar, et al.,2012). Previous studies have shown that higher sputum TIMP1 levels are associated with asthma airway remodeling (Vignola, et al.,1998; Chaudhuri, et al.,2014). A recent study has demonstrated that TIMP1 is increased in the serum of asthmatics and enhances eosinophilic airway inflammation in severe asthma (Cao, et al.,2023). Similarly, TIMP1 expression is upregulated in the serum and intestinal mucosa of IBD patients, and elevated TIMP-1 levels show a significant positive correlation with IBD disease activity (Louis, et al.,2000; von Lampe, et al.,2000; Kapsoritakis, et al.,2008; Lakatos, et al.,2012). Upregulation of TIMP1 has been validated in three experimental colitis models mimicking IBD, and genetic deletion of TIMP-1 attenuates fibrosis in dextran sodium sulphate-induced murine models of colitis (te Velde, et al.,2007; Breynaert, et al.,2016). Interestingly, the effect of TIMP-1 on COPD is inconsistent. Li et al. reported that serum expression of TIMP-1 is increased in COPD and correlated with worsening symptoms (Li, et al.,2020). However, Zhou et al. found that TIMP1 is decreased in the induced sputum of COPD and is associated with poor lung function (Zhou, et al.,2017). These results indicate that TIMP1 might exert tissue-specific effects. The above studies suggest that TIMP1 mainly regulates tissue remodeling and fibrosis. In the present study, we found that TIMP1 might have a common effect on barrier function in these diseases. We found that TIMP1 was upregulated in the epithelium or epidermis of asthma, allergic rhinitis, COPD, ulcerative colitis, Crohn's disease, atopic dermatitis, and psoriasis. The expression of TIMP1 was negatively correlated with the expressions of junction molecules (CLDN1, CLDN3, CLDN4, CLDN8, CLDN16, CLDN23, CDH1, and CTNNB1), suggesting that TIMP1 might be a key regulatory gene in barrier function. It is worth targeting TIMP1 to validate its effect on barrier function in future studies.

TCN1 belongs to the vitamin B12-binding protein family, which assists in cobalamin transportation into cells. Diseases associated with TCN1 include vitamin B12 deficiency and transcobalamin I deficiency (Hu, et al.,2018). Additionally, recent studies have indicated that high levels of TCN1 are associated with the progres-

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sion of lung and gastrointestinal malignancies (Li, et al.,2022; Zhu, et al.,2022, 2023). Previous studies have reported the upregulation of TCN1 in the airway epithelial cells of patients with asthma and chronic obstructive pulmonary disease (COPD) (Singhania, et al.,2018; Esteve-Codina, et al.,2021). A recent study has demonstrated that the expression of TCN1 was elevated in the sputum of asthma patients and exhibited a negative correlation with lung function (Xu, et al.,2023). Analysis of transcriptomes from single T helper (TH) cells derived from the nasal polyps of patients with chronic rhinosinusitis revealed that TH2 cells highly expressed TCN1 (Ma, et al.,2021). However, it is unclear whether TCN1 plays a role in epithelial barrier dysfunction in asthma. In the present study, we found that the expression of TCN1 was elevated in the epithelium or epidermis of diseases such as asthma, atopic dermatitis, ulcerative colitis, COPD, Crohn's disease, and psoriasis. The expression of TCN1 was negatively correlated with the expression of junction molecules (CLDN1, CLDN8, CLDN16, CLDN23, CDH1, and TJP1). Moreover, an analysis of the correlation between clinical characteristics of asthma and the expression of TCN1 revealed that the ACQ score or ICS dose was positively correlated with the expression of TCN1, while FEV1 or FEV1/FVC was negatively correlated with the expression of TCN1. Furthermore, the expression of TCN1 positively correlated with the severity of asthma. These results suggest that TCN1 might participate in the development of asthma, and the effects of TCN1 on epithelial barrier dysfunction diseases should be further validated in future studies.

The genes CDC7 and PXDN have been relatively underinvestigated in diseases related to epithelial barrier dysfunction. CDC7 is a protein kinase that plays an essential role in the transition stage of cell division and is critical for DNA replication, repair, and recombination pathways (Yang, et al.,2016; Iwai, et al.,2021; González-Garrido and Prado,2023). CDC7 has been demonstrated to be involved in the progression of various malignant tumors such as melanoma, cervical cancer, and esophageal squamous cell carcinoma (Cao and Lu,2019; Gad, et al.,2019; Wang and Zheng,2021; Liu and Huang,2022). PXDN is a heme-containing peroxidase that is secreted into the extracellular matrix and has been reported to be involved in fibrosis, host defense, atherosclerosis, endothelial dysfunction, and heart and vessel remodeling (Cheng and Shi,2022). However, CDC7 and PXDN have never been reported in relation to epithelial barrier dysfunction diseases. Although our findings suggest that CDC7 and PXDN might be involved in epithelial barrier dysfunction diseases, further validation of their effects is necessary.

Transcription factor–gene, miRNA–gene, and protein–protein interactions are crucial factors in regulating gene expression, and they have been reported to play a significant role in regulating epithelial barrier function (Glover and Colgan,2017; Mahurkar-Joshi, et al.,2021; Liang, et al.,2022). In the present study, we constructed networks of transcription factor–gene, miRNA–gene, and protein–protein interactions for key genes involved in this process. Several regulatory factors involved in epithelial barrier function regulation were identified in these networks. For example, it has been reported that the transcription factor JUND can repress the tight junction protein ZO-1 expression, leading to intestinal epithelial barrier dysfunction (Chen, et al.,2008). In our study, we found that JUND is a potential transcription factor of TIMP1, which is consistent with previous research that JUND can bind to the TIMP1 promoter and activate its transcription (Qing, et al.,2022). Additionally, we identified hsa-mir-29a-3p as a miRNA of CDC7, which has been demonstrated to be upregulated in the epithelium of AR and to promote epithelial barrier dysfunction (Wang, et al.,2023). Moreover, the interaction protein MMP-1 was induced by German cockroach extract, leading to tight junction disruption in human airway epithelial cells (Lee, et al.,2018). Other regulatory factors, such as transcription factors HOXA5, SRF, E2F1, HNF4A, RELA, FOXC1, TEAD1, NFKB1, GATA3, EGR1, CREB1, miRNA hsa-mir-1-3p, and interaction proteins MMP9, MMP3, and TIMP3 were also identified to be associated with epithelial barrier function regulation (de Guzman Strong, et al.,2006; Fréour, et al.,2009; Bin, et al.,2016; Fujino, et al.,2017; Sumitomo, et al.,2019; Zhang, et al.,2019; Kunkl, et al.,2020; Ma, et al.,2020; Montenegro-Miranda, et al.,2020; Al-Sadi, et al.,2021; Jin, et al.,2021; Mayangsari, et al.,2021; Muhammad, et al.,2021; Barroso, et al.,2022; Sun, et al.,2022). These results can help us further explore the regulation mechanisms of epithelial barrier function.

To identify potential chemicals that can regulate the expression of key genes, we constructed a chemical–gene interaction network using Gendoma. A number of chemicals identified in this have previously been

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reported to affect epithelial barrier function, such as smoke and tobacco smoke pollution (Nasreen, et al.,2014). Our network analysis suggested that tobacco smoke pollution may induce the expression of TCN1 and TIMP1 genes. We consistently observed higher levels of TCN1 and TIMP1 expression in the airway epithelium of current smokers compared to ex-smokers, indicating that smoking-induced disruption of the epithelial barrier may be mediated by these genes. Dexamethasone, a commonly used glucocorticoid for asthma exacerbation, has been shown to improve the barrier function of the airway, intestine, and skin (Sekiyama, et al.,2012; Ribeiro, et al.,2018; Lu, et al.,2023). Our chemical–gene interaction network analysis indicated that dexamethasone can inhibit TIMP1 expression. Consistent with this, we found that TIMP1 expression was higher in the airway epithelium of asthmatic patients who did not receive inhaled glucocorticoid treatment compared to those who did receive it. In addition, our analysis identified that benzo[a]pyrene, a type of polycyclic aromatic hydrocarbon generated primarily through the burning of organic materials, can upregulate the expression of CDC7, PXDN, TCN1, and TIMP1 genes. It is a known carcinogen that has been associated with DNA damage, gene mutations, and cell death, as well as long-term immunotoxicity, reproductive and developmental problems, and damage to the nervous system (Bukowska, et al.,2022). Previous studies have also demonstrated that benzo[a]pyrene can worsen asthma airway inflammation and atopic dermatitis epidermal inflammation (Tajima, et al.,2019; Carrard, et al.,2021). Further investigation is warranted to determine whether benzo[a]pyrene contributes to epithelial barrier disruption through the modulation of the CDC7, PXDN, TCN1, and TIMP1 genes.

Asthmatic airways are characterized by the infiltration of various inflammatory cells, including T lymphocytes, eosinophils, mast cells, dendritic cells, macrophages, and others (Lambrecht, et al.,2019). Epithelial barrier disruption can promote immune cell activation and accumulation, while immune cell infiltration can exacerbate epithelial barrier disruption (Heijink, et al.,2020). Our immune infiltration analysis showed that PXDN, CDC7, and TCN1 are negatively correlated with M2 macrophages. Macrophage polarization states play an essential role in inflammation, with M1 macrophages induced by proinflammatory TH1 cytokines, displaying proinflammatory and TH1-oriented immunostimulatory properties, while M2 macrophages encompass several subgroups with different functions (Bosco,2019). M2a macrophages are induced by TH2 cytokines, promoting allergic responses (Zhang, et al.,2017; Liang, et al.,2023). M2b macrophages are induced by immune complexes, exhibiting immunosuppressive and anti-inflammatory effects (Bianchini, et al.,2019). M2c macrophages are induced by anti-inflammatory cytokines, such as interleukin-10 or glucocorticoids, inducing resolution of lung inflammation and tissue repair (Tang, et al.,2017; Tu, et al.,2017). M2d macrophages are induced by cytokines similar to IL-6, promoting immune tolerance and tumor growth (Wang, et al., 2010). Although we did not perform a subpopulation analysis on M2 macrophages in our study, our findings suggest that key genes may play a role in the disruption of airway epithelial barriers via the phenotypic transition of M2 macrophages. Future studies should investigate whether key genes are involved in regulating the M2 macrophages subtype. Furthermore, we found that PXDN, TIMP1, and TCN1 are positively correlated with plasma cells, which can produce immunoglobulin E (IgE), causing inflammatory reactions in tissues and cells and participating in allergic diseases such as asthma, allergic rhinitis, and atopic dermatitis (Rindsjö and Scheynius,2010). Additionally, PXDN and TIMP1 are negatively correlated with Tregs, which are negative regulatory cells of the immune response, suppressing excessive Th2 response in asthma (Zhao and Wang,2018). Overall, our research results suggest that the airway epithelium may interact with immune cells through these key genes, and this requires further investigation in the future.

Despite the robust nature of our study, we need to acknowledge certain limitations. Firstly, we relied exclusively on the GEO public database for our research data, which may not provide a comprehensive representation of all populations. Secondly, it is crucial to verify the biological functions of the key genes that we have identified in our study, and also ensure the safety and efficacy of any potential drug candidates through further basic experiments or clinical trials. Lastly, there is a need for a more in-depth exploration of the molecular mechanisms underlying both the key genes and epithelial barrier dysfunction diseases.

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5 Conclusion

In summary, our study has identified four crucial genes, namely CDC7, PXDN, TCN1, and TIMP1, which are associated with epithelial barrier dysfunction. Using asthma as an example, we have further confirmed the relationship between these key genes and clinical characteristics of asthma or immune infiltration. Our study also found that knocking down all four key genes can upregulate the expression of CLDN1 in 16HBE. This research will contribute to the comprehensive exploration of the specific role of key genes in the dysfunction of the epithelial barrier and the development of corresponding therapeutic drugs to treat diseases associated with epithelial barrier dysfunction in the future.

Data availability statement

The datasets generated during and/or analyzed during the current study are available in the GEO database repository, [https://www.ncbi.nlm.nih.gov/geo/].

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Author contributions

Shixiu LIANG: Conceptualization, Software, Methodology, Data Curation, Validation, and Writing-original draft preparation. Xiaodi ZHU: Data Curation, Validation, and Experiments. Siqi WANG: Visualization, Writing-Reviewing and editing. Xi CHEN: Writing- Reviewing and editing. Yanwen LI: Data Curation, Writing- Reviewing and editing. Shiting YI: Data Curation, Writing-Reviewing and editing. Yingjun CHEN: Resources, Writing-Reviewing and editing, and Supervision.

Compliance with ethics guidelines

Shixiu LIANG, Xiaodi ZHU, Siqi WANG, Xi CHEN, Yanwen LI, Shiting YI, and Yingjun CHEN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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