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Evaluation of the Potential Association Between Cannabinoid Receptor 2 Gene Q63R Polymorphism (Rs35761398) and Risk of RDS Development in Preterm Neonates

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Abstract

Background: Respiratory distress syndrome (RDS) is a major acute postnatal pulmonary disease that influences mostly preterm neonates. Recently, the polymorphism of immunomodulatory genes has been suggested to be associated with RDS development.
Objectives: We aimed at investigating the association of *CNR2* gene Q63R polymorphism with the development of RDS.
Methods: In this multicenter case series study, we enrolled 300 preterm newborns. The RDS was diagnosed based on the clinical and radiographic findings. The polymerase chain reaction with sequence-specific primer method was used for genotyping.
Results: 140 neonates out of 300 were diagnosed with RDS. The overall frequency of the QQ, QR, and RR genotypes of rs35761398 was 23.7%, 50.7%, and 25.6%, respectively. In the present study, the differences in birth weight, birth height, gestational age (GA), and the severity of respiratory distress were statistically significant between the two groups. We found no statistically significant differences in either allele (P = 0.624) or genotype (P = 0.461) distributions between the RDS patients and the healthy group.
Conclusions: Gestational age of < 28 weeks has the highest impact on predisposition to RDS. No association was found between rs35761398 and RDS in a group of Iranian preterm infants.

Keywords: Respiratory Distress Syndrome, RDS, SNP, Cannabinoid Receptor 2, *CNR2*

1. Background

Respiratory distress syndrome (RDS) is a major concern in the management of preterm newborns (1, 2). In spite of extensive efforts and improvement in therapeutic strategies, it has remained an important cause of neonatal mortality and morbidity (3, 4). Considering the multifactorial etiology of RDS, genetic factors have been suggested as the molecular contributors of RDS pathogenesis (5). Since surfactant deficiency has been thought as the primary cause of RDS, most studies assessing the association of genetic factors with RDS development have focused on surfactant gene polymorphisms and valuable results and insights have been obtained (5-8).

There is growing evidence that shows some aspects of fetal lung maturation were mediated by inflammatory cells (9). Bry et al. reported the increased expression of SP-A and SP-B mRNA after the injection of Interleukin-1a (IL-1a), a proinflammatory cytokine, to the amniotic fluid sacs might accelerate lung maturation in preterm labor (10). They also concluded that the presence of IL-1 in amniotic fluid might be involved in the host-defense mechanisms. On the other hand, the increased level of IL-10, a crit-

ical immunoregulatory cytokine, has been documented in preterm neonates with RDS compared to those without RDS and full-term newborns (9). In addition, the association of IL-10-1082 polymorphism with RDS development has been shown in two different studies in Italy and Iran (11, 12). Taking together, the balance between proinflammatory and anti-inflammatory cytokines has been proposed as another important factor in acute RDS (8).

A novel family of immunological mediators (Endocannabinoids) controls immune functions and plays a role in immune homeostasis. The ability of cannabinoid receptor 2 (CB2) to decrease the inflammatory response has been shown in different studies. It is well known that a missense polymorphism at *CB2* gene (*CNR2*) position 63 (Q63R) can result in a twofold decrease in CB2 receptor function (13). The association of this polymorphism with several immunity-associated disorders including celiac disease (14) and childhood immune thrombocytopenic purpura (15), as well as with the risk of hospitalization in children with acute respiratory tract infection (ARTI) (16, 17), has been reported previously. Despite the considerable attention given to the role of the endocannabinoid system in human diseases, their effects on pulmonary disease are still unclear.

2. Objectives

In this study, we sought to investigate whether the *CNR2* gene Q63R polymorphism (rs35761398) is associated with the development and severity of RDS in a group of Iranian preterm neonates.

3. Methods

The Human Subjects Committees (HSC) at the Maternal, Fetal, and Neonatal Research Center (MFNRC) and the Tehran University of Medical Sciences (TUMS) approved this study (Code No. 94-01-91-28579), and all parents provided informed consent and signed HSC-approved consent forms. This study was conducted according to the Declaration of Helsinki.

3.1. Population Study

300 preterm Caucasian infants born before 34 weeks were recruited into the study. All of the participants were admitted to the neonatal intensive care unit (NICU) at three university hospitals in Tehran, Iran, from May 2013 to April 2015. Those with either known genetic disorder(s) or congenital anomaly (ies) were excluded from the study.

The diagnosis of RDS was made by our team neonatologist as previously described (6, 7). Based on the standard RDS diagnosis protocol, those neonates who developed RDS in the first six hours of life were considers as the RDS group. The severity of respiratory distress was scored according to the Dawnes' scoring system (18). Neonates with a Dawens' score of < 8 were considered as "mild" and those with a Dawnes' score of ≥ 8 were regarded as "severe." Moreover, infants with GA of < 28 weeks, 28 - 31 weeks + 6 days, and 32 - 34 weeks were categorized as extremely preterm, very preterm, and moderate to late preterm, respectively (5).

3.2. Genotyping of CNR2 Q63R Polymorphism

Peripheral blood was collected from the study participants into EDTA-containing tubes. Genomic DNA was extracted from the whole blood samples by a modified salting-out extraction and stored at -20°C until use. Genotyping of rs35761398 was performed by PCR with a sequence-specific primer (SSP-PCR) assay. Briefly, in two PCR tubes, either the AA-specific primer 5'-TATCTGATCCTGTCCTCCCACCAA-3' or GG-Specific primer 5'-TATCTGATCCTGTCCTCCCACCGG-3' in combination with reverse primer 5'-TAGTCACGCTGCCAATC-3' were used to amplify a 178 bp product, which corresponded to the glutamine (Q) and arginine (R) alleles, respectively. The PCR products were electrophoresed in 2% agarose gel (PCR condition is available on request).

3.3. Statistical Analysis

Statistical Package for Social Sciences (SPSS 13.5) was used for statistical analysis. Genotype and allele frequencies of rs35761398 and deviations from Hardy-Weinberg equilibrium (HWE) were assessed using the Chi-square and Fisher's Exact tests. Continuous variables such as birth weight and gestational age were compared by using the Student *t* test. The logistic regression analysis was applied to assess the independent relationship between RDS development and other variables. All statistical tests were two-sided and a P value of < 0.05 was considered significant. The power of the study was 80%.

4. Results

The participants included 300 preterm infants, comprising 152 (50.7%) males and 148 (49.3%) females. A total of 140 (46.6%) newborns were diagnosed with RDS and the remaining 160 (53.4%) were not. The GA ranged from 23 weeks + 5 days to 33 weeks + 6 days (mean: 30 weeks + 6 days) and the birth weight ranged from 0.500 g to 3100 g (mean: 1573 g). The participants involved 93 (30.66%) twin or multiple pregnancies that showed no association with RDS. Moreover, there was no significant difference in the distribution of monozygous twins between the two groups (P = 0.579). More details about the characteristics of participants are shown in Table 1. We could not find any significant differences in preterm birth risk factors including

preeclampsia, maternal diabetes, and preterm premature rupture of membranes (PROM) between the two groups. Moreover, the differences between cases and controls in terms of maternal medical history, antenatal glucocorticoid treatment, mode of delivery, birth order, and gender were not statistically significant.

The RDS rate in extremely preterm, very preterm, and moderate to late preterm neonates was 85.7%, 40%, and 34.3%, respectively (P = 0.0001). The mortality rate was significantly higher in RDS affected infants than in the controls (14.3% vs. 2.5%, respectively) (P = 0.0001).

4.1. Polymorphism Analysis

The total frequency of the QQ, QR, and RR genotypes of rs35761398 was 23.7%, 50.7%, and 25.6%, respectively. The observed distribution of QQ, QR, and RR genotypes showed no significant difference between the RDS group and the control group (24.3%, 47.1%, and 28.6% vs. 23.1%, 53.8%, and 23.1%, respectively) (P = 0.461). In addition, the frequency of Q or R alleles did not significantly differ between RDS patients and controls (49.7% and 52.1% vs. 50.0% and 50.0%).

In RDS patients, neither allele nor genotype distribution of rs35761398 was associated with severity of respiratory distress (data not shown). Similarly, no significant association was obtained between response to therapy and either genotype or allele distribution of rs35761398 (data not shown).

5. Discussion

Here, we determined *CNR2* gene rs35761398 polymorphism and investigated its potential association with the occurrence of RDS in a group of premature neonates. As recent evidence suggests, different genetic susceptibility factors are involved in extremely preterm and near-term delivery (5). Therefore, in this study, only preterm infants were enrolled. In addition, cases and controls were matched in terms of RDS risk factors such as gender, antenatal glucocorticoid therapy, and maternal diseases to decrease their confounding effect, which is a common problem in association studies.

In the present study, the RDS infants showed significantly lower birth weight and gestational age compared to those without RDS (Table 1). The rate of RDS was in a reverse correlation with GA so that GA of lower than 28 weeks was the main factor in RDS development. The RDS rate in this group was 85.7%. This result is in accordance with most previous reports. The reasons for preterm delivery are very heterogeneous and still unclear. A few studies suggested some gene polymorphisms were associated with preterm birth, but these findings were not confirmed in the other studies (6, 19). According to our results, no association was observed between *CNR2* rs35761398 and the risk of extremely premature birth.

It is now evident that prematurity alone does not determine the risk of developing RDS and the associations of *SP-A*, *SP-B*, *SP-C*, and *SP-D* gene polymorphisms with RDS have been shown in different populations (5, 8, 20). In the Iranian preterm infants, we also reported the association of *SFTPB* gene 9306 A/G polymorphism (rs7316) and *SFTPC* gene codon 186 G/A polymorphism (rs1124) with RDS development (6, 7). In addition to the genes related to the surfactant structure and function, the polymorphisms of genes contributed to the inflammatory response have been proposed as the other candidate genes for the development of RDS (11, 12). CNR2 is a G-protein-coupled cannabinoid receptor that is mainly expressed by immune cells. The inducible expression of this receptor in inflammatory conditions suggests CB2-dependency of anti-inflammatory and immunomodulatory action of cannabinoids (17). The polymorphism at *CNR2* gene position 63(Q63R) that substitutes glutamine by arginine can result in a twofold decrease in CB2 receptor function, leading to a reduced immune modulation function when activated by cannabinoids (21). Moreover, the association of *CNR2* RR genotype with immune-mediated conditions like celiac disease and childhood immune thrombocytopenic purpura has been shown in different studies (14, 15). In addition, the association between CB2 Q63R variation and susceptibility to hospitalization in children with ARTI has been shown recently. Tahamatn et al. reported that children carrying the QQ genotype were more prone to developing severe ARTI. They also reported that the risk of developing severe ARTI following RSV infection increased more than two folds in children carrying the Q allele (17). Taken together, the rationale of this study is based on the assessed association between the *CNR2* Q63R polymorphism and several immunity-associated diseases, as well as on the well-known anti-inflammatory and immunomodulatory effects of CB2 signaling.

According to our results, no significant differences were observed in either *CNR2* Q63R polymorphism allele or genotype distributions between patients and controls (Table 2). This finding may suggest that the implication of CB2 in the development of RDS is less likely. This result might be warranted by knowing that the action of the CB2 receptor is cannabinoid-dependent (22). Previous studies showed that the CB2 receptor was localized only to placental macrophages and the placenta may form a barrier preventing the maternal-fetal transfer of endogenous cannabinoids (23). This may consequently result in the lack of stimuli for CB2 during the pregnancy period. This idea worth to be investigated in future studies.

This study suffers from a relatively small sample size. In

Table 1. Characteristics of Preterm Infants with or Without RDS

Feature	RDS	Control	P Value
Gender, No. (%)			0.908
Female	70 (50)	78 (48.7)	
Male	70 (50)	82 (51.3)	
Gestation age			0.0001
Mean ± SD	30 wk ± 18.7 d	31 wk 6 d ± 14.1 d	
Minimum	23 wk 5 d	25 wk	
Maximum	33 wk 6 d	33 wk 6 d	
Birth weight, g			0.0001
Mean ± SD	1410.56 ± 500	1731.74 ± 478	
Minimum	500	750	
Maximum	2750	3100	
Birth height, cm			0.0001
Mean ± SD	39.83 ± 4.845	42.33 ± 4.645	
Minimum	27	30	
Maximum	50	52	
3-minute apgar score	6.22 ± 2.156	7.55 ± 1.769	0.0001
5-minute apgar score	7.78 ± 1.964	8.73 ± 1.335	0.0001

Table 2. Association of CNR2 (Q63R) Polymorphism with RDS in Male and Female Preterm Neonates

	RDS[a]	Control[a]	Total[a]	P Value
Female				
Genotype				0.896
GG/GG	16 (22.9)	17 (21.8)	33 (22.3)	
AA/GG	36 (51.4)	43 (55.1)	79 (53.4)	
AA/AA	18 (25.7)	18 (23.1)	36 (24.3)	
Allele				0.908
GG	68 (48.6)	77 (49.4)	145 (49)	
AA	72 (51.4)	79 (50.6)	151 (51)	
Male				
Genotype				0.368
GG/GG	24 (34.3)	20 (24.4)	44 (29)	
AA/GG	30 (42.9)	43 (52.4)	73 (48)	
AA/AA	16 (22.9)	19 (23.2)	35 (23)	
Allele				0.420
GG	78 (55.7)	83 (50.6)	161 (53)	
AA	62 (44.3)	81 (49.4)	143 (47)	

[a] Values are expressed as No. (%).

a multifactorial trait such as RDS, most of the genetic polymorphisms will have minor effects that are not detectable in association studies with small sample sizes (6). Since this is the first study to assess the potential association of *CNR2* Q63R polymorphism with RDS development, knowing whether this polymorphism is associated with RDS de-

velopment remains to be elucidated in future studies.

5.1. Conclusion

According to our results, the GA below 28 weeks was the main risk factor for RDS. Although no significant association between *CNR2* Q63R polymorphism and the risk of RDS was found, further studies are needed to verify this point.

Footnotes

Authors' Contribution: Study concept and design: Ali Rashidi-Nezhad, Fatemeh Nayeri , Sima Binaafar and Shiva Irani; acquisition of data: Sima Binaafar, Nasrin Ghasemi-Fakhr, Javad Tavakkoly-Bazzaz and Ali Rashidi-Nezhad; analysis and interpretation of data: Sima Binaafar, Majid Kalani, Fatemeh Nayeri, Nikoo Niknafs, Hosein Dalili, Elaheh Amini, Tahereh Esmaeilnia Shirvani, Amir-Kamal Hardani, Roya Taheritafti and Ali Rashidi-Nezhad; drafting of the manuscript: Ali Rashidi-Nezhad and Sima Binaafar; critical revision of the manuscript for important intellectual content: Vahid Salimi; statistical analysis: Ali Rashidi-Nezhad and Mamak Shariat; administrative, technical, and material support: Ali Rashidi-Nezhad, Sima Binaafar and Javad Tavakkoly-Bazzaz; study supervision: Ali Rashidi-Nezhad, Mohsen Ghadami and Fatemeh Nayeri.

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