



DNA Methylation Profiling in a Cigarette Smoke-Exposed Mouse Model of Airway Inflammation

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Purpose: DNA methylation, a major epigenetic modification, has been documented to play an important role in chronic obstructive pulmonary disease (COPD). In this study, we aimed to profile the DNA methylation patterns in a mouse model of airway inflammation induced by cigarette smoke (CS), a foremost risk factor of COPD.

Material and Methods: To establish a model of airway inflammation, wild-type mice were exposed to mainstream CS or room air for 2 hours twice daily, 6 days per week for consecutive 4 weeks. Lung tissues of the mice were collected for genome-wide DNA methylation analysis by liquid hybridization capture-based bisulfite sequencing, which were used for intersection analysis with gene expression by cDNA microarray to identify candidate methylated genes. Then, functional enrichment analyses with protein-protein interaction (PPI) network regarding these genes were conducted to explore the potential mechanisms.

Results: After 4-week CS exposure, the level of DNA methylation accompanied by a subacute airway inflammation was markedly enhanced, and 2002 differentially methylated genes (DMGs) were annotated, including 565 DMGs contained methylations in gene promoters, which were used for intersection with the differentially expressed genes. Then, 135 candidate methylated genes were further selected by the intersection, among which 58 genes with functional methylated modification were finally identified. Further analyses revealed candidate methylated genes were significantly enriched in a complicated network of signals and processes, including interleukins, toll-like receptors, T-cells differentiation, oxidative stress, mast cells activation, stem cells proliferation, etc., as well as the 58 functional methylated genes were partially located at key positions in PPI network, especially CXCL1, DDX58 and JAK3.

Conclusion: This study suggests CS exposure significantly enhances DNA methylated level, and the potential functional methylated genes are closely related to complicated inflammatory-immune responses, which may provide some new experimental evidence in understanding the epigenetic mechanisms of CS-induced airway inflammation in COPD.

Keywords: chronic obstructive pulmonary disease, airway inflammation, cigarette smoke, DNA methylation, liquid hybridization capture-based bisulfite sequencing

Introduction

Chronic obstructive pulmonary disease (COPD) is a common, heterogeneous chronic respiratory disease characterized by persistent respiratory symptoms and airflow limitation.¹ Cigarette smoke (CS) has been regarded as a major environmental risk factor for COPD. CS-induced airway inflammation significantly contributes to COPD pathogenesis,¹ but its molecular mechanisms are not fully understood.

DNA methylation, as a crucial epigenetic modification, plays a key role in regulating tissue-specific gene expression and complex traits.² In recent years, some studies have documented that abnormal DNA methylation may lead to alternative

expression of specific genes, especially the pro- and anti-inflammatory genes,³ which is closely associated with COPD susceptibility, lung function decline and exacerbation.^{4,5} Moreover, it is increasingly recognized that DNA methylation could be an important link between environmental and genetic factors.^{6,7} Emerging evidences have demonstrated environmental stimuli such as CS, could alter gene methylation patterns,⁸ and CS-induced DNA methylated modification was reported in several studies.^{9,10} However, the DNA methylation patterns in CS-induced airway inflammation are not well-profiled.

Therefore, we established a CS-exposed mouse model of airway inflammation to profile the genome-wide DNA methylation by liquid hybridization capture-based bisulfite sequencing (LHC-BS) and uncover the underlying mechanisms.

Materials and Methods

Animal Model

In our former article, a CS-exposed mouse model of airway inflammation has been established.¹¹ Briefly, wild-type (WT) C57BL/6 mice (7–9 weeks old, 20–22 g weight) were divided into two experimental groups: WT (control) group and CS group, n=10 mice per group. All mice were specific pathogen-free and kept on a 12-h light/12-h dark cycle, at a room temperature of 22 ±2°C, with free access to food and water. The mice in CS group were exposed to Marlboro cigarettes (Marlboro[®], Philips Morris, United States, with 1.0 mg nicotine and 11 mg tar per cigarette) with mainstream CS for 2 hours twice daily, 6 days per week for consecutive 4 weeks using a Baumgartner-Jaeger CSM2082i automated cigarette smoking machine (CH Technologies, West-Wood, NJ, USA), which output a smoke concentration of 320–340 mg TPM/m³. The control mice were exposed to filtered air according to the same schedule. Thereafter, all mice were anesthetized intraperitoneally with pentobarbital sodium and sacrificed by femoral artery transection. Hematoxylin-eosin (HE) stain in mouse lung tissues was used to display the CS-induced airway inflammation. The study protocol was in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (1986) and approved by the Panel on Laboratory Animal Care of West China School of Medicine of Sichuan University (approval number: 2020394A).

LHC-BS

Lung tissues from the mice (n=3 per group) were randomly selected for genomic DNA extraction by DNeasy Blood Tissue Kit (Qiagen, Germany) and subsequently used for library construction. In brief, 1 µg genomic DNA was sonicated to random fragments with approximately 200–300bp. After purification, the DNA library was prepared using SureSelect^{XT} Mouse methyl-seq Library Prep Kit (Agilent Technologies, USA). Afterward, DNA was bisulfite-treated using EZ DNA Methylation-GoldTM Kit (Zymo Research, Cat.D5006) according to the supplier's instruction. Finally, purified libraries were quantified by the Bioanalyzer analysis system (Agilent) and real-time PCR assay. The libraries were then sequenced on Illumina Novaseq PE150, the detailed process was described previously.¹²

DNA Methylation Data Analyses

The DNA methylation data were analyzed as described in detail previously.¹³ Briefly, after removing low-quality reads, then the cleans LHC-BC reads were aligned to the reference genome by Bismark software v0.19.0 with bowtie2 (version 2.3.4.2).¹⁴ The DNA methylation rate of cytosine was assessed by the number of supporting methylated readings divided by the total number of readings covering the cytosine. Methylation levels were analyzed using the R package, methylKit (version v1.6.1)¹⁵ and eDMR¹⁶ for differentially methylated regions (DMRs) of interest (eg, promoters, CpG islands), which may reveal more biological relevance. DMR was defined as adjusted $P < 0.05$ and the absolute differential methylation levels (absolute meth.diff >5%). Finally, the related differentially methylated genes (DMGs) were located and annotated in the DMRs by the ChIPseeker software.

cDNA Microarray

The data of gene expression by cDNA microarray have been informed in our former study.¹¹ Briefly, according to the manufacturer protocols, the total RNA from mouse lung tissue was extracted using the miRNeasy kit (Qiagen). After purification, the total RNA was generated to cDNA, which was biotin-labeled library to be hybridized on GeneChip Mouse Gene 2.0 STArray (Affymetrix, USA) covering more than 39,000 transcripts. Next, Affymetrix GeneChip

Scanner 3000 7G (Affymetrix) was used for signal detection. The microarray results were assessed by Expression Console software (Affymetrix) and Transcriptome Analysis Console software (Affymetrix). Then, the expression of each gene was estimated by the lognormal-normal model. Only differentially expressed genes (DEGs) at least 1.2-fold upregulated or downregulated were used for the further study.

Intersection Analyses

To select the candidate genes with potential functional methylated modification, CS-induced (CS vs WT) DMGs located in promoters and CS-induced DEGs were intersected. The functional methylated genes were finally identified according to the reversed effects of methylation in promoters on gene transcription.

Functional Enrichment Analyses

To identify biological function of the candidate genes, especially those with functional methylated modification, also called functional methylated genes, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, using ClueGO (Version 2.5.8)¹⁷ which integrates GO terms as well as KEGG pathways and creates a functionally organized GO/pathway term network within Cytoscape (Version 3.9).¹⁷ The GO and KEGG enrichment analyses were used with a kappa score of 0.5, showing ontologies with *P* values <0.05. Other settings were all default.

Protein–Protein Interaction (PPI) Network

To further identify the interactions among the candidate genes, especially the functional methylated genes, PPI network analysis was performed using STRING¹⁸ and displayed using CytoScape (Version 3.9).¹⁹ To calculate the degree of connectivity among the candidate genes in PPI network, the plug-in “Cytoscape” of Cytoscape²⁰ was conducted to score each node gene, using 10 different algorithms, and the top 10 node genes by each algorithm were obtained, respectively ([Table S1](#)).

Statistical Analysis

Data for the methylation level (%) were presented as median (interquartile range, IQR). All the statistical analyses in this study were performed with R software packages (R Foundation for Statistical Computing, Vienna, Austria). *P*<0.05 was considered statistically significant.

Results

DNA Methylation Data

Based on the LHC-BS method, we generated 15 Gbp raw sequence data on average for each sample ([Table S2](#)). Over 79% of mapped reads covered ~77% of the target regions, with an average of 57× sequencing depth per CpG and 13% duplication rate. For the downstream analysis, the duplicated sequence reads were filtered.

CS Exposure Induced Airway Inflammation and DNA Functional Methylated Modifications

The 4-week consecutive CS exposure significantly induced a subacute airway inflammation ([Figure 1A](#)) and meanwhile increased the DNA methylation level ([Figure 1B](#)). Then, 2002 DMGs were annotated with 1009 hypomethylated, 853 hypermethylated and 140 both hypo- and hypermethylated ([Figure 1C](#)). It is well-known that DNA methylation occurs almost in CpG islands that are primarily located in promoters, which is closely correlated with gene expression regulation according to a counter-regulation rule.²¹ Subsequently, among these 2002 DMGs, 565 DMGs (363 hypomethylated, 196 hypermethylated, 6 both hypo- and hypermethylated, [Figure 1D](#)) contained methylations in gene promoters, which were intersected with the DEGs. Finally, 135 candidate methylated genes were selected after the intersection analysis ([Figure 1E](#)), and 58 genes from the 135 candidate genes, with functional methylated modification in promoters were identified.

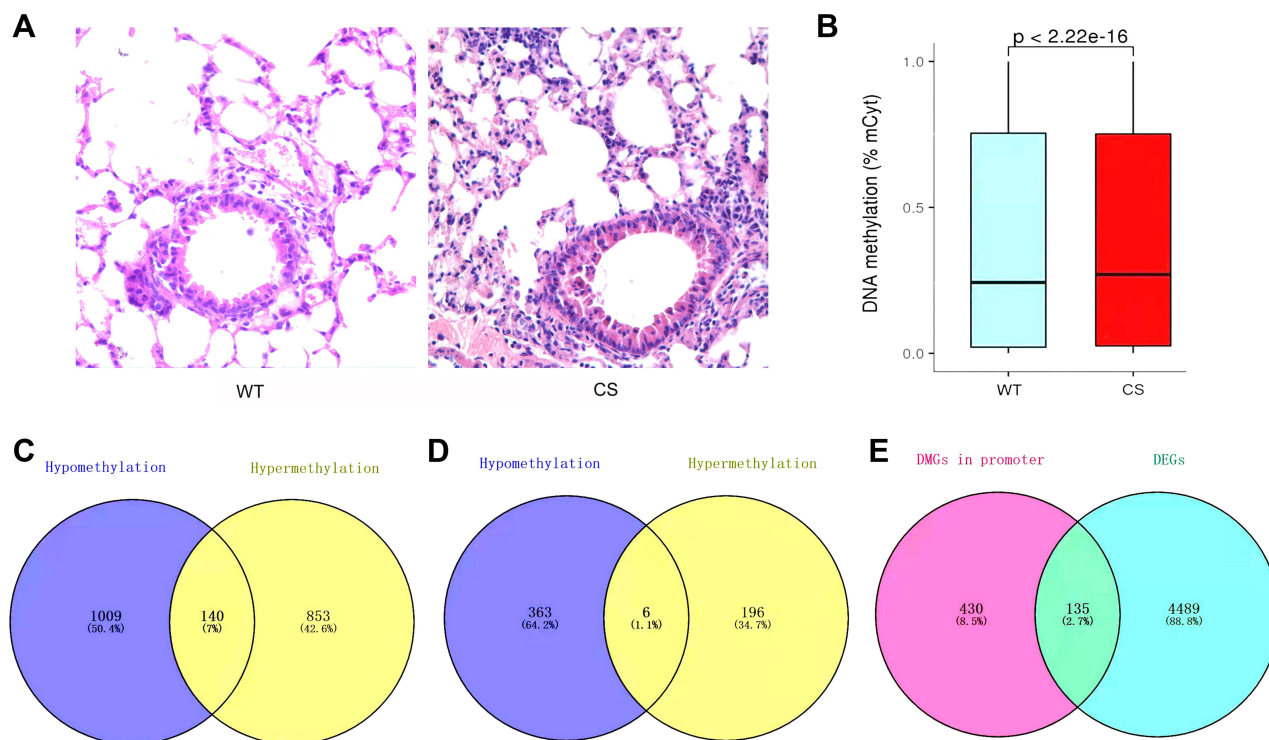


Figure 1 (A) The representative images of hematoxylin-eosin (HE) stain in mouse lung tissues ($\times 400$). (B) The methylation level (%) in wild-type (WT) and cigarette smoke (CS) groups. The medians with interquartile ranges (IQRs) for the WT and CS groups were 0.243 (0.173) and 0.27 (0.176) respectively. (C) Venn diagram of DMGs between WT and CS groups. (D) Venn diagram of the differentially methylated genes (DMGs) in promoter. (E) Venn diagram of intersection between DMGs in promoter and differentially expressed genes (DEGs).

The Functional Methylated Genes Were Correlated with CS-Induced Inflammatory-Immune Responses

Functional enrichment analyses indicated that the 135 candidate genes were significantly correlated with immune-inflammatory responses via a complicated network of signals and processes (Table S3), including interleukins, toll-like receptors, T-cells differentiation, oxidative stress, mast cells activation, stem cells proliferation, etc (Figure 2), and the candidate genes, especially the 58 functional methylated genes were in part located at key positions in PPI network (Figure 3), and further calculation by 10 different algorithms indicated the top 10 candidate genes, respectively. After taking intersection of the top 10 candidate genes in each algorithm, CXCL1, DDX58 and JAK3 were identified as the hub genes with functional methylated modification (Table S1).

Discussion

CS, as a foremost risk factor for COPD, has been shown to alter DNA methylation, which is associated with the initiation and progression of COPD.^{9,10} CS-induced DNA methylation plays a regulatory role in COPD, whereas the mechanisms remain not fully explained. In the present study, 135 overlapping candidate genes, regarding airway inflammation induced by CS exposure, were initially selected using an intersection model, and the functional DNA methylated modification in 58 genes were subsequently identified, which might significantly contribute to CS-induced airway inflammation in COPD. In addition, functional enrichment analyses suggested these candidate genes were significantly related to the immune-inflammatory responses via a complicated network of signals and processes, including interleukins, toll-like receptors, T-cells differentiation, oxidative stress, mast cells activation, stem cells proliferation, etc. All these signals and processes were reported to be contributors to inflammatory injury in COPD,^{10,22,23} and our data further indicated a potentially important role of methylated modifications in these signals/processes in CS-induced airway inflammation.

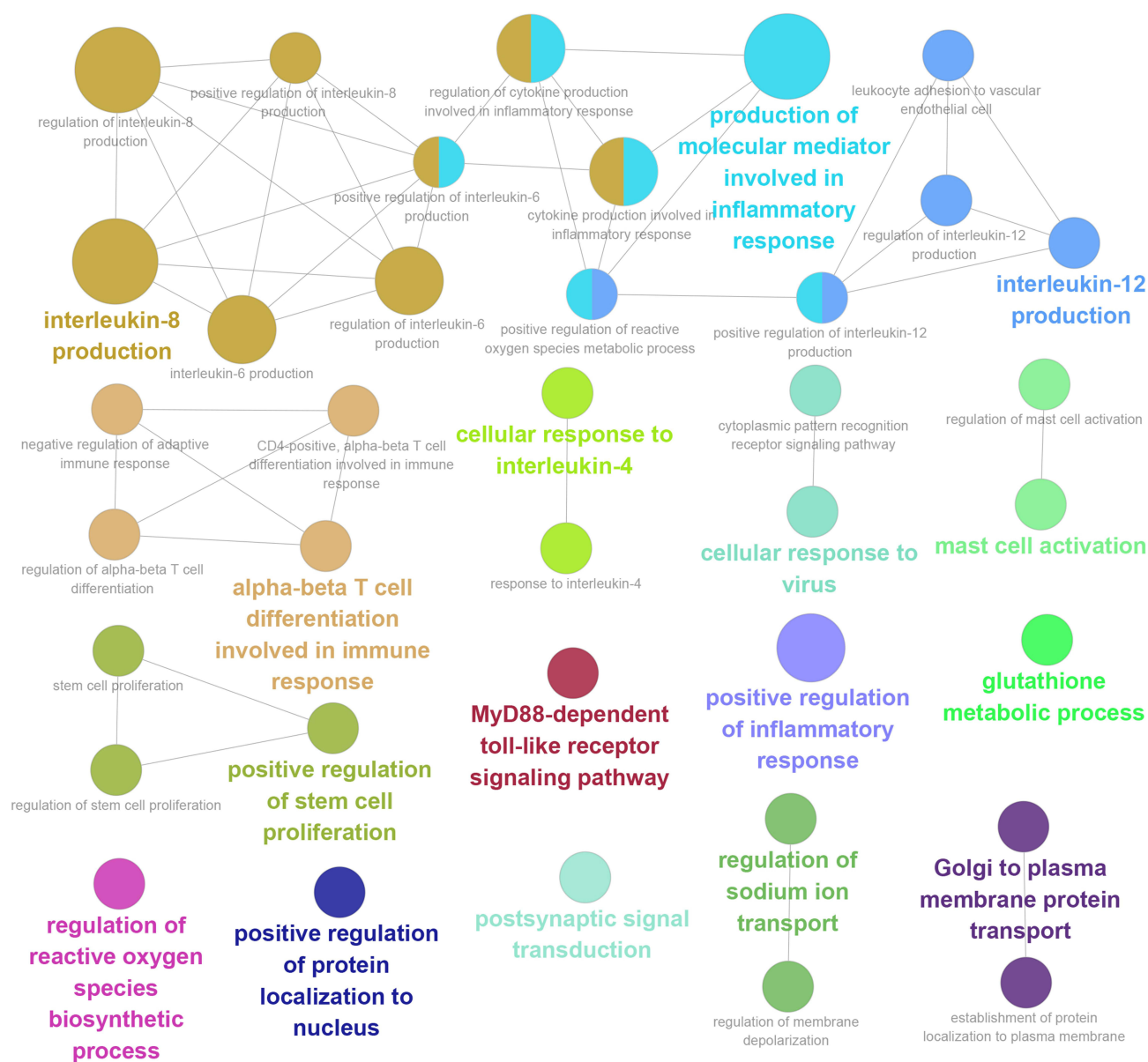


Figure 2 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) in the candidate genes. Each node represented a signal/process, and the same signals/processes were showed with the same colors. Cluster analyses were performed on these signals/processes, and the significant signals/processes were indicated with bold and bigger nodes in the same cluster.

Noticeably, the present study novel reported the functional methylated modifications in genes related to mast cells activation and stem cells proliferation induced by CS exposure. It has been documented that CS exposure can lead to an increase in the number of mast cells in the bronchial mucosa,²⁴ which plays a crucial role in excessive activation of innate immune system and is associated with lung function decline and airway/vascular remodeling in COPD.^{25–27} On the other hand, since the failure of lung regeneration is considered as a major mechanism of inflammatory injury in COPD,²⁸ stem cells significantly contribute to the maintenance and repair of lung tissue,²⁹ partly owing to the anti-inflammatory effects of stem cells via an increase in M2 macrophages, resulting in inflammation resolution and repair enhancement.³⁰ However, the mechanisms regarding mast cells and stem cells in COPD have not been well-elucidated. As suggested in this study, functional methylated genes related to mast cells activation and stem cells proliferation were identified, which would be potential targets for intervention in CS-induced airway inflammation in COPD.

In addition, based on the PPI network analysis with 10 different algorithms, the hub genes with functional methylated modification were further identified in this study, including CXCL1, DDX58 and JAK3, which might significantly contribute

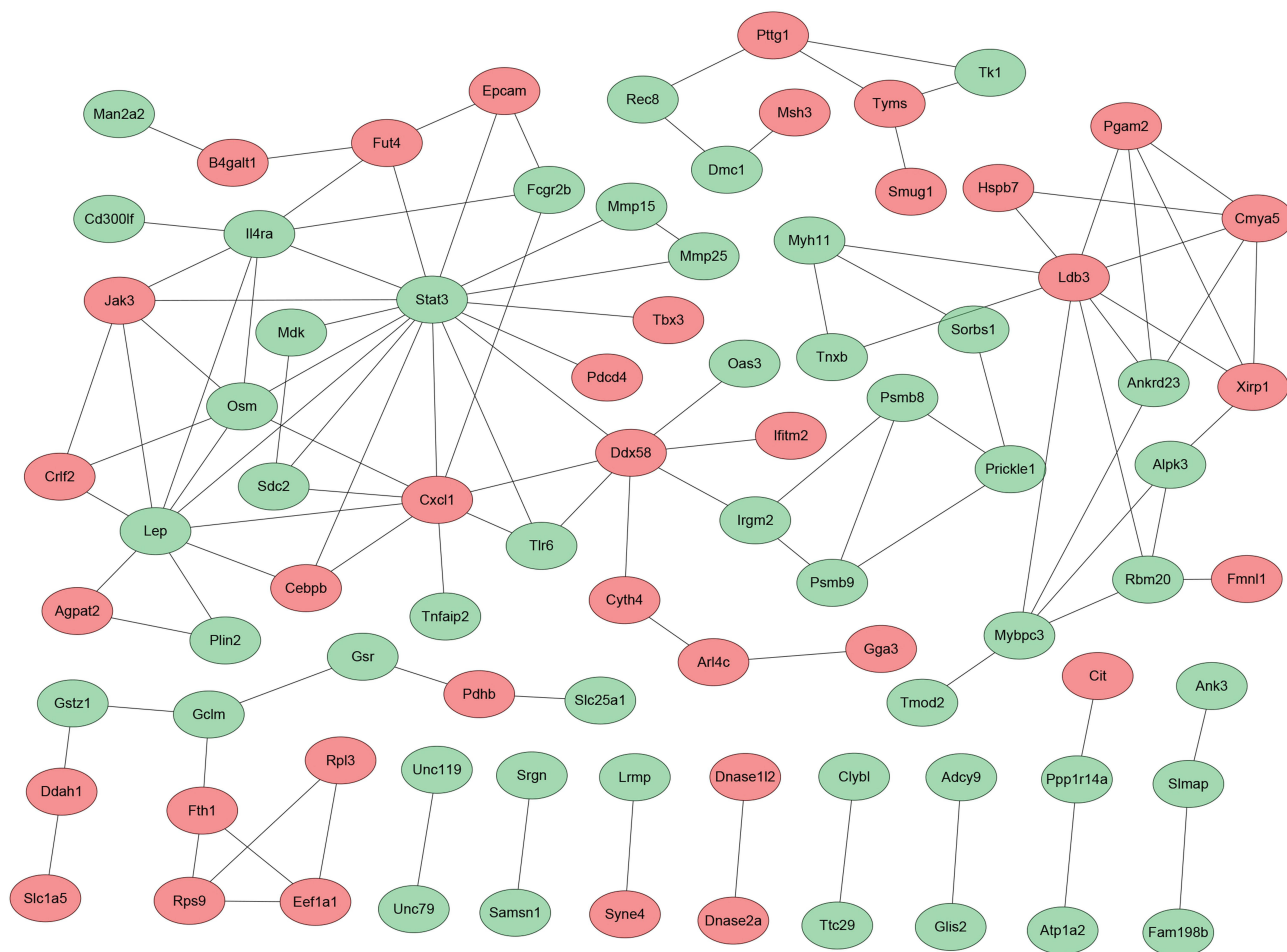


Figure 3 Protein-protein interaction network of the candidate genes. Each node represented a candidate gene and the undirected link between two nodes indicated an edge. Red circles pointed the functional methylated genes in promoter.

to CS-induced airway inflammation in COPD. CXCL1, a member of the chemokine subfamily of CXC, is markedly increased in the lungs of COPD patients, which is correlated with the degree of airflow limitation and the increased proportion of neutrophils, facilitating neutrophilic inflammation of COPD.^{22,31,32} In this study, functional methylated CXCL1 was enriched in positive regulation of stem cell proliferation and reactive oxygen species metabolic process. DDX58, also called RIG-I, is a pattern recognition receptor (PRR) involved in viral double-stranded (ds) RNA recognition and regulation of antiviral innate immune response,³³ which senses cytoplasmic viral nucleic acids and activates a downstream signaling cascade leading to the production of type I interferons and pro-inflammatory cytokines.^{34,35} In addition to the cellular response to virus, our study indicated functional methylated DDX58 was closely related to the production and regulation of IL-6 and IL-8. JAK3 is a member of Janus kinase (JAK) family of tyrosine kinases involved in cytokine receptor-mediated intracellular signal transduction. It is predominantly expressed in immune cells and participates in inflammatory-immune responses through tyrosine phosphorylation of interleukin receptors, which is associated with COPD.^{36,37} Functional methylated JAK3 participated in IL-12 regulation and production, cellular response to IL-4 and regulation of T cell differentiation. Moreover, functional methylated DDX58 and JAK3 might have potential crosstalk with another PRR, TLR6. Importantly, these hub genes (CXCL1, DDX58 and JAK3) have been reported to be targets of DNA methylated modification in schizophrenia,³⁸ hand, foot and mouth disease (HFMD),³⁹ and breast cancer.⁴⁰ The present results further suggested functional DNA methylation modification in these hub genes might play a crucial role in CS-induced airway inflammation in COPD.

However, two limitations in this study should be considered. First, the sample size of each group was relatively small, although the minimum requirement for biological repeat was reached. Second, the main findings were needed to be verified in COPD patients in the future.

Overall, in this CS-exposed mouse model of airway inflammation, the DNA methylated level was significantly enhanced, and the functional methylated genes were closely related to a complicated network of signals and processes associated with inflammatory-immune responses, which might provide some new experimental evidence regarding epigenetic mechanisms underlying CS-induced airway inflammation in COPD.

Abbreviations

COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CXCL1, C-X-C Motif Chemokine Ligand 1; DDX58, DExD/H-Box Helicase 58; DEGs, differentially expressed genes; DMGs, differentially methylated genes; DMRs, differentially methylated regions; GO, Gene Ontology; HFMD, hand, foot and mouth disease; IQR, interquartile range; JAK3, Janus Kinase 3; KEGG, Kyoto Encyclopedia of Genes and Genomes; LHC-BS, Liquid hybridization capture-based bisulfite sequencing; PPI, protein–protein interaction; PRR, pattern recognition receptor; TLR6, Toll Like Receptor 6; TPM, total particulate matter.

Data Sharing Statement

The datasets generated and analyzed during the present study are available from the corresponding authors on reasonable requests.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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