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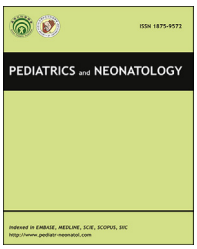

ORIGINAL ARTICLE

Association of Rho-kinase Gene Polymorphisms with Respiratory Distress Syndrome in Preterm Neonates

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Key Words
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Background: Respiratory distress syndrome (RDS) of the newborn is one of the most common causes of morbidity and mortality in preterm infants. Our objective was to determine the association between Rho-kinase (*ROCK1* and *ROCK2*) gene polymorphisms and RDS in preterm neonates.
Methods: A total of 193 preterm infants with RDS and 186 preterm infants without respiratory problems were included in this study. Polymorphisms were analyzed in genomic DNA using a BioMark 96.96 dynamic array system.
Results: We observed that *ROCK1* gene rs2271255 (Lys222Glu) and rs35996865 polymorphisms, and *ROCK2* gene rs726843, rs2290156, rs10178332, and rs35768389 (Asp601Val) polymorphisms were associated with RDS. However, no associations were found with rs73963110, rs1515219, rs965665, rs2230774 (Thr431Asn), rs6755196, and rs10929732 polymorphisms. Additionally, 12 haplotypes (6 in *ROCK1* and 6 in *ROCK2*) were found to be markedly associated with RDS.

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Conclusion: This is the first study to examine the involvement of *ROCK* gene variation in the risk of incident RDS. The results strongly suggest that *ROCK* gene polymorphisms may modify individual susceptibility to RDS in the Turkish population.

1. Introduction

Respiratory distress syndrome (RDS) of the newborn is the most common cause of respiratory distress in premature infants occurring as a result of surfactant deficiency and underdeveloped lung anatomy. The clinical presentation of respiratory distress in the newborn includes apnea, cyanosis, grunting, inspiratory stridor, nasal flaring, poor feeding, and tachypnea.[1] Respiratory distress occurs in approximately 7% of infants.[2] The pathophysiology of RDS is complex. Immature type II alveolar cells produce less surfactant, causing an increase in alveolar surface tension and a decrease in compliance. The resulting atelectasis causes pulmonary vascular constriction, hypoperfusion, and ischemia in lung tissue.[1]

Rho-kinase (ROCK) is a serine/threonine kinase that is activated by Rho proteins. Two ROCK isoforms have been described: ROCK1 and ROCK2. The ROCK isoforms are encoded by separate genes on human chromosomes 18q11 (ROCK1) and 2p24 (ROCK2). The Rho/ROCK pathway is thought to participate in a wide range of fundamental cellular functions including cell morphology, motility, adhesion, migration, proliferation, differentiation, and apoptosis.[3] ROCK can also regulate macrophage phagocytic activity and endothelial cell permeability, and it is known to play a role in inflammatory mechanisms and endothelial dysfunction.[4,5] The Rho/ROCK pathway plays an important role in the regulation of baseline tone and vasoconstrictor responses of the pulmonary vascular bed, and vascular remodeling occurring in pulmonary disorders.[6–8] ROCK is upregulated by inflammatory stimuli; inhibition of ROCK increases expression of endothelial nitric oxide synthase and inhibits inflammatory cell migration.[3,9]

There is considerable evidence indicating that mechanical stretch is essential for lung growth and development.[10–12] Mechanical stretch is also an important stimulus for activation of small GTPases RhoA in fetal type II epithelial cells.[13] There is evidence that the Rho/ROCK pathway plays an important role as mechanosensor acting *in vivo* either directly or indirectly for transforming increased distention into acceleration of lung growth.[14] In experimental studies, systemic administration of a ROCK inhibitor, Y-27632, substantially reduced pulmonary microvascular permeability, edema, and lung injury.[15,16] ROCK may play an important role in the pathogenesis of lipopolysaccharide-induced lung injury; and ROCK inhibition could attenuate cytoskeletal rearrangement of endothelial cells, leading to decreased neutrophil emigration into the lung parenchyma.[17] Exogenous human purified surfactant protein (SP)-A induced stress fiber formation in cultured human myometrial cells via a ROCK-related pathway. It was shown that pharmacological inhibition of ROCK resulted in a clear reduction of stress fiber formation induced by SP-A.[18] Although there is a strong genetic susceptibility to development of RDS in preterm infants,[19] no study has assessed the impact of *ROCK* gene polymorphisms on RDS development before. The purpose of this study was to determine the role of *ROCK1* and *ROCK2* gene polymorphisms in the development of RDS in preterm newborns.

2. Materials and methods

2.1. Patients

A total of 193 preterm infants with gestational age under 37 weeks and birth weight <2500 g who were admitted to the Neonatal Intensive Care Unit of Gaziantep University Hospital and Gaziantep Children Hospital between 2011 and 2012, were included in this study. RDS was diagnosed according to the following criteria: respiratory distress beginning in the first hours of life and lasting at least 24 hours, need for mechanical ventilation including continuous positive airway pressure, presence of typical radiological findings in chest X-ray, and abnormal arterial blood gas results. Tachypnea (>60 breaths/min), chest retractions, nasal flaring, grunting, need to maintain the oxygen saturation at $\geq 86\%$ with $Fio_2 \geq 0.40$ in addition to the chest radiograph results with ≥ 2 Grade 2 RDS findings confirmed the diagnosis of RDS. Classification of pulmonary X-ray findings for RDS was based on the following criteria: Grade 1—slight reticular (slightly granular) decrease in transparency of the lung with no certain difference from normal findings; Grade 2—soft decrease in transparency with an air bronchogram, which overlaps the heart; Grade 3—gradual stronger decrease in transparency, as well as a blurry diaphragm and heart; and Grade 4—practically homogeneous lung opacity.[20] In the RDS group, duration of hospitalization, mechanical ventilation and oxygen use, number of surfactants used, and mortality were recorded. Preterm infants without respiratory problems constituted the control group ($n = 186$; gestational age, 27–36 weeks; birth weight, 650–2700 g). The exclusion criteria were presence of congenital anomalies, sepsis, intrauterine infections, genetic disorders, and inherited metabolic disorders. Patients and controls came from the same ethnic group (Caucasians). This study was approved by the local Ethics Committee, and informed consent was obtained from all parents.

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2.2. Blood samples and DNA isolation

Venous blood sample was obtained from all study participants into EDTA-containing tubes. Immediately after collection, whole blood was frozen and stored at −20°C until the time of analysis. Genomic DNA was extracted from whole blood using the salting-out method and was stored at −20°C.

2.3. Genotyping

The genotype was determined in all patients and controls using the Fluidigm dynamic array system as previously described.[21,22] Polymorphisms were analyzed in genomic DNA using a 96.96 dynamic array on the BioMark HD system (Fluidigm, South San Francisco, CA, USA). The Digital PCR Analysis software (Fluidigm) was used to process the data after the reaction. Chambers that yielded signals were detected and counted. Genotyping was conducted in a blinded fashion.

The criteria for choice of single nucleotide polymorphisms analyzed in this study were as follows: (1) relatively high frequency of minor alleles in the Caucasian populations; (2) being located within the promoter region, and exonic and intronic sites that could potentially impact ROCK expression and function; and (3) suitability for the Fluidigm dynamic array chip design, i.e., with no high G/C levels. In the present study, 12 single nucleotide polymorphisms [ROCK1: rs2271255 (Lys222Glu) in exon 6, rs73963110 in intron 1, and rs35996865 in 5′ UTR; ROCK2: rs1515219 in intron 5, rs726843 in intron 13, rs2290156 in intron 29, rs965665 in intron 3, rs10178332 in intron 3, rs2230774 (Thr431Asn) in exon 10, rs6755196 in intron 1, rs10929732 in intron 1, rs35768389 (Asp601Val) in exon 16] were analyzed for *ROCK* gene polymorphisms.

2.4. Statistical analysis

Descriptive statistics are expressed as mean ± standard deviation or percentage. Statistical analysis was performed using GraphPad Instat (Version 3.05; GraphPad Software Inc., San Diego, CA, USA) and the SPSS statistical package (version 22.0; SPSS, Inc., Chicago, IL, USA). Gestational age, birth weight, Apgar score, and mode of delivery were adjusted in the binary logistic regression model for the association of ROCK polymorphisms with RDS. The odds ratio and 95% confidence intervals were also calculated using logistic regression analysis. The multivariate regression analyses were used for calculation of the significance of differences in genotype frequencies. For calculation of the significance of differences in allele frequencies, the Chi-square test (with Yate's correction) or Fisher's exact test was used. A statistical comparison of two groups was performed with unpaired Student *t* test. Apgar scores were compared with Mann–Whitney *U* test. The original significance level was set at a *p* value of 0.05. Haplotype analysis was performed using the online software, SHEsis (http://analysis.bio-x.cn/myAnalysis.php). Bonferroni correction for multiple testing was used for polymorphism studies, and a *p* value of <0.0042 (0.05/12) was considered statistically significant. All probability values were based on two-tailed tests.

3. Results

The demographic characteristics of the groups are shown in Table 1. Apgar score, mode of delivery, incidence of intubation, duration of ventilation, and incidence of bronchopulmonary dysplasia were found to be significantly different between patient and control groups. Average gestational age, birth weight, sex, use of antenatal steroid, and age of mother were similar in both groups.

Significant associations for genotype and allele frequencies of *ROCK1* gene polymorphisms are shown in Table 2. *ROCK1* gene polymorphisms rs2271255 (Lys222Glu) and rs35996865, but not rs73963110, were found to be significantly associated with RDS development. There were marked differences in both genotype (CC, 34.1%; TT, 37.2%) and allele (C, 48.5%; T, 51.5%) frequencies for the rs2271255 (Lys222Glu) polymorphism in the RDS group when compared to controls (CC, 66.7%; TT, 5.0%; C, 80.8%; T, 19.2%; $p < 0.001$). The presence of the TT genotype and T allele were associated with a 9.93- and 4.48-fold increased risk of RDS, respectively. GT genotype and T allele frequencies of the rs35996865 polymorphism were markedly high among cases with RDS (GT, 54.2%; T, 69.7%)

Table 1 Demographic characteristics of infants in control and RDS groups.

	Control group (n = 186)	RDS group (n = 193)	p
Gestational age (wk)	29.3 ± 2.3	29.5 ± 2.9	0.4585
Birth weight (g)	1408.9 ± 519.9	1339.1 ± 526.1	0.1948
Sex (male/female)	97/89	114/79	0.2107
Apgar score (5^{th} min)	9.1 ± 0.8	6.4 ± 1.4	<0.0001
Mode of delivery (NSD/C/S)	22/164	42/151	0.0145
Antenatal steroid use	24/162	19/174	0.4185
Age of mother (y)	29.6 ± 6.1	29.4 ± 5.8	0.7437
Incidence of intubation (n, %)	7 (3.8)	193 (100)	<0.0001
Exogenous surfactant use (n, %)	—	192 (99.5)	
Duration of ventilation (h)	14.6 ± 11.9	81.2 ± 75.8	<0.0001
Incidence of BPD (n, %)	1 (0.5)	8 (4.1)	0.0371

BPD = bronchopulmonary dysplasia; C/S = cesarean section; NSD = normal spontaneous delivery; RDS = respiratory distress syndrome.

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Table 2 Significant associations for genotype and allele frequencies of *ROCK1* gene polymorphisms among the RDS cases and controls.

Genotypes/alleles	Controls *n* (%)	RDS *n* (%)	*p*	OR (95% CI)
rs2271255 (Lys222Glu)	*n* = 159*	*n* = 164*		
C/C	106 (66.7)	56 (34.1)		
C/T	45 (28.3)	47 (28.7)	0.423	1.423 (0.601–3.371)
T/T	8 (5.0)	61 (37.2)	0.001	9.927 (3.094–31.849)
C	257 (80.8)	159 (48.5)		
T	61 (19.2)	169 (51.5)	0.001	4.478 (3.145–6.376)
rs35996865	*n* = 184*	*n* = 190*		
G/G	80 (43.5)	6 (3.2)		
G/T	32 (17.4)	103 (54.2)	0.001	82.578 (15.314–445.292)
T/T	72 (39.1)	81 (42.6)	0.001	46.857 (8.863–247.721)
G	192 (52.2)	115 (30.3)		
T	176 (47.8)	265 (69.7)	0.001	2.514 (1.863–3.392)

CI = confidence interval; OR = odds ratio; RDS = respiratory distress syndrome.
* Numbers do not always add up to total numbers because of missing values on the BioMark dynamic array system.

when compared to the control group (GT, 17.4%; T, 47.8%, $p < 0.001$). However, GG genotype (3.2%) and G allele (30.3%) were found to be low in the RDS group when compared to controls (GG, 43.5%; G, 52.2%). High odds ratios were observed with this polymorphism. The presence of GT genotype and T allele increased the risk of RDS by 82.58- and 2.51-fold, respectively (Table 2).

Significant associations for genotype and allele frequencies of *ROCK2* gene polymorphisms are presented in Table 3. Although there were marked changes in genotype distribution of the rs726843 polymorphism (TT, 44.7%; TC, 19.5% in the control group vs. TT, 13.6%; TC, 68.1% in the RDS group; $p = 0.001$), no significant differences were noted in allele frequency. The risk of RDS increased 16.73-fold in

Table 3 Significant associations for genotype and allele frequencies of *ROCK2* gene polymorphisms among RDS cases and controls.

Genotypes/alleles	Controls *n* (%)	RDS *n* (%)	*p*	OR (95% CI)
rs726843	*n* = 159*	*n* = 169*		
T/T	71 (44.7)	23 (13.6)		
T/C	31 (19.5)	115 (68.1)	0.001	16.728 (5.597–49.997)
C/C	57 (35.8)	31 (18.3)	0.083	2.746 (0.875–8.612)
T	173 (54.4)	161 (47.6)		
C	145 (45.6)	177 (52.4)	0.098	1.312 (0.965–1.783)
rs2290156	*n* = 139*	*n* = 156*		
C/C	78 (56.1)	19 (12.2)		
C/G	20 (14.4)	80 (51.3)	0.001	8.082 (2.593–25.193)
G/G	41 (29.5)	57 (36.5)	0.001	6.588 (2.314–18.752)
C	176 (63.3)	118 (37.8)		
G	102 (36.7)	194 (62.2)	0.001	0.353 (0.252–0.493)
rs10178332	*n* = 139*	*n* = 173*		
A/A	120 (86.3)	40 (23.1)		
A/C	18 (12.9)	128 (74.0)	0.001	13.750 (5.589–33.828)
C/C	1 (0.7)	5 (2.9)	0.428	2.874 (0.212–39.052)
A	258 (92.8)	208 (60.1)		
C	20 (7.2)	138 (39.9)	0.001	8.559 (5.174–14.159)
rs35768389 (Asp601Val)	*n* = 177*	*n* = 179*		
T/T	124 (70.1)	39 (21.8)		
T/A	19 (10.7)	99 (55.3)	0.001	20.696 (6.910–61.986)
A/A	34 (19.2)	41 (22.9)	0.001	8.151 (2.705–24.560)
T	267 (75.4)	177 (49.4)		
A	87 (24.6)	181 (50.6)	0.001	3.138 (2.282–4.316)

CI = confidence interval; OR = odds ratio; RDS = respiratory distress syndrome.
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Table 4 Insignificant associations for genotype and allele frequencies of *ROCK1* and *ROCK2* gene polymorphisms in RDS and control groups.

Gene SNP	Genotypes/alleles	Controls	n^*	RDS	n^*	p value
ROCK1	CC/CT/TT	39/97/9	145	71/84/18	173	0.0044
rs73963110	C/T	175/115		226/120		0.2256
ROCK2	CC/CT/TT	55/102/27	184	55/89/37	181	0.2978
rs1515219	C/T	212/156		199/163		0.5200
ROCK2	GG/GC/CC	111/70/5	186	114/78/1	193	0.2219
rs965665	G/C	292/80		306/80		0.8618
ROCK2	CC/CA/AA	52/96/38	186	51/90/52	193	0.3242
rs2230774 (Thr431Asn)	C/A	200/172		192/194		0.3006
ROCK2	GG/GA/AA	138/36/11	185	135/42/16	193	0.5348
rs6755196	G/A	312/58		312/74		0.2421
ROCK2	GG/GA/AA	123/54/9	186	128/61/4	193	0.3134
rs10929732	G/A	300/72		317/69		0.6673

RDS = respiratory distress syndrome; SNP = single nucleotide polymorphism.
* Numbers do not always add up to total numbers because of missing values on the BioMark dynamic array system.

the presence of TC genotype. CG genotype (51.3%) and G allele (62.2%) of rs2290156 polymorphism were more frequent among the RDS cases compared to controls (CG, 14.4%; G, 36.7%; $p = 0.001$). There were lower frequencies of the CC genotype (12.2%) and C allele (37.8%) in the RDS group when compared to controls (CC, 56.1%; C, 63.3%, $p = 0.001$). AC genotype (74.0% vs. 12.9%, $p = 0.001$) and C allele (39.9% vs. 7.2%, $p = 0.001$) of the rs10178332 polymorphism were more frequent in the RDS group. The presence of the AC genotype and C allele were associated with a 13.75- and 8.56-fold increased risk for RDS, respectively. For the rs35768389 (Asp601Val) polymorphism, high frequencies of TA genotype (55.3% vs. 10.7%) and A allele (50.6% vs. 24.6%) were noted in the patient group. TT genotype (21.8%) and T allele (49.4%) frequencies were lower in the RDS group when compared to controls (TT, 70.1%, T, 75.4%, $p = 0.001$). The presence of TA genotype and A allele increased the risk of RDS by 20.70- and 3.14-fold, respectively (Table 3). However, no associations were found with rs1515219, rs965665, rs2230774 (Thr431Asn), rs6755196, and rs10929732 polymorphisms (Table 4).

Haplotypes based on the *ROCK1* gene polymorphisms were constructed, and six haplotypes (CCG, CCT, CTG, TCT, TTG, and TTT) were detected to have significant association with RDS (Table 5). CCT, TCT, TTG, and TTT haplotype frequencies were higher in the RDS group. However, CCG and CTG haplotype frequencies were lower in cases with RDS ($p < 0.0001$). There were no marked associations between the CTT and TCG haplotype frequencies and RDS. Haplotypes based on the studied *ROCK2* gene polymorphisms were constructed, and 12 haplotypes with high frequency were detected (Table 5). Only six of these haplotypes were markedly associated with RDS. CCAT and TCAT haplotypes were less frequent, whereas CCAA, CGAA, TGCA, and TGCT haplotypes were more frequently found among cases with RDS compared to controls. Interestingly, TGCA and TGCT haplotypes were only observed among RDS cases. Although none of the controls had TGCT haplotype, it was seen in 26% of the infants with RDS.

4. Discussion

In this case-control study, we showed that *ROCK1* gene rs2271255 (Lys222Glu), rs35996865, and *ROCK2* gene rs726843, rs2290156, rs10178332, rs35768389 (Asp601Val) polymorphisms were significantly associated with RDS, and that they could be a risk factor for development of neonatal RDS. Our results suggest that polymorphisms may increase the susceptibility to RDS. Additionally, significant associations between *ROCK1* and *ROCK2* haplotypes and RDS were observed. To the best of our knowledge, this is the first study to examine the association of the *ROCK* gene polymorphisms with the risk of RDS development.

It has been demonstrated that cytoskeletal tension controlled by the Rho/ROCK pathway is a critical developmental regulator of branching morphogenesis in fetal mice lungs during the pseudoglandular phase.[23,24] ROCK2 protein levels were shown to be rapidly elevated following an increase in lung distension induced by tracheal occlusion, which suggests that ROCK2 plays a major role in the formation of the gas exchange units.[14] It has been reported that stretch-induced surfactant protein C gene expression is further enhanced when RhoA or ROCK activity are attenuated, suggesting that inactivation of the RhoA pathway may promote type II cell differentiation.[13] Hypoxia is often present in RDS.[1] Hypoxia also increases ROCK activity and downregulates endothelial nitric oxide synthase expression.[25,26] Collectively, these findings suggest that the RhoA/ROCK pathway is involved in the pathogenesis of RDS.

There are few studies related to *ROCK* gene polymorphisms in humans. rs35996865, rs2290156, rs10178332, and rs35768389 (Asp601Val) polymorphisms have been shown to be associated with colorectal cancer development and metabolic syndrome.[27,28] rs35768389 (Asp601Val) polymorphism was reported to be associated with Behçet's disease.[21] So far, rs2271255 (Lys222Glu) and rs726843 polymorphisms have not been associated with any disease. The structure or function of the ROCK enzymes affected by these polymorphisms is currently unknown, and further studies are necessary in order to clarify this issue.

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Table 5 Distribution of haplotype frequencies of *ROCK1* and *ROCK2* gene polymorphisms in RDS cases and controls.

ROCK1 rs2271255	rs73963110	rs35996865	Control n (%)	RDS n (%)	*p* value
C	C	G	66 (25.4)	15 (5.3)	**<0.0001**
C	C	T	54 (20.7)	97 (33.1)	**0.0010**
C	T	G	68 (26.1)	11 (3.6)	**<0.0001**
C	T	T	25 (9.4)	11 (3.9)	0.0086
T	C	G	18 (6.9)	16 (5.5)	0.4906
T	C	T	17 (6.7)	67 (22.9)	**<0.0001**
T	T	G	8 (3.2)	48 (16.5)	**<0.0001**
T	T	T	4 (1.7)	27 (9.2)	**0.0001**

ROCK2 rs726843	rs2290156	rs10178332	rs35768389	Control n (%)	RDS n (%)	*p* value
C	C	A	A	9 (4.6)	75 (27.7)	**<0.0001**
C	C	A	T	55 (27.1)	2 (0.9)	**<0.0001**
C	G	A	A	3 (1.4)	20 (7.3)	**0.0023**
C	G	A	T	14 (6.7)	23 (8.3)	0.4554
C	G	C	T	5 (2.6)	14 (5.2)	0.1398
T	C	A	A	9 (4.2)	7 (2.5)	0.3202
T	C	A	T	72 (35.2)	2 (0.6)	**<0.0001**
T	C	C	T	0 (0.0)	9 (3.2)	0.0153
T	G	A	A	5 (2.2)	9 (3.2)	0.4977
T	G	A	T	28 (13.8)	18 (6.6)	0.0108
T	G	C	A	0 (0.0)	11 (3.9)	**0.0038**
T	G	C	T	0 (0.0)	71 (26.2)	**<0.0001**

Frequencies <0.03 in both controls and cases have been dropped from analysis.
RDS, Respiratory distress syndrome.

In conclusion, the findings of the present study show that *ROCK1* and *ROCK2* gene polymorphisms might be a risk factor for RDS development. Rho/ROCK signaling plays an important role in pulmonary circulation, and ROCK inhibitors may be potential therapeutic applications in the treatment of RDS. Our findings show that *ROCK* gene may have a role in the pathogenesis of RDS, but further studies are required to validate these results.

Conflicts of interest

The authors declare no conflicts of interest.

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