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Original Article

The association between surfactant protein B gene variation and bronchopulmonary dysplasia in Chinese premature newborns

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Abstract: Objective: This study aimed to correlate the pulmonary surfactant B (SP-B) gene variation with bronchopulmonary dysplasia (BPD) in ethnic Han, Chinese, premature newborns. Method: 47 newborns with BPD and 55 controls without BPD were included. Genomic DNA was extracted from cord or artery blood. Genotyping for the SP-B gene was performed by polymerase chain reaction or gene sequencing, and the clinical characteristics were also analyzed. Results: Two types of SP-B gene variations in Exon 2 or Exon 5 were discovered, including V1 (Exon 2: c.[5A > C] + [5A > C] or c.[5A > C] + [=]) and V2 (Exon 5: c.[428C > T] + [428C > T] or c.[428C > T] + [=]). In the BPD group, there were 33 newborns with gene variations, of which type V1 and V2 accounted for 18 and 15 respectively. In the control group, there were 19 newborns with gene variations, of which type V1 and V2 accounted for 7 and 12 respectively. There was a significant difference between the two groups in type V1 variation (X^2=8.956, $P < 0.05$), and V1 variation was more likely associated with BPD occurrence. Logistic regression analysis showed that gene variation, premature rupture of membranes, birth weight, and the duration of mechanical ventilation were associated with BPD development. Among them, gene variation and premature rupture of the membranes were risk factors for BPD development. Conclusions: The exon 2 or 5 of SP-B gene variations were associated with the BPD in Chinese premature newborns, and the type V1: Exon 2: c.[5A > C] + [5A > C] or c.[5A > C] + [=] was a risk factor for the development of BPD.

Keywords: Very low birthweight premature newborns, pulmonary surfactant B, gene variation, bronchopulmonary dysplasia, Han Chinese

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease (CLD), which is defined as oxygen dependency at 36 weeks corrected gestational age [1]. BPD usually occurs in premature neonates who have received oxygen therapy, and mechanical ventilation and is associated with poor neurodevelopmental and medical outcomes [2]. Despite the great improvements that have been made in obstetric and neonatal care in very-low-birth-weight (VLBW) infants, BPD remains a major complication of the prematurity, resulting in significant morbidity and mortality. Its pathogenesis is multi-factorial, including fetal infection, inflammation, absence of antenatal steroids, oxidative stress, ventilator-induced lung injury, poor nutrition, and abnormal growth-factor signaling, which impairs the development of the alveoli and distal vessels [3]. Recently, genetic variance has emerged as a significant risk factor for BPD. In two studies of twins, the estimated heritability was greater than 50% or even close to 80%, despite slightly different definitions of BPD and cohorts with different gestational ages [4, 5]. A number of genes, including surfactant protein genes, have been found to be related to the risk of BPD [6, 7].

Pulmonary surfactant is a mixture of lipids and proteins, which contains four surfactant-associated proteins (SPs) referred to as SP-A, SP-B, SP-C and SP-D [8]. SP-B, in particular, is a very

Table 1. Patient clinical characteristics of BPD and Control groups

	BPD (n=47)	Control (n=55)	X^2/T	P
Gestational age (week)	30.98 ± 3.37	30.87 ± 1.69	-0.196	0.086
Birth Weight (g)	1115.17 ± 182.52	1235.89 ± 186.45	3.291	0.001**
Apgar score at 5 min	9.53 ± 0.65	9.49 ± 0.66	-0.313	0.755
Surfactant administration (n) (%)	41 (87.23)	47 (85.45)	0.068	0.795
Oxygen dependency (days)	45.53 ± 16.30	9.38 ± 8.07	-14.504	< 0.001**
Duration of mechanical ventilation (days)	22.70 ± 16.22	5.49 ± 5.00	-7.472	< 0.001**
Symptomatic patent ductus arteriosus (n) (%)	28 (59.57)	25 (45.45)	2.024	0.155
Respiratory distress syndrome (n) (%)	42 (89.36)	46 (83.64)	0.702	0.402
Premature rupture of the membranes (n) (%)	19 (40.43)	10 (18.18)	6.162	0.013*
Pregnancy-induced hypertension (n) (%)	13 (27.66)	21 (38.18)	1.263	0.261
Cesarean section (%)	53.19	73.58	4.496	0.034*
Primiparity (%)	44.68	41.82	0.805	0.771

*: P < 0.05, **: P < 0.001.

lipophilic protein with a molecular weight of 8 kD and is mainly responsible for the maintenance of alveolar stability [9]. The SP-B gene is located on the short arm of human chromosome 2 [10]. It is a relatively short gene (9500 bp) that consists of 11 exons, but the 11th exon is not translated [9]. The deficiency of SP-B, both in genetically engineered mice and in newborns with congenital alveolar proteinosis, has a devastating effect on respiratory function. Heterozygous (-/+) SP-B mice with half the amount of SP-B protein present decreasing compliance and increasing air trapping [11]. Knocking out the SP-B gene causes severe respiratory failure or even death in newborn mice [12]. Congenital alveolar proteinosis, the complete absence of SP-B, is a rare but fatal pulmonary disease in full-term newborns. The most common reason for this disease is the insertion of two nucleotides in codon 121 of exon 4, leading to early termination of translation [13].

In this study, we examined the gene variations of exons 2 and 5 of SP-B in Chinese Han premature newborns, and we attempted to explore the effect of SP-B genetic variants on BPD susceptibility.

Patients and methods

Patients

This study was approved by the ethical committee of The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, with written informed consent acquired from the parents of the patients. All the patients were of Chinese Han descent and were patients at the Neonatal Intensive Care Unit (NICU) of The Third Affiliated Hospital of Guangzhou Medical University, from Jan. 2013 to Oct. 2014. Patients with congenital or chromosomal abnormalities were excluded. The patients in our study were classified into newborns with BPD (BPD group, n=47) and newborns without BPD (control group, n=55), according to the diagnostic criteria for bronchopulmonary dysplasia [14].

DNA extraction and genotyping

Genomic DNA was extracted from the umbilical cord or cord blood, using a QIAamp DNA Mini Kit (Qiagen, Valencia, USA), according to the manufacturer's instructions. All coding exons of the SP-B gene were amplified by PCR using standard protocols. The primers for exons 2 and 5 of the SP-B gene were: 5' > GTG GGA TCA AGC ACC TGG < 3' sense, 5' > ATG CCT AGC ACA AAG CAG TG < 3' antisense, and 5' > GTC AGT CTG CCC TGG TGG < 3' sense, 5' > TGC TGT GTG TGT GGC TCC < 3' antisense respectively. The PCR mixture (total volume of 50 μL) consisted of 100 ng DNA, 1 × Pyrobest buffer II, 0.4 μM deoxyribonucleotide triphosphates (dNTPs), 0.4 μM of each primer, and 1.25 U Pyrobest DNA Polymerase (Takara Bio Tech, Dalian, China). The amplification reaction was carried out in the following conditions: an initial melting step of 2 min at 95°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 57°C and 1 min at 72°C with a final elongation of 7 min at 72°C. The PCR products were identified by aga-

Table 2. Distribution of the SPB gene variation in exons 2 and 5

	Control (n=55)	BPD (n=47)	X^2	OR (95% CI)	P
Gene variation (n) (%)	19 (34.55)	33 (70.21)	12.901	4.466 (1.935, 10.309)	< 0.001**
Gene normal (n) (%)	36 (65.45)	14 (29.79)			

**: P < 0.001.

Table 3. V1 and V2 variation between BPD and Control groups

	Control (n=55)	BPD (n=47)	X^2	OR (95% CI)	P
V1 variation (n) (%)	7 (12.73)	18 (38.30)	8.956	4.256 (1.586, 11.423)	0.003*
V1 normal (n) (%)	48 (87.27)	29 (61.70)			
V2 variation (n) (%)	12 (21.82)	15 (31.91)	1.327	1.680 (0.692, 4.075)	0.249
V2 normal (n) (%)	43 (78.18)	32 (68.09)			

*: P < 0.05.

rose gel electrophoresis. To confirm our results, the PCR products for each polymorphism were sequenced with the same control primers used for the amplification by the dideoxy chain termination method. The sequencing was performed by BGI-Huada, Shenzhen, China.

Statistics

All data were given as median, interquartile range or percentages. Analysis of continuous variables was performed by using the unpaired Student's t-test. Frequencies of genotypes were obtained by direct counting. Categorical variables were compared by Chi-square test. The Chi-square test was also used to identify departures from the Hardy-Weinberg equilibrium. The risk factors of BPD were analyzed by using Logistic regression. Differences were considered to be significant when the *P* values were less than 0.05. All analyses were performed using 15.0 SPSS software.

Result

Clinical characteristics of patients in BPD and non-BPD groups

47 premature infants with BPD and 55 premature infants without BPD were included in our research. Univariate and multivariate analyses were performed using the unpaired Student's t-test or Chi-square test. The birth weight in the BPD group was significantly lighter than in the control group (1115.17 ± 182.52 g VS. 1235.89 ± 186.45 g). The Oxygen dependency in the BPD group was significantly longer than in the control group (45.53 ± 16.30 day VS. 9.38 ± 8.07 day), and the duration of mechanical ventilation in BPD group was also longer than in the control group (22.70 ± 16.22 day VS. 5.49 ± 5.00 day). The premature rupture of the membranes and cesarean section also differed significantly between the two groups. The other tested factors including gestational age, male, Apgar score, surfactant administration, symptomatic patent ductus arteriosus, respiratory distress syndrome, pregnancy-induced hypertension, primiparity showed no significantly difference. The clinical characteristics of patients were shown in **Table 1**.

Distribution of the SPB gene variation in exons 2 and 5

The distributions of the variations in exons 2 and 5 of SP-B gene in our patients were shown in **Table 2**. Both groups showed no significant deviation from the Hardy-Weinberg equilibrium. Among the 47 cases in BPD group, 33 cases had SP-B gene variations in exons 2 and 5 and 14 cases were normal; however, among the 55 cases in the control group, 19 cases had SP-B gene variations in exons 2 and 5, and 36 cases were normal. There was a significant differen ce between the two groups in the SP-B gene variations (X^2=12.901, P < 0.001) using the Chi-square test. Moreover, the logistic regression analysis showed that SP-B gene variations increased the risk of BPD (OR=4.466, 95% CI=1.935, 10.309).

Genotyping of SP-B gene variations in exons 2 and 5

In our study, two types of SP-B gene variations, including V1 (Exon 2: c.[5A > C] + [5A > C] or c.[5A > C] + [=]) and V2 (Exon 5: c.[428C > T] +

Table 4. Logistic regression analysis of BPD risk factors

	β	SE	Wald	P	OR (95% CI)
Gene variation	3.183	1.066	8.911	0.003*	24.118 (2.983, 194.978)
Premature rupture of the membranes	2.694	1.003	7.214	0.007*	14.793 (2.071, 105.643)
Birth Weight	0.006	0.003	4.36	0.037*	1.006 (1.000, 1.012)
Duration of mechanical ventilation	0.522	0.122	18.265	< 0.001**	1.686 (1.327, 2.142)
Constant	15.506	4.786	10.495		

*: P < 0.05, **: P < 0.001.

[428C > T] or c.[428C > T] + [=]) were found using gene sequencing. The results are shown in **Table 3**. Among the 33 BPD newborns with SP-B gene variations in exons 2 and 5, 18 cases had type V1 and 15 cases had type V2; however, among the 19 control newborns with SP-B gene variations in exons 2 and 5, 7 cases had type V1 and 12 cases had type V2. There was a significant difference between the two groups in type V1 variation (X^2=8.956, P < 0.05) adopting the Chi-square test. Subsequent logistic regression analysis revealed that the V1 variation was a risk factor for BPD occurrence. However, there was no significant difference in the type V2 variation.

Risk factors analysis associated with BPD development

Exploratory analysis based on the two-category non-conditional logistic regression model was used to analyze the factors related to BPD development. Among the factors influencing BPD, five, such as gene mutation, premature rupture of membranes, birth weight, duration of mechanical ventilation and cesarean section, were selected using a chi-square test and a single factor logistic regression. As the results show in **Table 4**, four factors, including gene mutation, premature rupture of membranes, birth weight, and duration of mechanical ventilation, were associated with BPD development. Among them, gene variation and premature rupture of the membranes were risk factors, in which newborns were more likely to develop BPD.

Discussion

BPD is one of the most severe diseases in premature infants and has a poor prognosis. Multiple factors may contribute to the pathogenesis of BPD. Several common variants and rare mutations show associations with BPD in various strengths [15]. In our research, the variations in exons 2 and 5 of the SP-B gene were found both in the BPD group and the non-BPD group, but the total number of newborns with variations in the BPD group was much higher than in the control group. Moreover, two types of SP-B gene variations in exons 2 and 5 were discovered, i.e. type V1 (Exon 2: c.[5A > C] + [5A > C] or c.[5A > C] + [=]) and type V2 (Exon 5: c.[428C > T] + [428C > T] or c.[428C > T] + [=]). Among the newborns with variations in exons 2 and 5 of the SP-B gene, type V1 in the BPD group was more common than in the control group, so it may be a risk factor for BPD.

The human SP-B gene is located on chromosome 2, consisting of 11 exons and 10 introns. The SP-B gene is characterized by its being highly polymorphic, and many polymorphic loci are located in its promoter, codons and introns [16, 17]. A study concerning the C/A-18 polymorphism of the SP-B gene association with COPD in the Chinese Han population suggested that the C/A-18 polymorphism was not a risk factor in COPD, but the frequency of the CC genotype was higher than that of the AA genotype [18]. Another in vitro experiment demonstrated that the length of the intron 4 of the SP-B gene affected the splicing of the exon 4/intron 4 junction, and a larger amount of incompletely spliced SP-B mRNA was produced in cancerous tissue with intron 4 deletion [19]. Recently there were two studies suggesting that the Surfactant Protein B gene polymorphisms were associated with a risk of bronchopulmonary dysplasia in the Chinese Han population. One study showed that SP-B -18C/A and 1580C/T polymorphisms were associated with BPD. The 1580C/T polymorphism was protective while the -18C/A polymorphism increased the risk for BPD. Moreover, the SP-B 4564T/C polymorphism was not associated with BPD [7]. The other study suggested that the polymorphisms of the SP-B intron 4 and C/A-18

could be associated with BPD, and the deletion of intron 4 and the allele of C/A-18 might be used as markers of susceptibility in BPD [20]. To our knowledge, it was the first report to describe the association of variations within the SP-B exons 2 and 5 with the risk for BPD in very preterm infants of Han ethnicity.

Our research was a retrospective study conducted at a single center. One obvious limitation of the present study was the relatively small sample size. However, the experimental errors can be strictly controlled as result of the smaller subjects and implementation at a single center. We therefore believe that our results based on 102 infants of a single Chinese ethnicity that were uniformly treated in a single institution are meaningful. Further studies of larger sample size are necessary to clarify the SPB genotype's specific effects on BPD pathogenesis.

In conclusion, we found that SP-B exon 2 and exon 5 variations were associated with the development of BPD, and the Exon 2: c.[5A > C] + [5A > C] or c.[5A > C] + [=] may be a risk factor for the pathogenesis of BPD in Chinese Han premature newborns. It is hoped that this study will provide a new therapeutic target for neonatal BPD and improve the survival rate and quality of life in BPD patients.

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Disclosure of conflict of interest

None.

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