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Association of Surfactant Protein B Gene Polymorphisms (C/A-18, C/T1580, Intron 4 and A/G9306) and Haplotypes with Bronchopulmonary Dysplasia in Chinese Han Population*

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Summary: This study aimed to investigate the association between surfactant protein B (SP-B) polymorphisms and bronchopulmonary dysplasia (BPD) in Chinese Han infants. We performed a case-control study including 86 infants with BPD and 156 matched controls. Genotyping was performed by sequence specific primer-polymerase chain reaction (PCR) and haplotypes were reconstructed by the fastPHASE software. The results showed that significant differences were detected in the genotype distribution of C/A-18 and intron 4 polymorphisms of SP-B gene between cases and controls. No significant differences were detected in the genotype distribution of C/T1580 or A/G9306 between the two groups. Haplotype analysis revealed that the frequency of A-del-C-A haplotype was higher in case group (0.12 to 0.05, P=0.003), whereas the frequency of C-inv-C-A haplotype was higher in control group (0.19 to 0.05, P=0.000). In addition, a significant difference was observed in the frequency of C-inv-T-A haplotype between the two groups. It was concluded that the polymorphisms of SP-B intron 4 and C/A-18 could be associated with BPD in Chinese Han infants, and the del allele of intron 4 and A allele of C/A-18 might be used as markers of susceptibility in the disease. Haplotype analysis indicated that the gene-gene interactions would play an important part in determining susceptibility to BPD.
Key words: bronchopulmonary dysplasia; surfactant protein B; polymorphism

Bronchopulmonary dysplasia (BPD) is defined as a status requiring for oxygen supplementation for at least 28 days after birth. The condition is the most common chronic lung disease in premature infants and is characterized by disordered lung development and is associated with respiratory and neurodevelopmental morbidities[1]. Despite considerable improvements in obstetric and neonatal care of very-low-birth-weight (VLBW) infants, BPD remains a major complication of the prematurity resulting in substantial morbidity and mortality. It appears to result from multiple factors that can injure the immature lung and interrupt normal development of alveoli and distal vessels[2]. Recently, genetic variance has emerged as a significant risk factor for BPD development. After controlling for covariates, genetic factors account for 53% to 82% of the variance in BPD[3–5]. This suggests that genetic background may play a vital role in the susceptibility to BPD, and might be useful in personalized medicine. A variety of genes, including surfactant protein genes, have been found to be linked with the risk of BPD[6, 7].

Pulmonary surfactant consists of a variety of lipids and a number of proteins. The surfactant proteins (SPs) are a complex mixture of lipid-associated proteins, which are critical determinants of alveolar stability and functions[8]. Surfactant protein B (SP-B) is one of the four known SPs in humans, a hydrophobic protein contributing to the lowering of the surface tension at the air-liquid interface of the alveolus that prevents the lung from collapsing. This protein is essential for the biogenesis of pulmonary surfactant and formation of lamellar bodies[9, 10]. Heterozygous (–/+) SP-B mice with half the amount of SP-B protein showed decreased compliance and increased air trapping[11]. Results from the knockout mice indicated that life couldn't be supported in the absence of SP-B[12]. Moreover, genetic markers in the SP-B gene have been associated with a spectrum of pulmonary diseases including BPD[13].

In this study, we examined four polymorphisms of SP-B (C/A-18, C/T1580, intron 4 and A/G9306) in Chinese Han infants, with an attempt to understand the influence of SP-B genetic variants on the BPD susceptibility.

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1 SUBJECTS AND METHODS

1.1 Subjects

This study was approved by the local ethics committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, China. Informed consent was obtained from the parents of all infants enrolled in this study. All individuals were Chinese Han descent from the Neonatal Intensive Care Unit (NICU) of Tongji Hospital of Tongji Medical College, HUST, between Jan. 2008 and June 2012. This was a case-control study, in which the blood samples and information of the infants were collected prospectively. A total of 242 infants were divided into 2 groups, with 86 with BPD included in case group and 156 without BPD serving as control group. The case group and control group were matched for gender, gestational age, birth weight and the steroid treatment and no significant differences in these respects were found between the two groups (table 1).

Table 1 Characteristics of infants with or without BPD

Items	BPD	Control	*P*
n	86	156	
Gender (male, %)	67.4	65.4	0.747
Mean of GA[a]	30±1.8	30±1.3	0.459
Mean of BW (g)	1440±558	1490±259	0.349
Steroid treatment[b] (*n*)	64	113	0.740

GA: gestational age in weeks; BW: birth weight; [a]: $\bar{x}\pm s$; [b]: The subjects received maternal steroid therapy 24−168 h before infant birth.

1.2 Inclusion and Exclusion Criteria

Individuals who survived a BPD were eligible in this study. Diagnosis of BPD was confirmed according to the NICDH consensus definition: Clinical symptoms and radiographic findings correspond to anatomic abnormalities[1]. Preterm infants without BPD were included in control group. Infants with fatal congenital anomalies, such as central nervous system malformations, diaphragmatic hernia, serious congenital heart diseases and chromosome abnormalities, were excluded from this study.

1.3 DNA Extraction and Genotyping

Blood samples were harvested in compliance with the institutional guidelines for human studies. They were placed in tubes containing EDTA and kept at –80°C until genomic DNA was extracted using a Genomic DNA Purification Kit@ provided by Qiagen (Shanghai, China). The extraction was performed by following the manufacturer's instructions. The polymorphism sites of SP-B and conditions for the PCR-RFLP analysis (primer pairs, PCR products size, restriction enzymes and restriction fragments) are given in table 2.

Table 2 Primers and restriction enzymes used for genotyping of SP-B

Polymorphism	Primers	PCR fragment	Allelic products	Restriction enzymes	Restriction fragment (bp)
Intron 4	TGTGTGTGAGAGTGAGGGTGTAAG	604 bp (inv)			
	CTGGTCATCGACTACTTCCA				
C/A-18	GTCCAGCTATAAGGGGCCGTG	168 bp	GTGCAC	*Apa*L I	149, 19
	GTGAGTGGTGAGCTGCCTA		GTGCCC		168
C/T1580	CTCGAATTCTCTCGTAACTCCAGCACCC	278 bp	CTCAG	*Dde* I	164, 94, 20
	GTGAGCTTGCAGCCCTCTCA		CTCAA		184, 94
A/G9306	CTGTGTAATACAATGTCTGCACTA	137 bp	CTAG	*Bfa* I	113, 24
	CTCGAATTCTGCTGGATTGCAGGTGGA		CTAA		137

inv: invariant genotype type

To genotype intron 4 variation, a specific PCR was carried out in a total volume of 20 μL for the specific segment amplification with primers, which included Taq plus PCR Mastermix (Shanghai Xinghan Sci & Tec Co., Shanghai, China) 10 μL, 0.2 μmol/L of each primer, 100 ng/μL genomic DNA as template. Thermal cycle profile was as follows: 95°C for 2 min followed by 35 cycles of 95°C for 45 s, 63°C for 45 s, 72°C for 2 min. This was followed by one cycle of 72°C for 10 min. The PCR-products were run on 1.7% agarose gel for electrophoresis [1×Tris-borate EDTA (TBE), 0.2 μg/mL ethidium bromide) with 0.5× TBE running buffer at 60 V for 45 min (fig. 1). The SP-B genotyping of other three SNPs (C/A-18, C/T1580 and A/G9306) was performed by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis as described by Lin *et al*[14] (fig. 2–4).

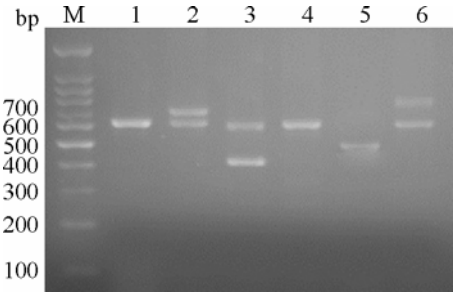

Fig. 1 Genotyping of intron 4

M: 100 bp ladder marker; lanes 1, 4: wild type; lanes 2, 6: insertion type; lanes 3, 5: deletion type

1.4 Sequencing

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[15]. The sequencing was performed by BGI-Huada, Wuhan, China.

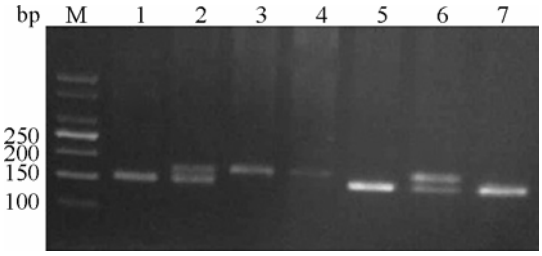

Fig. 2 Genotyping of C/A-18
M: 50 bp ladder marker; lanes 1, 5, 7: AA genotype; lanes 3, 4: CC genotype; lanes 2, 6: AC genotype

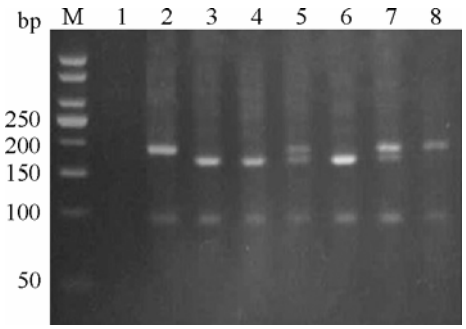

Fig. 3 Genotyping of C/T1580
M: 50 bp ladder marker; lanes 2, 8: TT genotype; lanes 3, 4, 6: CC genotype; lanes 5, 7: CT genotype

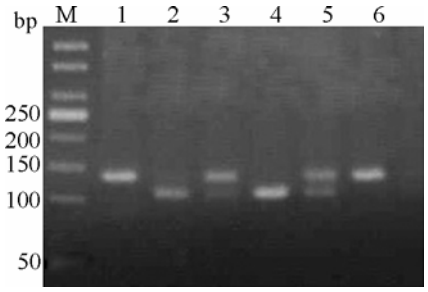

Fig. 4 Genotyping of A/G9306
M: 50 bp ladder marker; lanes 1, 6: AA genotype; lanes 3, 5: AG genotype; lanes 2, 4: GG genotype

1.5 Statistical Analysis

Descriptive statistics were used to describe the categorical and numerical variables of the infants' characteristics, and all data were expressed as $\bar{x}\pm s$ or as 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 analyzed by *Chi*-square test. The *Chi*-square test was also used to identify departures from the Hardy-Weinberg equilibrium. This study took into account the genotypes rather than just the alleles, following the guidelines proposed by Sasieni[16]. Analysis of genotypes used the Armitage's trend test. The fastPHASE software was employed for the reconstruction of haplotypes and estimation of the frequencies within the study population[17]. A two-tailed *P* value <0.05 was considered to be statistically significant. Retrospective statistical powers were calculated by using QUANTO software package.

2 RESULTS

2.1 Genotyping of SP-B Intron 4

The SP-B intron 4 variants are size variants as described by a previous study[18]. The invariant (inv) allele is approximately 604 bp, consisting, in part, of repetitive motifs that include a 20 bp conserved sequence followed by various dinucleotide repeats (CA-repeats). The variant alleles consist of either insertion of motifs or deletion of motifs. In this study, we designated all intron 4 alleles with size lower than that of the inv allele as deletion (del) alleles and those with size larger than the inv allele as insertion (ins) alleles. In the present study, there were 173 cases of invariants (homozygous wild-type), 52 heterozygous and 17 homozygous variants of SP-B intron 4 in all of the 242 infants. There were no homozygous ins variants in all the infants of our series. The frequencies of homozygous wild-type, heterozygous variant and homozygous del variant genotypes were 58.1%, 25.6% and 16.3% in case group. In control group, the frequencies were 78.8%, 19.2% and 1.9% for homozygous wild-type, heterozygous variant and homozygous del variant genotypes, respectively. There was significant difference in the genotype distribution between case group and control group (χ^2=18.365, *P*=0.000) (table 3).

2.2 Genotyping of Three SP-B Single Nucleotide Polymorphisms (SNPs)

The genotype distribution of three SP-B SNPs (C/A-18, C/T 1580 and A/G 9306) in two groups was studied. Not all the genotype frequencies were in accordance with the Hardy-Weinberg equilibrium, so the Armitage's trend test was conducted to analyze genotypes. As for the C/A-18 polymorphism, the frequencies of CC, AC and AA genotypes were 23.3%, 44.2% and 32.6%, respectively, in BPD infants. In control group, the frequencies were 38.5%, 42.3% and 19.2% for CC, CA and AA genotypes, respectively. There was a marked difference in the genotype distribution between the two groups (χ^2=8.031, *P*=0.018). No significant differences in genotype distribution for the C/T1580 and A/G9306 polymorphisms were found between case group and control group (table 3).

2.3 Haplotype Analysis

Of the possible 24 haplotypes of these four polymorphisms, 16 were found in reconstructed haplotypes on the basis of genotyping and sequencing in case group and 13 in control group. Table 4 lists haplotypes and their frequencies. Frequencies of six haplotypes in case group were less than 3%, and the frequencies of other six haplotypes were less than or equal to 3% in control group. Significant differences in haplotype distribution were observed between BPD subjects and controls. The most distinct difference was found in the haplotype C-inv-C-A bearing four wild-type alleles. Frequency in control group (0.19) was higher than that in case group (0.05) (*P*=0.000). The haplotype A-del-C-A bearing one C/A-18 mutated allele, one intron 4 del allele and two wild-type alleles also showed a marked difference in frequency between the two groups (frequency of 0.12 in case compared to 0.05 in control group, *P*=0.003). The frequency of haplotype C-inv-T-A with a C/T mutated

allele was significantly lower in case group, as compared with control group (P=0.008). The haplotypes C-del-T-A, C-del-C-G and C-del-T-G were not found in the control group, but they were observed in case group accompanied by intron 4 del allele. No significant differences were observed in other haplotypes between the two groups (table 4).

Table 3 Genotype distribution of four SP-B polymorphisms [*n* (%)]

Groups	Genotype of intron 4			Genotype of C/A -18			Genotype of C/T1580			Genotype of A/G 9306		
	inv/inv	inv/#	del/del	CC	AC	AA	CC	CT	TT	AA	AG	GG
BPD	50 (58.1)	22 (25.6)	14 (16.3)	20 (23.3)	38 (44.2)	28 (32.6)	42 (48.8)	34 (39.5)	10 (11.6)	58 (67.4)	20 (23.3)	8 (9.3)
Control	123 (78.8)	30 (19.2)	3 (1.9)	60 (38.5)	66 (42.3)	30 (19.2)	78 (50.0)	54 (34.6)	24 (15.4)	99 (63.5)	48 (30.8)	9 (5.8)
	χ^2=18.365	P=0.000		χ^2=8.031	P=0.018		χ^2=0.073	P=0.787		χ^2=0.003	P=0.957	

Inv: invariant allele; del: deletion variant allele; #: deletion variant allele or insertion variant allele

Table 4 Haplotypes and frequencies in two groups

Haplotype				Frequency		*P* values
C/A -18	Intron 4	C/T 1580	A/G 9306	BPD group	Control group	
A	inv	C	A	0.31	0.25	
A	**del**	**C**	**A**	**0.12**	**0.05**	**0.003**
C	inv	C	G	0.10	0.14	
C	**inv**	**T**	**A**	**0.09**	**0.18**	**0.008**
C	inv	T	G	0.08	0.05	
A	inv	T	A	0.06	0.06	
C	**inv**	**C**	**A**	**0.05**	**0.19**	**0.000**
C	**del**	**T**	**A**	**0.04**	**0.00**	
C	del	C	A	0.03	0.03	
C	**del**	**C**	**G**	**0.04**	**0.00**	
A	ins	C	A	0.02	0.02	
A	ins	T	A	0.01	0.01	
A	del	T	A	0.02	0.01	
C	ins	C	A	0.01	0.01	
C	**del**	**T**	**G**	**0.01**	**0.00**	
A	inv	T	G	0.01	0.01	

ins: insertion variant allele. Fonts in bold showed the frequencies between two groups were significantly different.

3 DISCUSSION

Multiple factors may contribute to the pathogenesis of BPD. The current consensus is that BPD is an involved condition, with the interaction of a susceptible host with a multitude of external risk factors underlying its pathogenesis[19]. Although exposure of immature lungs to various insults is thought to play a pivotal role in the onset of BPD, significant genetic susceptibility to BPD was recently demonstrated in preterm infants[20, 21]. Hereditary differences in the expression of genes critical for lung development may therefore play a part in BPD pathogenesis. Genetic foundations for the development of BPD are implicated in twin studies, which revealed highly significant concordance rates for BPD: 3.69-fold in monozygotic and 1.4-fold in dizygotic twins[22]. So far, some researches have demonstrated that polymorphisms in SP-B gene are associated with chronic obstructive pulmonary disease (COPD), respiratory distress syndrome (RDS), asthma and so forth[23–25]. However, the association between haplotype in SP-B and BPD was not well studied.

In the present study, we explored the relationship between four polymorphisms/haplotypes of SP-B gene and BPD in Chinese Han infants. Results showed that the frequencies of AA genotype for C/A-18 and homozygous del variant genotype for intron 4 were significantly higher in case group than in control group, but polymorphisms of C/T1580 and A/G9306 were not associated with BPD. Frequency of haplotype A-del-C-A bearing one C/A-18 mutated allele, one intron 4 del allele and two wild-type alleles were also higher in BPD infants than in control group.

The human gene encoding SP-B is located on chromosome 2, consisting of 11 exons and 10 introns. SP-B gene is characterized by being highly polymorphic and many polymorphic loci are located in its promoter, codons and introns[26–28]. C/A-18 of SP-B is located on the region 5'UTR, 11 nucleotides below the TATAAA box and 18 nucleotides to the left of the transcription initiation site[29]. Because of the critical location of the polymorphism site in the promoter, it could functionally impact on the promoter. The polymorphism C/A-18 has not been extensively studied. A study concerning the association between SP-B polymorphisms and COPD in Chinese Han population demonstrated that the C/A-18 polymorphism was not a susceptibility gene in COPD, but the frequency of CC genotype was higher than that of AA genotype[30]. Another experimental study suggested that the C allele of the SP-B promoter increased transcription more effectively than A allele, and AA genotype was associated with reduced SP-B expression in bronchoalveolar lavage fluid (BALF)[31]. This indicated that A allele of C/A-18 might be a risk factor for BPD. The length of SP-B intron 4 variation is relatively constant. Moreover, another *in vitro* experiment showed that the length of the human SP-B intron 4 gene influenced

splicing of the exon 4/intron 4 junction, and a larger amount of incompletely spliced SP-B mRNA was present in cancerous tissue with an intron 4 deletion genotype[32]. We are led to propose that the intron 4 size variant plays a role in the transcriptional regulation of SP-B gene, resulting in altered mRNA. Our results suggested that the homozygous del variant genotype of intron 4 might be associated with BPD.

The polymorphism C/T1580 of SP-B, present at the end of exon 4, on nucleotide 1580, affects the amino acid 131, causing a substitution from threonine amino acid (ACT) to isoleucine (ATT)[33]. One research confirmed that this alteration removes a potential site of glycosylation, and the N-linked glycosylation site at the N-terminal fragment does not exist when the allele is T on nucleotide 1580[34]. The polymorphism is associated with certain pulmonary pathological changes. For example, a recent study exhibited that the T allele was considered to be a protective factor against RDS, whereas the allelic variant C is a risk factor for RDS[35]. Our study failed to reveal the association between the polymorphism and BPD. A/G9306 of the SP-B gene is located on region 3'UTR, just 4 nucleotides upstream of the TAATAAA polyadenylation signal[14]. Although A/G9306 is located outside the protein translation site, this nucleotide change has the potential to affect signal transduction and RNA processing[35]. A case-control study about Brazilian preterm babies with RDS[36] showed that AG genotype was a protective factor against RDS. In another study, the AG genotype of the A/G 9306 marker appeared to be a susceptibility factor for RDS[37]. In this study, we did not observe the association between A/G9306 polymorphism and BPD.

The discrepancy between this study and previous reports may be due to several reasons. First, the difference might, in part, come from ethnic difference of the research population. It is well-known that ethnicity and population stratification may strongly influence the role of genetic risk factors in pulmonary diseases[38]. Liu *et al* also reported that ethnic background was an important factor in analytical studies of allele and genotype frequencies[39]. Furthermore, another important factor that may be responsible for the inconsistency in the findings is the selection criteria adopted for cases and controls, especially clinical features, severity of disease, age, race and the gene-gene and/or gene-environment interactions.

The present study, for the first time, examined the association between haplotypes in SP-B and BPD. A gene-specific allelic haplotype is the specific combination of the nucleotides, and the haplotype from each of the polymorphic sites is present on an individual chromosome. Haplotypes generally contain more information than individual SNPs do. If a specific disease-related allele is dependent on *cis* interaction with other loci, the association between the allele and the disease may not be detected unless the functional haplotype unit itself is analyzed for predicting genetic risk factors of the disease[40]. In addition, differences in haplotype diversity and frequency among populations may be of value in identifying the variants that are most likely primary etiological determinants of a common disease[41]. This study showed that frequency of haplotype A-del-C-A was significantly higher in BPD infants than in controls and this might be due to the presence of the variant alleles A for C/A-18 and del for intron 4, which could decrease expression of SP-B. Down-regulated SP-B expression and impaired pulmonary function may lower the amount of pulmonary surfactant, thereby resulting in BPD.

In conclusion, we observed that the polymorphisms of SP-B intron 4 and C/A-18 were associated with BPD in Chinese Han individuals. Haplotype A-del-C-A analysis showed that A allele for C/A-18 and del allele for intron 4 might be risk factors for BPD. Further large-scale epidemiological studies in various ethnic groups and experimental studies are needed to better understand the link between the polymorphisms and BPD.

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