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Study on JNK/AP-1 signaling pathway of airway mucus hypersecretion of severe pneumonia under RSV infection

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Abstract. – OBJECTIVE: To investigate the JNK/AP-1 signaling pathway of airway mucus hypersecretion of severe pneumonia under respiratory virus (RSV) infection.

PATIENTS AND METHODS: Total of 56 severe pneumonia children under RSV infection were selected. Reverse transcription polymerase chain reaction (RT-PCR) was performed to measure the expression quantity of MUC5B mRNA and MUC5AC mRNA, and ELISA was used to measure the expression quantity of MUC5AC and MUC5B proteins. Following that, the children were divided into airway mucus hypersecretion group (n = 37) and non-hypersecretion group (n = 19). Western blotting was performed to detect the expression levels of JNK1/2, p-JNK1/2 and AP-1 proteins.

RESULTS: Expression of MUC5AC and MUC5B proteins, and MUC5AC mRNA and MUC5B mRNA in the airway mucus hypersecretion group were significantly higher than those in the non-hypersecretion group ($p < 0.05$). The expression levels of JNK1/2, p-JNK1/2 and AP-1 proteins in airway mucus hypersecretion group were higher than those in the non-hypersecretion group ($p < 0.05$).

CONCLUSIONS: MUC5AC and MUC5B can be used as marker molecules of airway mucus hypersecretion. Airway mucus hypersecretion of severe pneumonia induced by RSV might be related to the activation of JNK/AP-1 signaling pathway.

Key Words:
Respiratory syncytial virus, Severe pneumonia, Airway mucus hypersecretion, JNK/AP-1 signaling pathway, Mucin MUC5AC.

Introduction

Airway mucus is an important part of the first line of airway defense. It plays an important role in maintaining the airway humidification and assisting the function of airway epithelial cells. Respiratory virus (RSV) infection is the most common viral infection of children's lower respiratory tract. It has been reported that every year about 5% children would die from RSV infection-induced severe pneumonia[1]. The main pathological changes of the lower respiratory tract infected by RSV are manifested in the necrosis and loss of bronchiolar epithelium, the increase of mucus secretion, inflammatory cell infiltration, mucosal congestion and elevated airway reactivity. The lower respiratory tract under RSV infection is also an important cause of asthma and bronchial dilation[2]. Airway mucus hypersecretion would block the airway lumen, induce and aggravate the bronchial and lung infection or more seriously results in the damages of bronchial and pulmonary parenchyma, reduce the irreversibility of pulmonary functions, even respiratory failure and death[3].

Studies have confirmed that under RSV infection, the expression of mucin MUC5AC and MUC5B were significantly elevated, indicating that RSV infection and MUC5AC and MUC5B were correlated[4]. But the mechanism has not been not fully understood. C-Jun N-Terminal Kinase (JNK) channel could be activated by multiple cytokines to induce the activity of activator protein-1 (AP-1), the binding site of MUC5AC and MUC5B promoter, thus promoting the gene transcription of MUC5AC and MUC5B[5]. Some studies have tried to verify the signaling pathway by cell model[6]. Our study would be focused on further investigating the role of RSV infection in airway mucus hypersecretion by clinical cases.

Patients and Methods

Patients

A total of 56 children that were diagnosed with RSV infection-induced severe pneumonia in our

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hospital from August 2013 to August 2015 were enrolled in our study. RSV diagnosis was made on the basis of airway throat swab and serum virus examination. RSV (++ and +++) was taken as the inclusion criteria, and excluded definite bacterial infectious. Diagnostic criteria for severe pneumonia: (1) breathing rate ≥ 30 times/min, PaO_2 < 60 mmHg, $PaCO_2$ > 50 mmHg, PaO_2/FiO_2 < 300, in need of mechanical ventilation therapy; (2) X-ray chest radiograph showed that pulmonary lobes on both sides or multiple sides were involved, or the lesion was expanded > 50% within 48h after admission; (3) Consciousness disturbance, arterial systolic pressure < 90 mmHg, in need of dialytic treatments due to oliguria or acute renal failure, with septic shock, etc. Children patients with the symptoms of 1 and 2, accompanied with or without 3 were diagnosed as severe pneumonia. All patients were treated according to standard medical therapy.

After obtaining the approval of our Ethics Committee and the informed consent of the children's guardians, the expression quantity of MUC5AC mRNA and MUC5B mRNA was measured by real-time fluorescence quantitative PCR, the expression of MUC5AC and MUC5B proteins was estimated by ELISA. The children were divided into airway mucus hypersecretion group (n = 37) and non-hypersecretion group (n = 19). Hypersecretion group includes 18 males and 19 females, ranging from 1-7 years and on average (4.2 ± 1.3) years. Non-hypersecretion group includes10 males and 9 females, with age ranging from 1.5-8 years, on average (4.5 ± 1.4) years. There was no significant difference in gender and age between the two groups ($p > 0.05$).

Reagents and Equipment

Reagents used were reverse transcription-polymerase chain reaction kit (Thermo Fisher, Waltham, MA, USA), SYBR Green I Master (Roche, Indianapolis, IN, USA), rabbit anti human SAPK/JNK antibody (Cell Signaling, Danvers, MA, USA), rabbit anti human AP-1 antibody (Sigma, St. Louis, MO, USA).

Table I. RT PCR primers used in this study. The primers were designed and synthesized by Shanghai GeneCore BioTechnologies Co. Ltd.

Gene	Sequence
MUC5AC	5'GACACCAAATACGCCAACAA 3'
	5'GGGTCGTCCATCTTCTGC 3'
MUC5B	5'CTGCGAGACCGAGGTCAACATC 3'
	5'TGGGCAGCAGGAGCACGGAG 3'
GAPDH	5'GTGGATATTGTTGCCATCAATGACC 3'
	5'GCCCCAGCCTTCATGGTGGT 3'

Real Time PCR

Total RNA was extracted by Trizol method, and concentration and purity of RNA were determined. 2 μg of RNA was reverse transcribed into cDNA according to the instructions of reverse transcription kit, amplified by 10 μl reaction system, set 3 complex holes on each sample. PCR conditions include, pre-denaturation at 95°C for 90s, annealing at 95°C for 5s, extension at 58°C for 30s, for 45 cycles [LightCycler 480 real-time fluorescent quantitative PCR instrument (Roche, USA)]. Subsequently, Ct value of gene was used to calculate relative quantification of the target gene according to computational formula $2^{-\Delta\Delta Ct}$.

Enzyme Linked Immunosorbent Assay

1500 g of the supernatant was taken and centrifuged for 5 min. The method was followed according to ELISA kit. (Santa Cruz Co, Santa Cruz, CA, USA) The concentration of protein was measured in each pore at ELISA 450 nm.

Western Blotting

Western blotting was performed to detect the expression levels of JNK1/2, JNK1/2 and AP-1 proteins. Samples were cleaned by cold phosphate buffered saline (PBS) solution for 3 times, blotted up the liquid by filter paper and then 100 μl of cold RIPA buffer was added for lysis for 15 min. The cells were transferred to 1.5 ml tube and proteins were extracted by ultrasound (100-200W) for 5s×3 times. The solution was centrifuged at 4°C at 15000 rpm for 20 min, and the supernatant was collected. Protein concentration was estimated by bicinchoninic acid (BCA) method and samples were loaded onto sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 5 replicates per group. The protein concentration in each sample was adjusted to 2 μg/μl and placed at 100°C for 10 min to denature the protein. 35 μg protein from each group was taken and added to 5% stacking gel for electrophoresis at 70V into 12% separation gel, afterward electrophoresis was continued at 120V for 55 min. The target protein was transferred to 0.45 μm PVDF, transferred under 300 mA for 60 min, incubated in 5% skim milk for 1h, followed by repeated washing of

membrane in Tris-Buffered Saline and Tween (TBST) solution. The primary antibody was added to incubate for overnight (1:750 diluted rabbit anti human JNK, p-JNK antibody; 1:200 diluted rabbit anti human AP-1 antibody; 1:1000 diluted rabbit anti human β-Actin antibody). Next morning, TBST solution was washed for 3 times and secondary antibody was added (1:2000 diluted horseradish peroxidase labeled sheep anti rabbit IgG antibody) to incubate at room temperature for 1h. PVDF membrane was taken and washed with TBST solution for 5 min×6 times. The chemiluminescent reaction was prepared with ECL luminescence kit for developing protein bands on X-ray film.

Statistical Analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was applied to make the statistical analysis. The measurement data were presented by means ± standard deviation; independent sample *t*-test was used in the comparison between groups. The data was presented by case or percentage; X^2 test was applied in intra-group comparisons. $p < 0.05$ was considered as statistically significant.

Results

Expression Quantity of mRNA of MUC5AC and MUC5B

The expression levels of MUC5AC mRNA and MUC5B mRNA in the airway mucus hypersecretion group were significantly higher than those in the non-hypersecretion group, and the difference was statistically significant MUC5AC mRNA (Figures 1-2).

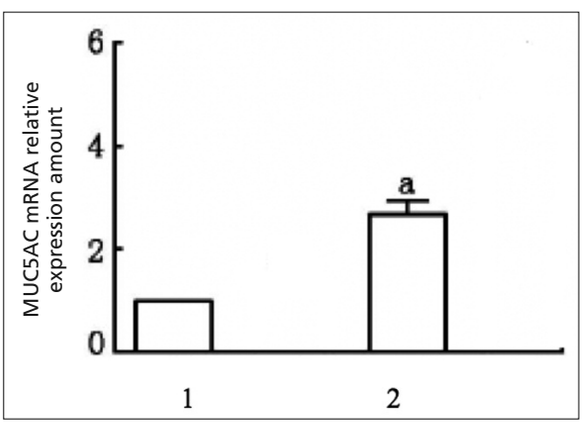

Figure 1. Relative MUC5AC mRNA levels for 1) Non-hypersecretion group 2) Hypersecretion group.

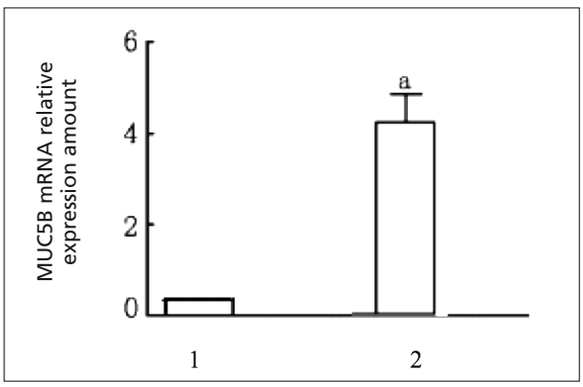

Figure 2. Relative MUC5B mRNA levels for 1) Non-hypersecretion group, 2) Hypersecretion group.

Expression Quantities of MUC5AC and MUC5B Proteins

The expression levels of MUC5AC and MUC5B proteins in the airway mucus hypersecretion group were significantly higher than those in the non-hypersecretion group with $p < 0.05$ (Figures 3-4).

Expression Levels of JNK1/2, p- JNK1/2 and AP-1 Proteins

Levels of JNK1/2, p- JNK1/2 and AP-1 proteins in the airway mucus hypersecretion group were higher than those in the non-hypersecretion group (Figure 5).

Discussion

Airway mucus is a kind of viscous colloidal liquid composed of water, ion, secretion, plasma

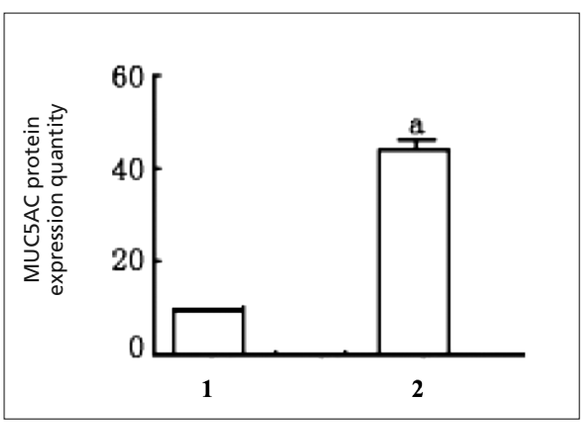

Figure 3. Relative MUC5AC protein levels for 1) Non-hypersecretion group, 2) Hypersecretion group.

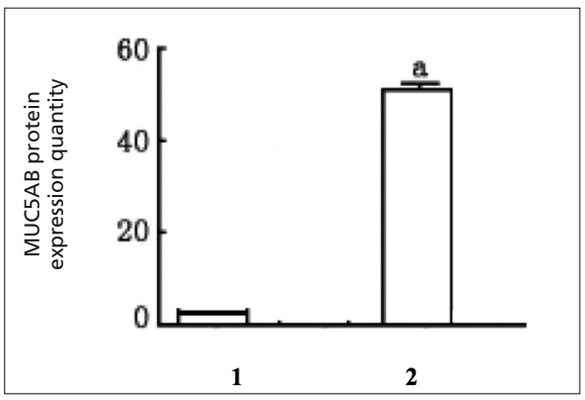

Figure 4. Relative MUC5B protein levels for 1) Non-hypersecretion group, 2) Hypersecretion group.

protein exudation and mucin MUC secreted from airway epithelial cells. It could humidify the airway mucosa and help discharge the pathogenic microorganisms timely. Excessive mucus secretion would cause cilia clearance barrier, goblet cell proliferation, accessory mucosa gland glands hypertrophy, airway obstruction, airflow limitation, ventilation/perfusion ratio imbalance, pulmonary function progressive decline, bacterial infection increase, leading to repeated and aggravated chest infection. Studies have shown that 50%-70% RSV infection was related to airway mucus hypersecretion[7]. In our study, the ratio was 66.07 (37/56). Airway mucus hypersecretion was closely related to inflammatory reaction. The ratios of IL-6, CRP, procalcitonin and neutrophils in patients with a recurrent cough and expectoration were increased significantly[8]. Meanwhile, it was found that airway mucus hypersecretion was also correlated with the decrease of lung function, the drop of arterial blood gas oxygen partial pressure, and the increase of carbon dioxide partial pressure, thus significantly improving the clinical curative effects and prognosis[9].

Most of the current studies deemed that[10] MUC5AC and MUC5B were the major increased airway mucins under inflammatory response. However, the specific mechanism of its expression was still unknown and there was still no consensus on the signaling cascade reaction and pathway involved. MUC promoter had two binding sites, AP-1 and NF-κB. AP-1 was an important site for regulating the expression of MUC, including homodimer and heterologous dimer C-jun and c-Fos protein. JNK signaling pathway can be activated by various cytokines, and the phosphorylated c-Jun could induce the formation of homodimer or heterologous dimers, combine with the binding site of cis-acting element of intranuclear AP-1, and start the gene transcription of MUC5B and MUC5AC. Choi et al[11] have found that SP600125, the inhibitor of JNK pathway could inhibit the phosphorylation of JNK

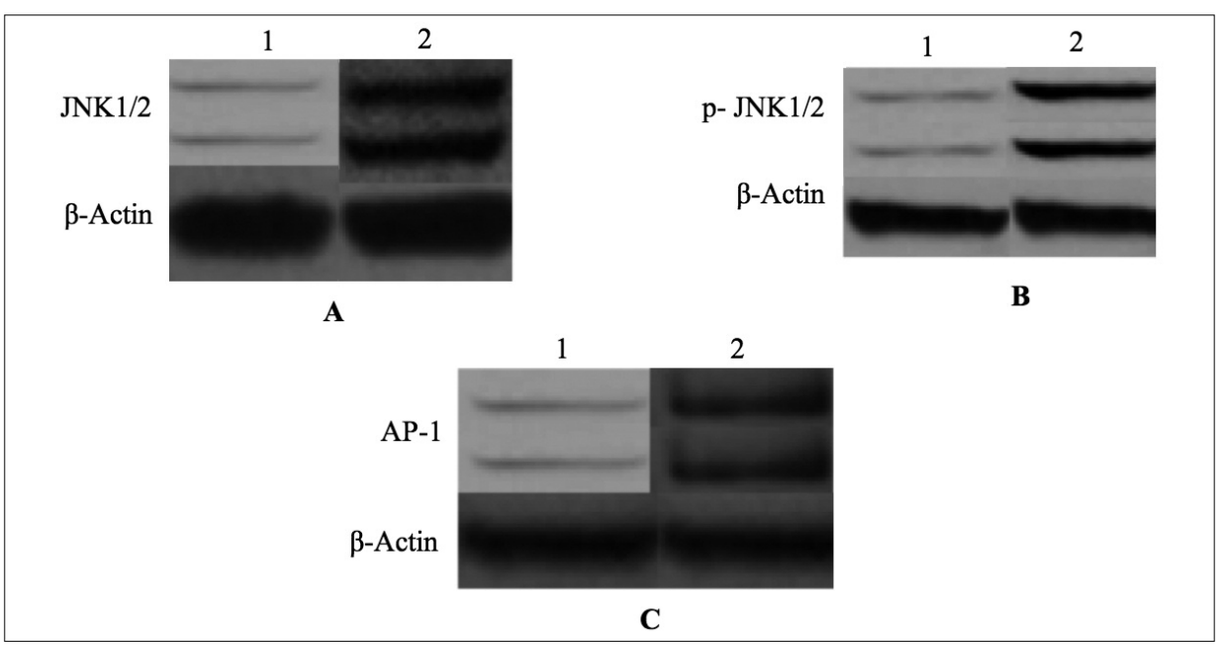

Figure 5. Western Blotting for ***(A)*** JNK1/2, ***(B)*** p-JNK1/2, ***(C)*** AP-1. Lane 1 is non-hypersecretion group and lane 2 is hypersecretion group. β-Actin was used as internal control.

and the activation of AP-1, and also inhibit the A549 cells stimulated by acrolein from secreting MUC5AC. Nie et al[12] have found that A549 cell stimulated by EGF can induce the phosphorylation of ERK1/2, p38-MAPK and JNK mitogen-activated protein kinase family, then leading to the activation of AP-1 and NF-κB, finally starting the gene transcription of MUC.

Some other studies also deemed that mice calcium-activated chloride channels-3 (mCLCA-3), human calcium-activated chloride channels-1 (CLCA-1), IL-4, IL-9, IL-13, IL-1β, TNF-α, cyclooxygenase-2 (COX-2) were all involved in mucus hypersecretion[4,5]. IL-9 could regulate the expression of MUC by activating the calcium chloride channel, IL-13 could up-regulate the expression of MUC5AC by up-regulating the phosphorylation of signal transducer and activator of transcription-6 (STAT6) and inhibiting the transcription factor (FoxA2), then resulting in mucus hypersecretion, airway structural changes, and airway remodeling. TNF-α could regulate mucus secretion by regulating the signaling channels of NF-κB, IL-1β, COX-2, EGFR, and FoxA2.

Conclusions

Through a clinical comparative analysis, our study has found that the expression levels of MUC5AC and MUC5B proteins, and MUC5AC mRNA and MUC5B mRNA in the airway mucus hypersecretion group were significantly higher than those in the non-hypersecretion group (Figures 1 to 4). The expression levels of JNK1/2, p-JNK1/2 and AP-1 proteins in the airway mucus hypersecretion group were higher than those in the non-hypersecretion group (Figure 5). Hence forth, MUC5AC and MUC5B can be used as marker molecules of airway mucus hypersecretion. Airway mucus hypersecretion of severe pneumonia induced by RSV might be related to the activation of JNK/AP-1 signaling pathway.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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