RESEARCH PAPER

Human Surfactant Proteins A2 (SP-A2) and *B (SP-B)* Genes as Determinants of Respiratory Distress Syndrome

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Objective: To study the relationship between *SP-A2* and *SP-B* gene polymorphisms and respiratory distress syndrome in preterm neonates.

Design: Cross-sectional.

Setting: Neonatal intensive care unit and the Molecular Biology unit of the Chemical Pathology Department, Kasr Alainy hospital, Cairo University.

Participants: Sixty-five preterm infants with respiratory distress syndrome and 50 controls. The genomic DNA was isolated using DNA extraction kits. SYBR Green-based real-time PCR was used to determine the variant genotypes of *SP-A2* c.751 G>A and *SP-B* c.8714 G>C single nucleotide polymorphisms.

Results: Homozygosity of *SP-A* (OR 46, 95% CI 14-151) and *SP-B* (OR 5.2, 95% CI 2.3-11.4) alleles increased the risk of respiratory distress syndrome. The logistic regression model showed that genotypes *SP-A2* (OR 164) and *SP-B* (OR 18) were directly related to the occurrence of respiratory distress syndrome, whereas gestational age (OR 0.57) and 5-minute Apgar score (OR 0.19) were inversely related to its occurrence.

Conclusions: There is a possible involvement of *SP-A2* and *SP-B* genes polymorphisms in the genetic predisposition to respiratory distress syndrome.

Keywords: *Neonate; Polymorphisms; Respiratory distress syndrome; Surfactant protein.*

According to Egypt Demographic and Health Survey 2008 [1], neonatal mortality was 16 per 1000 live births. Identifying the causes of neonatal morbidity and mortality is essential for planning to its reduction, as one of the Millennium Development Goals [2]. The inability of premature neonates to produce surfactant and immaturity of the lung constitute the primary etiologies of respiratory distress syndrome (RDS). The *surfactant protein* (*SP*) genes have been used as candidate genes. So far, five human proteins have been identified: SP-A1, SP-A2, SP-B, SP-C, and SP-D [3]. The *SP-A* gene is located in chromosome 10q21 with two 99% homologous genes– *SP-A1* and *SP-A2* — in which a large number of single-nucleotide polymorphisms (SNPs) exist [4-6]. *SP-B* is secreted by type II cells in the lung and is essential for its normal function. Its absence results in respiratory failure and death shortly after birth [7-11]. Several studies have evaluated the association of *SP-A* and *SP-B* SNPs with RDS [12-14], but the functional consequences of their allelic variations are not well understood. The study of their polymorphisms aids in understanding the susceptibility of various individuals to RDS. The aim of this study was to investigate the association between *SP-A2* and *SP-B* genes polymorphisms and the risk of RDS in preterm neonates.

METHODS

We conducted this cross-sectional study at Children's Hospital, Faculty of Medicine, Cairo University, during the period between May 2013 and January 2014. Sixty-five preterm infants with RDS and 50 without RDS as controls were recruited from the neonatal intensive care unit (NICU). The study protocol was approved by the Ethics committee of Faculty of Medicine, Cairo University. Written informed consent was obtained from the legal guardians.

The criteria for the diagnosis of RDS included the presence of respiratory rate >60/min, dyspnea, grunting, cyanosis, respiratory acidosis in addition to the presence of typical chest *X*-ray findings [15-17]. Bronchopulmonary dysplasia (BPD) was diagnosed according to the National Institute of Health (US) criteria [18]. Neonates with genetic syndromes, congenital malformations, and other associated pathologies were excluded.

We c ollected 3 mL of blood in EDTA vacutainers, kept at 4ºC until DNA was extracted. Genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche Applied Science, USA).

Genotype analysis was carried out at the Molecular Biology unit of the Chemical Pathology Department, Faculty of Medicine, Cairo University. The c.751 G>A of *SP-A2* and c.8714 G>C in the 3'UTR of *SP-B* genes were genotyped using SYBR Green-based polymerase chain reaction (PCR) and consequent melting curve analysis was performed using LightCycler 2.0 Instrument (Roche Applied Science, Germany). PCR amplification was done using the Primers sequences as previously reported for *SP-B* [19, 20] and *SP-A2* [21] genes. The 20 µL reaction mixture included 15 µL of master mix with the following components: 9µL water, PCR grade; 1 µL forward Primers, 10×conc.; 1 µL reverse Primers, 10× conc.; 4µL 5×conc, LightCyclerFastStart DNA Master^{PLUS} SYBR Green I (Roche Applied Science, Germany); and 5 µL of 50 ng genomic DNA, under the following conditions: initial denaturation at 95ºC for 10 min, followed by 45 cycles of denaturation at 95 ºC for 10 s, annealing at 59ºC for 20s, and extension at 72ºC for 25s. After amplification, melting curve analysis was performed by heating the reaction mixture from 65 to 95 ºC at a rate of 0.1ºC/s. LightCycler 2.0 PCR Systems automatically calculated the negative derivative of the change in fluorescence and generated a melting curve for each sample ***Web Fig*. 1.**

Statistical analysis: Data analysis was done using SPSS Version 15, and Epicalc version 2000. Pearson Chi-Square, Fisher's Exact, and z tests were used; the predictors for RDS were tested using logistic regression analysis. P<0.05 was considered significant.

TABLE I DEMOGRAPHIC AND CLINICAL DATA OF THE STUDIED GROUPS

Variables	*RDS (n = 65)*	*Controls (n=50)*	*P-value*
Gestational age (wks)*	29.4 (2.42)	32.5 (2.02)	<0.001
Birth weight (kg)*	1.2 (0.33)	1.6 (0.32)	<0.001
Apgar at 5 min*	4.9 (1.07)	6.7 (1.32)	<0.001
#Males	37 (57)	28 (56)	0.92
#Caesarian delivery	47 (72)	35 (70)	0.79
#Antenatal steroid usage	22 (33.8)	18 (36)	0.81
#Multiple pregnancies	21(32.3)	12 (24)	0.22
#Premature rupture of membranes	8 (12.3)	3 (6)	0.25
#Maternal hypertension	20 (30.8)	17 (34)	0.71
#Maternal diabetes mellitus	3 (4.6)	7 (14)	0.07

*Values in * mean* (SD) *or* # No. (%).

RESULTS

Mean (SD) gestational age, birth weight, and Apgar score were significantly (P<0.001) lower in the RDS group compared to controls. Otherwise, there was no significant difference between the two groups regarding the remaining demographic and clinical data (***Table* I)**.

The amplification of the genomic DNA segments with subsequent genotyping analysis was successful in 115 samples. There were statistically significant differences in genotypes between the RDS and control groups (***Table* II**). Preterm neonates with *SP-A2* (AA and GA) genotypes and *SP-B* (CC and GC) genotypes were more prone to have RDS compared to neonates with the GG genotype. A and C alleles of *SP-A2* and *SP-B* genes, respectively, were significantly higher (P<0.001) in the RDS group. There was no significant association between variant genotypes

TABLE II GENOTYPE AND ALLELE FREQUENCIES IN THE STUDIED GROUPS

Variables	*RDS (n=65)*	*Controls (n=50)*	*P value*	*OR (95% CI)*
Genotype frequencies				
SP-A2				
GG	13 (20)	46 (92)	<0.001	
GA	1 (1.5)	2 (4)	0.82	
AA	51(78.5)	2 (4)	<0.001	
SP-B				
GG	19 (29.2)	34 (68)	<0.001	
GC	5 (7.7)	16 (32)	0.001	
CC	41(63.1)	0 (0)	<0.001	
Risky and Protective genotypes				
*SP-A2**				
GA+AA	52 (80)	4 (8)	<0.001	46 (14-151)
GG	13 (20)	46 (92)		
*SP-B**				
GC+CC	46 (70.8)	16 (32)	<0.001	5.2 (2.3-11.4)
GG	19 (29.2)	34 (68)		
Alleles				
*SP-A2**				
G	25 (19.2)	94 (94)	<0.001	65 (26 -167)
A	105 (80.8)	6 (6.0)		
*SP-B**				
G	44 (33.8)	84 (84)	<0.001	10.3 (5.4-19.6)
C	86 (66.2)	16 (16)		

Data expressed as number and percent.

WHAT IS ALREADY KNOWN?

- Low gestational age, maternal diabetes and perinatal asphyxia are risk factors for respiratory distress syndrome.

WHAT THIS STUDY ADDS?

- *SP-B and SP-A* polymorphisms are associated with increased risk of respiratory distress syndrome.

of both *SP-A2* and *SP-B* and severity of RDS or BPD among our patients. These variant genotypes also did not differ between the infants who died *versus* those who survived (***Table* III**).

In our study, the distribution of the combined genotypes of *SP-A2* and *SP-B* between RDS and the control as (AA and CC) represents 49.2% of cases and 0% of the control, while (GG and GG) represents 60% of the control and only 6.2% of cases ($P<0.001$).

For the regression analysis, the variables included were: maternal hypertension, maternal diabetes mellitus, multiple pregnancies, maternal use of antenatal steroids, premature rupture of membrane, mode of delivery, gestational age, birth weight, sex, Apgar score at 5 min, and *SP-A2* and *SP-B* genotypes. There were four significant predictors for RDS; two were directly related to the occurrence of RDS (the *SP-A2* genotype with OR =164; $P<0.001$; and *SP-B* genotype with OR=18; $P=0.008$), while gestational age (OR=0.57; $P=0.01$) and 5-minute Apgar score (OR=0.19; $P=<0.001$) were inversely related.

DISCUSSION

Factors affecting the development of RDS include specific SNPs of SPs which affect the protein structure and function [22], degree of prematurity, sex, and ethnicity [23]. In this study, we found that preterm neonates with *SP-A2* (AA) or SP-B (CC) genotypes had higher odds of RDS compared to neonates with GG genotype. However, Lyra, *et al*. [24] reported that there was no statistically significant difference in the distribution of the genotypes of the G/C polymorphism at nucleotide 8714 in patients with and without RDS. Our results are in concordance with an earlier study showing that the homozygous genotype for *SPA* was over represented in RDS [23].

Studying the distribution of the combined genotypes of *SP-A2* and *SP-B* between RDS cases and the control showed that combined (AA & CC) were present in higher number of cases than controls, while combined (GG and GG) represented more of the control than cases. This indicates that homozygous wild genes are essential for surfactant function. Meanwhile, there was no statistically significant synergistic effect of either *SP-A2* or *SP-B* on the severity of RDS, BPD, or neonatal mortality.

The limitations of present study include small sample size and no matching of cases and controls for gestational age and birth weight.

In conclusion, this study identified that the *SP-A2* [AA] and SP-B [CC] genotypes are risk factors of RDS. Further studies on a larger group of patients in different populations are required to confirm these findings.

Contributors: AWA: conceived and designed the study, and revised the manuscript for important intellectual content. She will act as guarantor of the study; ZN, SW: collected data, and drafted the paper; RW: laboratory tests and interpretation, data analysis and manuscript writing; RH: data analysis. The final manuscript was approved by all authors.
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TABLE III RELATION BETWEEN *SP-A2* AND *SP-B* GENOTYPES AND RDS GRADE, BPD AND OUTCOME

Variables	*SP A2 genotypes*		*P value*	*SP B genotypes*		*P value*
RDS grade	CA+AA	CC		GC+CC	GG	
I and II	15 (88.2)	2 (11.8)	0.49	12 (70.6)	5 (29.4)	0.99
III and IV	37 (77.1)	11 (22.9)		34 (70.8)	14 (29.2)	
BPD	10 (76.9)	3 (23.1)	0.76	9 (19.6)	4 (21.1)	0.89
Death	29 (55.8)	9 (69.2)	0.38	28 (60.9)	10 (52.6)	0.54

**Value in No. (%).*

from: *http://www.who.int/mediacentre/factsheets/fs290/en/* Accessed June 2, 2014
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