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Original Article

Surfactant protein B gene polymorphisms is associated with risk of bronchopulmonary dysplasia in Chinese Han population

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Abstract: Objective: To investigate the relationship between Surfactant protein B (SP-B) gene polymorphisms and bronchopulmonary dysplasia (BPD) development in preterm infants of China Han ethnic population. Methods: SP-B gene polymorphisms were studied in 134 neonates who were born at < 32 weeks of gestation, with the diagnosis of BPD and in a control group of 168 preterm infants without BPD. Genotyping for SP-B was performed by polymerase chain reaction (PCR) and gene sequencing. Results: In this study, three of the SNP genotypes, -18C/A, 1580C/T and 4564T/C were common identified in SP-B gene. The -18C/A genotype was found to be significantly associated with BPD (χ^2 = 10.741, P < 0.01), with P < 0.01 for the dominant model (OR = 1.712, 95% CI = 1.228-2.3894) and the allelic model (OR = 1.787, 95% CI = 1.276-2.502). The 1580C/T genotype was found to be associated with BPD (χ^2 = 7.014, P < 0.05), with P < 0.05 for the dominant model (OR = 0.752, 95% CI = 0.593-0.954) and P < 0.01 for the allelic model (OR = 0.706, 95% CI = 0.548-0.909). The 4564T/C genotypes and alleles were found not to be associated with BPD (χ^2 = 3.399 and 3.227, P > 0.05). Conclusion: SP-B -18C/A and 1580C/T polymorphisms are associated with BPD. The 1580C/T polymorphism was protective while the -18C/A polymorphism increased the risk for BPD. SP-B 4564T/C polymorphism is not associated with BPD.

Keywords: Surfactant protein B, gene polymorphism, bronchopulmonary dysplasia

Introduction

Bronchopulmonary dysplasia (BPD), first described by Northway and colleagues in 1967 [1], is a chronic lung disease which develops in premature infants. The incidence of BPD increase progressively as advances in neonatal intensive care improved the survival rate of premature infants particularly those of extremely low birth weight over past decades. BPD remains a major cause of pulmonary morbidity and mortality during infancy [2]. Previous epidemiological studies have identified prematurity, low birth weight, oxygen toxicity and mechanical ventilation as major risk factors associated with BPD [3-5], but recent studies suggested that genetic susceptibility plays a vital role in BPD [6, 7], in a study of 450 sets of twins born at less than 32 weeks of gestation, after controlling for covariates, genetic factors accounted for 53% to 82% of the variance in liability for BPD [7-9]. A number of studies have shown an association between BPD and potential candidate genes [10-14], including polymorphisms in surfactant proteins [15-17].

Surfactant proteins (SPs) are components of pulmonary surfactant, a lipid-protein complex essential for lung function [18, 19]. Surfactant proteins (SP-A, SP-B, SP-C, and SP-D) are divided into two groups, by their hydrophobicity properties. SP-B plays important roles in surfactant structure and surface tension lowering properties to prevent of alveolar collapse at low lung volumes [20, 21]. Heterozygous (–/+) SP-B knockout mice showed decreased compliance and increased air trapping [22]. Moreover, SP-B gene polymorphism is associated with respiratory distress syndrome (RDS) and BPD has been demonstrated in preterm babies by both case control and family-based linkage studies [16, 17, 23-25].

Table 1. Primers used in the present study

SP-B exons	Primes	Size
exon 1 and 2	F 5'-AAGACAAACACTGAGGTCGC-3'	659 bp
	R 5'-AGCACCCTTCATTTCAGACC-3'	
exon 3 and 4	F 5'-AGAACCTCCCCATTGGAGC-3'	699 bp
	R 5'-CTCCCCATGGGTGGGCAC-3'	
exon 5 and 6	F 5'-TCCCTCCCAGACCCTAACAC-3'	623 bp
	R 5'-GAGAGAGGTGGGAGCTGC-3'	
exon 7 and 8	F 5'-TGGGCAGAGAGTGGAGGCTTG-3'	642 bp
	R 5'-GCAAAGCCTTTCCTGGGCTC-3'	
exon 9 and 10	F 5'-CCTAGAGTGATCCAGAGATG-3'	804 bp
	R 5'-CCTGATGGTCAGGACCTG-3'	

Table 2. Demographic characteristics of BPD infants and controls

	BPD infants (n = 134)	Controls (n = 168)	χ^2 or t	*P* value
Male:Female	81:53	97:71	0.226	0.634
GA (week)	28.18 ± 1.39	28.29 ± 1.52	0.649	0.517
BW (kg)	1.16 ± 0.22	1.18 ± 0.21	0.805	0.421
Apgar 1 min	7.10 ± 3.05	7.21 ± 2.65	0.335	0.737
Apgar 5 min	8.02 ± 2.01	8.11 ± 1.78	0.412	0.681

In this study, we examined the 10 encoding exons sequences of SP-B in Chinese Han preterm infants and sought to test the hypothesis that the SP-B genetic polymorphisms affect susceptibility towards BPD.

Materials and methods

Study population

Subjects in this study were infants receiving obstetrical care at the BaYi Children's Hospital at General Hospital of Beijing PLA, which were recruited sequentially between Jan 2009 and Dec 2011. All subjects were unrelated ethnic Han Chinese neonates in Beijing and its surrounding regions. The entry criteria were: (i) birth weight 500–1499 g; (ii) preterm birth at < 32 weeks of gestational age; and (iii) absence of major congenital defects. The BPD group consisted of 134 neonates, all of them at the 28 days category met the Bancalari et al [26] criteria of: (i) supplemental oxygen requirement 28 days after birth; (ii) persistent abnormalities in the chest radiograph; and (iii) tachypnea in the presence of rales or retractions. The control group consisted of 168 neonates with non-pulmonary diseases such as congenital heart disease, persistent pulmonary hypertension and shock. Both groups matched with Baseline demographic data like gestational age (GA), birth weight (BW), 1-minute Apgar scores, 5-minute Apgar scores and sex.

For each participant, a neonatal peripheral blood sample was collected into tubes containing EDTA immediately after delivery. We stored the whole blood samples at 4°C upon collection. Then, all whole blood samples were stored at -80°C until genomic DNA extraction. This study was performed with the approval of the Ethical Committee of General Hospital of Beijing PLA and was conducted according to the principles expressed in the Helsinki Declaration. At recruitment, written informed consent was obtained from all participants' guardians.

Laboratory tests

DNA extraction and PCR amplification: The genomic DNA of the neonate was purified from total blood using the QIAamp® DNA Blood Mini Kit (QIAGEN, Valencia, USA) according to manufacturer instructions. All coding exons and the exon–intron boundaries of the SP-B gene were amplified by PCR using standard protocols. The primers for exons of SP-B genes were design by Primer 5 software (see **Table 1**). The PCR mixture (total volume of 50 μL) consisted of 100 ng DNA, 1 × Pyrobest buffer II, 0.4 μM deoxyribonucleotide triphosphates (dNTPs), 0.4μM of each primer, and 1.25 U Pyrobest DNA Polymerase (Takara BioTech, 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 1min at 72°C with a final elongation of 7 min at 72°C. The PCR products were identified by agarose gel electrophoresis. PCR products were sequenced using the Big Dye Terminator Cycle Ready Reaction Kit V.1.0 (Applied Biosystems, Foster City, CA) on an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA) and analyzed with Chromas software (http://www.technelysium.com.au/chromas.html).

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) and compared with student's *t*-test. The fitness to Hardy-Weinberg

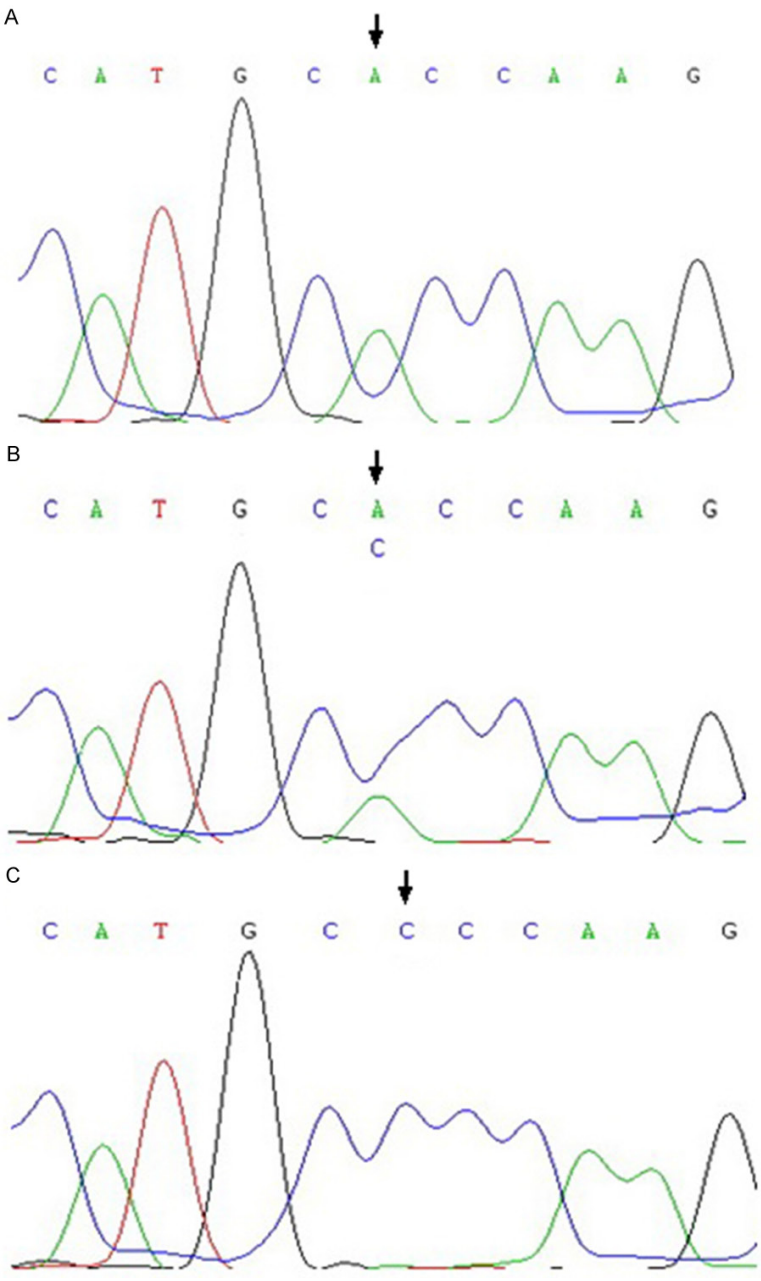

Figure 1. Sequence chromatograms of SP-B -18C/A genotypes. A. A/A homozygous genotype. B. C/A heterozygous genotype. C. C/C homozygous genotype. Arrows indicate the positions of altered nucleotides.

equilibrium was tested using the random-permutation procedure implemented in the Arlequin package (http://lgb.unige.ch/arlequin). The single allelic and single genotype frequencies in each of the two groups were analyzed using χ^2 test. We used both a dominant and an allelic model to assess BPD neonates and control neonates. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to measure the strength of association. An association was considered significant at a *P* value of < 0.05, and all statistical tests were two-sided. These analyses were performed using SPSS software (version 15.0; SPSS Inc.).

Results

Demographic characteristics

All subjects were of Chinese Han descent. The baseline characteristics of the study population, including 134 BPD infants and 168 controls. There was no significant difference between BPD patients and controls in term of sex, gestational age, birth weight, Apgar 1 min, and Apgar 5 min (**Table 2**).

Distribution of genotypic and allelic frequencies

In this study, three single nucleotide polymorphisms (SNP), -18C/A (rs2077079), 1580C/T (rs1130866), and 4564T/C (rs762548) (**Figures 1-3**) were common identified in SP-B gene.

-18C/A polymorphism

In -18C/A polymorphism, the frequencies of A/A, C/A and C/C genotypes among BPD neonates varied significantly from those among controls (**Table 3**, χ^2 = 10.741, *P* = 0.005 < 0.01). To investigate genotype effects, we used both a dominant model (frequency of the A/A plus C/A genotypes vs. frequency of the C/C genotype) and allelic model (frequency of the A allele vs. frequency of the C allele). For the dominant genetic model, subjects carrying the -18A allele (A/A + C/A genotypes) had an elevated risk of BPD compared to those non-carrying the -18A allele (C/C genotype) (**Table 4**, OR = 1.712, 95% CI = 1.228-2.389, *P* = 0.001 < 0.01). For

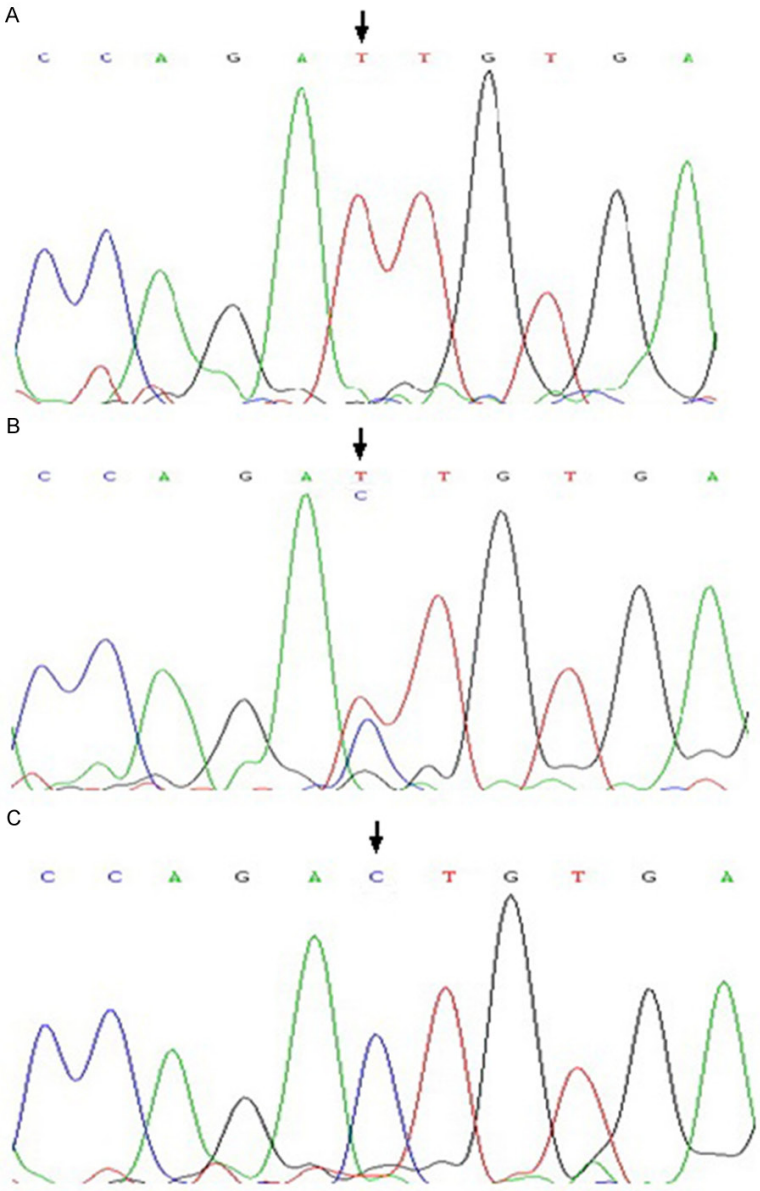

Figure 2. Sequence chromatograms of SP-B1580C/T genotypes. A. T/T homozygous genotype. B. C/T heterozygous genotype. C. C/C homozygous genotype. Arrows indicate the positions of altered nucleotides.

the allelic model, comparing the A allele to the C allele, the -18C/A site also showed an increased risk of BPD (**Table 4**, OR = 1.787, 95% CI = 1.276-2.502, $P = 0.001 < 0.01$).

1580C/T polymorphism

In 1580C/T polymorphism, the frequencies of T/T, C/T and C/C genotypes among BPD neonates varied significantly from those among controls (**Table 5**, $\chi^2 = 7.014$, $P = 0.030 < 0.05$). For the dominant model and the allelic model, subjects carrying the 1580T allele (T/T + C/T genotypes) had a reduced risk of BPD compared to those non-carrying the 1580T allele (C/C genotype) (**Table 6**, OR = 0.752, 95% CI = 0.593-0.954, $P = 0.016 < 0.05$). For the allelic model, comparing the T allele to the C allele, the 1580C/T site also showed a decreased risk of BPD (**Table 6**, OR = 0.706, 95% CI = 0.548-0.909, $P = 0.006 < 0.01$).

4564T/C polymorphism

Although the frequencies of the T/T, C/T and C/C genotypes among BPD neonates were different from the controls in 4564T/C polymorphism, there was still no significant difference between them (**Table 7**, $\chi^2 = 3.399$, $P = 0.183 > 0.05$). And it is also no significant differences between BPD neonates and controls in the allelic frequencies (**Table 7**, $\chi^2 = 3.227$, $P = 0.072 > 0.05$).

Discussion

The human gene encoding SP-B is located on the short arm of chromosome 2, consisting of 11 exons with the first 10 encoding the precursor protein [27]. Translation of SP-B mRNA yields a proprotein (proSP-B) that is processed to a 79-amino acid mature SP-B protein encoded by exons 6 and 7 [28]. Different polymorphisms within the SP-B gene have been described [29]. In the present study, we explored the relationship between polymorphisms of SP-B gene and BPD. Results showed that -18C/A, 1580C/T, and 4564T/C polymorphisms were common identified in Chinese Han infants.

-18C/A polymorphism of SP-B is located in the region 5'UTR between the TATA box and the transcription initiation site, which is in a GC-rich repeat that flanks the TATAA box [29]. Because of the critical location of the polymorphism site

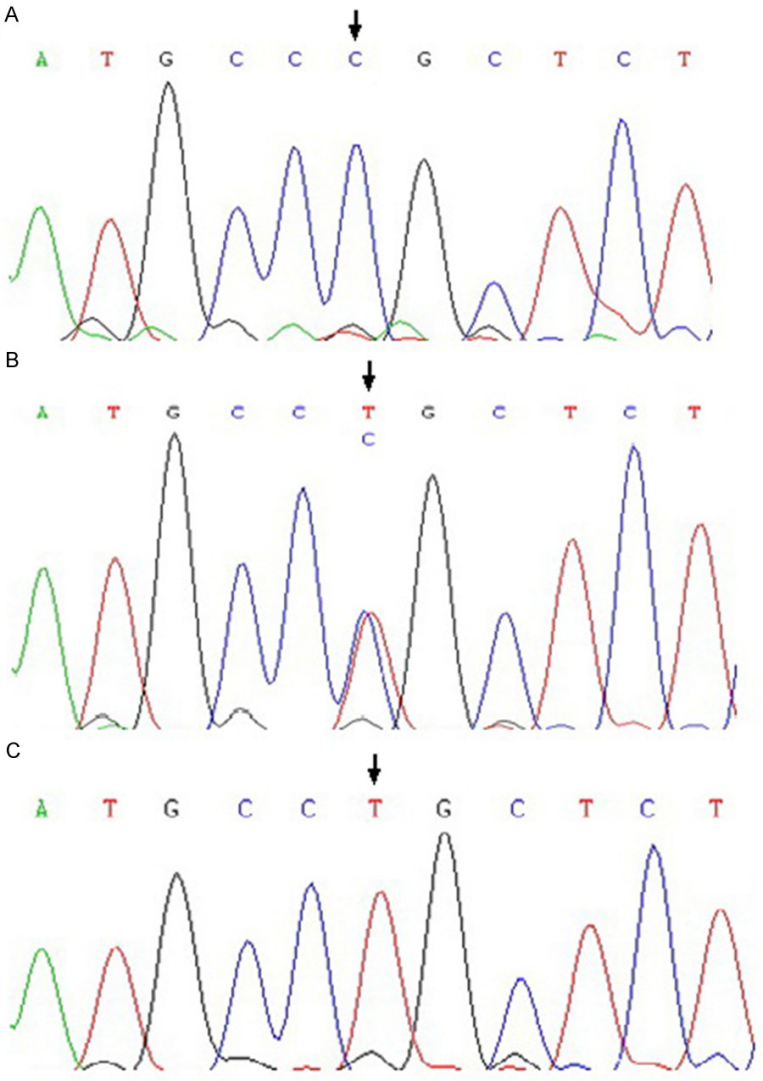

Figure 3. Sequence chromatograms of SP-B 4564T/C genotypes. A. C/C homozygous genotype. B. T/C heterozygous genotype. C. T/T homozygous genotype. Arrows indicate the positions of altered nucleotides.

Table 3. Distribution of the -18C/A genotypes in BPD infants and controls

Groups	Cases (n)	Genotypes		
		AA	CA	CC
BPD infants	134	11 (8.2%)	45 (33.6%)	78 (58.2%)
Controls	168	6 (3.6%)	35 (20.8%)	127 (75.6%)

*χ^2 = 10.741, P = 0.005.

in the promoter, it could functionally impact on the promoter. In the study by Steagall et al [29], Sp1 bound more tightly to the C allele sequence than to the A allele sequence by EMSA. Transcriptional analysis and ELISA on bronchoalveolar lavage fluid revealed that the presence of the C allele correlated with more SP-B promoter activity and protein. There was approximately threefold difference in amounts of SP-B in bronchoalveolar lavage fluid from C/A(-18) and A/A(-18) individuals. SP-B -18C/A polymorphism interfere with the effects of transcription level, resulting in BPD. 1580C/T polymorphism of SP-B is located in the end of exon 4, which alter the translation of amino acid 131 through a substitution from Thr (ACT) to Ile (ATT) [30]. The Thr-Ile change at amino acid 131 can block potential N-linked glycosylation sites [31]. Wang et al [32] showed that the C allele variant is indeed glycosylated at the Asn129-Gln-Thr131 site, whereas the T allele variant is not. N-linked glycosylation would interfere with SP-B processing, secretion and folding, resulting in modulating protein levels or functions of SP-B protein. In the literature, most studies suggest that the C/C genotype might be associated with a greater risk of pulmonary disease. There are considerable ethnic differences in allele frequency of 1580C/T of the SP-B gene. The frequency of the C allele is reported to be 0.3 in black subjects, 0.5 in white and Hispanic subjects, 0.73 in Japanese population, 0.71 in our study, which suggest that Asian people are at a genetic disadvantage for pulmonary defense [33]. 4564T/C of SP-B is located in the splice site of exon 7, so it would cause abnormal splicing and promote mRNA degradation.

In conclusion, we found that SP-B -18C/A and 1580C/T polymorphisms were associated with the risk of BPD in a Chinese population but not in 4564T/C polymorphism. The 1580C/T polymorphism protect against the development of BPD while the -18C/A polymor-

Table 4. Relative risk estimate on the basis of the distribution of the -18C/A genotypes and alleles in BPD infants and controls

	BPD infants (%) (n = 134)	Controls (%) (n = 168)	*P* value	Crude OR (95% CI)
Genotypes				
Carrying A (AA + CA)	56 (41.8)	41 (24.4%)	0.001	1.712 (1.228-2.389)
Not-carrying A (CC)	78 (58.2)	127 (75.6%)		
Alleles				
A allele	67 (25.0%)	47 (14.0%)	0.001	1.787 (1.276-2.502)
C allele	201 (75.0%)	289 (86.0%)		

Table 5. Distribution of the 1580C/T genotypes in BPD infants and controls

Groups	Cases (n)	Genotypes		
		TT	CT	CC
BPD infants	134	10 (7.5%)	47 (35.1%)	77 (57.5%)
Controls	168	24 (14.3%)	71 (42.3%)	73 (43.5%)

*$\chi^2 = 7.014$, $P = 0.030$.

Table 6. Relative risk estimate on the basis of the distribution of the 1580C/T genotypes and alleles in BPD infants and controls

	BPD infants (%) (n = 134)	Controls (%) (n = 168)	*P* value	Crude OR (95% CI)
Genotypes				
Carrying T (TT + CT)	57 (42.5%)	95 (56.5%)	0.016	0.752 (0.593-0.954)
Not-Carrying T (CC)	77 (57.5%)	73 (43.5%)		
Alleles				
T allele	67 (25.0%)	119 (35.4%)	0.006	0.706 (0.548-0.909)
C allele	201 (75.0%)	217 (64.6%)		

Table 7. Distribution of the 4564T/C genotypes and alleles in BPD infants and controls

	BPD infants (%) (n = 134)	Controls (%) (n = 168)	χ^2	*P* value
Genotypes			3.399	0.183
CC	6 (4.5%)	3 (1.8%)		
TC	41 (30.6%)	42 (25.0%)		
TT	87 (64.9%)	123 (73.2%)		
Alleles			3.227	0.072
C Allele	53 (19.8%)	48 (14.3%)		
T Allele	215 (80.2%)	288 (85.7%)		

phism increased the risk for BPD. Further studies using a larger population in investigating the role for polymorphisms in SPs as contributors to the development of BPD remain necessary. If confirmed by larger sample sizes studies, our findings of genetic factors contributing to the pathogenesis of BPD may have implications for the screening and treatment of this disorder.

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Disclosure of conflict of interest

None.

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