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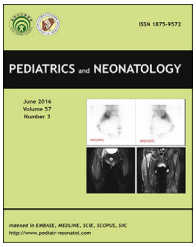

ORIGINAL ARTICLE

The Haplotype TGGAG in the *ABCA3* Gene Increases the Risk of Respiratory Distress Syndrome in Preterm Infants in Southern China

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Background: Rare mutations in the ATP-binding cassette (ABC) transporter A3 (*ABCA3*) gene are associated with neonatal respiratory distress syndrome (RDS). The contribution of common single nucleotide polymorphisms (SNPs) to preterm RDS differs between ethnicities and remains unclear in Chinese infants. This study evaluated whether common SNPs and consequent haplotypes increase susceptibility to RDS in a population of preterm infants from the Guangxi Zhuang Autonomous Region of China.
Methods: Using a tagging SNP (tSNP) strategy and real-time polymerase chain reaction, we genotyped four tSNPs (i.e., rs150929, rs4787273, rs11867129, and rs17135889) and one coding SNP (p.F353F) of the *ABCA3* gene in preterm infants with RDS ($n = 83$) and without RDS ($n = 83$). We predicted the haplotypes. Minor allele frequencies (MAFs) and haplotype distributions were compared between the two groups. We analyzed correlations between the clinical data and the genotypes.

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Results: Seven haplotypes existed at a frequency of 0.01 or greater. The haplotype TGGAG was significantly more frequent in RDS infants than in non-RDS infants ($p = 0.026$; odds ratio 3.41; 95% confidence interval 1.088—10.685). The MAF of rs17135889 SNP, a crucial SNP of the haplotype TGGAG located in the transcription factor binding site of *ABCA3*, was significantly higher in RDS infants ($p < 0.05$); however, the Bonferroni correction test showed no significant difference ($p > 0.05$). No significant correlation existed between the rs17135889 genotypes (AG/GG) and any clinical characteristic (e.g., oxygen supplementation duration and hospitalization, requirement for ventilation, bronchopulmonary dysplasia complications, and mortality rate).
Conclusion: The TGGAG haplotype may be a risk factor for RDS in preterm infants in this Chinese population. Further study is needed with a larger sample size to verify the association between the rs17135889 SNP and increased risk of RDS in preterm infants, and to determine whether rs17135889 can be a reference in further population-based studies of *ABCA3*.

1. Introduction

Respiratory distress syndrome (RDS), also known as hyaline membrane disease, is caused by pulmonary surfactant deficiency.[1] A surfactant is a phospholipid/protein mixture that reduces surface tension. Pulmonary surfactants prevent alveolar collapse at the end of expiration. The pulmonary surfactant metabolic cycle maintains alveolar homeostasis and includes synthesis, trafficking, processing, secretion, and recycling.[2] Developmental insufficiencies of pulmonary surfactants and genetic factors have been implicated as an important underlying cause of neonatal RDS.[3–8] ATP-binding cassette (ABC) transporter A3 (ABCA3) is a member of a large family of ABC transporters that are localized to the limiting membranes of the lamellar bodies in alveolar type II cells. ABCA3 transports lipids such as phosphatidylcholine, cholesterol, sphingomyelin, and phosphatidylglycerol into the lamellar bodies where surfactant complexes are assembled, processed, and stored.[9–14] Most mutations that are associated with neonatal RDS and chronic lung disease in children affect the *ABCA3* gene, and reports have revealed that most *ABCA3* mutations are rare and specific.[8,15,16] In addition to mutations in this gene, common polymorphisms that can exert minor effects on ABCA3 protein expression or function have been controversially associated with an increased risk of neonatal RDS. Haplotype analysis has revealed that p.F353F, a common synonymous *ABCA3* variant in the transmembrane domain, has been associated with a prolonged course of RDS in very premature Finnish infants.[17] By contrast, common or rare synonymous *ABCA3* variants have no contribution to the risk of neonatal RDS in infants of European or African descent in Missouri.[18] To date, it is unclear whether common *ABCA3* single nucleotide polymorphisms (SNPs) contribute to neonatal RDS in Chinese preterm infants, although a study of eight *ABCA3* SNPs has been conducted in eastern China and revealed that the rare synonymous variant p.P585P is associated with RDS susceptibility.[19] Because the selected SNPs in this previous study had a limited representation, further study is necessary to understand how *ABCA3* SNPs contribute to the risk of preterm RDS in Chinese infants.

China has the largest population in the world, and consists of 56 different ethnic groups. The Han are the largest ethnic group in China, and the population-based common variants in the Han population have been described and cataloged (which is available at HapMap.org site: http://www.stats.gov.cn/tjsj/pcsj/rkpc/6rp/indexch.htm,www.HapMap.org). Because genetic susceptibility may vary in different populations and because the contribution of *ABCA3* polymorphisms to RDS has yet to be characterized in preterm infants in southern China, we assessed the contribution of *ABCA3* polymorphisms to the risk of neonatal RDS in a native preterm cohort from the Guangxi Autonomous Region in southern China. We examined tagging SNPs (tSNPs), which were selected based on pairwise r^2 linkage disequilibrium (LD) statistics, rather than sequencing the 65-kb *ABCA3* gene, which contains 33 exons and abundant SNPs.[10] The r^2 threshold was set to 0.8 for the tSNP selection to enable the identification of informative markers that were predominant in the study population.[20,21] Haplotype prediction analysis was then performed to examine the associations between these *ABCA3* polymorphisms and the risk of neonatal RDS.

2. Materials and methods

2.1. The RDS diagnostic criteria and the study cohort

We defined RDS by the clinical signs (which typically include early respiratory distress with cyanosis, grunting, retractions, and tachypnea that present soon after birth and increase in severity during the first 2 days of life) and by the chest x-ray findings (e.g., classic ground-glass appearance and air bronchograms).[22] Using a case-control cohort design, we recruited 83 preterm infants with RDS (RDS+) and 83 preterm infants without RDS (RDS−) as the controls from five tertiary hospitals in the Guangxi Zhuang Autonomous Region: First Affiliated Hospital of Guangxi Medical University (Guangxi, China); Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region (Guangxi, China); Nanning Women and Children's Hospital (Guangxi,

China); Qinzhou Maternal and Child Health Hospital (Qinzhou, China); and Eighth Affiliated Hospital of Guangxi Medical University (Guangxi, China). All infants were ethnic Han, and the RDS+ and RDS− groups were matched according to gestational age (with a difference of less than 1 week, $p > 0.05$) and sex (Table 1).

This study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (Guangxi, China). The data were analyzed anonymously. The Ethics Committee approved a waiver of written consent.

2.2. DNA preparation

Genomic DNA was extracted from whole blood samples using a whole blood DNA extraction kit (RelaxGene Blood DNA System DP319; TIANGEN Biotech Co., Ltd, Beijing, China).

Table 1 Demographic characteristics of infants with and without respiratory distress syndrome.

	RDS+	RDS−	*p*
Total no. of infants	83	83	NS
Gestational age, wk *	32.15 ± 2.18	32.96 ± 2.67	NS
Birth weight (g)	1723.90 ± 466.95	1849.70 ± 470.07	NS
Men/women (*n*)	54/29	54/29	NS

NS = not significant; RDS+ = with respiratory distress syndrome; RDS− = without respiratory distress syndrome.
* The data are presented as the mean ± the standard deviation.

2.3. Selection of the tSNPs

We selected *ABCA3* gene SNPs among Han Chinese in Beijing, China from the HapMap database. All SNPs displayed a validated minor allele frequency (MAF) of 0.1 or greater (including intronic and exonic SNPs). The SNPs identified in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) under the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) entry NT_010393 were screened by using HaploView v. 4.2 software (Broad Institute, Cambridge, MA, USA).[23] In total, 13 SNPs displaying a MAF of 0.1 or greater were identified: 12 intronic SNPs—rs2238464, rs2014467, rs17183533, rs4787273, rs150929, rs150926, rs2302035, rs323073, rs323072, rs11867129, rs4787277, and rs17135889—and one coding SNP (cSNP; rs13332514), which corresponds to the F353F residue (Table 1). We then selected tSNPs using a method based on pairwise r^2 LD statistics with the r^2 threshold set to 0.8. The tSNP approach is a powerful means of reducing the number of examined SNPs to decrease the cost of association studies. Therefore, this approach is especially advantageous for genotyping large genes containing many SNPs. The HaploView v. 4.2 software (Broad Institute, Cambridge, MA, USA) uses an accelerated expectation maximization algorithm to calculate haplotype frequency estimates from unphased genotype data. A value of $p < 0.05$ was considered significant. Four tSNPs were selected: rs150929, rs4787273, rs11867129, and rs17135889, which are located in introns 29, 13, 10, and 1, respectively. The average intermarker distance was 14.65 kb, the largest distance between adjacent SNPs was 58.61 kb, and the smallest distance was 2.048 kb. An additional cSNP (rs13332514 in the p.F353F residue) was included as a target SNP because it was correlated with an increased RDS risk in a Finnish premature infant cohort.[17] All selected SNPs are listed in Table 2.

Table 2 Selected single nucleotide polymorphisms with a minor allele frequency of 0.1 or greater in the Han Chinese in Beijing population.

SNP no.*	Allele	Position †	Location	MAF	HWpval	tSNP	r^2 value	Corresponding tSNP ‡
rs17135889	G/A	2337259	Intron 1	0.19	0.076	tSNP1		
rs4787277	G/A	2318168	Intron 8	0.345	1		0.89	tSNP2
rs11867129	G/T	2315287	Intron 10	0.345	0.765	tSNP2		
rs323072	C/T	2310098	Intron 10	0.369	0.136		0.85	tSNP2
rs323073	G/A	2298761	Intron 10	0.369	0.136		0.85	tSNP2
rs13332514	G/A	2307337	Exon 10	0.351	1		0.92	tSNP2
rs4787273	G/T	2299759	Intron 13	0.416	1	tSNP3		
rs17183533	T/C	2295387	Intron 18	0.464	1		1	tSNP3
rs2014467	T/C	2286393	Intron 22	0.464	1		1	tSNP3
rs2238464	C/T	2282576	Intron 26	0.464	1		1	tSNP3
rs150926	C/G	2280168	Intron 28	0.416	1		0.98	tSNP4
rs2302035	C/T	2279260	Intron 28	0.399	0.7381		0.88	tSNP4
rs150929	T/G	2278649	Intron 29	0.417	1	tSNP4		

HW-pval: Hardy–Weinberg equilibrium value; MAF = minor allele frequency; SNP = single nucleotide polymorphism; tSNP = tagging single nucleotide polymorphism.
* The rs number IDs are indicated for SNPs with entries in the dbSNP [National Center for Biotechnology Information (NCBI; Bethesda, MD, USA)].
† These positions are reported in the NCBI database.
‡ The corresponding tSNPs are presented for non-tSNPs.

2.4. Genotyping

We used real-time polymerase chain reaction (PCR) technology (TaqMan real-time PCR) to genotype the five selected SNPs (i.e., rs150929, rs4787273, rs17135889, rs11867129, and rs13332514). The PCR primers and probes were designed and provided by Applied Biosystems (Foster City, CA, USA), and their respective assay IDs were as follows: C_3156769_10, C_27939939_10, C_34046137_10, C_31767685_10, and C_25970779_10 (www.lifetechnologies.com). The complete PCR products were analyzed using the ABI FAST (7500) system (Applied Biosystems, Foster City, CA, USA). The PCR cycle included a 95°C 10-minute hot start, followed by 50 cycles of two-step PCR (15 seconds at 95°C for denaturing and 1 minute at 60°C for annealing and extension). The 6-carboxyfluorescein (FAM) and VIC signals were recorded at the end of each PCR cycle. Digital PCR analysis software (TaqMan GenoTyper v1.3) was used to process the data.

2.5. Statistical analysis

The allele frequencies, Hardy–Weinberg equilibrium, pairwise LD assumptions for each polymorphism, and haplotype frequency estimations were analyzed using SHEsis software (Shanghai Jiao Tong University, Shanghai, China) (available at http://analysis.bio-x.cn/myAnalysis.php). The strengths of the associations between the alleles or genotypes and disease status were calculated using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). We used the Chi-square or Fisher's exact test to analyze the data. The *t* test was used for normally distributed data, and the Bonferroni correction test was used to confirm the frequency of each SNP. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Allele frequencies, Hardy–Weinberg equilibrium, pairwise LD, haplotype prediction, and data analysis

Five SNPs were genotyped, and their frequencies did not deviate from the Hardy–Weinberg equilibrium. The allele frequencies in the RDS+ and RDS− groups are listed in Table 3. The Chi-square test indicated that the MAF of rs17135889 was significantly higher in infants with RDS than in infants without RDS ($p < 0.05$); however, further analysis using the Bonferroni correction test revealed no significant differences ($p > 0.05$).

3.2. Haplotype prediction

The haplotype frequencies were estimated using SHEsis software/analysis (Shanghai Jiao Tong University, Shanghai, China). We identified seven haplotypes displaying frequencies of 0.01 or greater. Among them, the frequency of the TGGAG haplotype was significantly higher in the RDS+ group than in the RDS− group, based on the Chi-square test [$p = 0.026$, odds ratio (OR), 3.41; 95% confidence interval (Cl), 1.088–10.685]. All haplotypes are listed in Table 4.

3.3. Pairwise LD

Pairwise LD was detected between rs13332514 and rs11867129 according to the SHEsis analysis (Figure 1).

3.4. Genotype and clinical data analysis

In total, 1 AA, 31 AG and 51 GG rs17135889 genotypes were detected in premature infants with RDS, whereas 1 AA, 15 AG and 67 GG rs17135889 genotypes were detected in infants without RDS. The proportion of the homozygous AA genotype was very low (2 of 166), regardless of RDS status.

The frequency of AG in RDS+ and RDS− infants were 0.373 and 0.181, respectively, whereas GG were 0.614 and 0.807, respectively. The AG genotype was significantly more frequent among the RDS+ infants ($X^2 = 2.0$, $P = 0.023$).

Table 4 The *ABCA3* haplotypes (frequency ≥ 0.1) in infants with and without respiratory distress syndrome.

	RDS+	RDS−	*p*	OR	95% CI
TGGAG	0.078	0.025	0.026	3.410	1.088–10.685
GTTAA	0.041	0.066	0.325	0.611	0.227–1.645
GTGGG	0.123	0.183	0.134	0.627	0.340–1.645
TGGGG	0.308	0.345	0.493	0.850	0.533–1.354
TTGGG	0.050	0.037	0.554	1.381	0.472–4.039
GTTGA	0.243	0.237	0.878	1.040	0.625–1.732
TGTGA	0.049	0.074	0.345	0.645	0.472–1.616

CI = confidence interval; OR = odds ratio; RDS+ = with respiratory distress syndrome; RDS− = without respiratory distress syndrome.

Table 3 Minor allele frequencies of ABCA3 tagging single nucleotide polymorphism and p.F353F.

SNPs	Allele	RDS+ ($n = 83$)	RDS− ($n = 83$)	*P*	Bonferroni-corrected *p*
rs150929	T/G	0.45	0.51	0.23	1.15
rs4787273	G/T	0.53	0.45	0.12	0.60
rs11867129	G/T	0.42	0.41	0.91	4.55
rs17135889	A/G	0.19	0.10	0.02	0.10
rs13332514	A/G	0.41	0.40	0.46	2.30

RDS+ = with respiratory distress syndrome; RDS− = without respiratory distress syndrome; SNP = single nucleotide polymorphism; tSNP = tagging single nucleotide polymorphism.

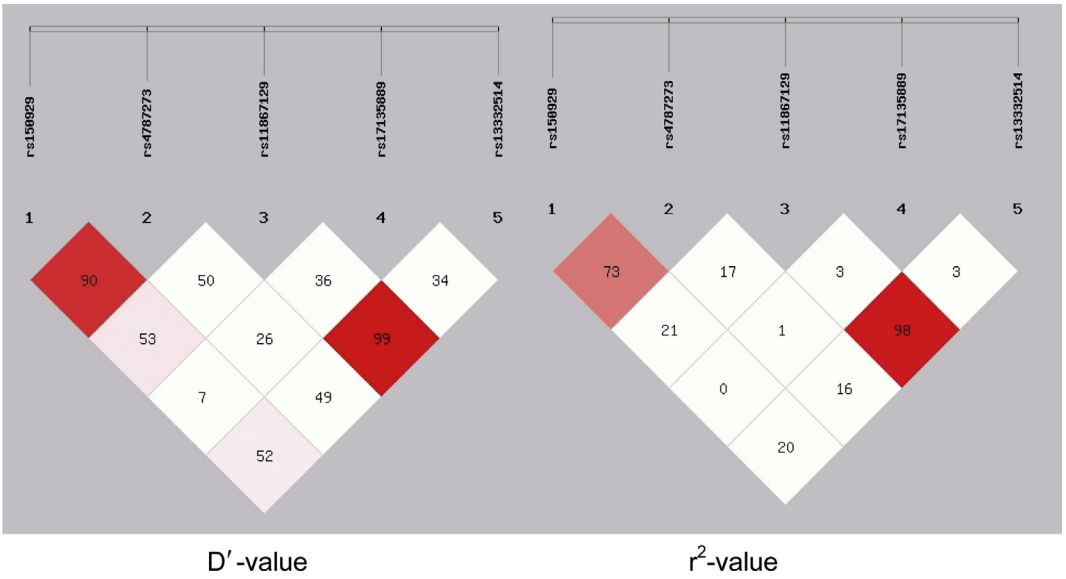

Figure 1 The IDs and relative positions of the examined single nucleotide polymorphisms are presented at the top. The numbers in the squares indicate the D′ values or the r^2 values for the pairwise comparisons. Pairwise linkage disequilibrium exists between rs13332514 and rs11867129.

We collected clinical data for all infants in our cohort to assess whether rs17135889 was associated with unique RDS characteristics. The clinical characteristics of the two groups of AG and GG carriers are presented in Table 5. We failed to detect a significant correlation between the AG genotype and any clinical characteristic such as duration of oxygen supplementation, requirement for ventilation, bronchopulmonary dysplasia (BPD) complications, hospitalization duration, and mortality rate.

4. Discussion

In our previous study, we used an Illumina next-generation sequencing platform (Illumina, San Diego, CA, USA) provided by Washington University in St. Louis, MO, USA to resequence the entire exonic and flank regions of *ABCA3* in 756 Han term infants. We identified the synonymous variant p.F353F at an MAF of approximately 0.40. However, we were unable to identify the synonymous variant p.P585P (data not shown), which is a rare variant according to the dbSNP database. This variant reportedly displayed a MAF of 0 in Han Chinese in a Beijing cohort consisting of 88 individuals, despite another report of its association with neonatal RDS risk in an eastern Chinese cohort (0.058 and 0.008 among preterm infants with and without RDS, respectively; $p < 0.05$).[19] The cohort in the present study exhibited a genetic background similar to that of our preliminary study cohort, both of which were from the Guangxi Zhuang Autonomous Region in southern China. Our list of tSNPs did not have p.F353F, although we genotyped this variant because it reportedly contributed to RDS susceptibility in a Finnish preterm population. However, we did not include p.P585P because it exists at extremely low frequencies in the HapMap database and in our preliminary study. As anticipated, we found similar p.F353F frequencies, compared to those in our previous study and compared to those of CHB (Han Chinese in Beijing, China)

Table 5 Clinical characteristics of respiratory distress syndrome infants with tagging single nucleotide polymorphisms rs17135889 (AG) and rs17135889 (GG).

Clinical characteristics	rs17135889 (AG)	rs17135889 (GG)	*p*
	$n = 31$	$n = 51$	
Gestational age, wk	32.08 ± 2.65	32.20 ± 1.87	NS
Birth weight (g)	1661.10 ± 425.85	1761.30 ± 48998	NS
Male/female patient (*n*)	16/15	34/17	NS
Ventilation, yes/no (*n*)	29/2	50/1	NS
Oxygen supplementation (median hours)	168.5	132.0	NS
BPD	1/30	3/48	NS
Hospitalization duration (median days)	27	26.5	NS
Prognosis			
Survival/death (withdrawn from study)	29/2	48/3	NS

BPD = bronchopulmonary dysplasia; NS = not significant.

individuals in HapMap. These results indicate that no selection pressure exists for this variant and demonstrate that the CHB and local Han populations in the Guangxi Autonomous Region have similar genetic backgrounds. We did not identify a significant difference in the p.F353F variant frequencies between the infants with and without RDS. This result is consistent with the findings of a previous study[18] of infants of European descent and of African descent in a Missouri population; however, the result differs from those of another study[17] of a cohort of Finish preterm infants. Differences in ethnicity may only partially explain the differences in the results obtained for these distinct populations.

We found that the MAF of the intronic SNP rs17135889 was interestingly higher among infants with RDS than among infants without RDS (0.19 vs. 0.10, $p < 0.05$), although no significant difference was found using the Bonferroni correction test ($p > 0.05$). The actual significance of this SNP is unknown because a small sample size was used in this study; thus, a further study with a larger sample size is required.

Seven haplotypes were present at a frequency of 0.01 or greater. The TGGAG haplotype frequency was significantly higher among the preterm infants with RDS than among infants without RDS. The rs17135889 SNP differentiated the TGGAG haplotype from the other haplotypes, and thereby explains the detected nominal haplotypic association. The nominal association between the TGGAG haplotype and RDS appears to depend exclusively on this SNP because this relationship disappeared after removing the SNP from haplotype analysis (data not shown). In this study, the TGGAG haplotype was observed more frequently in the case group. This finding may indicate that this haplotype is a risk factor for RDS among premature infants. To date, no published report is available regarding the association between the TGGAG haplotype and neonatal RDS. In the present study, this haplotype displayed a significantly higher frequency among premature infants with RDS. However, the mechanism by which the associated haplotype may predispose premature infants to RDS remains unknown.

Analysis of the association between the examined genotypes and clinical data did not reveal a significant correlation between the rs17135889 genotypes (AG/GG) and any clinical characteristic such as oxygen supplementation duration, requirement for ventilation, BPD complications, hospitalization duration, and mortality rate. This finding implies that this variant may exert a small effect on RDS or that gene interactions may be involved in the underlying mechanism. For example, the pulmonary surfactant A (*SP-A*) genotypes 6A2/6A2 (*SP-A1*) or 1A0/1A0 and 1A0/* (*SP-A2*) in conjunction with a specific *SP-B* genotype (9306 (A/G) or del/*) in certain white preterm infants are associated with an increased risk of RDS, whereas 6A3/6A3 or 6A3/* (*SP-A1*) in conjunction with the 1580 (T/T) (*SP-B*) genotype in black preterm infants is associated with a reduced risk of RDS.[24]

To our knowledge, this is the first study of the association between *ABCA3* gene polymorphisms and neonatal RDS in a Chinese population using tSNP. Four selected tSNPs in our study accounted for more than 90% of the haplotypes. However, our tSNP approach may contain some limitations. First, some rare variants were potentially overlooked because we selected tSNPs displaying an MAF of 0.1 or greater. The association between rare variants and an increased risk of preterm RDS among different ethnicities remains controversial. Second, our sample size was relatively small, and the results must be confirmed in replicate studies.

5. Conclusions

Our results suggest that the haplotype TGGAG may increase the risk of preterm RDS in the Chinese Han population. Other studies with a larger sample size are needed to verify an association between the rs17135889 SNP located in the transcription factor binding site of the *ABCA3* gene and an increased risk of RDS in preterm infants.

Conflicts of interest

All contributing authors declare that no conflicts of interest exist.

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