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Genetic Polymorphisms of *SP-A*, *SP-B*, and *SP-D* and Risk of Respiratory Distress Syndrome in Preterm Neonates

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Background: We examined selected polymorphisms in 3 pulmonary surfactant-associated proteins (*SP*) for their influence on serum *SP* levels and risk of respiratory distress syndrome (RDS) in preterm neonates.

Material/Methods: Premature infants from a Han population were enrolled, including 100 premature infants with RDS (case group) and 120 premature infants without RDS (control group). SNP genotyping for *SP-A* (+186A/G and +655C/T), *SP-B* (−18A/C and 1580C/T), and *SP-D* (Met11ThrT/C and Ala160ThrG/A) used polymerase chain reaction-restriction fragment length polymorphism. Haplotypes were calculated with Shesis software and serum *SP-A/B/D* levels were quantified by ELISA.

Results: Case and control groups exhibited significant differences in genotype and allele frequencies of *SP-A* (+186A/G, +655C/T) and *SP-B* (1580C/T). However, no statistically significant differences were observed in the allele and genotype frequencies of *SP-B* −18A/C, *SP-D* Met11ThrT/C, and *SP-D* Ala160ThrG/A. Importantly, serum *SP-A* and *SP-B* levels were reduced in RDS patients carrying *SP-A* (+186A/G, +655C/T) and *SP-B* (1580C/T) polymorphisms. AA genotype of +186A/G, *SP-A* level, and CC genotype of 1580C/T were independently correlated with increased RDS risk.

Conclusions: *SP-A* (+186A/G) and *SP-B* (1580C/T) polymorphisms are strongly associated with the risk of RDS in preterm infants. Notably, reduced serum *SP-A* levels were correlated with a high risk of RDS and may serve as novel biomarkers for RDS detection and monitoring.

MeSH Keywords: **Infant, Premature • Polymorphism, Single Nucleotide • Pulmonary Surfactant-Associated Proteins**

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Background

Respiratory distress syndrome (RDS) is an acute lung disorder caused by developmental insufficiency of surfactant production and structural immaturity of the lungs in preterm neonates [1,2]. Reduced surfactant level leads to insufficient surface tension in the alveolus during expiration, resulting in atelectasis, decreased gas exchange, severe hypoxia, and acidosis, clinically manifested in neonates as serious difficulty in breathing [3]. An epidemiological study showed that RDS risk is inversely correlated with gestational age [4]. RDS primarily occurs in infants with a gestational age of less than 32 weeks and weight of less than 1200 g [5]. In this context, the mortality rates in RDS are closely linked to disease severity, gestational age, and racial differences, although the average mortality rate is broadly placed at 20–50% [6]. Nevertheless, RDS affects approximately 7% of newborn infants and is the leading cause of preterm infant death worldwide [7]. ARDS is a common cause of respiratory failure in children and EuroNeoStat data shows that the incidence of RDS in infants is 52% at 30–31 weeks, 74% at 28–29 weeks, 88% at 26–27 weeks, and 91% at 23–25-week gestation [8,9]. Apart from gestational age, inherited and environmental risk factors impact RDS development, including sex, birth weight, asphyxia neonatorum, age of the pregnant woman, and gestational diabetes mellitus [10–12]. A previous document also reported that amantadine could induce respiratory failure, contributing to the occurrence of RDS [13]. Importantly, RDS is greatly influenced by genetic mutations that result in abnormal production or synthesis of surfactant-associated proteins [14–16].

Pulmonary surfactant (PS) is present at the alveolar surface and stabilizes the alveoli involved in gas exchange, preventing alveolar collapse at the end of expiration [17,18]. PS prevents airway collapse by decreasing the surface tension and also acts as a natural barrier for inhaled pathogens or other harmful aerosols and particles [19]. PS is mainly composed of 90% lipids and 10% protein, and contains 4 surfactant proteins: SP-A, SP-B, SP-C, and SP-D [20]. These surfactant proteins interact with surfactant phospholipids and are essential for the ultrastructure, metabolism, and surface tension in lungs [21,22]. SP-A and SP-D bind to pathogens and regulate microbial phagocytosis through activation or deactivation of inflammatory responses in alveolar macrophages [23]. Human SP-A is encoded by *SFTPA* gene located on chromosome 10q22.3 and is a member of a subfamily of C-type lectins known as collectins [24]. SP-A binds carbohydrate moieties found on lipids and on the surface of microorganisms, playing a vital role in surfactant homeostasis and in defense against respiratory pathogens [25]. Human SP-B protein is encoded by *SFTPB* gene located on chromosome 2p12-p11.2 and contains 14 exons [26]. SP-B increases the stability of surfactant monolayer and *SP-B* mutations resulting in SP-B protein deficiency

are associated with respiratory distress in neonates [15,17]. Human SP-D protein is encoded by *SFTPD* gene located on chromosome 10q22.2-23.1 and contains 8 exons [27]. Lung epithelial cells secrete SPs into the pulmonary airspaces and SPs are important in host defense against microorganisms inhaled into the lungs [22,28]. Mutations in *SP-A*, *SP-B*, and *SP-D* gene are also associated with idiopathic pulmonary fibrosis and other human pulmonary diseases [29–31]. However, the link between *SP-A*, *SP-B*, and *SP-D* polymorphisms and the risk of RDS in preterm neonates is not thoroughly established. In this study, we examined the association between *SP-A*, *SP-B*, and *SP-D* polymorphisms and the risk of RDS in premature infants to understand the contribution of *SP-A*, *SP-B*, and *SP-D* polymorphisms to RDS risk.

Material and Methods

Subject

Between July 2010 and November 2014, a total of 100 neonates with RDS from the Han population were recruited to this study as the case group at the General Hospital of the PLA Rocket Force, the 306 Hospital of PLA, and Haidian Maternal and the Child Healthcare Hospital. The case group contained 58 male infants and 42 female infants, with an average age of 30.80 ± 3.40 weeks. During the same period, a group of 120 age- and sex-matched preterm neonates without RDS were selected as the control group, with 72 male infants and 48 female infants and average age of 31.70 ± 4.10 weeks. The reasons for preterm delivery included twin pregnancy, placental abruption, gestational hypertension, and congenital heart disease. Patients enrolled in our study had no sibship, and no significant difference was found in comparisons of gestational age, sex, birth weight, mode of delivery, and glucocorticoid hormones used before delivery between the 2 groups (all $P > 0.05$). The study procedures were approved by the Ethics Committee of the General Hospital of the PLA Rocket Force. Parents of all enrolled subjects signed written informed consents before initiation of the study. This study conformed to the Declaration of Helsinki.

The diagnosis of RDS in preterm newborns was based on the standard clinical diagnostic criteria described in *Practical Neonatology (Third Edition)* [32]. The eligible RDS patients met the following inclusion criteria: (1) gestational age less than 37 weeks; (2) acute and sudden onset of respiratory distress appearing within 12 h after birth, manifested as shortness of breath, cyanosis, nasal flaring, and 3 depression signs; (3) a typical chest X-ray finding: reduction of universal radiolucency of lungs, mesh and granular shadows, air bronchogram, and consolidation observed as “white lung”. Subjects in the case and control groups were excluded if they: (1) were infants of

Table 1. Primers used for amplification of the *SP-A*, *SP-B* and *SP-D* SNPs.

SNP	Primer sequences
+186A/G	5'-GGAGAGAAGGGGGAGCCTGGCGAGAG-3' 5'-GGCCCTCCAGGTGAGCAGGGTGGGG-3'
+655C/T	5'-CTGGAGCCCCTGGTATCCCTGGAGAG-3' 5'-GTGGAGAGAAGGGGGAGCCTGGCGA-3'
-18A/C	5'-GTCCAGCTATAAGGGGCCGTG-3' 5'-GTGAGTGTGGAGCTGCCTA-3'
1580C/T	5'-CTCGAATTCGTGAACCTCCAGCACCAACC-3' 5'-GTGAGCTTCAGCCCTCTCA-3'
Met11ThrT/C	5'-AGACCTACTCCACAGAACAAT-3' 5'-ACCTACTCCACAGAACAAC-3'
Ala160ThrG/A	5'-CGTGGAGTCCCTGGAAACA-3' 5'-GTGGAGTCCCTGGAAACG-3'

SNP – Single nucleotide polymorphism.

diabetic mothers; (2) had a history of severe fetal distress or were born with severe asphyxia; (3) had used prophylactic surfactants in prenatal or postnatal period; (4) suffered from congenital malformations or severe intrauterine infection.

Specimen collection and baseline data collection

Peripheral venous blood samples (4 mL) were collected from all subjects within 3 days after birth. A portion of the collected blood (2 mL) was placed in tubes containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant and stored at -20°C until further use. This portion of blood was used for genomic DNA isolation using a whole-blood genomic DNA extraction kit (Tiangen Biotech CO., LTD). The remaining blood samples (2 mL) without anticoagulant were placed at room temperature for 1 h and centrifuged at 3000 rpm for 10 min to collect the serum. The collected serum was stored at -80°C until further use. Serum levels of IL-6 and TNF-α were measured to assess the inflammation status using enzyme-linked immunosorbent assay (ELISA). Blood gas analysis was performed in all subjects and the results were carefully recorded. The ventilator parameters in neonates were also collected, including fraction of inspired oxygen (FiO₂), peak inspiratory pressure (PIP), positive end-expiratory pressures (PEEP), and mean arterial pressure (MAP). Calculations were performed to obtain the values of compliance of respiratory system (C value), oxygenation index (OI), respiratory index (RI), and alveolar-arterial oxygen partial pressure difference [(A-a) DO₂].

The detection of SNP

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to analyze *SP-A*, *SP-B*, and *SP-D* genetic polymorphisms. PCR primers were designed using Primer Premier version 5.0 software (Premier Biosoft, Palo Alto, CA)

and synthesized by Shanghai Sangon Biological Engineering Technology Co., Ltd. (Shanghai, China). The primer sequences and their lengths are shown in Table 1. PCR reaction was carried out in 20-μl volume, containing 10 μl×PCR PLUS MIX (DBI), 1.2 μl DNA templates, 0.5 μl upstream primer, and 0.5 μl downstream primer. PCR conditions were: 95°C initial denaturation for 5 min, 95°C denaturation for 30 s, 60°C annealing for 45 s, and 72°C extension for 50 s. After 35 cycles, the final extension was at 72°C for 10 min. PCR amplification products (6 mL) were digested with 4 U Fsp I (Toyobo) in a 15-μl volume and incubated overnight at 37°C. A volume of 5 μl of the digested product was mixed with 3 μl 6×buffer solution and analyzed on 3% agarose gel, followed by UV photography to record the results. Locus-specific restriction enzymes HincII, XspI, and Ase I (TaKaRa) were also used to validate PCR products from different *loci*, and agarose gel electrophoresis was employed to analyze and record the results. All genotypes were finally confirmed by sequencing using a DNA sequencer (ABI370).

Detection of SP-A, SP-B, and SP-D levels in the serum

Serum levels of SP-A, SP-B, and SP-D were detected by ELISA. All related reagents were purchased from Immudiagnostik AG Bensheim, and the experimental procedures were carried out in strict accordance with kit instructions. Absorbance was read at 450 nm [optical density (OD₄₅₀)]. The concentrations were calculated by applying the linear regression equation of the standard calibration curve. All specimens were measured twice and averaged, and the procedures conformed to all clinical laboratory quality control standards.

Statistical analysis

Statistical analyses were conducted with SPSS 18.0 statistical software (SPSS, Chicago, IL). Quantitative variables are

Table 2. Baseline characteristics of the case group (premature infants with respiratory distress syndrome) and control group (premature infants without respiratory distress syndrome).

	Case group (n=100)	Control group (n=120)	P value
Gender (M/F)	58/42	72/48	0.580
Gestational age (weeks)	30.80±3.40	31.70±4.10	0.082
Birth weight (kg)	2.86±0.48	2.85±0.52	0.662
Mode of delivery (Cesarean/natural labor, n)	49/51	65/55	0.445
Hormones usage (n)	66	67	0.125
C value (ml/cmH ₂ O/kg)	0.29±0.05	0.62±0.21	< 0.001
OI value	13.93±1.99	6.32±0.78	< 0.001
RI value	5.27±1.02	3.25±0.90	< 0.001
(A-a)DO ₂ (mmHg)	309.48±49.20	268.79±57.77	< 0.001
IL-6 (pg/ml)	163.03±48.61	138.79±53.18	0.001
TNF-α (pg/ml)	62.59±17.26	70.24±24.34	0.068

M – male; F – female; C value – values of compliance of respiratory system; OI – oxygenation index; RI – respiratory index; (A-a)DO₂ – alveolar-arterial oxygen partial pressure difference; IL-6 – interleukin-6; TNF-α – tumor necrosis factor-α.

expressed as mean ± standard deviation (SD). Qualitative variables are expressed as percentage or ratio. Associations between quantitative variables were examined with the *t* test. Comparisons between qualitative variables were assessed with the chi-square test. Deviations from Hardy-Weinberg equilibrium of the genetic polymorphisms were evaluated by the chi-square test. The comparison of genotypic distribution and allele frequencies between groups was assessed by the R×C contingency table chi-square test. The odds ratio (OR) of different genotypes and 95% confidence interval (CI) were calculated to represent the relative risk. Haplotypes of different gene *loci* were calculated by Shesis software. All statistical tests were 2-sided, and *P*<0.05 was considered as statistically significant.

Results

Baseline characteristics

Baseline characteristics of preterm neonates with RDS and preterm neonates without RDS are shown in Table 2. We did not observe any statistically significant differences in sex, gestational age, or birth weight between the case group and control group (all *P*>0.05). Importantly, the C value in the case group was significantly lower than in the control group (*P*<0.05). Further, the average values of OI, RI, and (A-a) DO₂, and the mean serum levels of IL-6 were significantly elevated in the case group, in comparison to the control group (all *P*<0.001). However, TNF-α serum levels were similar between the 2 groups (*P*=0.068).

The distribution of genotypes and allele frequencies of SNP *loci* on *SP-A*, *SP-B*, and *SP-D* in the 2 groups

The distribution of genotypes and allele frequencies of *SP-A*, *SP-B*, and *SP-D* SNPs in the case and control groups are shown in Table 3. The test for Hardy-Weinberg equilibrium showed that the frequency of all 3 genes reached equilibrium, indicating that the selected sample was representative of the population. Notably, no significant differences between the case group and control group were observed in the genotype frequencies of *SP-B* –18A/C SNP and the 2 *SP-D loci*, Met11ThrT/C and Ala160ThrG/A (*P*>0.05). In addition, allele frequencies of *SP-B* –18A/C, *SP-D* Met11ThrT/C and *SP-D* Ala160ThrG/A did not exhibit statistically significant differences between the 2 groups (all *P*>0.05).

Further study revealed statistically significant differences between the case group and the control group in the genotype and allele frequencies of 2 SNPs of *SP-A*, +186A/G and +655C/T, and *SP-B* 1580C/T (all *P*<0.05). The frequency of AA genotype of *SP-A* +186A/G SNP, the CC genotype of *SP-A* +655C/T *loci*, and CC genotype of the *SP-B* 1580C/T *loci* were significantly higher in preterm neonates with RDS (case group) compared to the control group (all *P*<0.05). The risk of RDS in carriers of A allele of +186A/G *loci*, and C allele of +655C/T and 1580C/T *loci* was 3.117 times higher, 2.762 times higher, and 2.212 times higher, respectively, compared to the non-carriers (*SP-A*+186A/G: OR=3.117, 95%CI: 1.938–5.014, *P*<0.001; *SP-A*+655C/T: OR=2.762, 95%CI: 1.420–5.371, *P*<0.001; *SP-B* 1580C/T: OR=2.212, 95%CI: 1.479–3.309, *P*<0.001).

Table 3. Genotypes and allele frequencies of *SP-A* polymorphisms (+186A/G and +655C/T), *SP-B* polymorphism (–18A/C and 1580C/T) and *SP-D* polymorphisms (Met11ThrT/C and Ala160ThrG/A) in premature infants with respiratory distress syndrome (case group) and premature infants without respiratory distress syndrome controls (control group).

SNP		Case group (n=100)		Control group (n=120)		OR (95%CI)		P value	χ ²
SP-A +186A/G	AA	73	(73.0%)	54	(45.0%)	1	(Ref.)		
	AG	25	(25.0%)	49	(40.8%)	2.650	(1.459–4.812)	0.001	10.510
	GG	2	(2.0%)	17	(14.2%)	11.490	(2.546–51.87)	<0.001	14.590
	AG+GG	27	(27.0%)	65	(55.0%)	3.254	(1.840–5.757)	<0.001	17.020
	A	171	(85.5%)	157	(65.4%)	1	(Ref.)		
SP-A +655C/T	G	29	(14.5%)	83	(34.6%)	3.117	(1.938–5.014)	<0.001	23.190
	CC	75	(75.0%)	57	(47.5%)	1	(Ref.)		
	CT	22	(22.0%)	48	(40.0%)	2.871	(1.558–5.289)	0.001	11.810
	TT	3	(3.0%)	15	(12.5%)	6.579	(1.817–23.83)	0.001	10.230
	CT+TT	25	(25.0%)	63	(52.5%)	3.316	(1.861–5.907)	<0.001	17.190
SP-B –18A/C	C	172	(86.0%)	162	(67.5%)	1	(Ref.)		
	T	28	(14.0%)	78	(32.5%)	2.762	(1.420–5.371)	0.002	9.363
	CC	35	(35.0%)	57	(47.5%)	1	(Ref.)		
	CA	50	(50.0%)	49	(40.8%)	1.662	(0.934–2.958)	0.083	2.998
	AA	15	(15.0%)	14	(11.7%)	1.745	(0.752–4.048)	0.192	1.702
SP-B 1580C/T	CA+AA	65	(65.0%)	63	(52.5%)	1.680	(0.974–2.899)	0.061	3.503
	C	120	(60.0%)	163	(67.9%)	1	(Ref.)	0.084	2.979
	A	80	(40.0%)	77	(32.1%)	1.411	(0.954–2.088)	0.929	0.008
	CC	58	(58.0%)	38	(31.7%)	1	(Ref.)		
	CT	30	(30.0%)	56	(46.7%)	2.849	(1.558–5.209)	0.001	11.840
SP-D Met11ThrT/C	TT	12	(12.0%)	26	(21.6%)	3.307	(1.490–7.339)	0.003	9.074
	CT+TT	42	(42.0%)	82	(68.3%)	2.980	(1.714–5.180)	<0.001	15.380
	T	54	(26.9%)	108	(45.0%)	1	(Ref.)		
	C	146	(73.1%)	132	(55.0%)	2.212	(1.479–3.309)	<0.001	15.190
	TC	47	(47.0%)	62	(51.7%)	2.527	(0.658–9.699)	0.165	1.929
SP-D Ala160ThrG/A	CC	50	(50.0%)	48	(40.0%)	3.472	(0.900–13.390)	0.058	3.592
	TC+CC	97	(50.0%)	110	(60.0%)	2.939	(0.786–10.990)	0.095	2.791
	T	53	(26.5%)	82	(34.2%)	1	(Ref.)		
	C	147	(73.5%)	158	(65.8%)	0.69	(0.46–1.05)	0.083	3.015
	GG	56	(56.0%)	77	(64.0%)	1	(Ref.)		
SP-D Met11ThrT/C	GA	38	(38.0%)	40	(33.0%)	1.306	(0.745–2.291)	0.351	0.870
	AA	6	(6.0%)	3	(3.0%)	2.750	(0.659–11.470)	0.151	2.067
	GA+AA	44	(44.0%)	43	(36.0%)	1.407	(0.817–2.423)	0.217	1.522
	G	150	(75.0%)	194	(80.8%)	1	(Ref.)		
	A	50	(25.0%)	46	(19.2%)	1.406	(0.893–2.213)	0.140	2.176

SNP – single nucleotide polymorphism; OR – odd ratio; 95%CI – 95% confidence interval.

Table 4. Comparison of the SP-A, SP-B and SP-D serum levels in different genotypes of *SP-A*, *SP-B* and *SP-D* genes between the infants with respiratory distress syndrome (case group) and infants without respiratory distress syndrome (control group).

SNP	Genotypes	Case group (ng/ml)	Control group (ng/ml)
<i>SP-A</i> +186A/G	AA	110.67±2.52	112.64±2.13*
	AG	110.27±2.31	113.05±1.85*
	GG	111.20±2.16	114.70±1.75*
<i>SP-A</i> +655C/T	CC	110.68±2.51	112.67±2.25*
	CT	110.08±2.30	113.14±1.72*
	TT	111.70±1.76	114.60±1.70*
<i>SP-B</i> –18A/C	CC	109.74±3.52	110.74±2.47
	CA	109.69±5.34	111.22±2.16
	AA	109.66±5.67	111.10±2.26
<i>SP-B</i> 1580C/T	CC	109.53±4.45	111.42±1.99*
	CT	108.31±5.28	110.94±2.12*
	TT	114.01±1.94	110.41±3.01*
<i>SP-D</i> Met11ThrT/C	TT	112.27±4.99	111.16±2.00
	TC	111.43±3.60	111.10±2.79
	CC	110.08±2.65	111.73±3.30
<i>SP-D</i> Ala160ThrG/A	GG	110.88±3.30	111.71±2.62
	GA	111.96±3.00	110.54±3.45
	AA	110.75±2.72	112.92±2.23

* Compared with the control group, $P<0.05$; SNP – single nucleotide polymorphism.

The comparison of serum levels of SP-A, SP-B, and SP-D between the 2 groups

ELISA results revealed that serum levels of SP-A and SP-B in RDS neonates carrying the 2 SNPs of *SP-A*, +186A/G and +655C/T, and *SP-B* 1580C/T were significantly lower than in the control group (all $P<0.05$). However, serum levels of SP-B and SP-D in RDS neonates carrying *SP-B* (–18A/C) SNP and 2 SNPs of *SP-D* (Met11ThrT/C and Ala160ThrG/A) were not significantly different from the control group (all $P>0.05$), as shown in Table 4.

The expression of haplotypes of SP-A, SP-B, SP-D in the case group and the control group

The haplotypes of *SP-A*, *SP-B*, and *SP-D* are shown in Table 5. Shesis software was used to analyze the different haplotypes of *SP-A*, *SP-B*, and *SP-D* in preterm neonates of the 2 groups, and haplotypes with frequencies of less than 3% were excluded. The results revealed that, among a total of 10 haplotypes, the frequencies of 3 haplotypes exhibited statistically significant differences between the case group and the control group. The

frequency of AACTG in the case group was significantly lower than in the control group (OR=0.232, 95%CI: 0.076–0.712, $P=0.006$), suggesting the AACTG haplotype may be a protective factor against RDS. On the other hand, the frequency of ACACCA and ACACCG haplotypes in the case group was significantly higher than in the control group (OR=3.606, 95%CI: 1.220–10.660, $P=0.014$; OR=11.567, 95%CI: 3.341–40.045, $P<0.001$), indicating that ACACCA and ACACCG haplotype increases risk of RDS in preterm neonates.

Logistic regression analysis of risk factors for RDS

Bivariate logistic regression analysis was performed to evaluate the risk factors for RDS. The incidence of RDS was used as a dependent variable and AA genotype on *SP-A* +186A/G, CC genotype on *SP-A* +655C/T, CC genotype on *SP-B* 1580C/T, SP-A level, and SP-B level were tested as the independent variables. The results showed that the AA genotype of *SP-A* +186A/G, the CC genotype of *SP-B* 1580C/T, and SP-A level were independently associated with the risk for RDS (all $P < 0.05$) (Table 6).

Table 5. Comparison of frequency distributions of haplotypes in *SP-A*, *SP-B* and *SP-D* genetic polymorphisms in both the infants with respiratory distress syndrome (case group) and infants without respiratory distress syndrome (control group).

Haplotypes						Case group	Control group	χ^2	P value	OR (95%CI)
+186A/G	+655C/T	-18A/C	1580C/T	Met11	Ala160	(n=100)	(n=120)			
A	C	A	C	C	A	14.60 (0.073)	4.42 (0.018)	6.061	0.014	3.606 (1.220~10.660)
A	C	A	C	C	G	27.44 (0.137)	2.83 (0.012)	22.597	<0.001	11.567 (3.341~40.045)
A	C	A	C	T	G	3.94 (0.020)	16.33 (0.068)	7.618	0.006	0.232 (0.076~0.712)
A	C	C	C	C	G	58.32 (0.292)	65.57 (0.273)	0.346	0.556	0.879 (0.571~1.352)
A	C	C	C	T	G	29.26 (0.146)	28.40 (0.118)	0.062	0.803	1.074 (0.613~1.882)
A	C	C	T	C	G	0.83 (0.034)	8.19 (0.034)	0.088	0.766	0.855 (0.303~2.410)
A	C	C	T	T	G	12.37 (0.062)	13.88 (0.058)	0.050	0.823	0.913 (0.413~2.022)
G	T	A	T	C	A	17.29 (0.086)	24.14 (0.101)	1.048	0.306	0.712 (0.370~1.368)
G	T	A	T	C	G	6.93 (0.035)	5.42 (0.023)	0.244	0.621	1.333 (0.425~4.178)
G	T	C	T	C	G	1.77 (0.009)	9.98 (0.042)	5.663	0.621	0.175 (0.035~0.877)

OR – odd ratio; 95%CI – 95% confidence interval.

Table 6. The bivariate logistic regression analysis for the related risk factors of the premature infants with respiratory distress syndrome (RDS).

Variables	S.E	Wald	df	Sig	Exp (B)	95%CI	
						Inferior limit	Superior limit
SP-A+186A/G AA	0.521	4.429	1	0.035	2.991	1.078	8.297
SP-B1580C/T CC	0.404	4.129	1	0.042	2.275	1.030	5.026
SP-A	0.088	31.900	1	<0.001	0.609	0.855	1.023

S.E. – standard error of regression; Wald – Wald; df – degree of freedom; Sig – significance, P value; Exp (B) – adjusted odds ratio, OR value; 95%CI – 95% confidence interval.

Discussion

In the present study we examined multiple SNPs of *SP-A* (+186A/G and +655C/T), *SP-B* (–18A/C and 1580C/T) and *SP-D* (Met11ThrT/C and Ala160ThrG/A) for their association with RDS risk in preterm infants. Our findings suggested that *SP-A* +186A/G and *SP-B* 1580C/T polymorphisms are strongly correlated with an increased risk of RDS, while *SP-B* –18A/C, *SP-D* Met11 ThrT/C, and Ala160 ThrG/A polymorphisms were not associated with RDS in preterm infants. The results suggest a possibility that *SP-A* +186A/G and *SP-B* 1580C/T polymorphisms and *SP-A* level could serve as biomarkers for RDS for population-based screening.

SP is important for lung health and normal lung function throughout life, because of its surface tension lowering property and its critical function in innate immunity [18,33]. *SP-A*, *SP-B*, and *SP-D* are major components of SP and are the

essential players in SP homeostasis [17,34,35]. *SP-A* reflects the status of alveolar epithelial barrier injury [36]. *SP-A* and *SP-D* participate in host defense and inflammation, which are important in clearing a variety of lung pathogens such as respiratory syncytial virus, mycobacterium tuberculosis, bacteria, viruses, and fungi, and are also involved in the aggregation, agglutination, and inhibition of pathogen growth, which is a major component of pulmonary host defense mechanisms [21,37,38]. As such, *SP-A* is the most abundant surfactant protein and regulates phospholipid insertion into the monolayer as well as the uptake and secretion of phospholipids by type II cells [39]. Previous studies found that *SP-A* deficiency affects normal lung function and surfactant metabolism, and leads to immunopathological defects [40,41]. Consistent with a previous study, logistic regression analysis showed that *SP-A* was independently correlated with risk of RDS. Similarly, a previous study demonstrated that significant increases in plasma *SP-A* were observed during acute lung injury, suggesting the role of

SP-A in lung function [42]. On the other hand, SP-B has an essential role in the formation of tubular myelin, and, along with SP-C, promotes rapid phospholipid insertion into the air-liquid interface [43,44]. A C/T polymorphism at nucleotide 1580 alters amino acid 131 by substituting threonine with isoleucine, eliminating a potential N-linked glycosylation site [14]. Previous studies showed that neonates carrying *SP-B* gene deletions or genetic mutations exhibit lethal respiratory distress after birth [34,45]. Yin et al. showed that C/T polymorphism at nucleotide +1580 is linked with neonatal respiratory distress syndrome in the Han population [46]. Consistent with previous studies, our study also found that *SP-A* and *SP-B* polymorphisms are involved in the development of RDS in preterm neonates, and AA and CC genotypes of *SP-A*, and CC genotype of *SP-B* were independently associated with RDS risk.

Another important finding is that the serum levels of SP-A and SP-B in RDS patients carrying *SP-A* (+186A/G) and *SP-B* (1580C/T) SNPs were significantly lower than in the control group. *SP-A* and *SP-B* polymorphisms may result in decreased transcription/translation of SP-A and SP-B proteins and consequently decrease SP-A and SP-B serum levels, increasing the risk of RDS in preterm infants. As opposed to SP-A and SP-D, which are hydrophilic proteins and are primarily involved in innate host defense, the hydrophobic protein SP-B is essential for adsorption of surfactant film at the alveolar air-liquid interface and therefore is relevant to surfactant maturation [47–49]. Elevated expression of SP-A in amniotic fluid in late gestation is a biomarker for determining lung maturity of the fetus and lack of SP-A expression may predict RDS in infants [17]. Pérez-Gil et al. showed that lack of or reduced pulmonary surfactant proteins, including SP-A and SP-B, is a major factor in RDS development [21]. Similarly, decreased serum level of SP-B protein, caused by a hereditary deficiency in the *SP-B* gene, frequently results in unexplained respiratory distress in infants due to defective surfactant maturation [34,50]. The inability to produce multiple SPs due to autosomal recessive genetic alterations causes fatal respiratory insufficiency, indicating the fundamental role of SPs in normal pulmonary function [51]. Hallman et al. demonstrated that a major SP-A haplotype, interacting with SP-B Ile131Thr polymorphism and other environmental risk factors, increased the risk of RDS [52]. Clark et al. reviewed the association between multiple genetic factors and RDS, and showed that variations in the levels of SP proteins such as SP-A, SP-B, and SP-C, caused by corresponding genetic polymorphisms, elevated the risk of neonatal chronic lung diseases, including RDS [53]. Further, Yin et al. suggested that immature SP-B function is linked with the pathogenesis of neonatal RDS in the Chinese Han population and homozygous C/C genetic polymorphism in *SP-B* gene was the underlying defect [46]. Consistent with previous studies, our study showed that *SP-A* and *SP-B* polymorphisms increased the risk of RDS in preterm neonates, and decreased serum levels of

SP-A and SP-B are associated with elevated RDS risk and may serve as biomarkers for RDS. Importantly, we examined the association between genetic polymorphisms in 3 *SPs* and the risk of RDS in preterm infants. In addition, we studied the impact of the genetic polymorphism on the serum levels of *SPs*, and their overall correlation with the development of RDS in preterm neonatal. Kelli et al. found that *SP-D* rs1923537 polymorphism may be associated with a significantly increased risk of RDS in preterm infants with lower gestational ages [54], but such an association was not found in our study. On the other hand, Lyra et al. demonstrated that *SP-B* polymorphisms (–18A/C and 1580C/T) were not associated with RDS risk in premature infants, and the AG genotype in AG9306 polymorphism of *SP-B* gene is a protective factor against the development of RDS [15]. The conflicting results from previous studies suggest that allele and genotype frequencies of *SP* genes may differ between distinct human ethnic groups. Therefore, some differences between our study results and the results from previous studies may be attributed to ethnic differences or sample size. It is well known that ethnic background is an important interfering factor in studies involving analysis of allele and genotype frequencies. In this context, our observations will need to be confirmed in other populations.

Our study also showed that RDS patients had elevated serum levels of IL-6 in comparisons to the case group, which suggests the involvement of IL-6 in the development of RDS. However, further logistic regression analysis failed to confirm that serum IL-6 level was a risk factor for RDS. IL-6 is a pro-inflammatory cytokine and IL-6 measurement might reflect different biological actions. In contrast to our results, a previous study found no association between markers of placental or fetal infection and inflammation and CLD risk [55]. Additionally, a discrepancy was also found between our results and a previous study that suggested IL-6 accelerates fetal lung maturity, thereby decreasing the incidence of RDS in the preterm neonates [56]. One possible explanation for the difference between our observations and these reports is that we used peripheral venous blood rather than funisitis and cord serum IL-6. To some extent, this study was consistent with our result confirming the implication of IL-6 in RDS. Nevertheless, the exact role and mechanism of IL-6 in RDS still need further exploration.

Cortisol, produced in humans by the zona fasciculata, is a steroid hormone and functions to increase blood sugar through gluconeogenesis, to suppress the immune system, and to aid in the metabolism of fat, protein, and carbohydrates [57,58]. Evidence documented that diurnal cortisol rhythm predicts early lung cancer death, suggesting the role of cortisol in lung function [59]. Specifically, Tsuda et al. suggested that neonates who develop RDS/TTN have significantly lower cortisol levels in the umbilical cord at birth than neonates without RDS/TTN in twin pregnancies [60]. The investigation of cortisol is a promising

direction to pursue in order to better understand the possible mechanism of RDS, but limitations on time and budget do not allow us to address that topic in this article and we plan to investigate this interesting aspect in more detail in the future.

Conclusions

Our results provide strong evidence that polymorphisms of *SP-A* +186A/G, and *SP-B* 1580C/T increase the risk of RDS in preterm neonates. Notably, decreased *SP-A* and *SP-B* serum levels are associated with elevated RDS risk and may serve as important biomarkers for RDS. By contrast, *SP-B* -18A/C, *SP-D* Met11 ThrT/C, and Ala160 ThrG/A gene polymorphisms were not associated with the risk of RDS in preterm infants.

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Further studies using larger sample sizes are required to achieve more accurate outcomes and independently validate the associations between *SP-A*, *SP-B*, and *SP-D* gene polymorphisms and RDS risk.

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Competing interests

The authors have declared that no competing interests exist.

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