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Original Article

A gene polymorphism at SP-B 1580 site regulates the pulmonary surfactant tension of viral pneumonia through the cellular pyroptosis signaling pathway

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Abstract: Background: Viral pneumonias, such as SARS and MERS, have been a recurrent challenge for the public healthcare system. COVID-19 posed an unprecedented global crisis. The primary impact of viral pneumonia is pathologic changes of lung tissue. However, the effect of SP-B site gene polymorphism on alveolar surface tension in viral pneumonia remains unexplored. Objective: To explore the molecular mechanism of how the gene polymorphism at SP-B 1580 site regulates the pulmonary surfactant tension of viral pneumonia through the cellular pyroptosis signaling pathway using an *in vivo* animal experiment and a clinical trial. Methods: We constructed a genetically modified mouse model of viral pneumonia and administered H5N1 influenza virus through intratracheal injection. After 48 hours, the survival rate of each mouse group was evaluated. Lung tissue, blood, and bronchoalveolar lavage fluid samples were collected for histopathologic analysis. Inflammatory factor concentrations were measured using ELISA. The level of apoptosis was determined using TUNEL assay. Changes in the expression of cell death-related factors were assessed using qRT-PCR and protein blotting. Additionally, blood samples from patients with viral pneumonia were analyzed to detect single nucleotide polymorphisms and explore their correlation with disease severity, inflammatory factor levels, and pulmonary surfactant protein expression. Results: Following H5N1 infection of mice, the model group and hSP-B-C group showed high mortality rates within 24 hours. The survival rates in the blank control group, virus model group, hSP-B-C group, and hSP-B-T group were 100%, 50%, 37.5%, and 75%, respectively. Histologic analysis revealed significant lung tissue damage, congestion, alveolar destruction, and thickened alveolar septa in the model and hSP-B-C groups. However, these pulmonary lesions were significantly alleviated in the hSP-B-T group. Inflammatory factor levels were elevated in the model and hSP-B-C groups but reduced in the hSP-B-T group. TUNEL assay demonstrated a decrease in apoptotic cells in the lungs of the hSP-B-T group. Furthermore, the expression of SP-B and cell death-related proteins was downregulated in all three groups, with the lowest expression observed in the hSP-B-C group. The clinical trial found that patients with severe viral pneumonia exhibited a higher frequency of CC genotype and C allele in, along with increased inflammatory factor levels and decreased SP-B expression compared to those with mild-to-moderate viral pneumonia. Conclusion: SP-B polymorphism at the 1580 site regulates lung surfactant tension through the cell pyroptosis signaling pathway, thus affecting the progression of viral pneumonia.

Keywords: Viral pneumonia, cell pyroptosis, genetic polymorphism, SP-B, lung surfactant

Introduction

From Severe Acute Respiratory Syndrome (SARS) to Middle East Respiratory Syndrome (MERS), viral pneumonia has remained a persistent threat. The global outbreak of COVID-19 also presented an unprecedented challenge to public healthcare system [1]. Based on current

evidence, viral pneumonia has emerged as a systemic disease, causing damage to multiple organs, with lungs serving as the primary target [2]. Upon invasion, the virus triggers an immune response, activating immune cells and releasing inflammatory factors [3]. Within the lungs, these inflammatory factors impact the respiratory membrane, resulting in lung pathology characterized by pulmonary edema and extensive inflammatory exudate [4]. The interplay between pulmonary edema, inflammatory exudate, and surfactant can lead to a depletion of surfactant on the alveolar surface, increasing the surface tension at the liquid-gas interface of the alveoli. As a result, lung compliance decreases, which restricts alveolar expansion [5]. Additionally, infected lung epithelial cells and macrophages undergo necrosis/ apoptosis, initiating further immune reactions and releasing inflammatory factors such as TNF-α, IL-1β, IL-6, and chemokines. These factors can induce substantial infiltration of mononuclear macrophages and neutrophils, while also recruiting a large number of T lymphocytes from peripheral blood into infected lung tissue. Therefore, alveolar epithelial cells experience further damage, leading to decreased secretion of pulmonary surfactant and exacerbating lung dysfunction [6]. Various effector cells and molecules participate in this process, including mucosal epithelial cells, macrophages, natural killer cells, cytokines, and complement components. The development of this series of immune reactions ultimately determines the severity of the disease.

Based on epidemiological data released by the Chinese Medical Association, it has been observed that mild/moderate cases constitute 80.9% of confirmed cases, while severe cases make up 13.8%, and critically ill cases account for a small proportion of only 4.7%. However, once the condition deteriorates into a critically ill state, the fatality rate increases significantly, with a crude fatality rate as high as 49%. Viral pneumonia primarily leads to pathological changes in lung tissue. Clinical symptoms are relatively severe, including high fever, dry cough, fatigue, and dyspnea. Severe pneumonia can also lead to complications such as pulmonary edema and acute respiratory distress syndrome, particularly among the elderly and immunocompromised individuals, posing a serious threat and potentially resulting in death [7]. Early identification and intervention in severe cases hold the key to reducing the fatality rate. Therefore, the discovery of reliable methods or biological markers for screening or predicting severe viral pneumonia cases holds immense significance in terms of achieving patient classification management, advancing the management checkpoint for severe cases, and enhancing the success rate of rescuing critically ill patients.

Pulmonary surfactant proteins (SPs), comprising SP-A, SP-B, SP-C, and SP-D, are synthesized by type II alveolar epithelial cells, and, in conjunction with phospholipids, form the pulmonary surfactant. The primary role of this surfactant is to decrease the surface tension of the gas-liquid interface within the alveoli, thereby preventing alveolar collapse and maintaining normal lung function [8]. Among these four pulmonary SPs, SP-B plays the most crucial role [9-11]. Recent studies have identified a single nucleotide polymorphism (SNP) at position 1580 of the SP-B gene. Specifically, SP-B 1580 can be expressed as either C or T in normal human populations [12, 13]. This SNP, located in exon 4 of the SP-B gene, promote 129th asparagine glycosylation via C allele expression. This modification then affects the processing, folding, and secretion of SP-B, leading to decreased the production of mature SP-B protein [14]. Remarkably, various ethnic populations exhibit substantial variation in the expression of the 1580 C/T SNP, and this nucleotide polymorphism is closely linked to the incidence of neonatal respiratory distress syndrome. Premature infants carrying the C allele face a significantly higher risk of developing respiratory distress syndrome compared to those carrying the T allele [15]. The aforementioned research underpins the notion that the C allele of the SP-B 1580 site may act as a susceptibility factor for lung damage, while the T allele may have a protective effect on lung health.

However, the precise impact of the SP-B 1580 site on pulmonary surfactant tension during viral pneumonia, specifically concerning the activation of the cell death pathway, remains unestablished. Consequently, this study aimed to investigate the molecular mechanism underlying how the genetic polymorphism of the SP-B 1580 site regulates pulmonary surfactant ten-

sion in viral pneumonia through the cell death signaling pathway. In order to achieve this, an *in vivo* animal experiment and a clinical trial were conducted.

Materials and methods

Materials

Peripheral blood specimens were obtained from patients with viral pneumonia at the Respiratory Department of Jinan People's Hospital. Transgenic humanized mice were constructed in the Laboratory of Microbial Biosafety at Naval Medical University, expressing the SPB-C (hSP-B-C) and human SPB-T (hSP-B-T) genes instead of the mouse SP-B gene. The high-pathogenic avian influenza virus H5N1 strain, obtained from the Laboratory of Microbial Biosafety at Naval Medical University, was stored at -80°C after passage in our laboratory.

Construction of viral pneumonia model

After one week of adaptive feeding, the mice were weighed and divided into four groups: blank control, virus model, hSP-B-C, and hSP-B-T, with 8 mice in each group. Under pentobarbital anesthesia, the virus model group, hSP-B-C group, and hSP-B-T group underwent intratracheal injection of 0.05 mL LD50 virus solution [H5N1] to induce a viral pneumonia animal model, while the blank control group received an equal volume of PBS solution. The survival rate of mice in each group was observed after 48 hours of incubation. The mice were sacrificed under inhalation anesthesia with ether for sample collection. This experiment was approved by the Animal Research Ethics Committee of Jinan People's Hospital.

ELISA for measuring inflammatory factor concentrations

Forty-eight hours after intravenous injection of H5N1 virus, mice in each group were anesthetized using ether inhalation, and their eyeballs were enucleated for blood collection. The collected blood was preserved for future analysis. The chest cavity was opened to expose the trachea, and bronchoalveolar lavage fluid was obtained using a retention needle. Following centrifugation at 12,000 rpm for 10 minutes, the supernatant was collected and stored at -80°C. The levels of TNF-α, IL-1β, IL-18, and IL-6 were measured following the instructions of the ELISA kit (Beijing Zhonghao Biotechnology Co., Ltd.).

Hematoxylin-Eosin (H&E) staining

The lung tissues were fixed in 4% paraformaldehyde fixative, removed after 72 hours, rinsed with running water, dehydrated with ethanol gradient, rendered transparent with xylene, embedded in paraffin, and subsequently stained with H&E. Tissue pathological changes were evaluated using a light microscope and sealed with neutral gum for observation.

TUNEL assay for apoptosis detection

Following fixation, the lung tissue samples were embedded in paraffin and sectioned into 5 μm thick slices. TUNEL assays were conducted in accordance with the kit instructions, and TUNEL-positive cells were identified under an electron microscope as cells exhibiting yellow-brown granules or patches within the stained nuclei. The quantity of TUNEL-positive cells was counted and recorded.

qRT-PCR

The total RNA was extracted from the lung tissue samples using 1 ml Trizol reagent (Thermo Fisher Scientific Inc.) per well. The samples were lysed in 1.5 ml EP tubes for 10 minutes. To isolate RNA, 200 μl chloroform was added to each tube, followed by centrifugation at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase was carefully transferred to a new tube, and 400 μl of isopropanol was added. After multiple centrifugations and removal of the supernatant, the RNA pellet was dissolved in 20 μl of DEPC water. Reverse transcription (Takara Biomedical Technology (Beijing) Co., Ltd.) was performed at 25°C for 5 min, 50°C for 15 min, 85°C for 5 min, and 4°C for 10 min. The cDNA was subsequently diluted 10-fold and used for real-time fluorescence quantitative PCR. The primer sequences (Shanghai Shenggong Bioengineering Technology Services Company), including GAPDH as an internal reference gene, are provided in **Table 1**.

Western blot analysis

Approximately 100 mg of lung tissue samples were pulverized in liquid nitrogen and lysed with lysis buffer (Shanghai Biyun Tian Com-

Table 1. Primer sequences

Gene symbol	Forward primer	Reverse primer
GAPDH	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC
SP-B	ATGACCGCGCGCGGCGCGCGGAGG	CTAGGGGGAGGGCGGAGAGGTCCAGC
Caspase-1	GGCTCTGGTGGAAGAACCTGTAGC	GTGGAAGTCAGGGGGTGCTG
Caspase-11	GGCTCCTGGAGGGAAGAGTC	TCCCCTCACAGCTCCAGG

pany). The protein content was determined using the Bradford assay, and 40 μg of total protein was mixed with 1× sample buffer. The samples were boiled for 5 min and then loaded onto an SDS-PAGE gel for electrophoretic separation (80 V for the stacking gel, 100 V for the resolving gel). Subsequently, the gel was transferred to a membrane using a semi-dry transfer apparatus (30 mA, 90 min). Following blocking, the membrane was incubated with primary antibodies against SP-B, ICAM-1, caspase-1, and caspase-11 (ABmart Medical Technology (Shanghai) Co., Ltd.) overnight at 4°C. After washing the membrane three times with TBS-T wash buffer for 10 min each, an HRP-labeled secondary antibody was added and incubated at 37°C for 60 min. Finally, an ECL luminescence substrate was applied, and the X-ray film was exposed. The scanned band images were analyzed using Image-Pro Plus software, and the relative expression level of each target protein was determined by calculating the gray value ratio of each target band to β-actin.

Collection of clinical serum samples

A total of 80 patients admitted to the Respiratory Department of Jinan People's Hospital from May 2020 to May 2022 and diagnosed with influenza virus pneumonia were enrolled as participants in this study. Patients were excluded if they had bacterial or fungal pneumonia, severe cardiac, hepatic or renal dysfunction, severe immune system disorders, malignancies, or other serious illnesses, received immunosuppressive agents or glucocorticoids within the past six months, had known allergies or intolerance to the medications used in the study, or died during hospitalization. The included patients were divided into different groups based on the severity of illness, and two groups of patients with mild and severe illness were selected for the study after matching for age, gender, and other influencing factors. Blood samples were collected from the both groups and stored at -80°C. DNA was extracted using a DNA kit. The research protocol was approved by the Clinical Ethics Committee of Jinan People's Hospital, and written informed consent was obtained from each participant.

Association between SPB-1580 SNP and susceptibility to lung damage in COVID-19

Upon genetic identification, the critically ill patients were allocated into two groups, namely the SP-B-C and SP-B-T groups. The concentrations of inflammatory factors and the expression levels of SP-B were compared between the two patient groups by analyzing blood and bronchoalveolar lavage fluid samples.

Statistical analysis

All data were analyzed using GraphPad Prism 8.0 software. Descriptive statistics were used for general data. For quantitative data, independent sample t-tests were applied for comparisons between groups, and one-way ANOVA was utilized for comparisons among multiple groups. Then, Bonferroni post hoc test was performed for pairwise comparisons between groups. Statistical significance level was set at $\alpha=0.05$.

Results

Effect of SP-B 1580 C/T gene polymorphism on survival of mice

Following H5N1 influenza virus infection in mice, noticeable symptoms including emaciation, curled shivering, decreased reactivity, and clustering were observed in the model group and hSP-B-C group. These 2 groups also exhibited a higher mortality rate. The endpoint survival rates were 100% in the blank control group, 50% in the virus model group, 37.5% in the hSP-B-C group, and 75% in the hSP-B-T group. Notably, the survival time of mice in the hSP-B-T group was significantly prolonged compared to the model group and hSP-B-C group

Table 2. Survival of mice with SP-B 1580 C/T gene polymorphism at position 1580

Group	Endpoint survival rates/%	Survival time/d
Control group	100	14.00±0.00
Virus model group	50	12.04±1.45
hSP-B-C group	37.5	11.23±1.35
hSP-B-T group	75	12.75±1.02

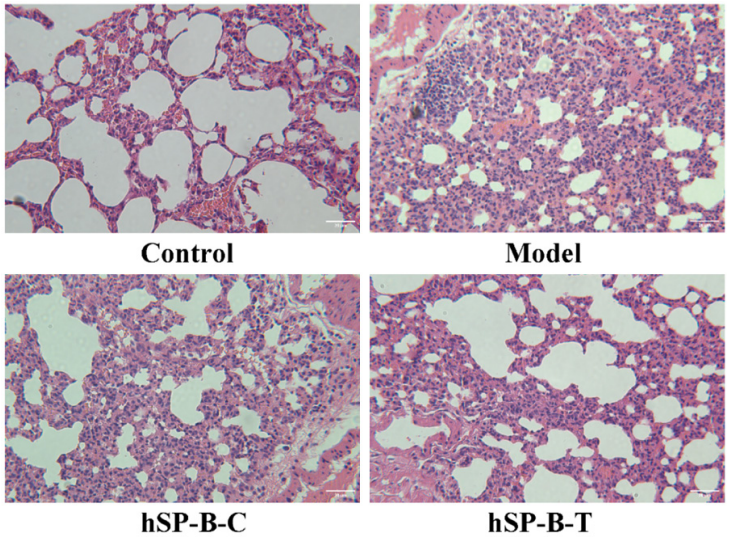

Figure 1. H&E staining of lung tissue of mice with SP-B 1580 C/T gene polymorphism at position 1580.

(P<0.01). Detailed results can be found in **Table 2**.

Influence of SP-B 1580 C/T gene polymorphism on lung tissue

H&E staining results revealed normal lung tissue in the blank group, whereas the lung tissue of mice in the model group and hSP-B-C group displayed congestion, destruction of alveolar structure, significant edema, thickening of alveolar septa, and diffuse inflammatory cell infiltration accompanied by shedding and necrosis of epithelial cells. However, in the hSP-B-T group, compared to the model group, lung congestion and edema were alleviated, inflammatory cell infiltration was significantly reduced, and the lung lesions were ameliorated. Nevertheless, some local thickening of the alveolar septa was still observed, as shown in **Figure 1**.

Effect of SP-B 1580 C/T gene polymorphism on inflammatory response

In comparison to the blank control group, the concentrations of IL-1β, IL-6, IL-18, and TNF-α in both serum and bronchoalveolar lavage fluid were substantially elevated in the model group and hSP-B-C group (P<0.01), as shown in **Figure 2**. Furthermore, the hSP-B-T group exhibited a significantly reduction in the levels of IL-1β, IL-6, IL-18, and TNF-α compared to the model group and the hSP-B-C group (P<0.01).

Effect of SP-B 1580 C/T gene polymorphism on cell apoptosis

Following DAPI staining and quantification of nuclear count in the lung cells of mice, it was observed that the SP-B-C and model groups exhibited only a few positively stained nuclei. Additionally, the nuclei displayed aberrant morphology, characterized by a smaller size, indicating substantial damage on the lung cells in these groups. In contrast, the SP-B-T group exhibited a significant increase in the number of nuclei, while the control group also demonstrated a large number of elliptical-shaped nuclei. The TUNEL assay revealed a higher number of red apoptotic cells in the lungs of the SP-B-C and model groups compared to the SP-B-T group. Only a minimal number of apoptotic cells were detected in the lungs of the control group, as shown in **Figure 3**.

Effect of SP-B 1580 C/T gene polymorphism on cell pyroptosis

To evaluate the impact of SP-B 1580 C/T gene polymorphism on active protein SP-B expression in the alveoli, total RNA and protein were extracted from the lung tissues of mice in each group. The expression of SP-B was assessed by RT-qPCR and western blot. The results indicated that the expression of SP-B was significantly reduced in both the SP-B-C and model groups compared to the control group (P<0.01), with the lowest expression of SP-B protein observed in the SP-B-C group. Additionally, to further confirm the effect of SP-B 1580 C/T gene polymorphism on cell pyroptosis, the levels of Caspase-1 and Caspase-11, which are associated with cell pyroptosis, were found to be significantly ele-

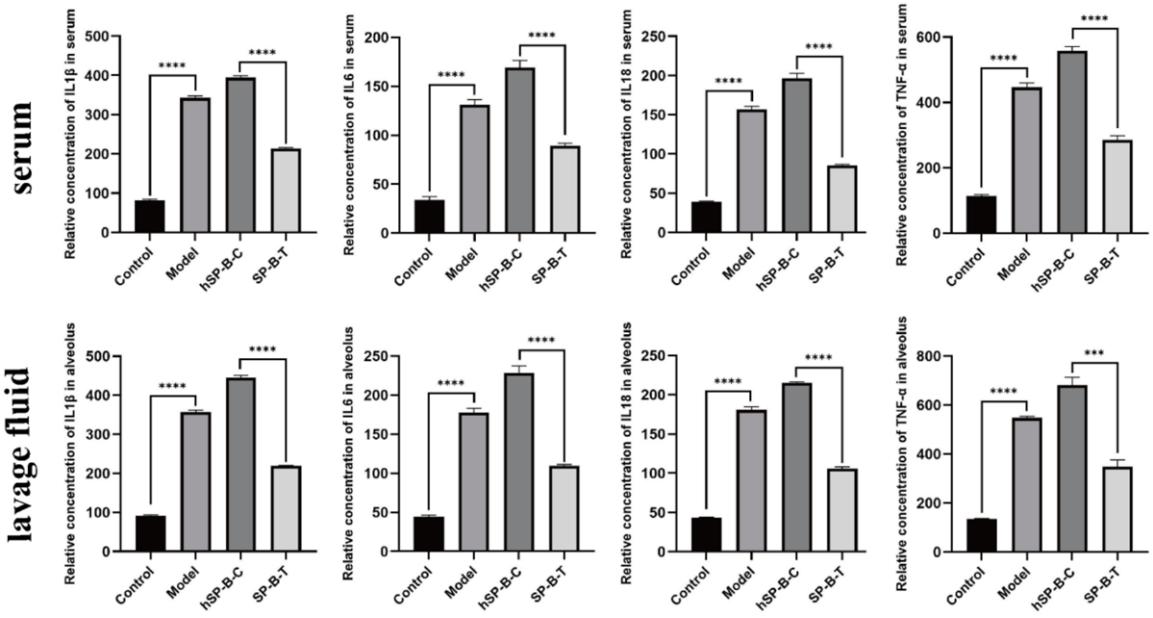

Figure 2. Changes in the expression of inflammatory factors in the serum and bronchoalveolar lavage fluid of mice with SP-B 1580 C/T gene polymorphism at position 1580.

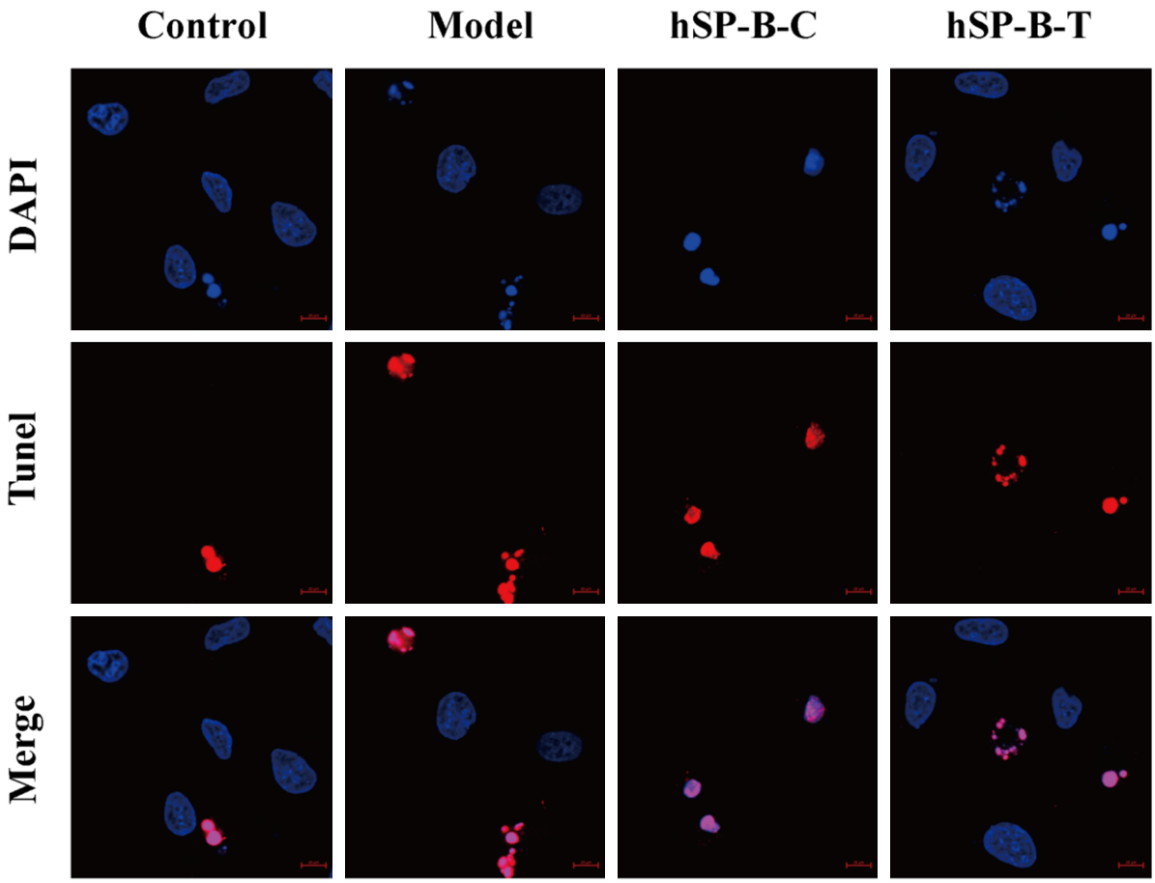

Figure 3. Immunofluorescence changes in lung cells of mice with SP-B 1580 C/T gene polymorphism.

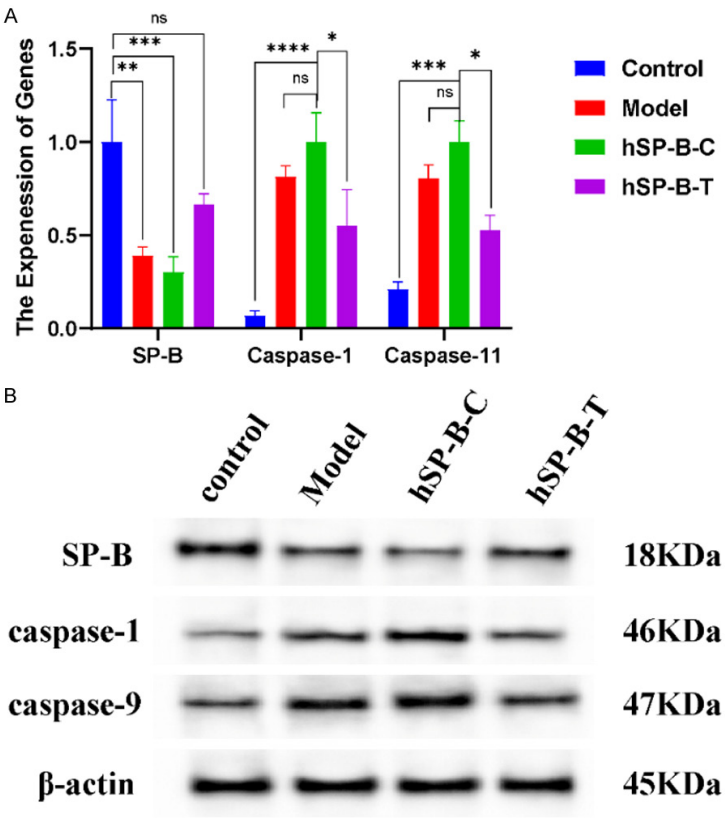

Figure 4. Effect of SP-B 1580 C/T gene polymorphism on mouse lung cell apoptosis. A. Apoptotic gene expression detected by PCR. B. Apoptotic protein bands detected by western blotting.

vated in the SP-B-C, SP-B-T, and model groups compared to the control group, with statistical significance ($P<0.01$). Notably, the SP-B-C group displayed the highest expression, as shown in **Figure 4**. These results suggest that SP-B 1580 C/T gene polymorphism may promote cell pyroptosis, resulting in decreased pulmonary surfactant surface tension in the alveoli.

Discussion

Viral pneumonia is a pulmonary infectious disease caused by viral invasion. Its pathogenesis involves various aspects, including virus invasion of the lungs, cellular damage, immune response, and abnormal coagulation function, all of which interplay and result in pulmonary tissue damage and severe clinical manifestations [16]. Additionally, viral pneumonia can directly or indirectly impact the content and properties of alveolar surfactant, leading to increased surface tension and disruption of normal expansion and contraction, thus affecting the respiratory function [17, 18]. The specific mechanisms encompass direct impairment of surfactant synthesis and secretion in the alveoli, inflammation-induced increase in alveolar wall and endothelial cell permeability, resulting in tissue fluid leakage into the alveoli and subsequent disruption of normal respiratory dynamics. Currently, the treatment for viral pneumonia primarily involves symptomatic management, oxygen therapy, antiviral agents, and immunotherapy [19, 20]. However, these treatments alone do not fully eradicate the virus, and rapid viral mutation poses challenges in achieving optimal therapeutic outcomes. Therefore, exploring the common pathogenic mechanisms underlying viral pneumonia may offer new directions and insights for effective therapeutic interventions.

This study identified an association between the C allele of the SP-B 1580 locus and early mortality in mice with viral pneumonia. Lung tissue analysis revealed congestion, alveolar structure destruction, significant edema, and thickening of alveolar septa, resulting in lung injury. Conversely, the T allele at the same locus demonstrated a significant reduction in lung lesions. SP-B plays a crucial role in maintaining alveolar surface tension [21]. The SP-B gene polymorphism has been identified as a predisposing factor for lung disease. Some studies have investigated the relationship between the C/T polymorphism at the SP-B1580 locus and the incidence and progression of lung disease [22, 23]. Clinical research has suggested that the C/T polymorphism at the SP-B gene locus may have predictive and diagnostic implications for the occurrence and severity of COVID-19. The study discovered a higher frequency of CT and TT genotypes at the C/T locus in COVID-19 patients, indicating an increased susceptibility to COVID-19 [24]. Furthermore, our study also noticed a heightened risk of respiratory dysfunction and prolonged hospital stays in COVID-19 patients with the CT genotype at the C/T locus. Although

this study indicates that the SP-B1580 C/T locus polymorphism may affect the occurrence and severity of viral pneumonia, further investigations are warranted to unravel the underlying biological mechanisms and the impact of other genetic and environmental factors on this correlation.

Furthermore, this study revealed that the C allele of the SP-B 1580 locus was linked to significantly elevated levels of inflammatory cytokines, including TNF-α, IL-1β, IL-18, and IL-6, in the bronchoalveolar lavage fluid of mice with viral pneumonia. Additionally, it was associated with an abnormal increase of alveolar surface tension and a notable rise in the number of apoptotic cells. Conversely, the T allele at the same locus exhibited the opposite effect. Recent research has demonstrated that SP-B 1580 C/T polymorphism can markedly enhance the expression of inflammatory cytokines, such as IL-6, IL-8, and TNF, which cannot be effectively cleared by the virus. It also upregulates the expression of apoptosis-related genes Caspase-3 and BAX [25]. Furthermore, another study uncovered higher levels of inflammatory cytokines, such as IL-6 and IL-8, in the blood of patients carrying the SP-B 1580 C/T genotype. Additionally, these patients exhibited increased expression of apoptotic factors in their lung tissue [26]. These findings suggest that SP-B 1580 C/T polymorphism may promote the progression of viral pneumonia by activating cellular apoptosis and the inflammatory response pathway.

Finally, this study observed significantly higher levels of caspase-1 and caspase-11, proteins related cell pyroptosis, in the lungs of viral pneumonia mice with the C allele at SP-B 1580 locus compared to those with the T allele. Moreover, the expression of pulmonary SP-B was significantly reduced. This indicates that the SP-B 1580 C/T polymorphism may promote cell death, increase alveolar surface tension, and accelerate the progression of viral pneumonia through activating the cell pyroptosis signaling pathway. Previous studies have documented signs of cell pyroptosis in lung tissue of viral pneumonia patients, which may be caused by oxidative stress, cell membrane rupture, or cytoplasm infiltration induced by virus infection [27]. Additionally, study also demonstrated that the inhibition of the cell pyroptosis pathway alleviated the severity of pneumonia and related symptoms in patients [28]. Furthermore, virus infection can activate the cell pyroptosis pathway, triggering an inflammatory response and cellular death. At the same time, blocking the cell pyroptosis pathway can impede virus replication, ameliorate inflammatory response, and alleviate pneumonia symptoms [29, 30]. However, it should be acknowledged that the sample size in our study was relatively small, which may limit the generalizability of the findings and introduce confounding factors. Additionally, our *in vitro* cellular experiments only focused on the detection of specific downstream proteins, without exploring the underlying molecular mechanisms. In future research, we plan to conduct further clinical studies with a larger sample size to elucidate the impact of SP-B 1580 gene polymorphism on the regulation of pulmonary surfactant through the apoptotic signaling pathway in viral pneumonia.

In conclusion, the gene polymorphism at the SP-B 1580 locus appears to modulate alveolar surface tension by influencing the cell pyroptosis signaling pathway, consequently impacting the progression of viral pneumonia. The presence of the C allele at the SP-B 1580 locus could serve as an indicator of alveolar surface tension and potentially a promising therapeutic target for viral pneumonia. Thus, the findings represent a significant research avenue for future investigations into the theory and treatment of viral pneumonia.

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Disclosure of conflict of interest

None.

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