Original Research

Involvement of B-cell CLL/lymphoma 2 promoter methylation in cigarette smoke extract-induced emphysema

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Abstract

Abnormal apoptotic events play an important role in the pathogenesis of emphysema. The B-cell CLL/lymphoma 2 (Bcl-2) family proteins are essential and critical regulators of apoptosis. We determined whether the anti-apoptotic Bcl-2 play a role in the cigarette smoke extract (CSE)-induced emphysema. Furthermore, given the involvement of epigenetics in chronic obstructive pulmonary disease, we hypothesized that the deregulation of Bcl-2 might be caused by gene methylation. The emphysema in BALB/C mice was established by intraperitoneally injection of CSE. 5-aza-2′-deoxycytidine (AZA; a demethylation reagent) and phosphate-buffered saline were also administered intraperitoneally as CSE. TUNEL assay was used to assess apoptotic index of pulmonary cells. The methylation status of CpG dinucleotides within the Bcl-2 promoter was observed in all groups by bisulfite sequencing PCR. Pulmonary expression of Bcl-2, Bax, and cytochrome C were measured after four weeks of treatment. The apoptotic index of pulmonary cells in CSE injection group was much higher than control ((25.88 ± 7.55)% *vs*. (6.28 ± 2.96)%). Compared to control mice, decreased expression of Bcl-2 and high methylation of Bcl-2 promoter was observed in CSE injected mice (0.88 ± 0.08 *vs*. 0.49 ± 0.11, (3.82 ± 1.34)% *vs*. (35.68 ± 5.99)%, $P < 0.01$).CSE treatment induced lung cell apoptosis and decreased lung function. AZA treatment increased Bcl-2 expression with Bcl-2 promoter demethylation. AZA also alleviated the lung cell apoptosis and function failure caused by CSE treatment. The decreased expression of anti-apoptotic Bcl-2 might account for the increased apoptosis in CSE induced-emphysema. Apparently, epigenetic alternation played a role in this deregulation of Bcl-2 expression, and it might support the involvement of epigenetic events in the pathogenesis of emphysema.

Keywords: Apoptosis, B-cell CLL/lymphoma 2, emphysema, methylation

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Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality, though it has been described as a preventable and treatable disease.[1] The characteristics of COPD are airflow limitation and destructive alveolar loss (emphysema), which are mainly caused by cigarette smoking. There are several mechanisms that contribute to the pathogenesis of emphysema, including persistent inflammation, proteolytic/antiproteolytic imbalance, oxidative stress, and apoptosis.[2–5] Apoptosis is a highly regulated cell suicide program, which could be regulated by the B-cell CLL/lymphoma 2 (Bcl-2) family proteins via mitochondrial maintenance.[6,7] These Bcl-2 family proteins consist of anti- and pro-apoptotic member. Interactions of them decide whether the mitochondria should release cytochrome C (cyt C), a pro-apoptotic and initial factor.[6] Cigarette smoke, the most causal risk factor of COPD, also causes pulmonary apoptosis in both *ex vivo* and *in vivo* studies.[8–10] Abnormal apoptotic events are involved in emphysema though unbalancing cellular homeostasis. It has been reported that the classic anti-apoptotic protein, Bcl-2, might present protective potential for emphysema.[8,11–13] whereas the pro-apoptotic protein, Bax, might contribute

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to emphysema progress.[14–16] Some studies described methylation, an important epigenetic event, participated in regulation of Bcl-2 and apoptosis.[17,18] Promoter methylation leads the condensation of chromatin into a compact state, which is inaccessible by transcription factors, causing a down-regulation of exon expression.[19] Recent studies also demonstrated the involvement of epigenetics in COPD,[19,20] and our previous study showed demethylation might protect models from emphysema.[21] Thus, the methylation-related apoptosis might play a role in emphysema pathogenesis. This study tried to examine whether methylation of Bcl-2 involved in the pathogenesis of emphysema.

Materials and methods

Preparation of CSE and AZA

Cigarette smoke extract (CSE) was prepared as we described previously.[22] Briefly, one cigarette (China Tobacco Hunan Industrial CO, Ltd. Tar: 12 mg, nicotine: 1.1 mg, carbon monoxide: 14 mg) was burned; the smoke was passed through 25 mL of phosphate-buffered saline (PBS) by a vacuum pump. This 100% CSE was adjusted to pH 7.4 and removed particles and bacteria by filter (Fisher, NH), and then injected into the peritonium (0.3 mL per 20 g). This CSE solution was prepared fresh for each set of experiments. 5-aza-2′-deoxycytidine (AZA, A3656, Sigma, USA) is a well-known methyltransferase inhibitor, causing DNA demethylation. AZA was dissolved in double distilled water, and then further diluted in PBS at 1 mmol/L. This AZA was also adjusted to pH 7.4 and filtered, and injected into the peritonium (2.5 mg per kg). For the unstable AZA, the preparation was conducted immediately at each set of experiments.

Experimental protocols

This animal protocol was approved by the Ethics Committee of the Second Xiangya Hospital, Central South University. Six-week-old male SPF BALB/c mice (21–23 g, Shanghai Animal Husbandry Advisory Services, China) were randomly divided into four groups ($n = 10$ per group): CSE group, CSE + AZA group, AZA group, and PBS group as control. The CSE group mice were injected intraperitoneally with CSE at day 0, 11, 22 of the study, and injected with 0.3 mL PBS at day 15, 17, 19 of study. While the control group mice were administrated with 0.3 mL PBS in the same way at day 0, 11, 15, 17, 19, 22. The CSE + AZA group was injected with intraperitoneally with 0.3 mL CSE at day 0, 11, 22, and AZA at day 15, 17, 19. Mice in AZA group were injected with 0.3 mL PBS at day 0, 11, 22, and AZA at day 15, 17, 19. All mice were fed and housed the same way for 28 days, and all mice anesthetized at day 28 to collect samples for further analysis.

Pulmonary function analysis

Mice were anesthetized by intraperitoneal injection of chloral hydrate (3 mL/kg). Plethysmograph (Buxco Research Systems, USA) was used to measure model pulmonary function. Respiratory frequency (*f*, bpm), tidal volume (TV, mL), dynamic compliance (Cdyn, mL/cm H_2O), and airway resistance (RI, cm H_2O/mL/min) were measured in each sample.

Lung tissue samples collection

Lung tissue was collected by following the protocol as we described previously.[22] The left lung tissues were inflated with 10% formalin at a constant pressure of 25 cm H_2O for 24 h, before fix and paraffin-embedding. The right upper lungs were homogenized immediately after harvest in a buffer containing 50 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid, 1 mmol/L dithiothreitol, 0.1% triton X-100, and 10% glycerol. The supernatant was separated after two cycles of centrifugation at 1000*g* for 10 min. Isolation of cytosolic fractions from the right lower tissues was performed by following the instruction of Mitochondria Isolation Kit for Tissue (Pierce, USA). The concentration of both total and cytosolic protein was determined via the bicinchoninic acid (BCA) protein assay (Pierce, USA). Total RNA was extracted from the right lower lungs as Rio and colleagues described.[23]

Morphological assessment

The imbedded left lung was cut and stained with hematoxylin and eosin (HE). The mean linear intercept (MLI) and the destructive index (DI) were calculated to assess the pulmonary architecture as the previous researches.[22] MLI was obtained under light microscopy at 100 × magnification. DI was measured under light microscopy at 200 × magnification. These assessments were coded with samples and assessors.

Apoptosis assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed to figure out the DNA-damage cells in the lungs. The In-situ Apoptosis Detection Kit (Nanjing Keygen Biotech, China) was used to this TUNEL experiment following the manufacturer's instruction. The apoptotic index (AI) was calculated as the percentage of TUNEL-positive nuclei randomly counted for each lung in a total of more than 3000 nuclei by light microscopy at 200 × magnification.

Immunohistochemistry for expression of Bcl-2 in endothelia

Paraffin-embedded sections were deparaffinized and rehydrated with xylene and ethanol. Antigens retrieval was performed via the microwave method with citrate buffer for 20 min. Endogenous peroxidase, avidine, and biotin were blocked by 3% hydrogen peroxide. Normal bovine serum (5%) was used for blocking. Following this, the samples were incubated with rabbit anti-human Bcl-2 antibodies (Bioworld Technology, USA) overnight at 4°C with a dilution of 1:50. After washing with PBS with 0.05% Tween, the bound primary antibodies were detected with Polink-2 Plus Polymer HRP Detection System (ZSGB-BIO, China) following the manufacturer's instruction. Negative control was established without adding the primary antibody. After 3,3′-diaminobenzidine (DAB) staining (DAB Detection Kit,

ZSGB-BIO, China), sections were then scanned for vessel and endothelial cells, and calculated numbers of Bcl-2 positive endothelial cells/100 endothelial cells by pathologists in blinded. In each case three sections were detected, and each section was analyzed for five areas.

Western blotting

A total of 30 μg of protein was separated on SDS-PAGE gel (Beyotime, China) and transferred to the nitrocellulose (NC) membranes (Millipore, USA). After transferring, these membranes were blocked by 5% nonfat milk, in which dry milk was diluted by PBS containing 0.05% Tween (PBST) for 1 h at room temperature. Following blocking, these membranes were washed and incubated overnight with rabbit polyclonal antibodies, respectively, at 4°C (Bcl-2, Bax, Cyt C, 1:1000, Cell Signaling Technology, USA). These membranes were washed four times with PBST and incubated with HRP-Conjugated Goat Anti-Rabbit IgG (Jackson Immuno Research Laboratories, USA) for 1 h at room temperature. Lighting was done using ECL kit (Thermo, USA), and film was developed and fixed by a developer and fixer kit (Beyotime). The expression of each protein was detected and quantitated with Quality-one software (Bio-Rad Laboratories, CA).

Real-time reverse transciptase-polymerase chain reaction

After extraction, RNA was reverse-transcribed using PrimeScript® RT reagent Kit (Takara, China), and assayed using SYBR®Premix Ex Taq™ following the manufacturer's instruction. All of the primers were obtained from Sangon Shanghai, China (Table 1). Real-time polymerase chain reaction (PCR) was conducted on the Step-one ABI Real-time RT-PCR system. All mRNA expression values were presented relative to β-actin.

Analysis of Bcl-2 promoter methylation status

Bcl-2 promoter was determined from −3000 bp to + 70 bp by retrieving Transcriptional Regulator Element Database (Accession Number 46672, NM 009741). After searching CpG island in UCSC Genome Browser, the CpG island in promoter (−1867 bp to −1541 bp) was detected and analyzed for methylation status using bisulfite sequencing PCR (BSP). Primers (Table 2) for BSP were designed through MethPrimer (http://www.urogene.org/methprimer/), and then were blasted and confirmed using methBLAST (http://medgen.ugent.be/methBLAST/). A Genomic DNA Extraction Kit (Takara) was used to extract DNA from tissue. The bisulfate conversation of DNA was performed with EpiTect Bisulfate Kits (QIAGEN, Netherlands) following instruction. After bisulfate modification, the nested PCR was performed. The premix solution for first round contained bisulfate-modified DNA (2 μL), Taq (0.2 μL) (Takara), a pair of primer (20 μm, 1 μL each), diethylpyrocarbonate (DEPC) H_2O (11.8 μL) (Takara), and dNTPs (10 mmol/L, 1 μL) (Takara). The PCR was run as follows: 94°C for 3 min denaturing; 30 cycles at 94°C for 30 s, at 50°C for 30 s, and at 72°C for 1 min; then extending at 72°C for 5 min. In the second round the primer was changed, though following the same PCR steps. After amplification, the PCR products were cloned into pMD-18T vector (Takara) and sequenced. The methylation status was presented by counting the number of methylated CpG in all clones, and expressing it as percentage of the whole CpG.

Table 1 Primer for real-time RT-PCR

Bcl-2	Forward	5′-GATTGTGGCCTTTGAGTTC-3′
	Reverse	5′-ACTCATTCAACCAGACATGCAC-3′
Bax	Forward	5′-GAGACACCTGAGCTGACCTTG-3′
	Reverse	5′-GAAGTTGCCATCAGCAAACAT-3′
Cyt C	Forward	5′-AGGCTGCTGGATTCTCTTACAC-3′
	Reverse	5′-TGCCCTTCCCTTCTTCTTA-3′
β-Actin	Forward	5′-ATATCGCTGCGCTGGTCGTC-3′
	Reverse	5′-AGGATGGCGTGAGGGAGAGC-3′

Statistical analysis

A software package (SPSS 16.0; Statistical Product and Service Solutions, USA) was used to perform all statistical analyses. One-way ANOVA and Kruskal–Wallis test were performed to evaluate each group data. P value less than 0.05 is considered statistically significant.

Result

Morphological findings and pulmonary function

All mice in PBS group survived. During experimental period, two mice in CSE and CSE + PBS died, and one in AZA group died. The CSE group showed increased MLI and DI, and worse Cdyn, TV and RI ($P < 0.01$, Figure 1). The CSE + AZA mice showed less destruction of pulmonary construction and function compared with CSE group ($P < 0.01$, Figure 1).

AZA decreases apoptosis in pulmonary endothelia

As previously described, the increased endothelial apoptosis was found after CSE injection.[24] The AI was increased in CSE group, compared to the controls ((25.88 ± 7.55)% vs. (6.28 ± 2.96)%, Figure 2). Compared to CSE injection mice, CSE + AZA mice had lower AI (5.85 ± 3.23)%, $P < 0.01$, Figure 2), indicating the demethylation might protect cells from CSE-induced-apoptosis.

AZA improves Bcl-2 expression in endothelial cells

CSE mice had lower Bcl-2 positive endothelial cells than control mice ($P < 0.05$, Figure 3). Mice in CSE + AZA

Table 2 Primers for BSP

Bcl-2 outer primers	Forward	5′-GAAAGGGTTTATTGGATTGTGT-3′
	Reverse	5′-AAAAAAAAAAAAAAAAACCCTCCT-3′
Bcl-2 inner primers	Forward	5′-GGTTTATGTGATTTGTATATGTTATATAGA-3′
	Reverse	5′-AAAAAAAACCCTCCTCTAAAC-3′

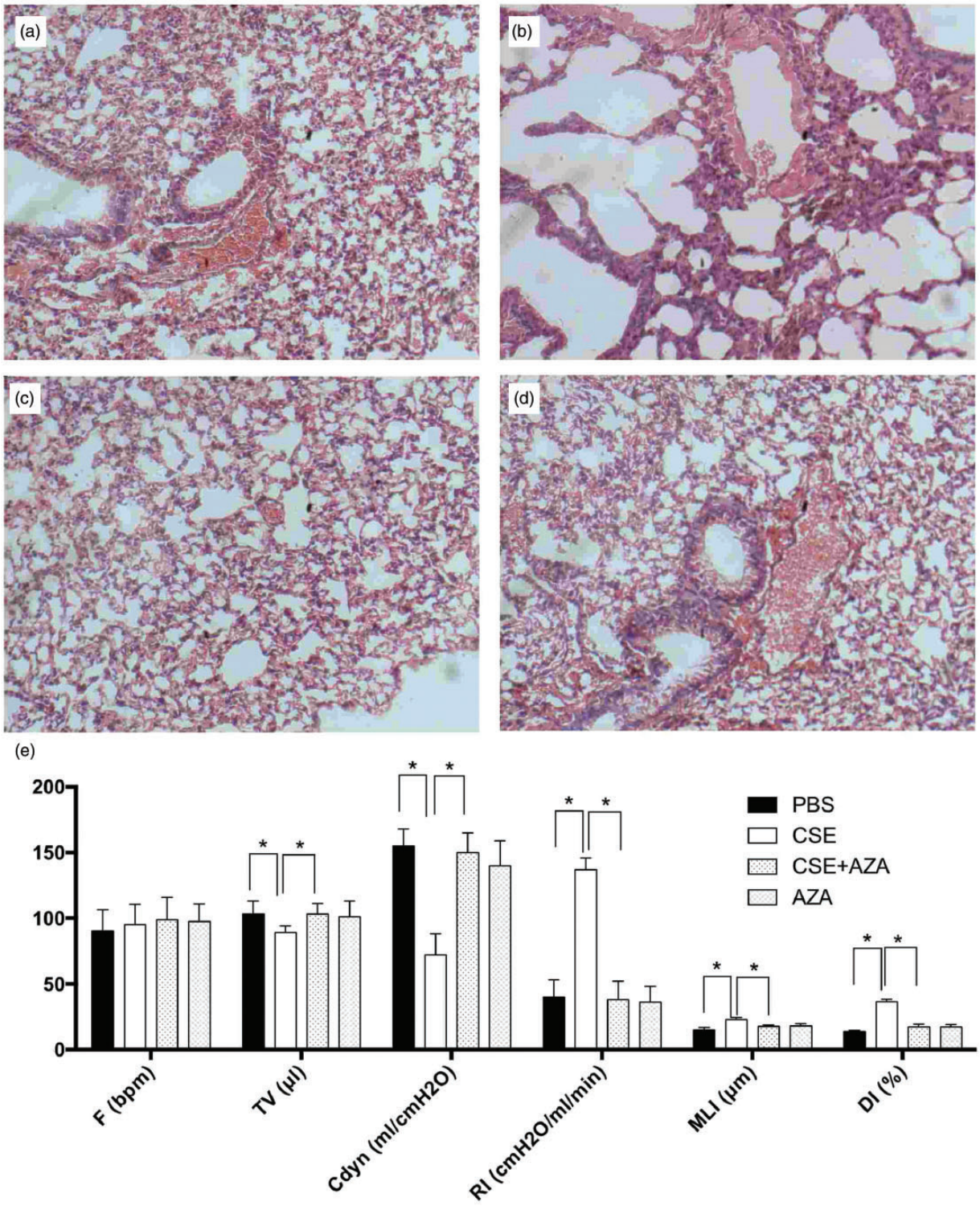

Figure 1 The morphological findings and pulmonary function in groups. (a) Histological image of lung in PBS group; (b) histological image of lung in CSE group, showing CSE injection built the morphological disorder of emphysema; (c) histological image of lung in CSE + AZA group, showing demethylation treatment improved morphological disorders in CSE injection model; (d) histological image of lung in AZA group; (e) the indexes of pulmonary function in groups, showing CSE injection deteriorated lung morphology and lung function, and demethylation treatment improved morphological and functional disorders in CSE injection model; the mean linear intercept (MLI) and the destructive index (DI) in each group, showing CSE injection deteriorated lung morphology, and demethylation treatment (AZA injection) improved morphological disorders in CSE injection model.*$P < 0.01$ *vs*. CSE group. (A color version of this figure is available in the online journal.)

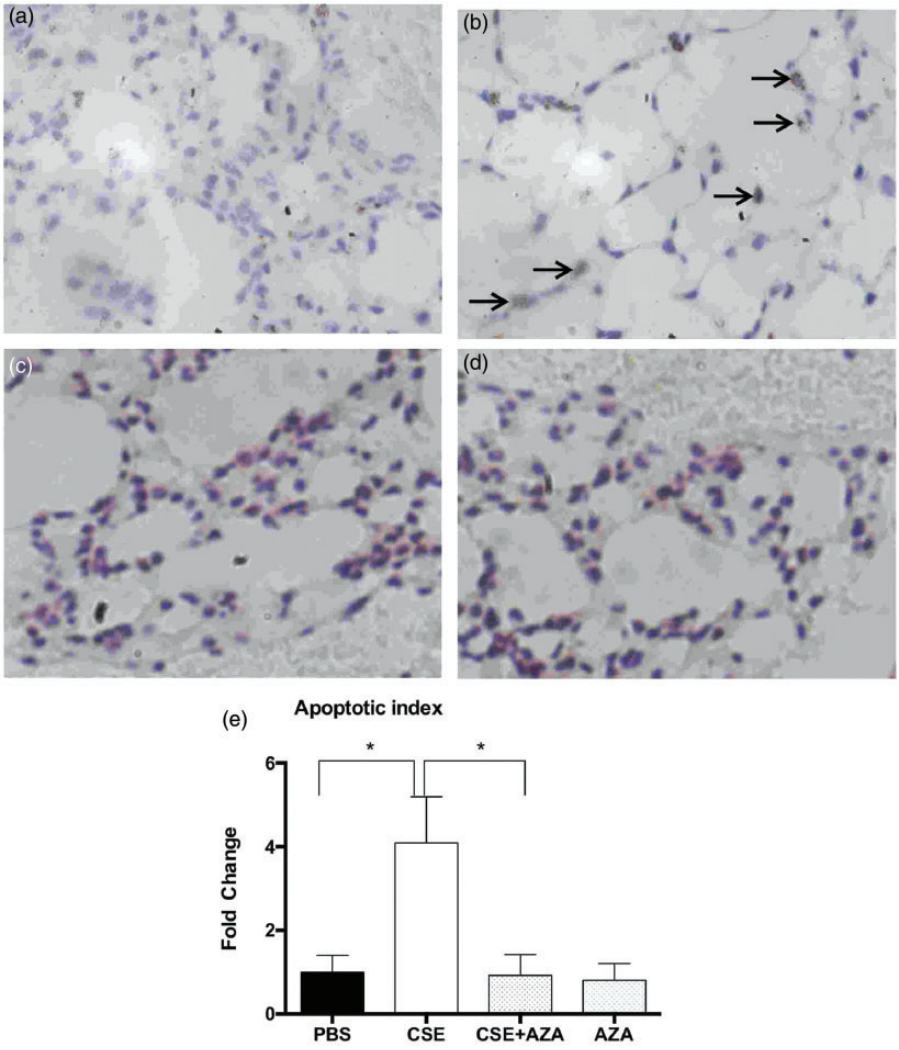

Figure 2 The TUNEL detection of apoptosis cells in lung. (a) The TUNEL detection in PBS group; (b) the TUNEL detection in CSE group; (c) the TUNEL detection in CSE + AZA group; (d) the TUNEL detection in AZA group; (e) apoptosis index in each group, showing CSE injection increased cell apoptosis, and AZA injection restored the deregulation; → the TUNEL-positive nuclei, magnificent 400×; *$P < 0.01$ *vs*. CSE group. (A color version of this figure is available in the online journal.)

group had higher expression of Bcl-2 than those mice treated with CSE ($P < 0.05$, Figure 3).

AZA regulates Bcl-2, Bax, and cyt C expression in the lung

Bcl-2/Bax is a pair of proteins associated with apoptosis with opposite effects. Bcl-2 is a key anti-apoptosis protein, which can stabilize the mitochondria membrane and prevent the leakage of cyt C. The CSE-injected group showed a decreased expression of Bcl-2 and an increased expression of Bax in the whole lung tissue, compared to control mice and CSE + AZA mice (Bcl-2: 0.49 ± 0.11, 0.88 ± 0.08, 0.77 ± 0.07, respectively; Bax: 0.88 ± 0.07, 0.49 ± 0.10, 0.53 ± 0.13, respectively; $P < 0.01$, Figure 4). No significant difference about cyt C was found in the total lung protein among four groups ($P > 0.05$). According to the process of apoptosis, the cyt C will be released to cytoplasm to induce apoptosis, so we detected the cyt C expression in cytosolic protein. CSE injection increased the expression of cytosolic cyt C, compared to control mice and CSE + AZA mice ($P < 0.05$, Figure 4). AZA alone did not affect the expression of Bcl-2, Bax, and cytosolic cyt C compared to the controls.

Restored Bcl-2 mRNA expression after AZA treatment

Bcl-2 mRNA expression was decreased after CSE injection, and the demethylation treatment with AZA restored the mRNA expression ($P < 0.05$, Figure 5). AZA alone did not affect the mRNA expression of Bcl-2, compared to the controls (Figure 5). The mRNA expression of Bax had no difference among groups (Figure 5).

Involvement of Bcl-2 promoter methylation in emphysema models

Compared to the controls, CSE treatment significantly promoted the methylation of Bcl-2 promoter ($(35.68 \pm 5.99)\%$ *vs*. $(3.82 \pm 1.34)\%$, $P < 0.01$, Figure 6). AZA treatment lowered the Bcl-2 promoter methylation induced by CSE ($(3.41 \pm 2.27)\%$ *vs*. $(35.68 \pm 5.99)\%$, $P < 0.01$, Figure 6). AZA

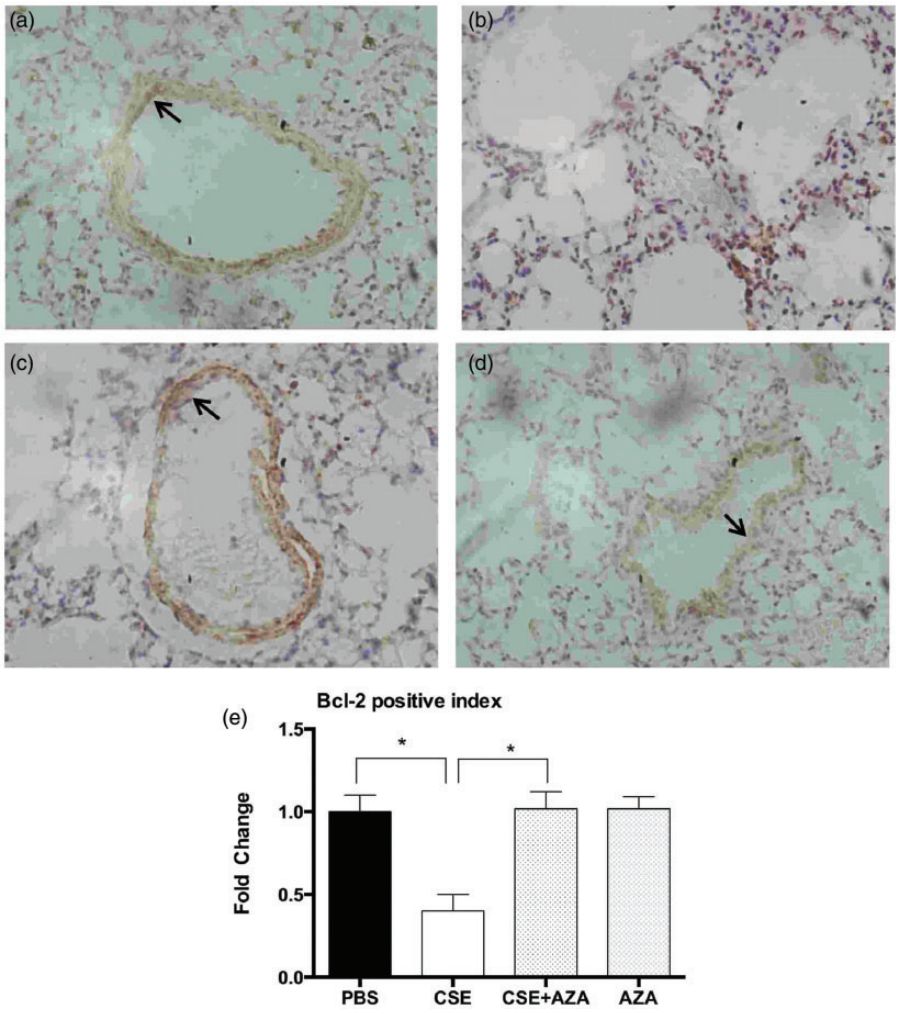

Figure 3 Expression of Bcl-2 in endothelia cells. (a) Immunohistochemistry image in PBS group; (b) immunohistochemistry image in CSE group; (c) immunohistochemistry image in CSE + AZA group; (d) immunohistochemistry image in AZA group; (e) Bcl-2 positive cells index in groups, showing CSE injection decreased Bcl-2 expression, and demethylation treatment (AZA injection) restored Bcl-2 expression; → positive of Bcl-2 expression, magnificent 200×.. $^*P < 0.05$ *vs*. CSE group. (A color version of this figure is available in the online journal.)

alone did not affect the mRNA expression of Bcl-2, compared to the controls (Figure 5).

Discussion

Our previous research[21,24] and this research found that CSE injection lead emphysematous change and abnormality of pulmonary function. Cigarette smoke was inhaled into the lung and diffused into the mucus and the blood as liquor phase. CSE could mimic this process, and the pathologic and pulmonary function data demonstrated that CSE injection induce the emphysema in mice.

Bcl-2, a principal anti-apoptotic protein, is agonist with the proapoptotic Bax. It was demonstrated that decreased Bcl-2/Bax caused apoptosis, by releasing cytosolic cyt C from mitochondria.[25] In consensus with other emphysema or COPD patients and models,[11,12,15] CSE injection showed the augment of apoptosis, deregulation of Bcl-2 and upregulation of Bax protein in the lung. Furthermore, the increased cytosolic cyt C was found in the lung. It might be deduced that the CSE-induced unbalance between Bcl-2 and Bax caused leakage of cytosolic cyt C, and initiated endothelia apoptosis.

There was obvious decrease in Bcl-2 mRNA expression in emphysema models. However, there was no difference about Bax mRNA expression between CSE treated mice and control mice. It seems that CSE-induced augment of Bax protein was under post-transcriptional regulation. On the contrary, the CSE-induced increase of Bcl-2 was under pre-transcriptional regulation. This study speculated the CSE-induced increase of Bcl-2 might be due to the epigenetic regulation. Epigenetic variation, especially the methyl groups attaching to cytosine bases neighbored by guanine (CpG sites), is an emerging and important pre transcription regulation.[26] The CpG island in promoter, which is rich of CpG sites and is likely to be methylated. The methylated CpG island induces the significant down-regulation of gene expression.[27] DNA methylation might answer the questions that could not be fully explained by DNA sequence in COPD, such as the variable disease susceptibility in smokers and persisting risk after smoking cessation. Previous researches found that cigarette smoke not only impacted

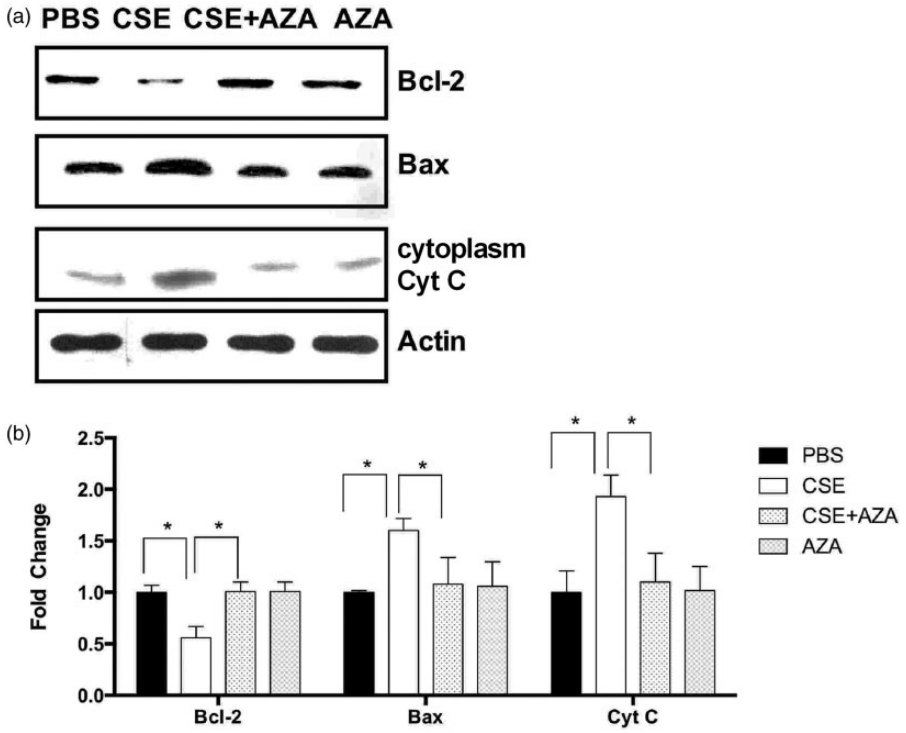

Figure 4 Bcl-2, Bax, and cytosolic cyt C protein expression in lungs. (a) Bcl-2, Bax, and cytosolic cyt C expression in lung of each group; PBS group, CSE group, CSE + AZA group, and AZA group. (b) Density of Bcl-2, Bax, and cytosolic cyt C protein was normalized against β-actin to obtain a relative blot density. CSE decreased the protein expression of Bcl-2, and increased the protein expression of Bax and cytosolic cyt C. The demethylation treatment, intraperitoneal injection of AZA, could restore all of these deregulations. $^{*}P < 0.01$ *vs*. CSE group, $\#P < 0.05$ *vs*. CSE group

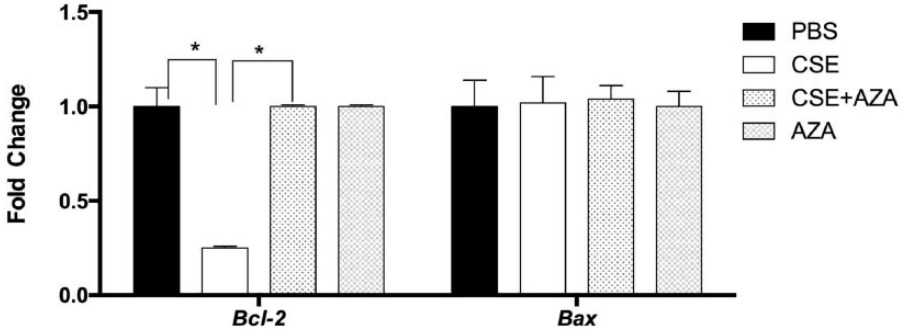

Figure 5 Bcl-2, Bax, and cyt C mRNA expression in lungs. The mRNA expression values of each group were presented relative to β-actin. The expression of Bcl-2 mRNA in emphysema models was significantly decreased, and the demethylation treatment could restore the Bcl-2 mRNA expression. There was no difference of Bax mRNA expression between each group. $^{*}P < 0.05$ *vs*. CSE group

the global epigenetic pattern, but also induced the methylation of individual genes.[28–30] Though DNA methylation is defined as a tissue specific program, there was limited data about pulmonary DNA methylation. So far, the presenting data described that methylation status of DNA from peripheral white blood cells was associated with COPD stage and lung function.[28] This work supported the role of methylation in emphysema by finding hyper-methylated Bcl-2 promoter in emphysematous lung. Previous researches found Bcl-2 promoter hyper-methylation conferred the silence to Bcl-2 expression.[31,32] DNA methyltransferase inhibitors (DNMTIs) could restore Bcl-2 expression.[33] This study also showed CES injection induced Bcl-2 promoter hyper-methylation and loss of Bcl-2 expression, and demethylation treatment recovered methylation status of Bcl-2 promoter and Bcl-2 expression in CSE injection mice.

Intraperitoneal injection of AZA restored methylation status of Bcl-2 promoter with recovered endothelia cell, improved pathological abnormality and pulmonary function in CSE injection mice. And relative analysis also found the anticipated negative correlation between methylation status of Bcl-2 promoter and morphological disorder and pulmonary function. Since it was described that the unbalance of Bcl-2/Bax was a decision regulator of endothelia apoptosis, and the pulmonary endothelia apoptosis could trigger emphysema process.[22,24,34] It suggested

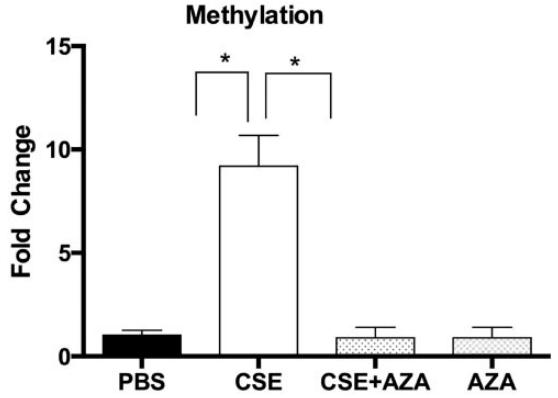

Figure 6 Methylation of Bcl-2 promoters in groups. The methylation status of Bcl-2 promoter in emphysema models was significantly higher than others, and demethylation treatment could reverse the increase. *$P < 0.05$ *vs*. CSE group

that hyper-methylation of Bcl-2 promoter might induce endothelia apoptosis by decreased Bcl-2/Bax ration, and then participate emphysema pathogenesis and damage of pulmonary function through endothelia apoptosis. According to this result, it was plausible that demethylation protect smoker from emphysema, thus it might be a promising treat strategy for COPD.

Intraperitoneal injection of AZA was genome wide demethylation treatment. It might demethylate numbers of genes rather than the unique Bcl-2 promoter. Methylation status of other genes might impact pulmonary construction and function. However, this study certainly found hyper-methylation of Bcl-2 promoter in emphysema model, and demethylation of Bcl-2 promoter showed protective potential for emphysema. The relationship analysis presented statistical significance between methylation statutes and morphologic and functional disorder. Thus it is convincing that the Bcl-2 promoter hyper-methylation took a role in emphysema pathogenesis.

Authors contribution: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; HZ, ZS, and XK conducted the experiments, HZ supplied critical reagents and animals for model building, HZ wrote the manuscript, YC, HP, and HL contributed to statistics analysis, and PC directed and corrected this research as corresponding author.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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