

Invited Paper

Surfactant protein (SP) B associations and interactions with SP-A in white and black subjects with respiratory distress syndrome

JOANNA FLOROS,^{1,2} RUZONG FAN,³ SUSAN DIANGELO,¹ XIAOXUAN GUO,¹ JOHN WERT¹
AND JUNMING LUO¹

Departments of ¹Cellular and Molecular Physiology, ²Pediatrics, and ³Health Evaluation Sciences, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania

Abstract

Background: The etiology of respiratory distress syndrome (RDS) is multifactorial and/or multigenic. Surfactant protein A (SP-A) and/or SP-B genetic variants have been identified as risk or protection factors for RDS.

Methods: We genotyped subjects with and without RDS for the SP-B intron 4 size variants (invariant (inv), deletion (del), insertion (ins) and for four (–18 (A/C), 1013 (A/C), 1580 (C/T), 9306 (A/G)) SP-B single nucleotide polymorphisms (SNP), to study case–control associations in black and white subjects. We also determined whether specific SP-B variants interact with RDS susceptibility or protective SP-A variants to enhance or reduce risk for RDS.

Results: Based on odds ratio: (1) the SP-B intron 4 del variant in white subjects is more of an RDS risk factor for males and for subjects of 28 weeks < gestational age (GA) < 33 weeks; (2) the SP-B intron 4 ins variant in black subjects is more of an RDS risk factor in females; (3) in white subjects, SP-A1 (6A²/6A²) or SP-A2 (1A⁰/1A⁰ or 1A⁰/*) genotypes in subjects of certain GA and with a specific SP-B genotype (9306 (A/G) or del/*) are associated with an enhanced risk for RDS; (4) in black subjects, SP-A1 (6A³/6A³ or 6A³/*) genotypes in subjects of 31 weeks ≤ GA ≤ 35 weeks and with the SP-B (1580 (T/T)) genotype are associated with a reduced risk for RDS.

Conclusions: The SP-B polymorphisms are important determinants for RDS. These may identify differences between black and white subjects, as well as, between males and females regarding the risk for RDS. Furthermore, SP-A susceptibility or protective alleles, in specific SP-B background, are associated, based on OR, with an increased or reduced risk for RDS.

Key words

gene interactions, genetic determinant, respiratory distress syndrome, surfactant protein B, synergism.

It has been well established that deficiency of pulmonary surfactant (a lipoprotein complex) leads to respiratory distress syndrome (RDS) in a prematurely born infant. It has also been well documented that although surfactant replacement therapy has reduced the number of deaths from RDS and/or has ameliorated the severity of symptoms, it has not eliminated the incidence of RDS. Thus, in spite of pulmonary surfactant replacement therapy and/or other therapies (i.e. maternal steroid treatment), RDS remains a cause for concern. This observation supports the notion that

the etiology of RDS is multifactorial and/or multigenic and that genetics may play a role in its pathogenesis.^{1–3}

Both lipid and protein components of surfactant have been studied extensively with regards to their role in the pathogenesis of RDS and have been used as markers for predicting the risk for RDS. In the last several years, the surfactant protein genes have been used as candidate genes in the study of the genetics of RDS. A number of case–control association studies of unrelated individuals or family-based association studies have identified surfactant protein A (SP-A) and SP-B genetic variants as risk or protective factors for RDS, and have linked the SP-A and SP-B loci to RDS.^{4–8}

Surfactant protein B has been shown to be essential for normal lung function by both animal studies and human studies.⁹ A number of variants and mutations have been

Correspondence: Joanna Floros PhD, Professor, Department of Cellular and Molecular Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, PO Box 850, 500 University Drive, H166, Hershey, PA 17033, USA.
Email: jfloros@psu.edu

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Table 1 Characteristics of the study group for SP-B case-control associations

Race	Group	No. subject	No. sex	No. steroid treatment [†]	GA range	Mean of GA	SD of GA
White	Control	331	145F:186M	194S:120NS [‡]	28 < GA	33.65	2.73
	RDS	203	77F:124M [§]	103S:90NS ^{‡‡}	28 < GA	32.60	2.73
Black	Control	38	25F:12M [§]	27S:10NS ^{§§}	28 < GA < 35	32.28	1.51
	RDS	40	18F:22M	22S:16NS ^{¶¶}	28 < GA < 35	30.59	1.56

F, female; GA, gestational age in weeks; M, male; NS, no steroid treatment; RDS, respiratory distress syndrome; S, steroid treatment; SP-B, surfactant protein B. [†]Maternal steroid therapy received 24–168 h before birth. [‡]The sex of two subjects is missing. [§]The sex of one subject is missing. [¶]Steroid status is missing for 17 subjects. ^{‡‡}Steroid status is missing for 10 subjects. ^{§§}Steroid status missing for one subject. ^{¶¶}Steroid status is missing for two subjects.

identified for SP-B.¹⁰ The SP-B genetic variants may compromise levels and/or functional capabilities of SP-B, as suggested by animal studies of heterozygous (+/–) SP-B knockout mice.¹¹ The levels of SP-B were lower than those in the wild type and small, but significant physiological abnormalities (i.e. decreased compliance and increased air-trapping in the distal airspaces) were observed under normal conditions. Surfactant protein B is encoded by a single gene and in humans the SP-B gene has been localized on the short arm of chromosome 2.¹² An SP-B intron 4 genetic size variant has been identified in a case-control study of mixed racial composition as a risk factor for RDS.⁴ This size variant characterized by insertion or deletion of certain motifs, appeared to have race-related characteristics. Although the insertion (ins) variant was more frequently found in black subjects and the deletion (del) variant was more frequently found in white subjects, no specific association between the ins variant and RDS in black subjects or between the del variant and RDS in white subjects could be made in the previous study. Furthermore, no association of the SP-B intron 4 variant and RDS could be made in a study group of Finnish subjects. Whether an inadequate sample size in the former and a low frequency of intron 4 variant in the Finnish population contributed to lack of association, is unknown.^{4,13}

The SP-A plays a role in aspects of surfactant physiology and structure,¹⁴ as well as in innate host defense and regulation of inflammatory processes in the lung.¹⁵ The human SP-A locus includes two linked functional genes, SP-A1 and SP-A2, in opposite transcriptional orientation.¹⁶ Several genetic variants (alleles) have been characterized for each SP-A gene with 10 of these found frequently in the general population.¹⁷ Certain SP-A variants have been identified as risk or protective factors for RDS in several studies of homogeneous or mixed ethnic origin. The SP-A2, 1A⁰ allele, and the SP-A1, 6A² allele, as well as the corresponding haplotype 1A⁰/6A² have been identified as risk factors in association studies of unrelated individuals and in family-based association using transmission disequilibrium test (TDT) analysis.^{5–7} Moreover, the SP-A1 and SP-A2 loci

have been linked to RDS by TDT and extended TDT analyses.⁷ Protective SP-A alleles have also been identified by either association studies of unrelated individuals^{5–7} or by family-based associations. These include alleles 6A³, 6A⁴, and 1A⁵.

Surfactant protein-A and SP-B may interact functionally, as assessed by *in vitro* studies, and may work synergistically to reduce surface tension.¹⁸ Both SP-A and SP-B are necessary for the tubular myelin (TM) formation, a structural form of surfactant.^{19,20} The levels of SP-A and SP-B, as well as those of TM, are significantly reduced or absent in babies with RDS.^{21–23} In one study, the combined frequency of certain SP-A and SP-B alleles was found to be significantly higher in babies with RDS than the frequency of either allele alone, suggesting a synergistic effect of the two marker loci.⁵ In a recent study, an SP-B variant, the frequency of which did not differ significantly between RDS and control, appeared to be a determinant for RDS with certain SP-A alleles in RDS.¹³ In the present study, we wished to investigate (a) associations between SP-B polymorphisms and RDS in white and black subjects and (b) interactions between SP-A and SP-B genetic variants with regards to their potential to modulate risk or protection from RDS in white and black subjects.

Methods

Samples

The samples of RDS and control white or black subjects analyzed in this paper were those of previous studies.^{4,5,7} The samples, as described previously, were obtained according to institutional guidelines for human studies. The characteristics of the study group for the case-control associations of SP-B polymorphisms are shown in Table 1. The characteristics of the study group where SP-A and SP-B marker alleles were studied to identify interactive alleles in the risk or protection from RDS are shown in Table 2.

Table 2 Characteristics of the study group for SP-A and SP-B interactions

Race	Group	No. subject	No. in sex	No. in steroid treatment [†]	GA range	Mean of GA	SD of GA
White	Control	301	129F:172M	181S:120NS	28 < GA	33.57	2.52
	RDS	210	84F:124M [‡]	102S:107NS [§]	28 < GA	32.44	2.57
Black	Control	34	21F:12M [¶]	26S:8NS	28 < GA < 35	32.16	1.59
	RDS	39	17F:22M	24S:15NS	28 < GA < 35	30.52	1.53

F, female; GA, gestational age in weeks; M, male; NS, no steroid treatment; RDS, respiratory distress syndrome; S, steroid treatment; SP-A, surfactant protein A; SP-B, surfactant protein B. [†]Maternal steroid therapy received 24–168 h before birth. [‡]The sex of two subjects is missing. [§]Steroid status is missing for one subject. [¶]The sex of one subject is missing.

Genotype analysis

SP-B intron 4 size variants

The SP-B intron 4 variants are size variants as described previously.⁴ The size of the invariant (inv) allele is approximately 620 bp, and consists in part (first half) of repetitive motifs that include a 20 bp conserved sequence and a variable number of CA dinucleotides. The variant alleles consist of either insertion of motifs or deletion of motifs. In this paper, we denote all intron 4 alleles with a size larger than that of the inv allele as insertion (ins) alleles and those with size lower than the inv allele as deletion (del) alleles. The intron 4 variants (inv, ins, del) were studied for case–control associations in black and white subjects and then for interaction with SP-A alleles. The characteristics of the study group used in the SP-B case–control association study are shown in Table 1.

With the exception of the subset of samples described previously,⁴ the genotyping of the majority of the samples was carried out as described here. Primers 161⁻ and 172⁺ were used in polymerase chain reaction (PCR) with genomic DNA from subjects under study. The sequence of primer 161⁻ is 5'-TGT GTG TGA GAG TGA GGG TGT AAG-3' and the sequence of primer 172⁺ is 5'-CTG GTC ATC GAC TAC TTC CA-3'. The PCR reaction was in 10 µL containing: 40 ng of genomic DNA, 1 × buffer 3 supplied by Roche (Indianapolis, IN, USA) as part of the Explant Long Template PCR kit, 0.625 mM of each dNTP, 10 ng of each oligo 172⁺ and 161⁻, and 0.25 Units of Taq DNA polymerase (Roche). Oligo 161⁻ was labeled with ³²P-γ-ATP by end-labeling and added in the PCR reaction at 1 × 10⁵ cpm/10 µL reaction. The cycling condition was 94°C for 2 min; followed by five cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min 30 s; 21 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min 30 s. The final extension step was at 72°C for 8 min. After PCR, the PCR products were run on 6% polyacrylamide gel electrophoresis gel (PAGE) (1 × Tris-borate EDTA (TBE), 5.6 M urea, 32% formamide) with 0.5x TBE running buffer under 1600 V for 5 h. The gel

was transferred to Whatman 3 mm chromatography paper (Whatman International, Maidstone, England) and covered with plastic wrap. The gel was dried under vacuum on a gel dryer and then exposed to X-ray film at – 80°C.

Single nucleotide polymorphisms of SP-A and SP-B

Genomic DNA samples from RDS and control subjects were used as templates for genotyping. The SP-A genotype analysis was performed as described by DiAngelo *et al.*¹⁷ The SP-B genotyping of the four single nucleotide polymorphisms (–18 (A/C), 1013 (A/C), 1580 (C/T) and 9306 (A/G)) was performed as described by Lin *et al.*^{24,25}

Statistical analysis

We used the SAS program in the Department of Health Evaluation Sciences at the Pennsylvania State University, College of Medicine. Fisher's exact tests and logistic regression analyses were performed to detect association between RDS and SP-B marker alleles, and interaction between SP-A and SP-B marker alleles. A result of *P*-value ≤ 0.05 is considered significant.

Results

Frequency of SP-B intron 4 variants in RDS and control white and black subjects

White subjects

When the del genotype (del/del or del/*) that contains at least one del variant was compared to genotypes (*/*) that did not include any del variant or the del allele was compared to all other alleles, the frequency of the del genotype or allele was significantly higher in subjects with RDS (Fig. 1). This applied to subjects with gestational age (GA) > 28 weeks (Fig. 1a) and 28 weeks < GA < 33 weeks (Fig. 1b) that included both sexes, and of mothers that either

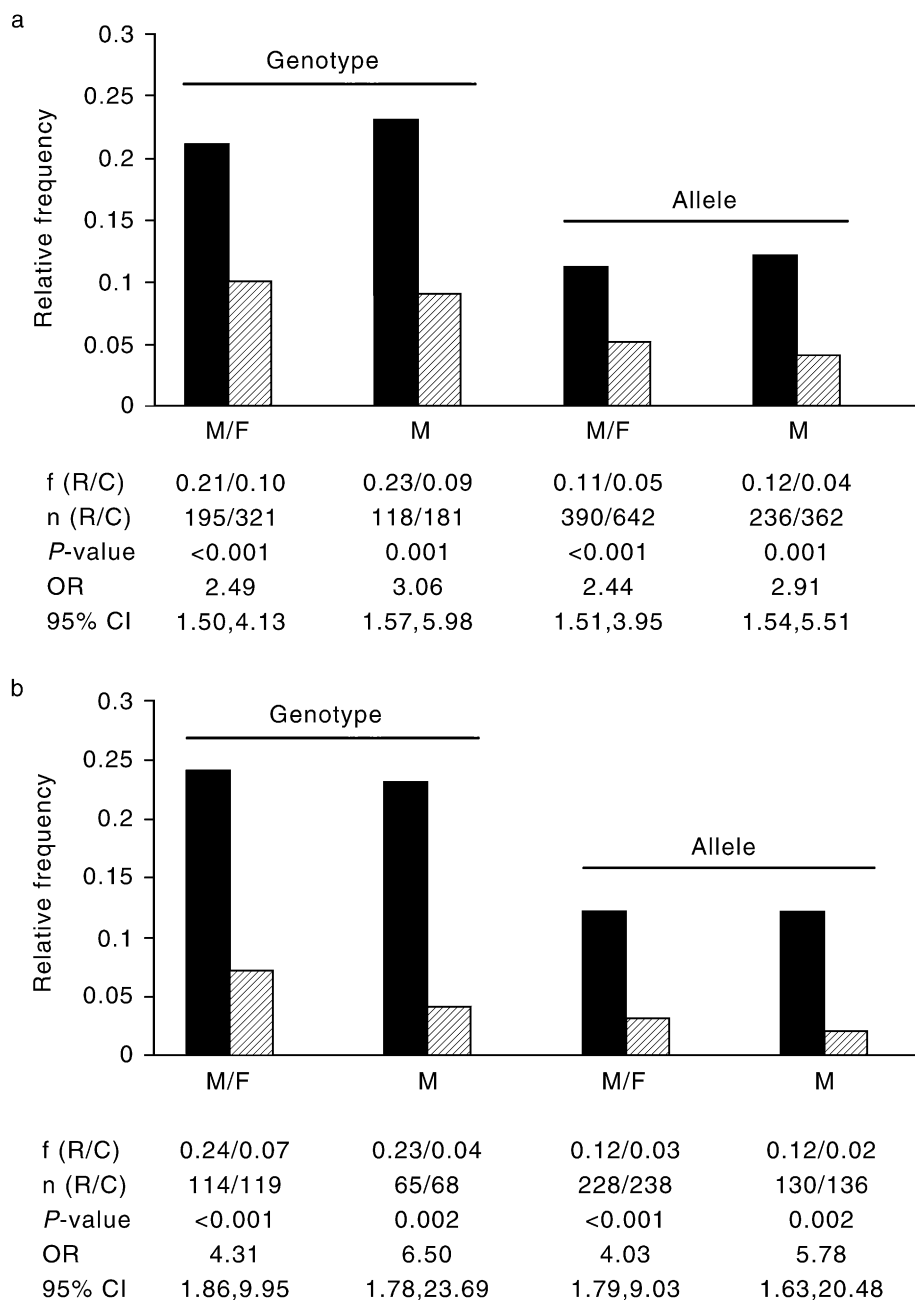
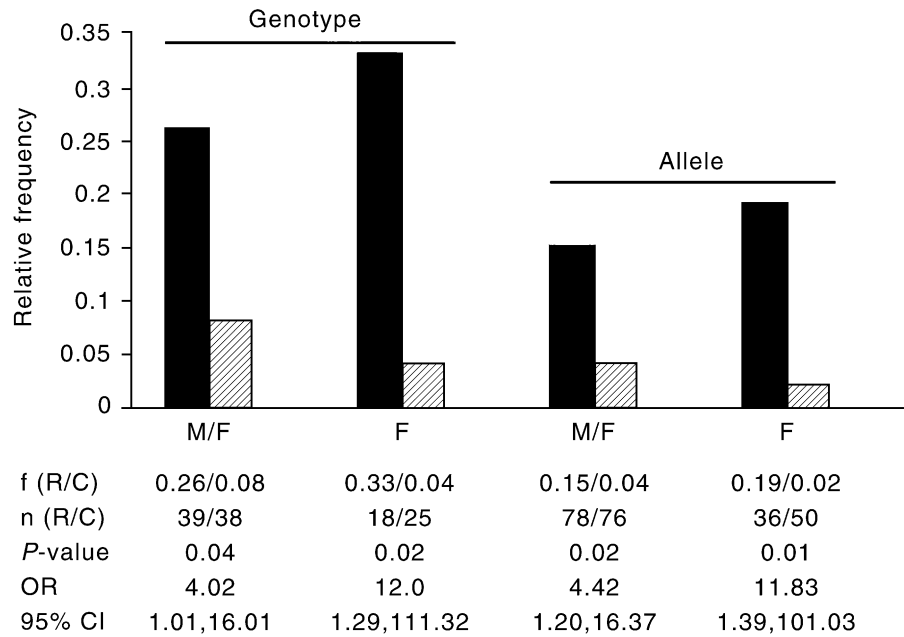


Fig. 1 Frequency of surfactant protein B intron 4 deletion (del) variant in white subjects with and without respiratory distress syndrome (RDS). Groups of two different gestational age (GA) were studied, GA > 28 weeks (Fig. 1a) and 28 weeks < GA < 33 weeks (Fig. 1b). The frequency (f) of the del genotype (del/del or del/*) versus */* or of the del allele versus all other alleles (*) was studied in RDS and the control that included both male (M) and female (F) or male subjects of the two GA. f (R/C), frequency of the given genotype or allele in RDS (R) and control (C); n (R/C), number (n) of genotypes or alleles studied in RDS and control; OR, odds ratio; CI, confidence intervals. A *P*-value of ≤ 0.05 is considered significant. (■), RDS; (▨), control.

had or did not have prenatal steroid therapy. For example, in Fig. 1a the frequency of the del genotype in RDS was 0.21 versus 0.10 in the control ($P < 0.001$) and the frequency of the del allele in RDS was 0.11 versus 0.05 in the control ($P < 0.001$). In younger subjects (28 < GA < 33) the frequency of the del genotype or allele was significantly increased in the RDS group that included both sexes with an odds ratio (OR) > 4.0 compared to an OR of ~ 2.5 when subjects of all GA (> 28 weeks) were included in the analysis. No significant differences were observed in RDS and control subjects with GA ≥ 33 weeks (not shown).

When the analysis was carried out as a function of sex, the frequency of the del genotype or del allele was significantly higher ($P = 0.001$) in males of GA > 28 weeks with RDS (Fig. 1a) and in males of the younger GA group (Fig. 1b). No difference of the del genotype or allele was observed between females of GA > 28 weeks with or without RDS, suggesting that when subjects of both sexes were grouped together, the observations made were accounted for entirely by the male subjects. In the younger age group the OR for the del genotype and del allele was increased (OR = 6.50 and OR = 5.78, respectively) when

Fig. 2 Frequency of surfactant protein B intron 4 insertion (ins) variant in black subjects of 28 weeks < GA < 35 weeks with and without respiratory distress syndrome (RDS). The frequency (f) of the ins genotype (ins/ins or ins/*) versus */* or of the ins allele versus all other alleles (*) was studied in RDS and the control that included both male (M) and female (F) or female subjects. f (R/C): frequency of the given genotype or allele in RDS (R) and control (C); n (R/C): number (n) of genotypes or alleles studied in RDS and control; OR: odds ratio; CI: confidence intervals. A P-value of ≤ 0.05 is considered significant. (■), RDS; (▨), control.



only males were considered in the analysis compared to that when both sexes were considered (Fig. 1b). In this group a trend of differences in the frequency of the del genotype ($P = 0.06$; OR = 2.81, CI: 0.90, 8.82) between RDS ($n = 47$) and control ($n = 51$) females and the del allele ($P = 0.04$; OR = 2.84; CI: 0.96, 8.39; RDS ($n = 94$), control ($n = 102$)) was observed. Also, the OR in the younger group (Fig. 1b) was generally higher than the OR in subjects with GA > 28 weeks (Fig. 1a).

The data in Fig. 1 indicate that sex and GA may be important contributors to RDS. The del variant may be more of a factor in males than in females, as well as in younger subjects. Furthermore, the frequency of the del variant allele was higher in RDS of GA > 28 weeks with ($P < 0.01$; OR = 2.64; CI: 1.37, 5.10, RDS ($n = 196$), control ($n = 372$)) or without ($P = 0.03$; OR = 2.10; CI: 1.01, 4.38; RDS ($n = 174$), control ($n = 236$)) maternal steroid therapy.

Black subjects

The frequency of the genotype (ins/ins or ins/*) with at least one ins allele was higher (Fig. 2) in babies of both sexes with RDS compared to controls of same GA (28 < GA < 35) ($P = 0.04$). Similarly, the frequency of the ins allele was higher in the RDS group ($P = 0.02$). When the analysis was performed as a function of sex and GA, the following observations were made. The frequency of the ins genotype and ins allele was significantly higher in females with RDS with an OR = 12.00 and OR = 11.83, respectively, compared to an OR = 4.02 and OR = 4.42, respectively, when both sexes were considered together. No significant differences were observed in males. Although no significant differences

were observed as a function of GA, this must be reconsidered given the small number of subjects in this study group.

Frequency of SP-B single nucleotide polymorphic (SNP) markers in RDS and control groups

The frequency of four SP-B SNP (- 18 (A/C), 1013 (A/C), 1580(C/T), and 9306 (A/G)) in RDS and control groups was studied. Alleles and genotypes, the frequency of which differed between RDS and control groups of certain GA in white subjects, are shown in Table 3. The A allele and/or A/A genotype of the 1013 (A/C) marker appear to be protective for RDS in subjects of GA > 28 weeks and the A/G genotype of the 9306 (A/G) marker appears to be a susceptibility factor for RDS in subjects of GA ≥ 33 weeks. In the presence of 9306 (A/G) genotype certain SP-A alleles appear to increase risk for RDS (see below). In black subjects, the frequency of the A/A genotype of - 18 (A/C) marker compared to A/C genotype was higher ($P = 0.04$) in subjects with RDS (28 weeks < GA < 31 weeks). However, the number of subjects in this comparison was very low ($n = 19$ RDS, $n = 4$ control). No significant interactions were observed between either one of the other loci and SP-A alleles.

SP-A and SP-B allele interactions

We focused our attention on SP-A and SP-B alleles or genotypes, the frequency of which differed between RDS and control either in previous studies or in the present study. When both male and female subjects with a given SP-B genotype were studied for differences in SP-A genotype in

Table 3 RDS versus control
Marker 1013 (A/C), RDS versus control in white subjects

Comparison	Sex	Steroids [†]	GA in weeks	RDS		Control		<i>P</i> -value	Odds ratio	95% CI
				f	n	f	n			
Allele A vs. allele C	F M	+ -	> 28	0.34	404	0.41	640	0.03	0.77	0.59, 0.99
Allele A vs. allele C	F M	+	> 28	0.35	206	0.44	376	0.02	0.67	0.47, 0.96
A/A vs. C/C	F M	+ -	> 28	0.21	109	0.33	174	0.02	0.55	0.31, 0.96
A/A vs. A/C or C/C	F M	+ -	> 28	0.11	202	0.18	320	0.03	0.59	0.35, 1.00
A/A vs. C/C	F M	+	> 28	0.22	58	0.39	103	0.02	0.46	0.22, 0.95

Marker 9306 (A/G), RDS versus control in white subjects

Comparison	Sex	Steroids [†]	GA in weeks	RDS		Control		<i>P</i> -value	Odds ratio	95% CI
				f	n	f	n			
A/G vs. A/A	F M	+ -	≥ 33	0.29	84	0.18	198	0.03	1.86	1.03, 3.39
A/G or G/G vs. A/A	F M	+ -	≥ 33	0.29	84	0.18	198	0.03	1.86	1.03, 3.39

CI, confidence intervals; F, female; f, frequency; GA, gestational age in weeks; M, male; n, number of alleles or genotypes; RDS, respiratory distress syndrome. [†]Maternal steroid therapy received (+) or was not received (-) 24–168 h before birth.

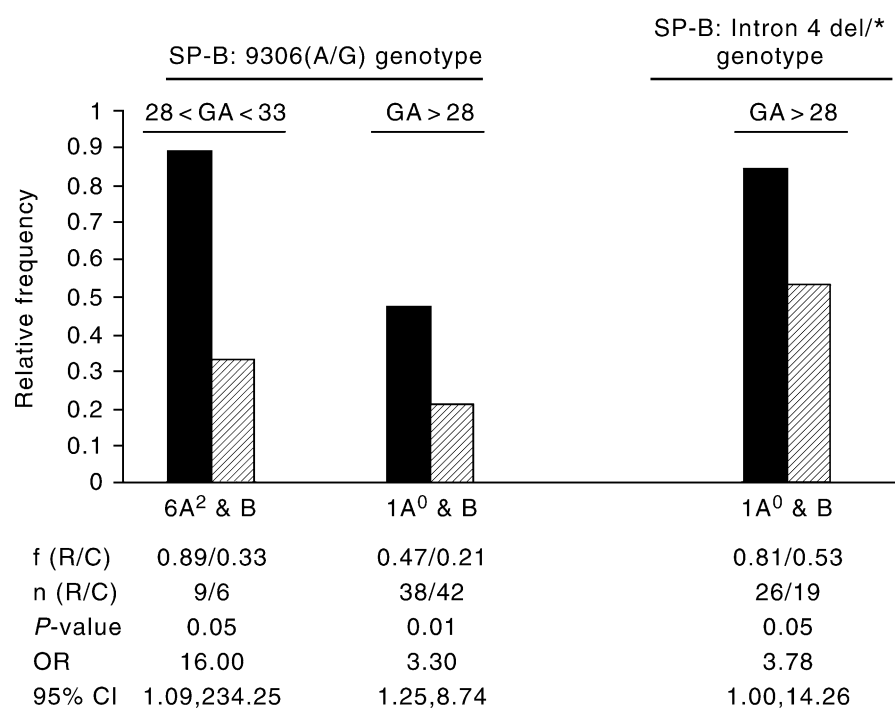
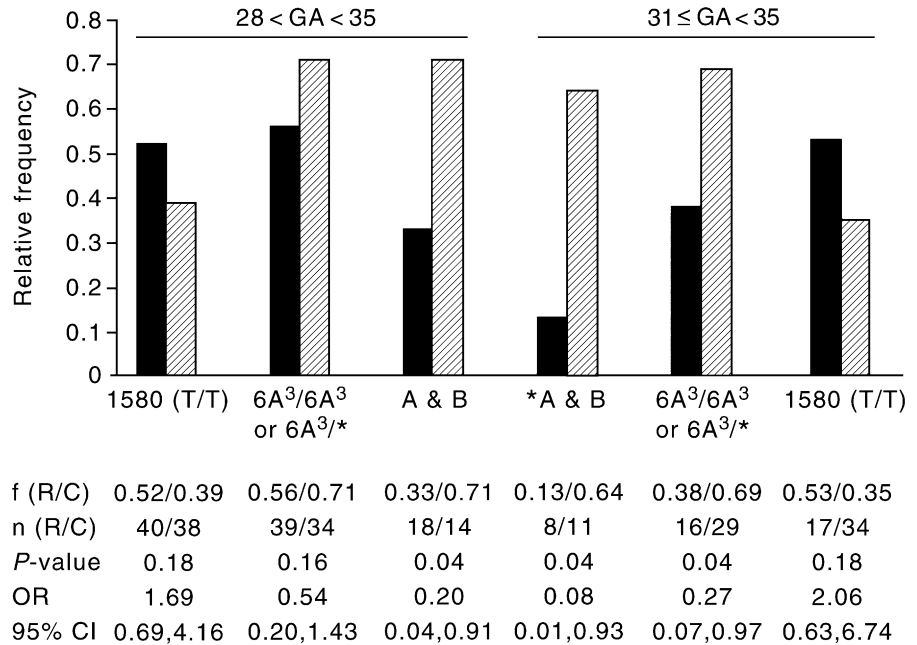


Fig. 3 Surfactant protein-A (SP-A) and SP-B interactions in white subjects with and without respiratory distress syndrome (RDS). The first two sets of comparisons depict the frequencies of the SP-A1 (6A²/6A²) genotype versus */* or the SP-A2 (1A⁰/1A⁰) versus all other genotypes (1A⁰/* or */*) in subjects of the indicated gestational age (GA) and with the SP-B 9306 (A/G) genotype. The third set of comparison depicts the frequency of 1A⁰/* versus */* in subjects of GA > 28 weeks and with SP-B intron 4 del/* genotype. f (R/C) frequency of the given genotype or allele in RDS (R) and control (C); n (R/C), number (n) of genotypes or alleles studied in RDS and control; OR, odds ratio; CI, confidence intervals. A *P*-value of ≤ 0.05 is considered significant. (■), RDS; (▨), control.

RDS and control subjects, three observations were made in white subjects (as described in Fig. 3) and one set of observations was made in black subjects (Fig. 4). In all these circumstances, the presence of specific SP-A and SP-B alleles in the same individual resulted in a considerable change of the *P*-value or the OR compared to values obtained with either genotype alone. No other major interactions were observed between SP-A and SP-B genotypes in

this study group, as assessed by a lack of a change in the *P*-value or the OR when compared to either genotype alone. Based on OR, the data presented indicate that the frequency of SP-A susceptibility genotypes in white subjects with RDS is increased when these subjects have a specific SP-B genotype, suggesting that the SP-B genotype helps determine risk for RDS. In black subjects, the frequency of SP-A protective genotypes in subjects with RDS, especially in older subjects,

Fig. 4 Surfactant protein-A1 (6A³/6A³ or 6A³/*) and SP-B (1580 (T/T)) interactions in black subjects with and without respiratory distress syndrome (RDS). Groups of two different gestational age (GA) were studied (28 < GA < 35 and 31 ≤ GA < 35). The frequency of the individual SP-A1 and SP-B genotypes as well as the combined (A & B and *A and B) frequency of the SP-A1 and SP-B genotypes are shown for both GA groups. The data of the 6A³/6A³ or 6A³/* comparison in subjects of 31 ≤ GA < 35 are from our previous study.⁷ f (R/C), frequency of the given genotype or allele in RDS (R) and control (C); n (R/C), number (n) of genotypes or alleles studied in RDS and control. OR, odds ratio; CI, confidence intervals. A P-value of ≤ 0.05 is considered significant. (■), RDS; (▨), control.



is decreased when these subjects have a specific SP-B genotype, suggesting that the SP-B genotype helps determine protection from RDS.

White subjects

Infants of 28 weeks ≤ GA ≤ 33 weeks with the SP-B 9306 (A/G) genotype

The frequency of the 6A²/6A² genotype versus genotypes (*/*) that did not include a 6A² allele was significantly higher in RDS compared to controls (Fig. 3). No significant findings were observed for the 9306 (A/G) genotype alone in this age group (P = 0.55; OR = 1.03; CI: 0.49, 2.17; RDS (n = 119); control (n = 122)). Although differences were observed in the frequency of the 6A²/6A² versus */* when subjects with all SP-B genotypes were included, the OR was 2.1⁷, whereas when the SP-B genotype was preselected (9306 (A/G)) the OR increased to 16.00. The interaction between these SP-A and SP-B genotypes appears to be synergistic. Synergism is said to occur when the observed OR of the 6A²/6A² and 9306 (A/G) minus one is higher (differs) from the calculated sum of the individual OR of 6A²/6A² minus one and of 9306 (A/G) minus one.²⁶ In this case OR of 6A² and A/G is 16 – 1 = 15 and the sum of the individual OR of 6A² and A/G is (2.1 – 1) + (1.03 – 1) = 1.13. As 15 > 1.13, it is suggested that the two loci interact synergistically.

Infants of GA ≥ 28 weeks with the SP-B 9306 (A/G) genotype

The frequency of the 1A⁰/1A⁰ versus all other genotypes (i.e. genotypes that included one or zero 1A⁰ alleles) was higher in RDS (OR = 3.30) compared to controls. Comparable findings were observed when the 1A⁰/1A⁰ genotype was compared to 1A⁰/* (OR = 3.07) and the 1A⁰ allele to all other alleles (OR = 2.23). No significant differences were observed for the 9306 (A/G) genotype alone in this age group (P = 0.16; OR = 1.29; CI: 0.82, 2.05; RDS (n = 203); control (n = 320)) or the 1A⁰/1A⁰ (P = 0.06; OR = 1.38; CI: 0.94, 2.02; RDS (n = 210); control (n = 301)) in subjects with no regard of the presence or absence of maternal steroid therapy. However, as shown previously the 1A⁰/1A⁰ genotype versus all other genotypes was increased (P = 0.05, OR = 1.6) in infants with RDS of mothers who had received steroid therapy.⁷

The interaction between 1A⁰/1A⁰ and 9306 (A/G) appears to be synergistic because the observed OR minus one for the combined A and B polymorphisms is larger than the sum of the individual OR each minus one (2.30 > 0.65). For 1A⁰/1A⁰ and 9306 (A/G), OR = 3.30 – 1 = 2.30 and the sum of individual OR of 1A⁰ and A/G is (1.36 – 1) + (1.29 – 1) = 0.36 + 0.29 = 0.65.

Infants of GA ≥ 28 weeks with the SP-B intron 4 del/ genotype*

The frequency of 1A⁰/* versus */* was higher in RDS compared to the control (OR = 3.78) (Fig. 3). A significant

Table 4 Logistic regression analysis of 6A³ genotype in black subjects with and without RDS and SP-B 1580 (T/T) genotype

Variables	Estimation	SD	Odds ratio	95% Confidence interval	P-value
Ind-GA	2.34	1.22	10.38	0.94, 114.10	0.06
Ind-male	0.80	0.98	2.22	0.33, 15.15	0.42
Ind-steroids	-1.38	1.05	0.25	0.03, 1.98	0.19
Ind-(6A ³ /6A ³ or 6A ³ /*)	-3.01	1.22	0.05	0.01, 0.54	0.01**

SP-B, surfactant protein B. The logistic regression model is given by $\log(P(\text{RDS})/P(\text{Control})) = \alpha_0 + \alpha_1 \text{Ind-GA} + \alpha_2 \text{Ind-male} + \alpha_3 \text{Ind-steroids} + \alpha_4 \text{Ind-(6A}^3/6\text{A}^3 \text{ or } 6\text{A}^3/*)$, where Ind-GA is 1 if $28 < \text{GA} < 33$ and 0 if $33 \leq \text{GA}$, Ind-male is 1 if male and 0 if female, Ind-steroids is 1 if steroid treatment is Yes and 0 if steroid treatment is No, Ind-(6A³/6A³ or 6A³/*) is 1 if the genotype is 6A³/6A³ or 6A³/* and 0 otherwise. * represent alleles other than 6A³. Number (n) = 18 cases; n = 14 controls. ** indicates significance at a level $P \leq 0.05$.

difference of the del/* versus */* alone was observed ($P = 0.001$; OR = 2.37, CI: 1.42, 3.95; RDS (n = 193); control (n = 321)) but no significant difference was observed for the 1A⁰/* versus */* comparison alone ($P = 0.25$; OR = 1.20; CI: 0.76, 1.92; RDS (n = 141); control (n = 222)). The OR minus one for 1A⁰ + del is $3.78 - 1 = 2.78$ and the sum of individual OR each minus one is $(1.20 - 1) + (2.37 - 1) = 1.57$. The combined presence of del/* and 1A⁰/* appears to also have a synergistic effect on RDS ($2.78 > 1.57$).

Black subjects

Male and female RDS and control subjects with 1580 (T/T) genotype

The frequency of the (6A³/6A³ or 6A³/*) genotype versus genotypes (*/*) that did not include a 6A³ allele was significantly lower in subjects with RDS of 28 weeks $< \text{GA} < 35$ weeks when the SP-B genotype of the marker locus 1580 was T/T (see comparison A and B, OR = 0.20, Fig. 4). Although the frequency of neither genotype (6A³ or T/T) alone differed in this age group, the frequency of the (6A³/6A³ or 6A³/*) genotype versus the (*/*) genotype was found previously (and shown here for emphasis) to be lower in subjects with RDS of 31 weeks $\leq \text{GA} < 35$ weeks.⁷ When the frequency of this 6A³ genotype was studied in subjects with the SP-B 1580 (T/T) genotype of 31 weeks $\leq \text{GA} < 35$ weeks, significant differences (see comparison *A and B, OR = 0.08, Fig. 4) were observed between RDS and the control. In the *A/B comparison where only older subjects ($31 \leq \text{GA} < 35$) were included, the OR was lower (OR = 0.08) compared to OR of the A and B comparison (OR = 0.20) where subjects of 28 weeks $< \text{GA} < 35$ weeks were included. Moreover, the OR of the 6A³ genotype (when a specific SP-B genotype was not considered) in the older group was also considerably higher (OR = 0.27) compared to that (OR = 0.08) observed in subjects with the specific SP-B 1580 (T/T) genotype. Interestingly, a lower OR = 0.05 was also observed for the 6A³ genotype in logistic regression

analysis when subjects were preselected for the SP-B 1580 (T/T) genotype (Table 4). Without SP-B genotype preselection, the OR for the 6A³ genotype in logistic regression is much higher (OR = 0.25).⁷ These data together indicate that the SP-B (1580 (T/T)) genotype in black subjects enhances the protective RDS effect of the 6A³ genotype in subjects of 31 weeks $\leq \text{GA} < 35$ weeks. Based on P-value or OR, no other observations were made in this study group where a specific SP-B genotype increased or decreased the frequency of an SP-A genotype more than what may have been observed for the SP-A genotype alone (i.e. without preselecting subjects with a given SP-B genotype).

The interaction of the 1580 (T/T) and 6A³ genotypes may be synergistic.²⁶ For subjects of 28 weeks $< \text{GA} < 35$ weeks the OR in A and B is $0.20 - 1.00 = -0.80$. The sum of OR of 1580 (T/T) minus one and of 6A³/6A³ or 6A³/* minus one is $(0.69 - 0.46) = 0.23$. Based on these findings ($-0.80 \neq 0.23$), the two loci may interact synergistically. Similarly, for subjects of 31 weeks $\leq \text{GA} < 35$ weeks the OR in *A and B is $0.08 - 1.00 = -0.92$ and the sum of the OR minus one of each marker is $(1.06 - 0.73) = 0.33$, indicating that a synergistic effect ($-0.92 \neq 0.33$) may exist for subjects of older GA.

Paradoxical observations were made in subjects with the heterozygous 1580 (C/T) genotype

Although 1A⁰ or 6A² genotypes have been shown previously to be overrepresented in black (1A⁰) and in white (1A⁰, 6A²) RDS subjects of certain GA, the frequency of the (1A⁰/1A⁰ or 1A⁰/*) or (6A²/6A² or 6A²/*) genotype was decreased in RDS subjects with the 1580 (C/T) genotype suggesting a protective role (data not shown).⁷ Similar paradoxical observations were made with intron 4 genotypes. The 1A⁰ allele, shown previously to be a risk factor, appears as a protective factor in infants with the SP-B intron 4 inv/ins genotype ($P = 0.01$, OR = 0.05). These paradoxical findings may be erroneous due to the small sample size and must be re-examined in a study of a considerably larger sample size.

Discussion

Multiple factors may contribute to the pathogenesis of RDS and surfactant protein genetic variants have been identified as risk or protective factors in RDS. In the present study, we investigated association of SP-B polymorphisms in white and black subjects and studied whether a specific SP-B genetic background increased or reduced the risk for RDS as assessed by SP-A susceptibility or protective alleles.

The results indicated that different polymorphisms of the SP-B intron 4 size variant identify RDS subgroups in white (del variant) and black subjects (ins variant). Within each race group, the corresponding variant appeared to be more of a risk factor for males in white subjects and for females in black subjects. As assessed by the SP-B intron 4 variant, subjects of younger GA appeared to be at a greater risk, especially white subjects. In white subjects, the SP-A RDS susceptibility 6A² or 1A⁰ genotypes in the presence of a given SP-B genotype (9306 (A/G) or intron 4 (del/*)) showed, based on OR, enhanced risk for RDS. In black subjects, however, the SP-A1 protective 6A³ genotype in the presence of the SP-B 1580 (T/T) genotype exhibited enhanced protection from RDS, especially in subjects of older GA.

A number of previous studies have implicated race, sex, and prematurity as contributing factors to RDS.¹⁻³ In the present study, the SP-B intron 4 size polymorphism appears to support this notion. The del and the ins variants were not only found in higher frequency in white and black subjects, respectively, as shown previously,⁴ but their frequency was found increased in RDS and in white males (del variant) or black females (ins variant) in particular. Younger infants with the intron 4 polymorphism appeared to associate with an increased risk for RDS especially white subjects, although no significant differences were observed for the black subjects with regards to GA differences. The latter may reflect the low number of subjects studied. Also, no significant differences of intron 4 polymorphism were observed between control and RDS in a Finnish study group,¹³ where the frequency of intron 4 variants is low (9% del and 1% ins). The present data indicate that the intron 4 polymorphism may be useful in identifying a subset of subjects in black and white people, and in males and females, that may be at a higher risk for RDS. Consistent with this is the finding that differences of SP-B levels in amniotic fluid between white and black subjects have been observed.²⁷ However, ethnic differences in the frequency of intron 4 may exist as indicated by the collective observations in this and other studies.^{5,13}

The SP-B polymorphisms may contribute to RDS by modulating the risk for RDS, in the presence of SP-A susceptibility or protective alleles. In the present study, differences were observed between white and black subjects

with regard to the combination of SP-A and SP-B variants that led to an increased or decreased risk for RDS. The interaction of specific SP-B genotypes (9306 (A/G) or intron 4 del/*) with SP-A susceptibility genotypes (6A² or 1A⁰) to increase the risk of RDS in white subjects appeared to be synergistic. Synergism between 1A⁰ and del variants has been previously observed in a study group that included both white and black subjects.⁵ Similarly, in black subjects the presence of the SP-B 1580 (T/T) genotype enhanced the protective effect of the 6A³ genotype, especially in older subjects. This interaction may be synergistic as well. Consistent results were obtained from the logistic regression analysis. In a recent study, the 1580 (C/C) genotype seemed to determine both, RDS risk for the SP-A susceptibility alleles and protection for the 6A³ allele in a Finnish study group.¹³ Of interest, in a preliminary study,²⁵ the 1580 (C/C) genotype appeared to associate with increased risk in a subgroup (i.e. of idiopathic etiology such as pneumonia) of acute RDS and the SP-B 1580 (C) allele was shown to associate with increased risk for chronic obstructive pulmonary disease in a Mexican study group.²⁸ Therefore, in the majority of available studies, the 1580 (C/C) genotype was shown to associate with risk of pulmonary disease. A corollary of this may be that the 1580 (T/T) enhances protection from disease, as shown here for RDS in black subjects. The SP-B 1580 (C/T) polymorphism affects amino acid 131, and changes it from a Thr (ACT) to Ile (ATT). The change from a Thr to Ile eliminates a potential N-linked glycosylation site. It has been shown previously that in heterozygous subjects both alleles are expressed.²⁵ Whether the presence or absence of this putative glycosylation affects protein processing, folding or other aspects that in turn may have an impact on lung health, remains to be determined.

In summary, we describe SP-B markers that may be useful in assessing risk in subsets of RDS with regards to race, sex, and GA. Moreover, we show that certain SP-A and SP-B variants interact synergistically to increase or reduce risk of RDS in certain subgroups. Racial differences in these interactions also appear to exist. The data together point to the possibility that the etiology of RDS is multifactorial and multigenic and that different mechanisms may lead to the pathogenesis of RDS. Therefore, different genetic markers or sets of markers may be appropriate for the study of risk of RDS in the various subgroups.

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