BMC Pulmonary Medicine

RESEARCH

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Increased Lipocalin 2 detected by RNA sequencing regulates apoptosis and ferroptosis in COPD

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Abstract

Background Chronic obstructive pulmonary disease (COPD) is a complex respiratory condition influenced by environmental and genetic factors. Using next-generation sequencing, we aimed to identify dysregulated genes and potential therapeutic targets for COPD.

Methods Peripheral blood leukocyte RNA profiles from COPD patients and healthy controls were analyzed using next-generation sequencing. Key genes involved in COPD pathogenesis were identified through protein–protein interaction network analysis. In vitro, bronchial epithelial cells treated with cigarette smoke extract (CSE) were used to study the effects on gene expression, cell viability, apoptosis, and ferroptosis. Additionally, Lipocalin 2 (LCN2) inhibition experiments were conducted to elucidate its role in COPD-related cellular processes.

Results Analysis of RNA profiles revealed consistent downregulation of 17 genes and upregulation of 21 genes across all COPD groups. Among these, Cathelicidin Antimicrobial Peptide(CAMP), Defensin Alpha 4(DEFA4), Neutrophil Elastase(ELANE), LCN2 and Lactotransferrin(LTF) were identified as potentially important players in COPD pathogenesis. Particularly, LCN2 exhibited a close association with COPD and was found to be involved in cellular processes. In vitro experiments demonstrated that CSE treatment significantly increased LCN2 expression in bronchial epithelial cells in a concentration-dependent manner. Moreover, CSE-induced apoptosis and ferroptosis were observed, along with alterations in cell viability, Glutathione content, Fe2 + accumulation, ROS: Reactive Oxygen Species and Malondialdehyde levels, Lactate Dehydrogenase(LDH) release and Glutathione Peroxidase 4(GPX4) expression. Inhibition of LCN2 expression partially reversed these effects, indicating the pivotal role of LCN2 in COPD-related cellular processes.

Conclusion Our study identified six candidate genes: CAMP, DEFA4, ELANE, LCN2, and LTF were upregulated, HSPA1B was downregulated. Notably, LCN2 emerges as a significant biomarker in COPD pathogenesis, exerting its effects by promoting apoptosis and ferroptosis in bronchial epithelial cells.

Keywords COPD, LCN2, Apoptosis, Ferroptosis

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Introduction

Chronic obstructive pulmonary disease (COPD) represents a multifaceted respiratory condition characterized by progressive airway obstruction and inflammation, posing a significant global health challenge due to its increasing prevalence and high morbidity and mortality rates [1]. The disease's pathogenesis is intricate, involving a cascade of molecular events influenced by both environmental exposures, such as cigarette smoke, and genetic predispositions, which collectively contribute to up to 60% of disease susceptibility [2].

The complexity of COPD pathophysiology encompasses several interrelated mechanisms, including chronic inflammation, oxidative stress, protease-antiprotease imbalance, and impaired host defense mechanisms [3]. These factors culminate in structural changes in the airways and alveolar destruction, leading to irreversible airflow limitation. Emerging research underscores the roles of programmed cell death pathways, specifically apoptosis and ferroptosis, in exacerbating COPD progression [4, 5]. Recent literature highlights their significance, pointing towards promising directions for COPD research and treatment [6, 7]. Apoptosis, as a fundamental process involved in maintaining tissue homeostasis, triggered by persistent oxidative stress and inflammatory mediators, contributes to epithelial and endothelial cell loss, disrupting lung tissue integrity and function [8, 9]. Concurrently, dysregulated iron homeostasis and accumulation, characteristic of ferroptosis, further aggravate cellular damage through lipid peroxidation and mitochondrial dysfunction [5, 10].

The bronchial epithelial cells, as a frontline barrier in the airways, play a pivotal role in COPD pathogenesis. Their susceptibility to apoptosis and ferroptosis significantly impacts disease progression [4]. Apoptosis of these cells, induced by oxidative stress and inflammatory cytokines, compromises epithelial barrier function and enhances inflammatory responses, perpetuating lung tissue damage [7, 11]. Similarly, ferroptosis in bronchial epithelial cells exacerbates oxidative stress and inflammation, exacerbating tissue injury and impairing respiratory function [12]. Given the critical involvement of bronchial epithelial cell apoptosis and ferroptosis in COPD [13, 14], identifying regulatory genes that modulate these pathways becomes essential. Several key genes are implicated in these processes. For instance, the BCL2(B-cell lymphoma 2) family of genes, including BCL2 and BAX(Bcl-2-associated X protein), are critical regulators of apoptosis; BCL2 inhibits apoptosis, while BAX promotes it [15]. TP53(Tumor Protein 53), another important gene, can induce apoptosis by upregulating pro-apoptotic genes and downregulating anti-apoptotic genes [16]. In the context of COPD, dysregulation of these genes can exacerbate epithelial cell death and tissue damage [17]. Regarding ferroptosis, genes such as GPX4 (Glutathione Peroxidase 4) and SLC7A11 (Solute Carrier Family 7 Member 11) are essential.GPX4 prevents ferroptosis by reducing lipid peroxides, and SLC7A11 is involved in cystine uptake for glutathione synthesis, crucial for defending against oxidative stress [18, 19]. These has become a topic of significant interest, especially in the fields of cancer, cardiovascular, and neuroscience research [20–22]. In COPD, the downregulation of GPX4 and impaired function of SLC7A11 enhance susceptibility to ferroptosis, contributing to cell death and disease progression [23]. Furthermore, Nrf2 (Nuclear Factor Erythroid 2-Related Factor 2) and HMOX1 (Heme Oxygenase 1) are also significant. Nrf2 is a transcription factor that regulates the expression of antioxidant proteins, including those that protect against ferroptosis and apoptosis [24]. HMOX1, upregulated in response to oxidative stress, helps degrade heme into biliverdin, iron ions, and carbon monoxide, reducing oxidative damage and preventing apoptosis and ferroptosis [25, 26]. However, research specifically focusing on genes that simultaneously regulate both apoptosis and ferroptosis in COPD is relatively scarce. This gap in knowledge highlights the need for comprehensive studies to uncover such dual-function genes and their roles in COPD pathogenesis.

Given these insights, our study aims to perform gene sequencing on patients with COPD to identify genes involved in regulating apoptosis and ferroptosis. Following sequencing, we will conduct network analysis to elucidate the interactions between identified genes and validate their functional roles through targeted experiments. This integrative approach will not only enhance our understanding of COPD mechanisms but also pave the way for developing targeted therapies to improve patient outcomes.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of the Shanxi Medical University (NO.2015053). Participants provided informed consent to participate in this study.

Patients

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guideline, the inclusion criteria, exclusion criteria and grouping of COPD patients were consistent with previous studies [27]. The patients included in our study were recruited between January 1, 2015, and November 30, 2015. A total of 20 male COPD patients were divided into 4 groups (A, B, C and D, 5 patients/group). Healthy, age-matched, male smokers (at least 20 packs per year of smoking history) were

recruited in this study, and they possessed normal lung function.

RNA extraction and sequencing

Peripheral blood was collected in EDTA anticoagulation tubes. The method for leukocyte isolation and RNA extraction was consistent with previous studies [27]. Following the manufacturer's protocol and methods described in earlier research [28], the steps for RNA sequencing were as follows:

1. RNA Isolation: Total RNA was treated with DNase I to remove any contaminating DNA.
2. mRNA Isolation: Magnetic beads with Oligo (dT) were used to isolate mRNA from the total RNA.
3. mRNA Fragmentation: The mRNA was fragmented into short segments using fragmentation buffer.
4. cDNA Synthesis: The fragmented mRNA served as templates for cDNA synthesis.
5. Fragment Purification and Treatment: The resulting short cDNA fragments were purified and treated with EB buffer for end repair and single nucleotide adenine addition.
6. Adapter Ligation: The fragments were then ligated with sequencing adapters.
7. Fragment Selection: After agarose gel electrophoresis, suitable fragments were selected for PCR amplification.
8. Library Preparation and Sequencing: The prepared library was sequenced using the Illumina HiSeqTM 2000 platform.

RNA sequencing data analysis

Raw reads were filtered to obtain clean reads, which were then aligned to the reference sequences. The alignment data was used to calculate the distribution of reads on reference genes and the mapping ratio. If the alignment results passed quality control (QC), downstream analyses were conducted, including gene and isoform expression analysis. Additionally, we performed in-depth analysis based on differential gene expression (DEG), which included Gene Ontology (GO) enrichment analysis, pathway enrichment analysis, protein–protein interaction (PPI) network analysis, and transcription factor identification.

Cell culture, cigarette smoke extract (CSE) treatment and transfection

The human bronchial epithelial cell line BEAS-2B was procured from ATCC (Manassas, VA, USA) and cultured in DMEM/F12 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (Gibco).

CSE was prepared as previously described [29] and used within 30 min. Smoke from a single cigarette was bubbled into 5 mL of serum-free DMEM/F12 medium to create 100% CSE. BEAS-2B cells were cultured in serum-free DMEM/F12 medium for 2 h and then exposed to various concentrations of CSE for 24 h.

Following transfection with negative control (NC) siRNA or LCN2 siRNA (GenePharma, Shanghai, China), BEAS-2B cells were treated with 5% CSE for 24 h.

Quantitative Real-Time PCR (qRT-PCR)

Total cellular RNA was isolated from leukocytes using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA according to the manufacturer's instructions. The expression of some genes was conducted using RT-PCR primers and conditions.

Western blot

Total proteins from BEAS-2B cells were extracted, separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk and then incubated with diluted primary LCN2 (1:1000, Abcam, China), glutathione peroxidase 4 (GPX4; 1:1000, Abcam) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000, Abcam) antibody overnight at 4 °C. After incubation with secondary antibody (1:2000, Abcam), the protein blot signals were measured by BeyoECL Plus (Beyotime, Shanghai, China).

Apoptosis analysis

BEAS-2B cells were suspended in 500 μL binding buffer and incubated at room temperature in the dark for 15 min after labeled with 5 μL Annexin V-fluorescein isothiocyanate (FITC) and 5 μL propidium iodide (BD Biosciences, San Jose, CA, USA). At last, the double staining cells were subjected to a flow cytometry (FACScan, BD Biosciences) to detect the apoptosis rate.

Cell viability assay

After completion of experimental treatment conditions, cell viability was detected by the cell counting kit-8 (CCK-8; Beyotime). BEAS-2B cells were incubated with 10 μL CCK-8 solution at 37 °C for 2 h and the absorbance at 450 nm was analyzed by a microplate reader. Cell viability was normalized to relative control.

Iron content assay

Intracellular ferrous iron level was measured with the iron assay kit (Abcam) based on the company's guide. BEAS-2B cells were homogenized with iron assay buffer,

and then 100 μL homogenate was incubated with 5 μL assay buffer at 37 °C for 30 min. Afterward, the above mixture was incubated with 100 μL iron probe at 37 °C for 60 min protected from light. At last, the optical density value at 593 nm was measured on a colorimetric microplate reader.

Evaluation of oxidative stress

BEAS-2B cells were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe in the dark for 30 min, and the ROS-mediated fluorescence was analyzed under a fluorescence microplate reader or a fluorescent microscope. BEAS-2B cells were homogenized with lysis buffer, and the supernatant was used to determine the malondialdehyde (MDA) or glutathione (GSH) content by the lipid peroxidation MDA assay kit (Beyotime) or GSH and GSSG assay kit (Beyotime) based on the manufacturer's protocol.

Lactate dehydrogenase (LDH) release assay

After completion of experimental treatment conditions, cell culture supernatants were collected and then used to assess LDH release with the lactate dehydrogenase assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the instruction of assay kit.

Statistical analysis

The software of SPSS 21.0 (Chicago, IL, USA) was used to analyze the statistical significance. The expressed difference of selected genes between COPD patients and control was analyzed by Student's t-test. The correlation analysis of fold change (FC) data between RNA-Seq and qRT-PCR was performed using Pearson correlation analysis. P value < 0.5 was considered as statistically significant difference.

Results

Differentially expressed genes (DEGs) between COPD patients and control

The characteristics of enrolled 20 subjects with COPD (A-D groups, 5 patients/group) and 6 control subjects were consistent with our previous study [27]. All subjects were male and the mean age and the mean pack years of cigarette smoking history in the COPD group were almost similar with the control group. The gene expression levels of each sample were calculated by fragments per kilobase of exon model per million mapped reads (FPKM), and the average number of genes expressed in 26 samples was 17,074 (Table s1). DEGs were detected by Possion Distribution method and Noiseq package, and the filter criterion was that the gene expression in at least one group between two groups was greater than or equal to 5 (≥ 5) and the absolute value of $\log_2$FoldChange was greater than or equal to 0.5 (≥ 0.5). The results showed that 44 genes were differentially expressed between the two groups among 17,074 genes (Fig. 1A, Table 1).

The comparison of differentially expressed mRNAs among A-D groups

Among the DEGs detected in the four groups, the down-regulated genes were more than the up-regulated genes (Fig. 1B). There were 44 interspecific genes in the ABCD groups, of which 17 were consistently down regulated genes and 21 were consistently up-regulated genes (Fig. 1A, Table 1). The expression of down-regulated LGALS9C, LGALS9B and the up-regulated RNASEK-C17orf49 was gradually decreased, and negatively correlated with disease severity (Fig. 1C, Table 1).

Gene-ontology (GO) and pathway-enrichment analyses between COPD and control

Functional enrichment analysis was conducted on 44 differentially expressed genes (DEGs) identified from the intersection of COPD and control groups using DAVID. Among these, one pathway was significantly enriched in the Kyoto Encyclopedia of Genes and Genomes (KEGG), with a p-value less than 0.05. Additionally, 31 terms were enriched in Gene Ontology (GO), comprising 17 Biological Process (BP), 11 Cell Component (CC), and 3 Molecular Function (MF) categories (Fig. 1D). KEGG analysis identified antigen processing and presentation as a representative signaling pathway enriched by these genes. Furthermore, DAVID analysis highlighted major GO terms associated with antibacterial humoral response, innate immune response in mucosa, extracellular space, innate immune response, killing of cells of other organisms, and extracellular exosome, among others.

Protein–protein interaction network of the DEGs

We utilized STRING to construct a protein–protein interaction (PPI) network to identify interactions among up-regulated, down-regulated, and inconsistently expressed genes/proteins from differentially expressed genes (DEGs). The resulting network consisted of 15 nodes and 21 edges (Fig. 2A). In the network, red symbols represent genes up-regulated in COPD patients compared to controls, blue symbols represent down-regulated genes, and light blue symbols represent genes with inconsistent expression trends. Based on the PPI scores, we identified the top five important genes potentially crucial in COPD progression: cathelicidin antimicrobial peptide (CAMP), defensin alpha 4 (DEFA4), elastase, neutrophil expressed (ELANE), lipocalin 2 (LCN2), and lactotransferrin (LTF). Among these, CAMP exhibited the highest centrality, suggesting its significant role in COPD pathogenesis. Conversely, heat shock protein

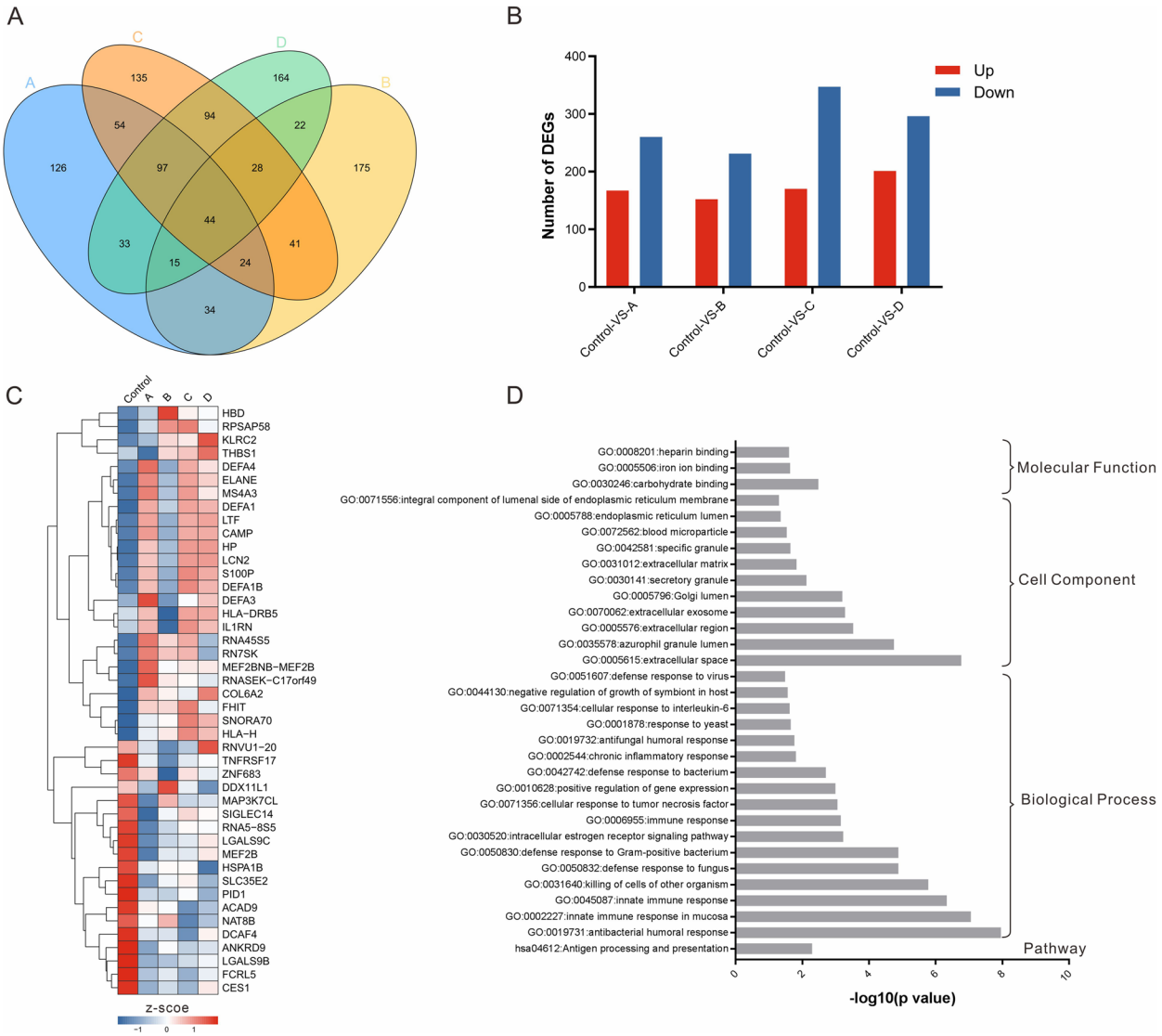

Fig. 1 Differentially expressed genes (DEGs) and Gene-ontology (GO) analyses. **A** DEGs between COPD patients and control; **B** DEGs among COPD **A**-**D** groups; **C**. Heatmap of gene expression; **D** GO and pathway-enrichment analyses between COPD and control

family A (Hsp70) member 1B (HSPA1B) was prominently down-regulated in COPD groups.

Candidate genes in COPD patients and healthy controls

The preceding findings indicated that several key genes may play pivotal roles in the pathogenesis of COPD. To validate their significance, we conducted fluorescence quantitative PCR on peripheral blood samples from COPD patients. The results demonstrated upregulation of CAMP, DEFA4, ELANE, LCN2, and LTF in COPD patients, consistent with RNA sequencing data (Fig. 2B). Conversely, the expression of HSPA1B, LGALS9B, and LGALS9C was downregulated in COPD patients compared to healthy controls. These findings underscore the potential importance of these genes in COPD pathophysiology.

CSE promotes apoptosis of bronchial epithelial cells through LCN2

Among the validated key genes mentioned above, current research indicates that LCN2 has emerged as a pivotal gene of interest in COPD research due to its significant implications in inflammatory responses and immune modulation within the lung microenvironment [30–32], which has also presented in our GO analysis (Fig. 1D). Understanding the role of LCN2

Table 1 The expression profiles of selected genes in ABCD group

Gene-id	Log2 ratio (cases/controls)			
	A	B	C	D
CAMP	2.74	0.73	2.58	2.61
DEFA4	3.18	0.45	2.55	2.03
ELANE	2.59	0.85	2.35	2.04
LCN2	1.95	0.78	2.26	2.29
LTF	2.82	0.67	2.76	2.82
DEFA1	2.97	1.49	3.14	2.89
DEFA1B	2.18	0.53	2.60	2.22
RNASEK-C17orf49	0.93	0.55	0.52	0.42
RNA45S5	3.02	2.10	2.51	0.84
RPSAP58	1.91	4.09	4.27	2.44
HP	3.24	2.52	2.69	0.79
LGALS9B	-1.12	-1.03	-0.92	-0.79
LGALS9C	-0.91	-0.64	-0.58	-0.44
HSPA1B	-0.71	-0.60	-0.62	-1.25
MAP3K7CL	-1.61	-0.44	-1.08	-0.98
DCAF4	-0.79	-0.79	-1.15	-0.62
SIGLEC14	-2.03	-0.93	-0.61	-0.81
RNA5-8S5	-2.49	-1.74	-1.28	-1.34
ZNF683	-0.47	-1.72	-0.51	-0.76

A-group (low risk, less symptoms GOLD 1–2 mild or moderate airflow limitation, 0–1 exacerbations per year, and mMRC grade 0–1 or CAT score < 10), B-group (low risk, more symptoms GOLD 1–2 mild or moderate airflow limitation, 0–1 exacerbations per year, and mMRC grade ≥ 2 or CAT score ≥ 10), C-group (high risk, less symptoms GOLD 3–4 severe or very severe airflow limitation, and/or ≥ 2 exacerbations per year, and/or ≥ 1 hospitalized exacerbation per year, and mMRC grade 0–1 or CAT score < 10), D-group (high risk, more symptoms GOLD 3–4 severe or very severe airflow limitation, and/or ≥ 2 exacerbations per year, and/or ≥ 1 hospitalized exacerbation per year, and mMRC grade ≥ 2 or CAT score ≥ 10)

Abbreviations: *CAMP* cathelicidin antimicrobial peptide, *DEFA4* defensin alpha 4, *ELANE* elastase, neutrophil expressed, *LCN2* lipocalin 2, *LTF* lactotransferrin, *RNA45S5* RNA, *45S* pre-ribosomal 5, *RPSAP58* ribosomal protein SA pseudogene 58, *HP* haptoglobin, *LGALS9B* galectin 9B, *LGALS9C* galectin 9C, *HSPA1B* heat shock protein family A (Hsp70) member 1B, *MAP3K7CL* MAP3K7 C-terminal like, *DCAF4* DDB1 and CUL4 associated factor 4, *SIGLEC14* sialic acid binding Ig like lectin 14, *RNA5-8S5* RNA, 5.8S ribosomal 5, *ZNF683* zinc finger protein 683

could provide crucial insights into the pathogenesis of COPD and potentially uncover novel therapeutic strategies targeting inflammation and tissue damage in the lungs. In our study, we investigated the effects of cigarette smoke extract (CSE) on bronchial epithelial cells, focusing on LCN2 expression and its consequences. Upon exposure to CSE, we observed a concentration-dependent increase in LCN2 expression (Fig. 3A). This upregulation was effectively attenuated when LCN2 siRNA was employed, leading to a notable decrease in LCN2 levels (Fig. 3B). Western blot analysis further confirmed the elevation of LCN2 protein levels upon CSE treatment (Fig. 3C). Importantly, CSE exposure significantly induced apoptosis of bronchial epithelial cells, as evidenced by experimental assays (Fig. 3D). In Fig. 3E, CSE treatment significantly increases apoptosis compared to the control, while LCN2 siRNA notably reduces this effect, suggesting LCN2 silencing protects against CSE-induced apoptosis. This indicates that CSE enhances bronchial epithelial cell apoptosis by upregulating LCN2 expression, and inhibiting this upregulation with LCN2 siRNA effectively mitigates the apoptosis.

CSE upregulates LCN2 expression to enhance ferroptosis in bronchial epithelial cells

Recent studies have also implicated LCN2 in the context of ferroptosis, a form of regulated cell death characterized by iron-dependent lipid peroxidation [33, 34]. Additionally, in our GO analysis(Fig. 1D) has identified enrichment in pathways related to iron ion binding, suggesting LCN2's potential involvement in iron homeostasis and oxidative stress regulation. Expanding on these findings, we explored LCN2's role in ferroptosis induction by CSE. Our results demonstrated that CSE exposure led to notable increases in intracellular Fe2 + levels, reactive oxygen species (ROS) production, and malondialdehyde (MDA) accumulation (Fig. 4B-D), which are hallmark features of ferroptosis. Additionally, CSE treatment resulted in elevated lactate dehydrogenase (LDH) release (Fig. 4E) and a reduction in cellular glutathione (GSH) levels (Fig. 4F), indicative of impaired antioxidant defenses. Furthermore, CSE downregulated the expression of glutathione peroxidase 4 (GPX4), a key regulator of ferroptosis (Fig. 4G-H). Interestingly, when LCN2 expression was suppressed using siRNA, the aberrant increases in Fe2 +, ROS, MDA, and LDH release induced by CSE were significantly attenuated (Fig. 4B-E). Moreover, GSH levels were restored, and GPX4 expression returned to baseline levels (Fig. 4F-H), suggesting a protective effect against ferroptotic cell death. These findings underscore LCN2's involvement in mediating ferroptosis and potentially regulating iron ion binding in bronchial epithelial cells under conditions of CSE exposure.

Discussion

The pathogenesis of COPD is multifaceted and involves intricate molecular mechanisms [1, 2]. Identifying biomarkers that reflect disease activity and severity remains pivotal for diagnosing COPD and monitoring therapeutic responses. Our previous studies have employed microRNA analysis to elucidate differential gene expression patterns in COPD patients compared to controls [27]. In this study, we conducted RNA sequencing to profile gene expression differences between COPD patients and healthy controls, focusing on identifying key genes and pathways associated with disease severity. Our

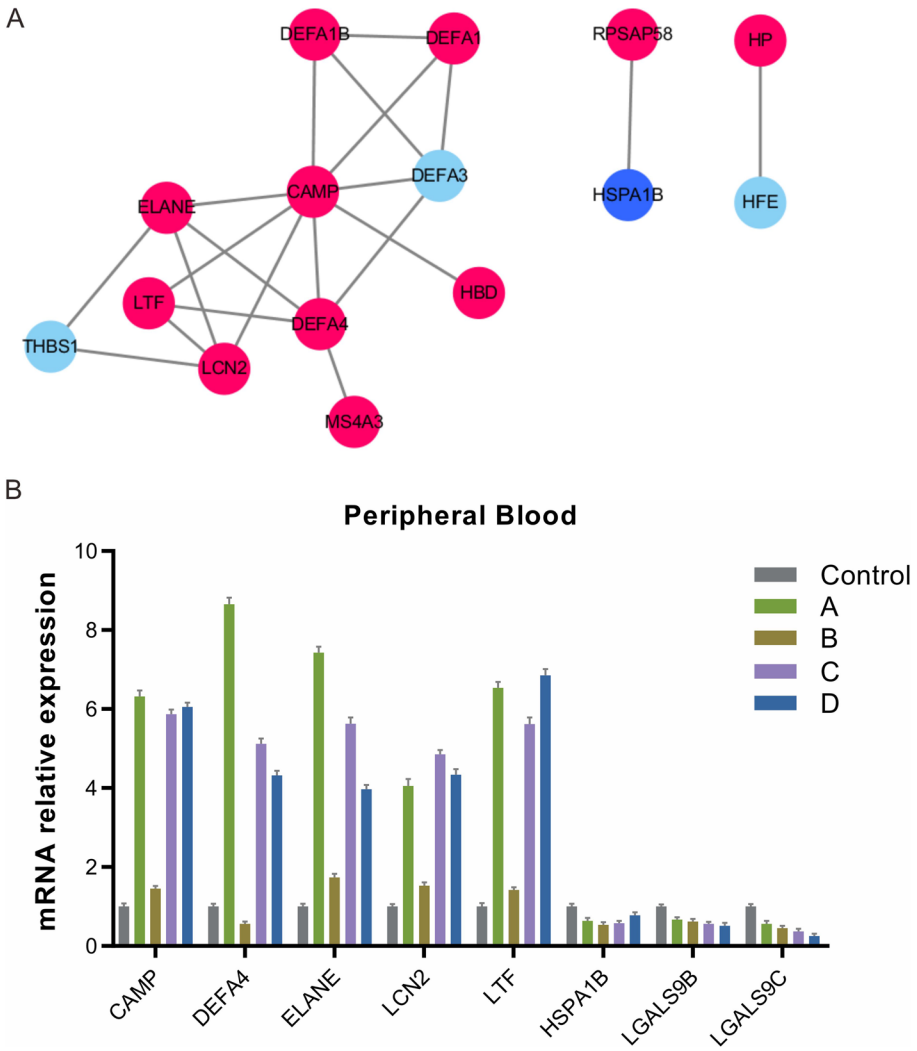

Fig. 2 Protein–protein interaction network of the DEGs and Candidate genes in COPD. **A** Protein–protein interaction network of the DEGs; **B** The qPCR results of candidate genes $^{*}P < 0.05$

investigation highlighted several significant findings, particularly regarding the role of LCN2 in bronchial epithelial cells, including its involvement in apoptosis and ferroptosis pathways.

We initially identified 44 significant genes associated with COPD severity, including 21 commonly upregulated and 17 downregulated genes. Notably, downregulation of LGALS9C and LGALS9B, along with upregulation of RNASEK-C17orf49, correlated negatively with disease severity. In humans, Galectin-9 (Gal-9) is encoded by the LGALS9 gene located on chromosome 17q12. The LGALS9 gene has two splice variants that produce different isoforms of Galectin-9: LGALS9B (short arm) and LGALS9C (long arm) [35]. These isoforms exhibit distinct structural characteristics and may have different functions, although their precise roles in various physiological and pathological conditions are still under investigation [35–37]. In our study, we observed a gradual decrease in the expression levels of LGALS9B and LGALS9C with increasing severity of COPD, and we found these genes involved in carbohydrate binding according to GO analysis. Horio et al. [38] demonstrated that Gal-9 can mitigate PPE-induced inflammation and emphysema in mice by reducing neutrophil infiltration and MMP-9 production, suggesting Gal-9 as a potential therapeutic agent for pulmonary emphysema and COPD.

Utilizing KEGG and GO analyses, as well as constructing a protein–protein interaction network, we identified six candidate genes among the differentially expressed genes (DEGs). Specifically, five genes (CAMP, DEFA4,

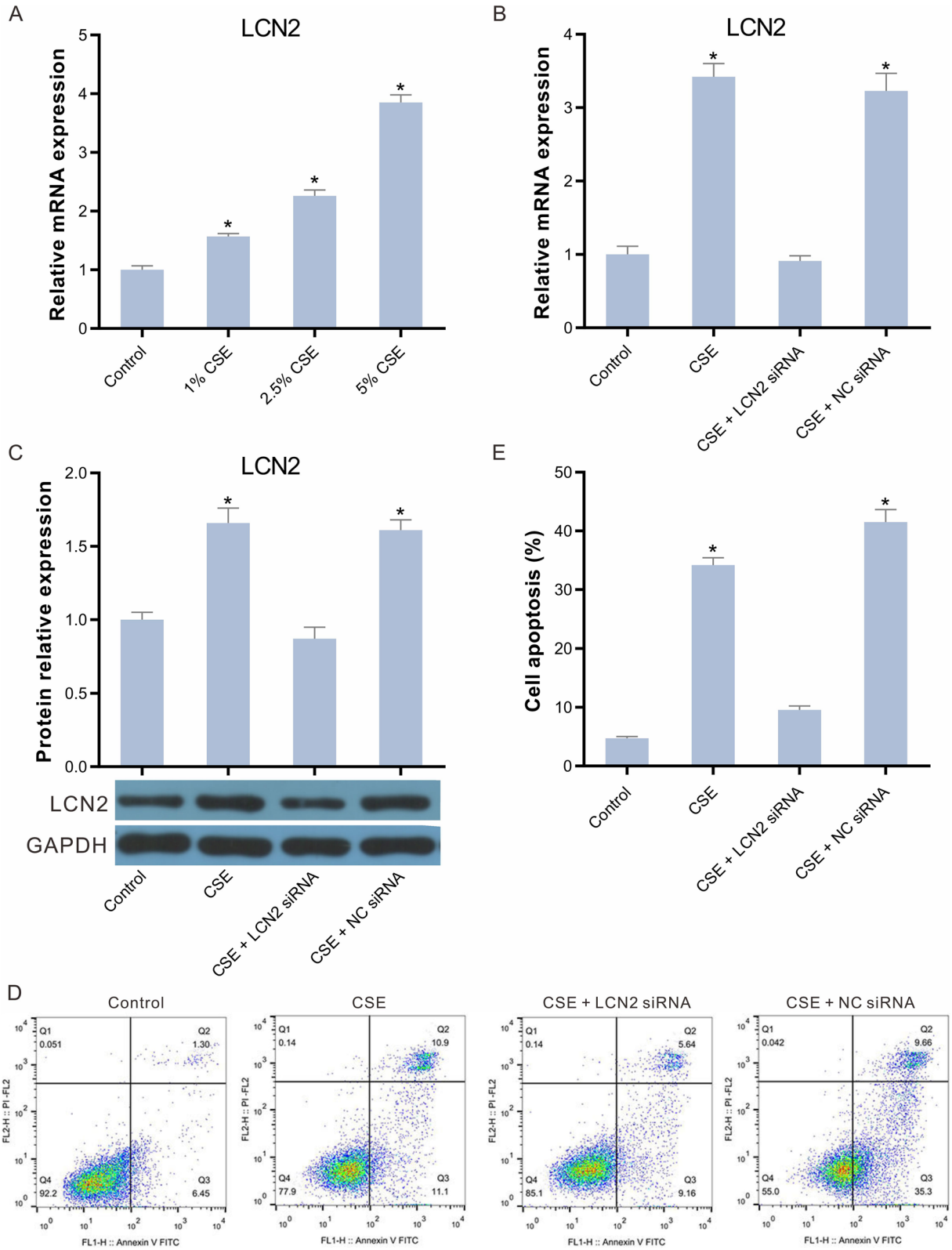

Fig. 3 CSE promotes apoptosis of bronchial epithelial cells through LCN2. **A** Expression levels of LCN2 varied with different concentrations of CSE. **B** QPCR analysis of LCN2 levels in BEAS-2B cells transfected with NC, CSE, CSE + LCN2siRNA, and CSE + NCsiRNA. **C** Western blot analysis of LCN2. **D** Assessment of cell apoptosis in BEAS-2B cells transfected with NC, CSE, CSE + LCN2siRNA, and CSE + NCsiRNA. **E** The apoptosis rate was detected by Flow cytometry in different groups (*$P < 0.05$)

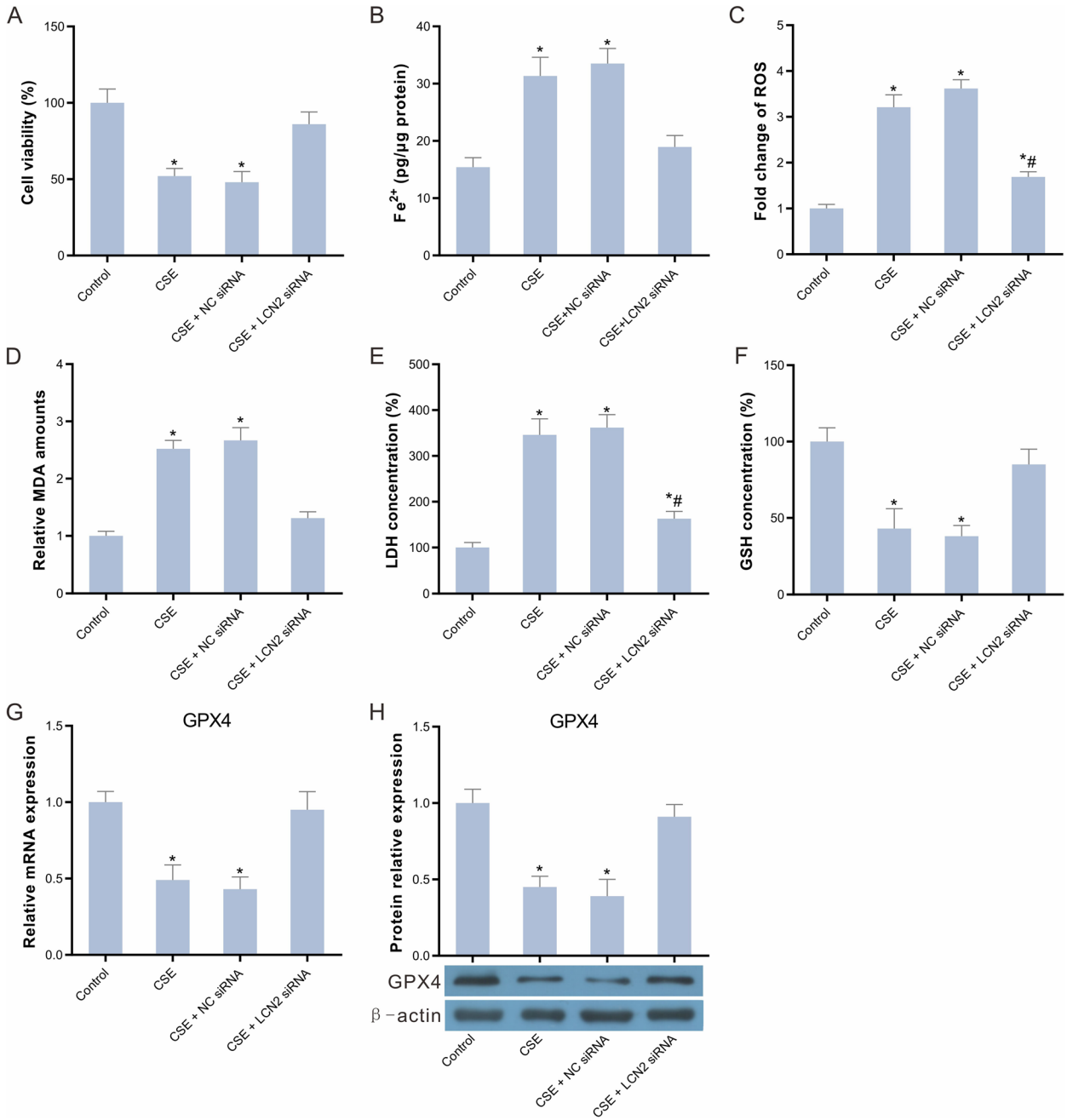

Fig. 4 CSE upregulates LCN2 expression to enhance ferroptosis in bronchial epithelial cells. **A** BEAS-2B cell viability was assessed by incubating cells with 10 μL CCK-8 solution. **B** Fe2 + levels in BEAS-2B cells transfected with NC, CSE, CSE + NCsiRNA and CSE + LCN2siRNA. **C** ROS levels in BEAS-2B cells transfected with NC, CSE, CSE + NCsiRNA and CSE + LCN2siRNA. **D** MDA accumulation levels in BEAS-2B cells transfected with NC, CSE, CSE + NCsiRNA and CSE + LCN2siRNA. **E** LDH leves in BEAS-2B cells transfected with NC, CSE, CSE + NCsiRNA and CSE + LCN2siRNA. **F** GSH levels in BEAS-2B cells transfected with NC, CSE, CSE + NCsiRNA and CSE + LCN2siRNA. **G** QPCR analysis of GPX4 levels. **H** Western blot analysis of GPX4 (*$P < 0.05$)

ELANE, LCN2, and LTF) exhibited significant upregulation, highlighting their roles in extracellular space dynamics and innate immune responses. Conversely, HSPA1B, as one of SNPs in HSP genes, implicated in antigen processing, gene expression regulation, and blood microparticle pathways, demonstrated downregulation in COPD. This finding aligns with previous research, which has extensively explored the role of

HSP genes in COPD. Specifically, all SNPs in HSP genes is associated with the risk of COPD and severe forms of the disease [39]. Similarly, the other upregulated five genes we identified have also been the subject of preliminary research in the context of COPD. For example, CAMP is a series of evolutionarily conserved molecules and are produced mainly by mucosal epithelial cells or phagocytes and are critically important components of respiratory innate immunity [40]. And it may potentially identify COPD patients at increased risk for more severe lung disease [41]. Paone et al. [42] found that sputum DEFA levels were also significantly higher in COPD patients with severe airway obstruction as compared to individuals with mild to moderate functional impairment and with symptomatic smokers and indicated DEFA concentrations correlate with the degree of airway obstruction. In addition, recent study confirmed PBMC ELANE expression of mRNA was significantly higher in COPD patients and an obvious negative correlation between NE and FEV1 in COPD patients [43]. LTF in COPD mainly focused on its antibacterial and anti-inflammatory activity [44, 45]. Elevated LCN2 promoted COPD airway remodeling, might be a potential target for reversing airway obstruction and remodeling in COPD [46], and a positive relationship with symptoms in patients with COPD [47]. These five important elevated genes are commonly associated with immune responses and might relate to the severity of lung function. Based on the pathways in which these genes participate, we speculate that they may be involved in resistance to bacteria, viruses, and immune response. As the disease progresses, the immune damage and inflammation mediated by exosome signals become more pronounced. Among these upregulated genes, LCN2 stands out due to its involvement in several crucial biological processes as revealed by GO analysis. Specifically, LCN2 is implicated in iron ion binding, response to oxidative stress, and regulation of apoptosis. The significance of LCN2's role is further highlighted by its potential involvement in both apoptosis and ferroptosis, two key forms of cell death that contribute to COPD pathogenesis. Apoptosis, a form of programmed cell death, and ferroptosis, a form of iron-dependent cell death characterized by the accumulation of lipid peroxides, are both implicated in the inflammatory and oxidative stress responses observed in COPD [4, 5]. Given these associations, studying LCN2 in the context of apoptosis and ferroptosis could provide critical insights into the mechanisms driving COPD progression. Therefore, understanding LCN2's role in these pathways might reveal new therapeutic targets and strategies for managing COPD.

To further explore the function of LCN2 in COPD, we conducted in vitro experiments with bronchial epithelial cells. Our findings showed that CSE induced LCN2 expression and enhanced cell apoptosis and ferroptosis. After downregulating LCN2 expression, CSE-induced cell apoptosis and ferroptosis were significantly reduced. This indicates that CSE increases apoptosis and ferroptosis in bronchial epithelial cells by promoting LCN2 expression, thereby contributing to the progression of COPD. We specifically selected bronchial epithelial cells for this study because they play a crucial role in the pathophysiology of COPD. As the first line of defense in the respiratory tract, these cells are directly exposed to inhaled harmful substances, such as cigarette smoke [4]. These harmful substances trigger inflammation and oxidative stress responses in bronchial epithelial cells, leading to apoptosis and ferroptosis, which subsequently cause pathological damage and functional impairment in lung tissues [7, 11, 12]. Studying bronchial epithelial cells allows us to observe the direct responses and changes in these critical cells during the development of COPD. Their unique role in lung defense and repair mechanisms makes them an ideal model to understand how LCN2 influences COPD progression through the regulation of apoptosis and ferroptosis. LCN2, known as neutrophil gelatinase-associated lipocalin, is an acute-phase protein secreted by immune and epithelial cells in various mucosal tissues, playing a crucial role in lung inflammation and iron homeostasis, and serving as a potential diagnostic biomarker for lung diseases [32]. Although there is limited research on the function of LCN2 in COPD, extensive studies have been conducted on its role in other lung diseases. Endogenous LCN2, but not exogenous LCN2, triggers NiCl2-mediated autophagy and apoptosis in bronchial epithelial cells [48]. Wang et al. [49] showed that LCN2 expression was significantly upregulated in newborn mice with acute respiratory distress syndrome (ARDS); LCN2 knockdown inhibited the MAPK/ERK pathway, thereby reducing the inflammation and oxidative stress associated with ferroptosis, and improving the pathological damage of lungs and cells. Zhang et al. [50] further pointed out that lipopolysaccharide (LPS) promoted the expression of LCN2 in BEAS-2B cells, while the ferroptosis inhibitor Ferrostatin-1 reversed the expression of LCN2; LCN2 knockdown reversed abnormal changes in LPS-induced lipid peroxidation, Fe2+, ACSL4, and GPX4 levels, indicating that LCN2 is an important gene associated with ferroptosis. Therefore, LCN2 is of great significance in pulmonary diseases such as COPD and can promote pathological progression through ferroptosis.

Several limitations should be considered when interpreting the findings of this study. Firstly, while peripheral blood leukocyte RNA profiles provide systemic insights, they may not fully capture the specific molecular changes

in COPD-affected lung tissues. Future research using lung tissue samples could offer more direct insights into disease pathogenesis. Due to difficulties in obtaining lung tissue samples, studies often rely on more accessible and less invasive peripheral blood samples from patients with COPD. Secondly, the in vitro model using bronchial epithelial cells exposed to CSE provides controlled conditions but lacks the complexity of the in vivo lung environment, potentially limiting its relevance to disease mechanisms involving interactions with other lung cell types and the microenvironment. Thirdly, the cross-sectional study design restricts the ability to establish causal relationships between LCN2 expression and COPD progression. Longitudinal studies are needed to explore the temporal dynamics of LCN2 expression in COPD. Moreover, while this study implicates LCN2 in apoptosis and ferroptosis pathways in COPD, the specific molecular mechanisms through which LCN2 exerts these effects remain inadequately discussed. Further detailed mechanistic studies are warranted to elucidate the precise roles and regulatory pathways of LCN2 in COPD pathophysiology.

Conclusion

In brief, our study identified differentially expressed genes between COPD patients and healthy controls using RNA-Seq analysis. KEGG, GO, and protein–protein interaction analyses highlighted six candidate genes. Among these, CAMP, DEFA4, ELANE, LCN2, and LTF were upregulated, while HSPA1B was downregulated. Notably, LCN2 is linked to apoptosis and ferroptosis, making it a potential target for COPD treatment. Further mechanistic studies are needed to explore these pathways.

Abbreviations

BP	Biological Process
BCL2	B-cell lymphoma 2
BAX	Bcl-2-associated X protein
COPD	Chronic obstructive pulmonary disease
CAMP	Cathelicidin Antimicrobial Peptide
CC	Cellular Component
CSE	Cigarette Smoke Extract
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DEFA4	Defensin Alpha 4
DEGs	Differentially Expressed Genes
ELANE	Neutrophil Elastase
Fe^{2+}	Ferrous Iron
GPX4	Glutathione Peroxidase 4
GO	Gene Ontology
GSH	Glutathione
HSPA1B	Heat Shock Protein Family A (Hsp70) Member 1B
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCN2	Lipocalin 2
LDH	Lactate Dehydrogenase
LTF	Lactotransferrin
MDA	Malondialdehyde
MF	Molecular Function
ROS	Reactive Oxygen Species

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12890-024-03357-3.

Supplementary Material 1.

Acknowledgements

Not applicable.

Authors' contributions

RY.W. had the idea and wrote the manuscript. JY.X. supervised the study. S.W. did the statistical analysis. XS.L. designed the study and offered the funding. All authors reviewed the manuscript.

Funding

This work was financially supported by Fundamental Research Program of Shanxi Province(No. 202303021221194), Four "batches"innovation project of invigorating medical through science and technology of shanxi province(2023XM003),and External Expert Workshop Fund Program of Shanxi Provincial Health Commission.

Data availability

Sequencing data have been deposited in the NCBI sequence read archive (SRA) under accession code SRR29503460-SRR29503485.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Shanxi Medical University (NO.2015053). Participants provided informed consent to participate in this study.

Consent for publication

All participants provided informed consent for the publication of their data as part of this study.

Competing interests

The authors declare no competing interests.

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Received: 5 August 2024 Accepted: 21 October 2024
Published online: 26 October 2024

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