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Is there any association between childhood cardiac septal defects and *ROCK2* gene polymorphism?

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ABSTRACT. Rho/Rho-kinase pathway plays a critical role in the regulation of cellular functions such as proliferation and migration. One of the possible theories of the development of ventricular septal defects is cell migration disorder. The aim of this study was to analyze the genotype distributions and allele frequencies for the *ROCK2* gene Thr431Asn polymorphisms in the development of cardiac septal defects in a Turkish population. In this case-control study, 300 patients with cardiac defects (150 patients with ventricular and 150 patients with atrial septal defects) and control group (150 healthy control subjects) were investigated. A single-nucleotide polymorphism in *ROCK2* gene Thr431Asn was analyzed by real-time PCR using a

Light-Cycler. Neither genotype distributions nor the allele frequencies for the Thr431Asn polymorphism showed a significant difference between the groups. These results suggest that there is no association of the *ROCK2* gene Thr431Asn polymorphism with the development of cardiac septal defects in pediatric patients.

Key words: Cardiac septal defects; Genetic polymorphisms; *ROCK2*; Rho-kinase

INTRODUCTION

Congenital heart defects (CHD) are the most prevalent heart diseases in infants and children, including mainly atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus, and other types. Cardiac development is a very complicated process, involving the expression of many genes at different times, space and order, related to cell migration and, differentiation, hyperplasia and acute interaction. Endocardial cushion septum is the main structure correlated with compartment separation, where an abnormality can lead to ASD and VSD. The formation of cushion septum is mainly associated with local thickening of the extracellular matrix and invasion of endocardium epithelial cell transformed by epithelium stroma. Although recent human genetic findings have gained novel insights into genetic etiology of cardiac malformations by identifying single gene defects as a pathogenic substrate for familial recurrence of CHD, the genetic etiology in the large majority of these cases remains entirely unknown (Lagendijk et al., 2010). ASD and VSD are considered to be multigenetic and multi-pathogenic, yet no susceptibility factors have been discovered (Lagendijk et al., 2010; Posch et al., 2010). However, TGF beta signaling and Bmp2 were found to be essential for developing atrioventricular cushion (Ma et al., 2005; Jiao et al., 2006). Rho-kinase (ROCK) is a serine/threonine kinase which is major downstream effector of small GTPase Rho. Rho-kinase has two isoforms (ROCK1 and ROCK2), and plays crucial roles in various cellular functions including the regulation of cellular contraction, motility, morphology, polarity, cell division, adhesion, migration, proliferation and gene expression (Amano et al., 1997; Chihara et al., 1997; Amano et al., 2010). ROCK also regulates myocardial differentiation. ROCKs also play a role in endocardial cell differentiation and migration (Rikitake and Liao 2005; Loirand et al., 2006). ROCK1 and ROCK2 are found in the endocardial cushions during development. It is likely that ROCK-dependent differentiation and migration of endocardial cells are critical for normal heart development (Zhao and Rivkees, 2003, 2004). ROCK inhibition decreased cardiac myocyte proliferation, suggesting that ROCK regulate cardiomyocyte division. ROCK blockade decreased expression of cell cycle proteins, cyclin D3, CDK6, and p27KIP1 in the heart and cardiomyocytes, which are required for initiation of cell cycle and G1/S phase transition (Zhao and Rivkees, 2003). These observations show that ROCK plays a role in cardiac development and that ROCK regulates cardiac cell proliferation and cell cycle protein expression. However, there is no study investigating the role of ROCK polymorphisms in patients with cardiac septal defects. Therefore, the purpose of this study was to investigate a possible association between *ROCK2* gene polymorphisms and cardiac septal defects in a Turkish population.

MATERIAL AND METHODS

Study population

To investigate whether polymorphisms are associated with cardiac septal defects, 300 patients with isolated VSD (N = 150) and ASD (N = 150) were enrolled in this study. Healthy control subjects (N = 150) were randomly selected from healthy children without a history of congenital cardiac defects. The study was approved by the local ethics committee, and it was conducted in accordance with the Declaration of Helsinki. All study participants and/or families gave informed consent. All subjects underwent a cardiac examination including electrocardiography and echocardiographic examination. The diagnosis of cardiac septal defects was confirmed by echocardiographic examinations in all patients. Information on demographic characteristics, height, body weight, cardiac defect types, and clinical features was collected. Patients were of the same ethnic origin and from the same geographical area (southeastern Turkey). The demographic and clinical characteristics of the study population are shown in Table 1.

Table 1. Demographic characteristics of the study participants.

	VSD group (N = 137)	ASD group (N = 123)	Control group (N = 128)	P
Gender				
Male (N)	74 (54%)	60 (49%)	66 (52%)	
Female (N)	63 (46%)	63 (51%)	62 (48%)	0.701[+]
Age (months)[1]	39.6 ± 39.8	62.3 ± 56.2	100.3 ± 54.2	
Defect type				
Perimembraneous	113 (82.5%)			
Muscular	24 (17.5%)			
Secundum		119 (96.7%)		
High venosum		4 (3.3%)		

VSD = ventricular septal defect; ASD = atrial septal defect. [1]Data are reported as means ± SD. [+]Chi-square test for independence.

Blood samples and DNA isolation

From all participants, 5-mL peripheral blood samples were collected by venous puncture into sterile siliconized Vacutainer tubes with 2 mg/mL disodium ethylenediaminetetraacetic acid. Immediately after collection, whole blood was stored at -20°C until use. Genomic DNA was extracted from whole blood by using a standard proteinase K digestion and the salting-out method and stored at -20°C (Miller et al., 1988).

Genotyping

Analysis of the polymorphism of the *ROCK2* gene was performed as previously described (Kalender et al., 2010). The detection of *ROCK2* gene polymorphisms was performed with real-time polymerase chain reaction (PCR) using a Light-Cycler Instrument (Roche Diagnostics, GmbH, Mannheim, Germany). The sequences and the hybridization probes and primers are shown in Table 2. Probes were designed by TIB (Molbiol, Berlin, Germany). The Light-Cycler instrument measures the emitted fluorescence of the light cycler-red 640. The hybridization probes in combination with the Light-Cycler DNA Master Hybridization Probes kit (Roche Diagnostics) were used to determine the genotype by using a melting curve analysis

after the amplification cycles were completed. All related gene regions were amplified in 20-µL PCR capillary tubes. After preparation of the master mixture, 18 µL reaction mixture and 2 µL (approximately 40 ng) genomic DNA or control template were added to each Light-Cycler capillary tube. For negative control, PCR grade water was added instead of the template. The cycling program was carried out after a denaturation step at 95°C for 10 min through 50 cycles (denaturation at 95°C for 10 s, annealing at 50°C for 10 s, extension at 72°C for 15 s), with a maximum ramp rate of 20°C/s. Fluorescence was measured at the end of the annealing period of each cycle to monitor amplification. After amplification was complete, a final melting curve was recorded by 90 s denaturation at 95°C followed by a continuous temperature increase from 45 to 85°C in increments of 0.2°C/s. The fluorescence signal was converted to a melting peak by plotting the negative derivative of the fluorescence with respect to temperature (-dF/dT) against temperature (T). The resulting melting peak allowed discrimination between the homozygous as well as the heterozygous genotypes for each exon. Genotyping was conducted in a blinded fashion. We could not find a suitable restriction enzyme to cut for the Thr431Asn polymorphism. Therefore, real time PCR analyses were performed for this SNP.

Table 2. Studied *ROCK2* gene Thr431Asn in exon 10 polymorphisms with nucleotide changes, their locations, primer, and probe sequences.

	Location	Reference SNP	Primer and probe sequences
Thr431Asn	Exon 10	rs2230774	Forward 5'-ATGAAACTAAAATAACTACAGCAG Reverse 5'-AACTTGAATAAAACAGCACATAG Probe1 5'-TTGTATGGAATCATTTTCTCTACAAGAT-FL Probe2 5'-LC640-AGAGTCACTTAATAATCTACATGGAGGGG- PH

Statistical analyses

Results are reported as means ± SD or percentage. Statistical analysis was performed using GraphPad Instat (version 3.05, GraphPad Software Inc., San Diego, CA, USA). Polymorphisms were tested for deviation from Hardy-Weinberg equilibrium by comparing the observed and expected genotype frequencies using the chi-square test. Statistical comparison of more than two groups was performed by a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. For calculation of the significance of differences in genotype and allele frequencies, the chi-square test (with Yate's correction) was used. For all statistical tests, P values were two-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

A total of 450 patients were enrolled in the study. Thirteen patients from the VSD group, twenty-seven patients from the ASD group and twenty-two patients from the control group were excluded because of some technical problem with DNA isolation and the other laboratory difficulties. Totally, 388 patients were included (137 from VSD, 123 from ASD, and 128 from the control groups). The characteristics of all these patients included in the study are given in Table 1.

All patients were Caucasians, and the genotype distribution of the *ROCK2* Thr431Asn polymorphism was in Hardy-Weinberg equilibrium among cases (P = 0.687

for the VSD group, and P = 0.968 for the ASD group) and controls (P = 0.452). Genotype and allele frequencies of *ROCK2* gene Thr431Asn are presented in Table 3. There were no statistical differences in genotype and allele frequencies between the control, VSD and ASD groups (P > 0.05).

Table 3. Genotype and allele frequencies of *ROCK2* polymorphism (Thr431Asn in exon 10) among cases and controls.

Genotypes/alleles	Control group (N = 128)	VSD group (N = 137)	P*	ASD group (N = 123)	P*
Thr/Thr	32 (25%)	44 (32.1%)	1.000	37 (30.1%)	1.000
Thr/Asn	54 (42.2%)	61 (44.5%)	0.609	59 (48%)	0.975
Asn/Asn	42 (32.8%)	32 (23.4%)	0.103	27 (21.9%)	0.125
Thr	118 (46.1%)	149 (54.4%)	1.000	133 (54.1%)	1.000
Asn	138 (53.9%)	125 (45.6%)	0.069	113 (45.9%)	0.090

VSD = ventricular septal defect; ASD = atrial septal defect. *Chi-square test (with Yate's correction).

DISCUSSION

In the present study, we investigated Thr431Asn polymorphism of the *ROCK2* gene for its association with cardiac septal defects. No association was observed with Thr431Asn polymorphism with cardiac septal defects. To our knowledge, this is the first study to examine the association of the *ROCK2* gene polymorphism with cardiac septal defects in pediatric patients. It is known that ROCK is likely to exist as dimers and the dimerized kinase domain of ROCK appears to be in an active conformation (Doran et al., 2004; Yamaguchi et al., 2006). It is likely that the Thr431Asn polymorphism may affect dimerization and Rho binding and thereby ROCK activation.

ROCK activity regulates major morphogenetic events during embryonic development including formation of the heart (Wei et al., 2001). ROCK1 and ROCK2 transcripts are enriched in cardiac mesoderm. In cultured murine embryos, inhibition of ROCKs decreases cell proliferation in the heart, but does not modify programmed cell death, suggesting that ROCK activity is not involved in cardiomyocyte apoptosis, but regulates cardiomyocyte division during heart development (Rikitake and Liao 2005). In cultured endocardial cushions, inhibition of ROCKs prevents the epithelial-mesenchymal transition and cell migration (Zhao and Rivkees, 2004).

There is only a limited number of published studies related to Rho-kinase polymorphism in humans and all of these studies are related to hypertension, chronic kidney disease, diabetic retinopathy, breast cancer, or Behçet's disease (Seasholtz et al., 2006; Zhao et al., 2008; Peterson et al., 2009; Yoshida et al., 2009; Demiryurek et al., 2010; Kalender et al., 2010; Oguz et al., 2012). In the present study, genotype and allele distributions of the *ROCK2* Thr431Asn polymorphism did not show a significant association with cardiac septal defects. Therefore, the results of the present study suggest that this polymorphism is not a contributing factor to the susceptibility of ASD or VSD.

In conclusion, our results showed that genotype and allele distributions of the *ROCK2* gene Thr431Asn did not indicate a significant association with cardiac septal defects. Epidemiologic studies in patients with polymorphism of the *ROCK* gene may help clarify whether ROCK is an important mediator or marker of cardiac diseases. Further studies are also required to verify these findings in different ethnic groups.

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