

Comparison of surfactant protein B polymorphisms of healthy term newborns with preterm newborns having respiratory distress syndrome

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Abstract

Polymorphisms and mutations in the surfactant protein B (SP-B) gene have been associated with the pathogenesis of respiratory distress syndrome (RDS). The objective of the present study was to compare the frequencies of SP-B gene polymorphisms between preterm babies with RDS and healthy term newborns. We studied 50 preterm babies with RDS (inclusion criteria - newborns with RDS and gestational age between 28 and 33 weeks and 6 days), and 100 healthy term newborns. Four SP-B gene polymorphisms were analyzed: A/C at nucleotide -18, C/T at nucleotide 1580, A/G at nucleotide 9306, and G/C at nucleotide 8714, by PCR amplification of genomic DNA and genotyping by cRFLP. The healthy newborns comprised 42 female and 58 male neonates; 39 were white and 61 non-white. The RDS group comprised 21 female and 29 male preterm neonates; 28 were white and 22 non-white. Weight ranged from 640 to 2080 g (mean: 1273 g); mean gestational age was 31 weeks and 2 days (range: 28-33 weeks and 6 days). When white children were analyzed separately, a statistically significant difference in the G/C polymorphism at 8714 was observed between groups ($P = 0.028$). All other genotype frequencies were similar for both groups when sex and race were analyzed together. Analysis of the SP-B polymorphism G/C at nucleotide 8714 showed that among white neonates the GG genotype was found only in the RDS group at a frequency of 17% and the GC genotype was more frequently found in healthy term newborns. These data demonstrate an association of GG genotype with RDS.

Key words

- Polymorphisms
- Surfactant protein B
- Respiratory distress syndrome

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Publication supported by FAPESP.

Received July 13, 2006
Accepted February 27, 2007

Introduction

Pulmonary surfactant is a lipid-protein complex essential for normal lung function, responsible for reducing the superficial tension of the air-liquid interface of the alveoli, thus preventing lung collapse at the end of

expiration (1). Avery and Mead (1) showed in 1959 that pulmonary surfactant deficiency is a major factor in the pathophysiology of respiratory distress syndrome (RDS).

Surfactant is a mixture of lipids (~90%) and proteins (~10%). Phospholipids represent 80-90% of the surfactant lipids, of which

phosphatidylcholine is the most important, accounting for 70-80% of the total. Dipalmitoylphosphatidylcholine represents about 60% and is the principal surface tension-lowering component of surfactant (2). Four proteins are associated with the surfactant complex: SP-A, SP-B, SP-C, and SP-D, playing important roles in surfactant function and metabolism (3).

Surfactant function is severely impaired in life-threatening lung diseases, including neonatal RDS. Clinical, epidemiological and biochemical evidence has strongly suggested that RDS is a multifactorial and multigenic disease, and the surfactant proteins might be implicated in this genetic variation (4-6).

SP-B is a hydrophobic protein secreted by type II cells in the lung and is essential for normal pulmonary function (7,8). It is required for synthesis of lamellar bodies and the reduction of surface tension at the air-liquid interface. SP-B is necessary for the formation of the lungs and tubular myelin, and for the processing of SP-C. Absence of SP-B in animals and humans results in respiratory failure and death shortly after birth (9-13).

Several studies have evaluated the association of SP-A and SP-B gene polymorphisms with RDS (14-21), but the functional consequences of the allelic variations of the SP genes are not well understood, and clarification of the genetic diversity is a challenge for the future (5).

The study of the genetic variation of surfactant proteins can help understand individual variability in the susceptibility to the development of pulmonary pathologies. These genetic variants can be valuable markers in the mapping of several pathologies, particularly for the respiratory distress syndrome.

The aims of the present study were to determine and compare the frequencies of four SP-B gene polymorphisms (A/C at nucleotide -18, C/T at nucleotide 1580; A/G at nucleotide 9306, G/C at nucleotide 8714)

in Brazilian preterm babies in RDS and healthy term newborns.

Subjects and Methods

Study population

This is a sectional study in which the samples and information were prospectively collected. The study population consisted of 100 healthy term newborns with no apparent disease and 50 preterm newborns with a diagnosis of RDS. Informed consent was obtained from the parents. The study protocol was approved by the Ethics Committees of the participating centers: Neonatal Intensive Care Unit of the Children's Institute, University of São Paulo, Brazil and Santa Marcelina Maternity, a collaborative center in São Paulo, during the period from June 2001 to July 2004.

The inclusion criteria for the term newborn group were healthy term neonates with a gestational age ≥ 37 weeks, with no respiratory insufficiency or other sign or symptom of disease at delivery. The inclusion criteria for the RDS group were preterm newborns with a diagnosis of RDS (22) and gestational age between 28 and 33 weeks and 6 days.

The diagnosis of RDS was made on the basis of clinical and radiological criteria: presence of signs and symptoms of respiratory distress (grunting, intercostal retractions, nasal flaring, cyanosis, and tachypnea), a chest radiograph with a diffuse reticulogranular pattern, and air bronchograms.

Term newborns with early respiratory distress, genetic syndromes, congenital malformations, and mothers with infection were excluded, as were preterm newborns with other associated diseases, including genetic syndromes, congenital malformations and mothers with infection.

We studied 150 neonates who were classified in two groups: healthy term newborns and RDS preterm newborns. The first group consisted of 100 apparently healthy term

newborns; 42 (42%) were females and 58 (58%) males; 39 (39%) were white and 61 (61%) non-white. Weight ranged from 2280 to 4740 g (mean: 3239.9 g), and gestational age ranged from 37 to 41 weeks and 6 days (mean: 39 weeks and 3 days).

The RDS group consisted of 50 preterm neonates; 21 (42%) were females and 29 (58%) males; 28 (56%) were white and 22 (44%) non-white. Weight ranged from 640 to 2080 g (mean: 1273 g); mean gestational age was 31 weeks and 2 days (range: 28 to 33 weeks and 6 days).

The maternal diseases identified in the RDS group are pregnancy-induced hypertension, systemic hypertension, diabetes mellitus, uterine anomalies, and systemic lupus erythematosus. Forty-four (88%) mothers of preterms with RDS did not use steroids, while only 6 (12%) used this medication.

Laboratory tests

Blood sample collection. Total blood from the term newborn group was drawn from the umbilical cord after clamping at the time of delivery. This procedure did not change the quality of the assistance provided to the baby and the mother. We collected 3 mL of blood that was placed in a tube with EDTA.

Blood collection from newborns of the RDS group was done in the neonatal care unit at the time of other routine exams. Blood samples were placed in tubes with EDTA. All samples were kept at 4°C until DNA extraction.

DNA extraction and PCR amplification. Genomic DNA of the newborns was purified from total blood using the Wizard Genomic DNA Purification Kit® (Promega, Madison, WI, USA) according to the manufacturer specifications. DNA amplification from the blood samples of patients and healthy newborns was done using PCR amplification protocols, as described by Lin et al. (23).

A DNA sequence of 10,751 bp spanning

all SP-B genes, including the 5' and 3' flanking regions, was amplified using the "Expand Long Template PCR system" (Roche, Mannheim, Germany). The primers used were specific for SP-B - sense primer 536 and antisense primer 535 (23,24). The PCR assay mixture (total volume of 50 µL) contained 100 ng/µL DNA, 1X "PCR buffer", 2.0 mM MgCl₂, 1.5 mM dNTPs (Promega), 150 ng of sense primer 536 and of anti-sense primer 535, and 0.75 µL of the enzyme Expand. PCR cycles consisted of one cycle at 95°C for 2 min, followed by 10 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 10 min, and then 20 cycles at 95°C for 30 s, 62°C for 10 min, and 68°C for 12 min, with a final 20-min extension at 68°C.

For amplification of the segments that span the polymorphisms cited above we used primers and protocols described in the literature (23,24). All primers are listed in Table 1.

The PCR product obtained in the first PCR was used as substrate for amplification of fragments with the polymorphic sites (Table 2). The PCR mixture (total volume of 30 µL) consisted of 1 µL of the 11-kb PCR product, 0.2 µM of each primer, 0.15 mM dNTPs, 1X PCR buffer, and 0.15 µL AmpTaq (Roche). The cycles consisted of 95°C for 2 min followed by 5 cycles of 95°C for 30 s, 50°C for 1 min, and 70°C for 1 min, followed by 30 cycles of 95°C for 30 s, 55°C for 1 min,

Table 1. Primers used in the present study.

Primers	Direction	Sequence 5' → 3'
535	Anti-sense	GCGACTAGTCTATGACGTCTGCTTCTCTGCCAAGGGAGT
536	Sense	GCGGTGCACTCATCATGGTACTAATTTGCCCGTCCA
556	Sense	GTCCAGCTATAAGGGGCCGTG
95A	Anti-sense	GTGAGTGGTGAGCTGCCTA
133	Sense	CTCGAATTCAGTCTGTAAGTCCAGCACCC
584	Anti-sense	GTGAGCTTGCAGCCCTCTCA
101	Sense	CTCGAATTCAGGACATACACACAGTCCCT
102	Anti-sense	CCAGCTGAGCTTTCAGCAGA
557	Sense	CTGTGTAATACAATGTCTGCACTA
121	Anti-sense	CTCGAATTCAGTCTGATTGCAGGTGTGA

Nucleotide positions according to Pilot-Matias et al. (25). The sequences of the primers have been reported by Lin et al. (23,24).

and 70°C for 1 min. The final extension was at 72°C for 2 min.

Genotyping of SP-B polymorphisms. We analyzed four SP-B polymorphisms: A/C at nucleotide -18, C/T at nucleotide 1580, A/G at nucleotide 9306, and G/C at nucleotide 8714. The genotypes were defined on the basis of the analysis of the PCR products obtained from the restriction enzyme reactions (PCR-based converted restriction fragment length polymorphism), as described by Lin et al. (23,24). Six microliters of the second PCR product was subjected to digestion with the restriction enzymes *ApalI*, *NlaIII*, *DdeI*, *BfaI*, according to manufacturer specifications. The digested products were identified after 8 or 10% polyacrylamide gel electrophoresis.

Statistical analysis

Descriptive statistic was used to describe categorical and numerical variables: frequency distribution for categorical variables and central trend measures, and variability measures for numerical variables.

Inferential analysis was done taking into account three possibilities: first, both variables (sex and race) were considered while comparing the RDS and healthy newborn groups; second, only sex was considered as an important variable while comparing both groups; third, only race was the important variable. The two groups were compared by the chi-square test or Fisher exact test when

appropriate. The chi-square test was also used to identify which groups were not in Hardy-Weinberg equilibrium. We considered both groups with no control variable and then with race being the control variable.

Results

Polymorphisms

G/C at nucleotide 8714. The amplification of the genomic DNA segment with subsequent genotyping was successful in the 100 healthy term newborns and in 43 patients. The reaction was not successful in 7 samples. Overall, there were no statistically significant differences in polymorphisms between the healthy term group and RDS patients (Figure 1A). Nevertheless, when the distribution of the polymorphism was stratified according to race, there was a statistically significant difference among individuals of the white race: the GC genotype was present in 67% of the healthy term group and in 50% of RDS preterm newborns, while the GG genotype was not detected in the healthy term babies and was present in 17% of the RDS newborns ($P = 0.028$; Figure 1B). There were no statistically significant differences between groups when the analysis was done after stratification according to sex.

C/T at nucleotide 1580. The reaction for the analysis of this polymorphism was successful in the 100 healthy babies and in 45 of 50 patients. Overall, there were no statistically significant differences among genotypes between groups (Figure 1C). When the distributions of the polymorphisms were stratified according to race and/or sex there were also no significant genotype differences.

A/G at nucleotide 9306. We were able to genotype 100 term babies and 49 RDS patients for this polymorphism. There were no statistically significant genotype differences

Table 2. Location of the polymorphic sites analyzed.

Polymorphism	Localization in the gene	Nucleotide		Primers	PCR fragment	Restriction enzyme
		Position	Change			
A/C -18	5'flanking	18	A/C	556/95A	167 bp	<i>ApalI</i>
C/T 1580	Exon 4	1580	C/T	133/584	270 bp	<i>DdeI</i>
A/G 9306	3'UTR	9306	A/G	557/121	129 bp	<i>BfaI</i>
G/C 8714	3'UTR	8714	G/C	101/102	784 bp	<i>HinfI</i>

Nucleotide positions are according to Pilot-Matias et al. (25). The sequences of the primers have been reported by Lin et al. (23,24).

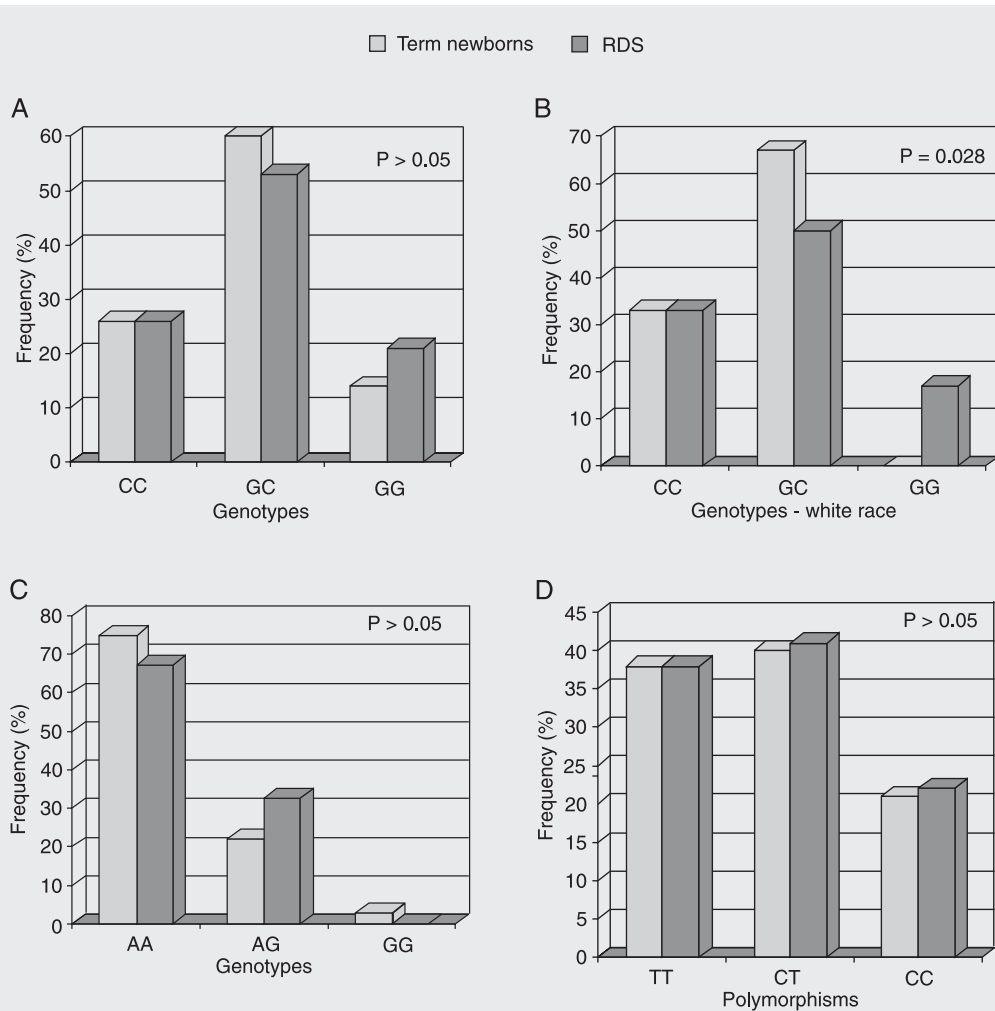


Figure 1. Frequencies of the G/C 8714 SP-B genotypes. **A**, Comparison of newborns with respiratory distress syndrome (RDS) and healthy term newborns. The relative frequencies of the SP-B genotypes (C/C, G/C, and G/G) are shown for the groups under study. **B**, Frequencies of the G/C 8714 SP-B genotypes. Comparison of white newborns with RDS and white healthy newborns. The relative frequencies of the SP-B genotypes (C/C, G/C, and G/G) are shown for the two groups under study. **C**, Frequencies of the C/T 1580 SP-B genotypes. Comparison of newborns with RDS and healthy term newborns. The relative frequencies of the SP-B genotypes (T/T, C/T, and C/C) are shown for the two groups under study. **D**, Frequencies of the A/G 9306 SP-B genotypes. Comparison of newborns with RDS and healthy term newborns. The relative frequencies of the SP-B genotypes (A/A, A/G, and G/G) are shown for the two groups under study. **E**, Frequencies of the A/C at -18 SP-B genotypes. Comparison of newborns with RDS and healthy term newborns. The relative frequencies of the SP-B genotypes (A/A, A/G, and G/G) are shown for the two groups under study.

between the healthy term group and the RDS group (Figure 1D) even when the distributions of the polymorphisms were stratified according to race and/or sex.

A/C at nucleotide -18. The reaction for the analysis of polymorphism A/C at nucleotide -18 was successful in the 100 term babies and in 45 patients and was unsuccessful in 5 patient samples. There were no statistically significant differences in genotypes between the healthy group and the RDS group (Figure 1E).

Discussion

RDS represents the most frequent form of respiratory insufficiency in the preterm newborn, and is still a major cause of morbidity and mortality in this group of patients (26,27). However, significant variations in pulmonary outcomes of similar infants with comparable exposures to oxygen, mechanical ventilation and nutritional deficiencies suggest that genetic factors also contribute to different pulmonary outcomes (28). RDS is considered to be a result of complex interactions between several environmental and genetic factors associated with prematurity, sex, race, and the presence of maternal diseases (5,29). Some genetic variants of surfactant proteins, particularly SP-A and SP-B, were identified as risk factors or protectors in the etiology of RDS (11,14,15,30).

The present study analyzed four SP-B polymorphisms (G/C at nucleotide 8714; C/T at nucleotide 1580; A/G at nucleotide 9306, and A/C at nucleotide -18) in healthy term newborns and in preterm babies with RDS in a Brazilian sample, as well as the association of these polymorphisms with sex and race.

The G/C polymorphism at nucleotide 8714 is located in the 3'UTR of the SP-B gene, corresponding to the flanking region. Although it is located out of the protein translation site, this region can somehow have an impact on the gene expression and/

or protein function (24).

We did not find a statistically significant difference in the distribution of the genotypes of polymorphism G/C at nucleotide 8714 between healthy term newborns and the RDS group when the variables sex and race were analyzed together or when sex was analyzed separately. However, when race was analyzed separately, in the white individuals the GG genotype was only found in 17% of the RDS group. These findings suggest that the GG genotype might be a risk factor for RDS and that the GC genotype might be a protective factor against the development of the disease in the white race. On the other hand, Lin et al. (24), in a study of a family in which 14 newborns had died because of early respiratory insufficiency, observed that the G/C polymorphism at nucleotide 8714 seemed not to be responsible for any important alteration with a physiopathologic impact in this population. Therefore, our results indicate the need for other studies to identify the role of this genotype in RDS.

The C/T polymorphism at nucleotide 1580 is located at the end of exon 4, at nucleotide 1580 and can alter the translation of amino acid 131 through a substitution from threonine (ACT) to isoleucine (ATT) (23). This change eliminates a potential N-linked glycosylation site but the real consequences of this alteration are not known (17,31). In the literature, most studies suggest that the C/C genotype might be associated with a greater risk of pulmonary disease. We did not find any differences in the frequencies of genotypes CC, CT and TT when the healthy term group and the RDS group were compared. This might be attributed either to ethnic differences between the studied population and individuals analyzed in other countries, or to sample size. Liu et al. (32) have reported that ethnic background is an important risk factor to be considered in analytical studies of allele and genotype frequencies. These investigators analyzed

the similarity of genetic markers among populations of three different ethnic groups (Caucasian, Black, and Hispanic) in order to determine whether individuals of different races or ethnic groups could be grouped together in linkage studies. The results showed that the allele and genotype frequencies can differ among distinct ethnic groups, especially between ethnic groups of different races.

The A/G polymorphism at nucleotide 9306 is located in the 3'UTR. Previous studies have suggested that SP-A and SP-B may functionally interact *in vitro* in a synergistic way that reduces alveolar tension (33). The analysis of the interaction between SP-A and SP-B might be useful to evaluate the presence of a greater or smaller risk to develop RDS in association with the A/G 9306 genotype in the studied population (17). The frequency of the A/G genotype in white male and in non-white female preterm neonates with RDS compared to term newborns was not different.

The A/C polymorphism at nucleotide -18 is located in the 5'UTR and may have an impact on the initiation of mRNA transcription. Floros et al. (16) evaluated the association of SP-A and SP-B polymorphisms in white and African-American newborns with RDS in a case-control study and observed a higher frequency of genotype A/A of the A/C -18 polymorphism in African-American neonates. This observation was made in preterm newborns with RDS and a gestational age of 28 and 31 weeks (17). We could not find any statistically significant difference in the distribution of the genotypes of the A/C -18 polymorphism between healthy term newborns and RDS patients.

In summary, this study showed a higher frequency of genotype GG at nucleotide 8714 in the white newborns with RDS compared to white healthy term newborns, while genotype GC was more frequent in healthy term neonates. This suggests that genotype GG might be associated with RDS and reinforces the need for other studies to address this issue.

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