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EXPRESSION OF PULMONARY SURFACTANT-ASSOCIATED PROTEIN B IN NEONATAL RESPIRATORY DISTRESS SYNDROME

EKSPRESIJA PLUĆNOG SURFAKTANT-VEZANOG PROTEINA B U SINDROMU NEONATALNOG RESPIRATORNOG DISTRESA

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Summary

Background: The aim of this study was to investigate the role of pulmonary surfactant-associated protein B (SP-B) expression in the pathogenesis of neonatal respiratory distress syndrome (RDS) via detecting the protein and mRNA expression of SP-B.
Methods: A total of 60 unrelated neonates who died of RDS were chosen as the RDS group and then subgrouped into ≤32 weeks group, 32~37 weeks group and ≥37 weeks group (n=20). Sixty neonates who died of other diseases were enrolled as controls and subdivided into 3 matched groups based on the gestational age. Western blot assay and RT-PCR were employed.
Results: In the RDS group, SP-B protein expression was reduced or deficient in 8 neonates of which 6 had no SP-B protein expression. In the control group, only 1 had reduced SP-B protein expression. The reduced or deficient SP-B protein expression in 9 neonates of both groups was noted in the ≥37 weeks group. In the RDS group, the SP-B mRNA expression was significantly lower than that in the control group. In the ≤37 weeks group, SP-B mRNA expression was comparable between the RDS group and control group. In the 32~37 weeks group, the SP-B mRNA expression in the RDS group was significantly reduced when compared with the control group. In the ≥37 weeks group, the SP-B mRNA expression in the RDS group was dramatically lower than that in the control group.
Conclusions: Alteration of SP-B expression is present at transcriptional and translational levels. Reduction of SP-B mRNA and protein expression is involved in the pathogenesis of RDS.

Keywords: respiratory distress syndrome, neonate, pulmonary surfactant-associated protein B, mRNA

Kratak sadržaj

Uvod: Ova studija imala je za cilj ispitivanje uloge ekspresije plućnog surfaktant-vezanog proteina B (SP-B) u patogenezi sindroma neonatalnog respiratornog distresa (NRD) putem određivanja ekspresije SP-B u proteinima i mRNK.
Metode: Ukupno 60 novorođenčadi koja su umrla od NRD činilo je eksperimentalnu grupu koja je potom podeljena na grupu ≤32 nedelje, grupu 32~37 nedelja i grupu ≥37 nedelja (n=20). Šezdeset novorođenčadi koja su umrla od drugih bolesti činilo je kontrolnu grupu, takođe podeljenu na tri podgrupe prema gestacijskoj dobi. Primenjeni su testovi Western blot i RT-PCR.
Rezultati: U grupi NRD proteinska ekspresija SP-B bila je snižena ili deficitarna kod 8 novorođenčadi, od kojih je kod 6 utvrđeno odsustvo proteinske ekspresije SP-B. U kontrolnoj grupi samo 1 novorođenče imalo je sniženu proteinsku ekspresiju SP-B. Snižena ili deficitarna proteinska ekspresija SP-B kod 9 novorođenčadi iz obe grupe zabeležena je u grupi ≥37 nedelja. U grupi NRD ekspresija SP-B u mRNK bila je značajno niža nego u kontrolnoj grupi. U grupi ≤37 nedelja, ekspresija SP-B u mRNK bila je slična u kontrolnoj i grupi NRD. U grupi 32~37 nedelja, ekspresija SP-B u mRNK bila je značajno niža u grupi NRD u poređenju s kontrolnom grupom. U grupi ≥37 nedelja ekspresija SP-B u mRNK bila je upadljivo niža u grupi NRD nego u kontrolnoj grupi.
Zaključak: Promena ekspresije SP-B prisutna je na nivoima transkripcije i translacije. Redukcija ekspresije SP-B u proteinima i mRNK ima ulogu u patogenezi NRD.

Ključne reči: sindrom respiratornog distresa, novorođenče, plućni surfaktant-vezani protein B, mRNK

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Introduction

Pulmonary surfactant is a surface-active lipoprotein complex and plays an important role in the lung function of neonates. Pulmonary surfactant is crucial to reduce the surface tension at the air–water interface. Pulmonary surfactant-associated proteins (SP) include SP-A, SP-B, SP-C and SP-D which play pivotal roles in the function and metabolism of pulmonary surfactant. SP-B is one of the important SPs and can reduce or alter the surface tension via changing the surface area, which prevents the alveolar collapse (1–5). Respiratory distress syndrome (RDS) is a multifactorial and multi-gene disease, and a genetic factor has been found to be involved in the pathogenesis of RDS. Studies showed SP-B deficiency was closely related to the neonatal RDS (5–7). It is well-known that the protein expression is regulated by the up-stream genes. To explore the role of abnormal SP-B expression in the pathogenesis of neonatal RDS, the protein and mRNA expression of SP-B were detected in the lung of neonates who were admitted to the neonatal intensive care unit (NICU) from July 2006 to October 2011 and died of RDS.

Materials and Methods

Patients and samples

RDS was diagnosed according to the criteria for RDS in the Practical Neonatology (3rd edition). A total of 60 unrelated Han neonates in Beijing who died of RDS were recruited from July 2006 to March 2012 and further subdivided into ≤32 weeks group, 32~37 weeks group and ≥37 weeks group (n=20 per group) on the basis of gestational age. In addition, 60 neonates who died of other diseases were enrolled as controls, and the diseases included congenital heart disease, bronchopulmonary dysplasia and persistent pulmonary hypertension. The controls were also subdivided into gestational age-matched groups. The neonates developed RDS within 30 min to 6 h after birth and progressive dyspnea was the major clinical manifestation. Blood gas analysis showed hypercapnia and hypoxemia. These findings together with chest X-ray were employed to confirm the diagnosis of Grade III or IV RDS. During the hospitalization, RDS patients were repeatedly treated with pulmonary phospholipid (200 mg/kg) from swine (a total of 3 or 4 times) and high frequency oscillatory mechanical ventilation was simultaneously performed. These patients died within 14 d. Informed consent was obtained from relatives and the whole study was approved by an Ethics Committee.

Main reagents

Total RNA extraction kit (Inritrogen), Trizol (Inritrogen, USA), reverse transcription kit (TaKaRa, USA), DL 2000 DNA Marker (TaKaRa, USA), rabbit anti-human SP-B polyclonal antibody (Abcam, USA) and alkaline phosphatase conjugated goat anti-rabbit IgG (Beijing Zhongshan Biotech) were used in the present study. The primers for human SP-B were synthesized at Shanghai Sangon Biotech Co., Ltd and were as follows: 5′-GGACACGATGAGGAAGTTC-3′ (forward), 5′-GTCTGGTTCTGGAA GTAGTC-3′ (reverse) and the anticipated size was 126 bp. The primers for GAPDH (an internal reference) were as follows: 5-TGACTTC AACAGCGACACCCA-3′ (forward), 5′-CACCCTGTTGCTGTAGCCAAA-3′ (reverse) and the anticipated size was 121 bp.

Sample collection

The lung tissues were collected within 30 min after death. Samples were collected from the 5 lobes of RDS patients. In the control group, the lung tissues were randomly selected. The lung tissues and bronchoalveolar lavage fluid (BALF) were immediately stored at –80 °C for use.

Western blot assay

Lung tissues were homogenized in the lysis buffer at a ratio of 1:5 followed by centrifugation at 4 °C for 1 h at 12000 r/min. The supernatant was collected and 10 μL of supernatant was subjected to SDS gel electrophoresis. Before electrophoresis, the supernatant was mixed with loading buffer. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane which was then washed in Tween 20 in Tris buffer (TBS). Subsequently, the membrane was blocked in 3% bovine serum albumin (BSA) for 1 h. The membrane was then treated with rabbit anti-human SP-B polyclonal antibody (1:3000) in TBST at 4 °C overnight under continuous shaking. After washing in 1XTBST, this membrane was treated with alkaline phosphatase conjugated goat anti-rabbit IgG (1:5000) in TBST at room temperature for 2 h under continuous shaking. After washing in TBS, visualization was done followed by observation. Rabbit anti-human β-actin was used to detect the expression of β-actin as an internal reference.

RT-PCR

The lung tissues were homogenized and total RNA was extracted with Trizol reagent and RNA extraction kit. The RNA concentration was determined and RNA was employed for reverse transcription into cDNA according to the manufacturer's instructions. Fluorescence quantitative PCR was done to screen the concentration of template and quantitatively determine the mRNA expression of SP-B.

Screening of SP-B mRNA deficiency subjects

The lower limit of normal for relative SP-B mRNA expression = mean – 2 standard deviations ($\bar{x}$–2SD). An SP-B mRNA expression lower than the lower limit of normal in the control group was defined as SP-B mRNA deficiency.

Statistical analysis

SPSS version 13.0 was employed for statistical analysis and data were expressed as mean ± standard deviation ($\bar{x}\pm$SD). Comparisons between two groups were done with the t test and those among different groups with one-way analysis of variance. The comparisons of rate were done with the chi-square test. A value of $P<0.05$ was considered statistically significant.

Results

Clinical information

The RDS developed within 30 min to 12 h after birth and dyspnea progressed rapidly. Blood gas analysis showed hypercapnia and hypoxemia. Once RDS was confirmed, pulmonary phospholipid from the swine was administered at 200 mg/kg. The clinical manifestation improved gradually and the concentration of administered oxygen was reduced to 20~30%. Blood gas analysis showed hypercapnia and hypoxia were improved. However, 12 h later, the disease condition aggravated and a second administration of pulmonary phospholipid ensued. Pulmonary phospholipid was administered for a maximum of 5 times (mean: 3~4 times). At the same time, high frequency oscillatory mechanical ventilation was performed. Thirty patients had concomitant pulmonary hypertension and were treated with additional NO. The oxygen concentration was higher than 50%. Although comprehensive therapy was performed, these patients finally died. Among these RDS patients, 35 neonates died of RDS and the parents of 25 neonates refused further treatment due to economic concerns. The minimal hospital stay was 3 days and the maximal hospital stay was 14 days.

SP-B protein expression

In the RDS group, the SP-B protein expression was reduced or deficient in 8 patients, of whom 6 had complete deficiency (*Figure 1–1 lane 1~8*). In the control group, the reduced SP-B protein expression was found in only 1 patient (*Figure 1–2, lane 4*). Of note, the reduced or deficient SP-B protein expression in these 9 patients was found in the ≥37 weeks group.

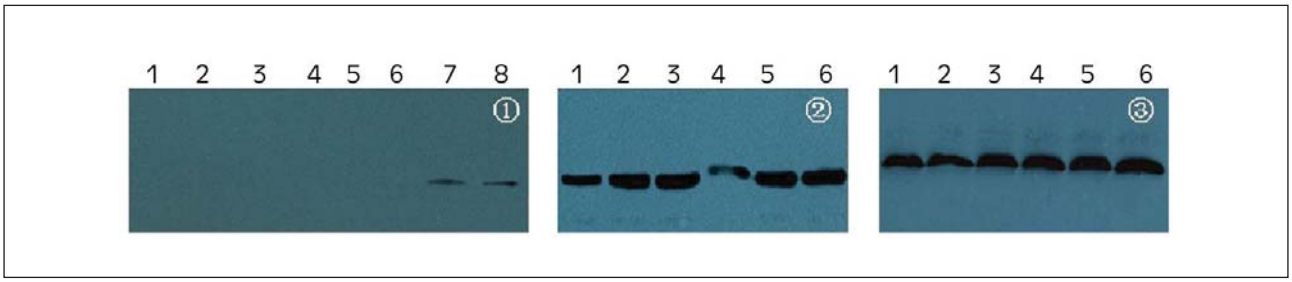

Figure 1 SP-B protein expression in the lung of two groups. 1: RDS group; 2: control group; 3: β-actin.

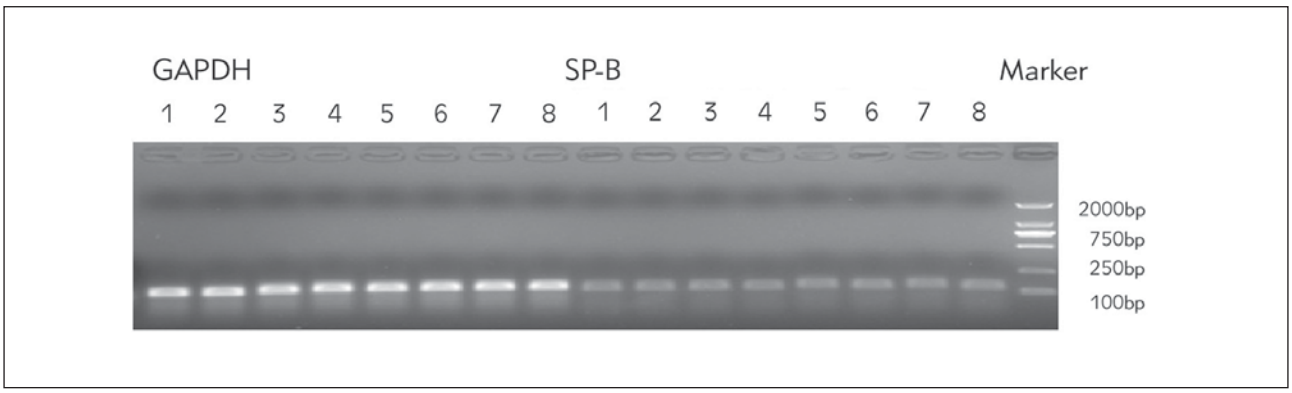

Figure 2 mRNA expression of SP-B and GAPDH in two groups.

SP-B mRNA expression in the lung

The total RNA was extracted and used as template for reverse transcription into cDNA. The size of target gene was 126 bp and that of GAPDH was 121 bp. Visualization was done by addition of Golden view (10 μL) into 10% agarose gel (*Figure 2*). RT-PCR showed the SP-B mRNA expression was 2.55±1.90 in the RDS group, which was markedly lower than in the control group (3.74±1.12; t=4.145, P<0.001) (*Figure 3*).

In the control group, the SP-B mRNA expression raised with the increase in gestational age, which was not observed in the RDS group. In the≤32 weeks group, the SP-B mRNA expression was comparable between the control group and RDS group. In the 32~37 weeks group and ≥37 weeks groups, there was a significant difference in the SP-B mRNA expression between the control group and RDS group (*Table I* and *Figure 4*).

Protein and mRNA expression of SP-B in the two groups

In the RDS group, the deficiency rate of SP-B protein was 13.3% (8/60) which was markedly higher than that in the control group (1.7%, 1/60; χ^2= 4.324, P<0.05). In the RDS group, the deficiency rate of SP-B mRNA was 43.3% (26/60) which was also markedly higher than that in the control group (6.7%, 4/60; χ^2=21.511, P<0.001). The changes in SP-B in the RDS neonates are present at the transcriptional and translational levels. The reduction of SP-B protein expression might be secondary to the reduction of SP-B mRNA expression.

Discussion

Although steroids, exogenous surfactant and mechanical ventilation have been applied in the clinical treatment of RDS, it is still a major cause of death in neonates (8, 9). It has been found that RDS is mainly observed in premature neonates. With the development of newborn medicine, the incidence of RDS is increasing in full-term neonates. Studies have demonstrated that RDS is a multifactorial and multi-

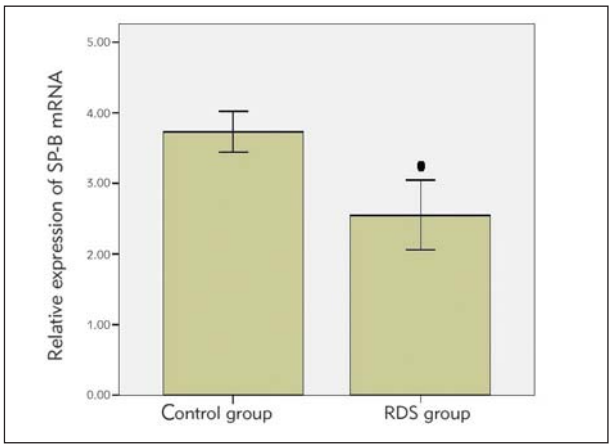

Figure 3 SP-B mRNA expression in RDS group and control group.

•P<0.001 vs control group

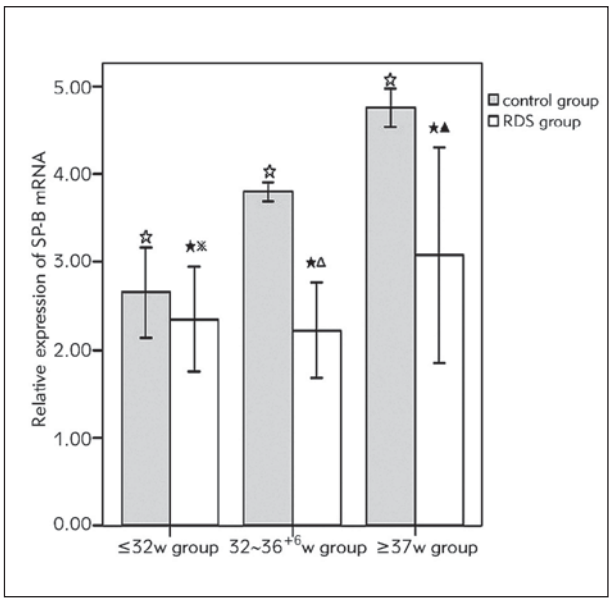

Figure 4 SP-B mRNA expression in different subgroups.
☆P<0.001 among subgroups in the control group
★P=0.312 among subgroups in the RDS group
※P=0.043 vs control group
△P<0.001 vs control group
▲P=0.014 vs control group

Table I SP-B mRNA expression in different groups ($\bar{x}$±SD).

Group	≤32w group	32～37w group +6w group	≥37w group	*F*	*P*
Control	2.66±1.14	3.80±0.25	4.75±0.49	41.361	<0.001
RDS	2.35±1.34	2.23±1.22	3.08±2.74	1.187	0.312
t	0.776	5.661	2.682		
P	0.443	<0.001	0.014		

gene disease in which there is interaction among genetic factors, environmental factors, preterm, gender, race and maternal factors (9). Some surfactant-associated proteins (especially SP-A and SP-B) are susceptible to mutation, and detrimental or protective in the RDS (1). The relationship between deficiency in the surfactant and neonatal RDS has been confirmed (10–12). On the basis of the antibacterial effect of SP-B and the significance of SP-B in maintaining normal lung function, the association between a abnormal SP-B expression and the pathogenesis of neonatal RDS has been receiving increasing attention (1, 2, 7). However, the mechanism causing abnormal SP-B expression is still uncertain. Whether it is the SP-B gene mutation leading to abnormal transcription, abnormal translation leading to primary deficiency in SP-B protein, environmental factors (such as inflammation) causing inactivation of SP-B protein or abnormal production of other substances such as SP-C that attributes to the reduction in SP-B production is still unclear, and more studies are required to clarify this.

In the present study, fluorescence quantitative RT-PCR was performed to measure the mRNA expression of SP-B in the lung of 60 RDS neonates and 60 neonates with other diseases. In the RDS group, the mRNA expression of SP-B was markedly lower than that in the control group. This indicates that gene alteration might lead to abnormality in SP-B transcription and subsequent reduction of SP-B mRNA expression. This is only a speculation. Environmental factors such as inflammation may also inactivate SP-B leading to secondary reduction of SP-B protein expression, or resulting in abnormalities of other substances. These require studies to confirm. Results showed the SP-B mRNA expression in the RDS patients was markedly lower than that in the control group. This suggests that abnormal transcription of SP-B is attributed to the change in the gene. Further analysis showed there was no marked difference in the SP-B mRNA expression between RDS patients and controls in the ≤32 weeks group, which suggests RDS in premature neonates is related to the immature lung development and the insufficient SP-B synthesis. In the 32~37 weeks group, there was significant difference in the SP-B mRNA expression between RDS patients and controls, which indicates that not only immature lung development results in insufficient SP-B synthesis, but other factors cause the inactivation of SP-B. In the ≥37 weeks group, significant difference in the mRNA expression of SP-B was also noted between the RDS patients and controls, which suggests genetic factors causing abnormal SP-B synthesis is a major cause of RDS in full-term neonates. In addition, other factors such as infection are also involved in the pathogenesis of RDS. Further screening of subjects with SP-B mRNA expression lower than the lower limit of normal showed the deficiency frequency of SP-B mRNA expression in the RDS group was significantly higher than that in the control group. This further supports that the genetic factor results in abnormal SP-B mRNA expression and subsequent abnormal SP-B protein expression.

In the present study, Western blot assay was employed to qualitatively determine the protein expression of SP-B in 60 RDS neonates and 60 controls. In the RDS group, the SP-B expression was reduced or deficient in 8 patients, of whom 6 had complete deficiency. Reduction or deficiency of SP-B expression was found in 8 patients of the RDS group and 1 subject of the control group, all of whom had the gestational age of ≥37 weeks. Moreover, the reduction or deficiency in SP-B protein expression in 9 patients was found in the ≥37 weeks group. These findings further demonstrate that the translation of SP-B mRNA into SP-B protein is also abnormal, which leads to the reduction or deficiency in SP-B. This also supports that genetic factors are involved in the reduction or deficiency in SP-B protein expression. Further analysis of the SP-B expression trend showed the protein and mRNA expression of SP-B reduced simultaneously and the changes in SP-B expression occurred at the transcriptional and translational levels. The alteration in mRNA and protein expression of SP-B is involved in the pathogenesis of RDS. The reduction of both SP-B mRNA and protein expression might be involved in the pathogenesis of RDS.

In the present study, 60 neonates who died of RDS were recruited, and SP-B deficiency was found in only 8 patients. Thus, the cause of death in the remaining 53 patients was attributed to multiple factors including alteration in environment, diseases, infection, gestational age, genetic factors and others. The partial or complete deficiency in SP-B may be attributed to the reduction in the SP-B mRNA expression and/or SP-B protein expression which may cause disturbance in the formation of lamellar bodies or tubular myelin. Thus, the mature SP-B in the lung reduces and the secondary mature SP-C subsequently declines. The spread of surfactant at the air – water interface reduces and then compromises the activity of surfactant. Thus, the surfactant induced reduction in surface tension is compromised and the alveoli collapse. The fluid exudes from the capillary into the alveoli. At the same time, the reduction in SP-B may compromise the host defense and the SP-B induced antibacterial effect is attenuated or disappears (13). In the control group, 1 subject developed congenital heart disease (ventricular septal defect) and pulmonary edema in whom the SP-B protein expression in the lung was reduced, which may be attributed to the dilution of SP-B in the lung by the liquid due to pulmonary edema, or even the SP-B enters the capillaries or small blood vessels in the lung and finally the blood (14).

Although pulmonary surfactant replacement therapy and other therapies have been developed for the treatment of RDS, RDS is still a challenge in clinical practice and has become a hot topic in research.

The RDS is a multifactorial and multi-gene disease and gene regulation plays an important role in the pathogenesis of RDS. The surfactant-associated protein gene has been used as candidate gene in studies on the genetics of RDS. The polymorphism and mutation of SP-B have been found to be related to the RDS (15, 16). SP-B is a hydrophobic protein synthesized in type II alveolar cells. SP-B has been demonstrated to play an important role in maintaining normal lung function and can reduce the surface tension at the air–water interface. SP-B is required for the structure of the lung and the synthesis of tubular bone marrow in which SP-C is also involved. The deficiency in SP-B may cause death due to respiratory failure in animals and humans (17).

It has been demonstrated that RDS is closely related to the polymorphism of SP-B gene. However, the changes in the SP-B allele are still unclear and thus the polymorphism of SP-B gene is still a challenge (18). The polymorphism and mutation of surfactant proteins are helpful for the understanding of the susceptibility to neonatal RDS. The polymorphisms of these genes may become valuable markers of diseases including RDS. Although our study demonstrated that the changes in the protein and mRNA expression of SP-B are involved in the pathogenesis of RDS, further studies with gene sequencing are required to confirm our findings.

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Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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