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Single-nucleotide polymorphism of the pri-miR-34b/c gene is not associated with susceptibility to congenital heart disease in the Han Chinese population

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ABSTRACT. Recent evidence has shown that the microRNA polymorphism may play an important role in the susceptibility to congenital heart disease (CHD). A potentially functional SNP rs4938723 (T>C) in the promoter region of pri-miR-34b/c might affect transcription factor GATA binding and therefore pri-miR-34b/c expression. We genotyped the pri-miR-34b/c polymorphism in a case-control study of 590 patients and 672 controls in a Han Chinese population and assessed the effects of the pri-miR-34b/c polymorphism

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on CHD susceptibility by TaqMan SNP genotyping assay. There was no association between the pri-miR-34b/c polymorphism and the risk of CHD in both genotype and allelic frequency. In a subsequent analysis of the association between this polymorphism and CHD classification, there was still no significant difference in both genotype and allelic frequency. Our results suggest that the pri-miR-34b/c polymorphism rs4938723 is not associated with susceptibility to sporadic CHD in the Han Chinese population.

Key words: Congenital heart disease; pri-miR-34b/c; Susceptibility; Single-nucleotide polymorphism

INTRODUCTION

Congenital heart disease (CHD) is the most common type of birth defect, with an incidence of about 19 to 75 per 1000 live births, depending on the categories and severity of the defects that are included, and it is the number one cause of noninfectious infant mortality (Hoffman and Kaplan, 2002). Thus, there is a dire need for new diagnostic and therapeutic strategies in the treatment of CHD.

The etiology of CHD is multifactorial, with environmental and genetic factors playing important roles. Environmental insults during fetal development are known to increase the risk of CHD and include viral infections with rubella (Kohl, 1985), exposure to chemical teratogens such as retinoic acid and lithium (Singh et al., 2000), and maternal diseases that include diabetes and systemic lupus erythematosus (Hoffman and Kaplan, 2002; Kumar et al., 2007). The etiological factors of many genetic syndromes and familial CHD have been identified, but the genetic basis of the majority of "sporadic" CHD remains unknown. Single nucleotide polymorphisms (SNPs) are the most common form of human genetic variation and may contribute to an individual's susceptibility to CHD.

MicroRNAs (miRNAs) are single-stranded, nonprotein-coding small RNA molecules that regulate gene expression by binding to target mRNAs and suppressing their translation or initiating their degradation. miRNAs are involved in the control of various aspects of cardiac function and dysfunction, including myocyte growth, integrity of the ventricular wall, contractility, gene expression, and maintenance of cardiac rhythm (Carè et al., 2007; van Rooij et al., 2007; Yang et al., 2007; Zhao et al., 2007). Dysregulation of miRNAs may result in CHD in humans, considering that miR-1 controls cardiac muscle development (Yang et al., 2007) and that deletion of miR-1-2 results in heart defects including ventricular septal defect, where surviving mice have conduction system defects and increased cardiomyocyte proliferation (Zhao et al., 2007).

Recent evidence suggests that rs11614913 SNP in miR-196a2 is associated with the susceptibility of CHD (Xu et al., 2009), so the miRNA polymorphism may play an important role in susceptibility to CHD. Studies have reported that a potentially functional SNP rs4938723 (T>C) in the promoter region of pri-miR-34b/c may affect transcription factor GATA binding and therefore pri-miR-34b/c expression (Xu et al., 2011). In our study, we hypothesized that SNP rs4938723 (T>C) in the promoter region of pri-miR-34b/c is associated with susceptibility to CHD. To test this hypothesis, we genotyped the SNP in a

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case-control study of 590 CHD patients and 672 CHD-free controls frequency-matched to the cases according to age, gender, and residence area in a Chinese population. To our knowledge, this is the first study evaluating the association between the pri-miR-34b/c polymorphism and susceptibility to CHD.

MATERIAL AND METHODS

Subjects

Subjects were recruited from the Affiliated Nanjing Children's Hospital of Nanjing Medical University, Nanjing, China, between March 2009 and July 2011. All cases had non-syndromic CHD diagnosed by ultrasound and confirmed during surgical treatment. Cases who had structural malformations involving another organ system or known chromosomal abnormalities were excluded. Exclusion criteria also included a positive family history of CHD (parents and siblings), maternal diseases (diabetes mellitus and phenyl ketonuria), maternal teratogen exposures, and maternal therapeutic drug exposures during the intrauterine period. Controls were non-CHD patients from the same geographic area who were frequency-matched to the cases by age and gender. They were recruited from the hospitals listed above during the same time period; controls with congenital anomalies were excluded. All subjects were genetically unrelated ethnic Han Chinese. After written informed consent was obtained, each subject's parents were scheduled for an interview using a structured questionnaire to collect information on demographic data. After interview, a 5-mL venous blood sample was collected from each participant for DNA extraction for genetic testing. Blood samples were collected in EDTA-containing tubes. The study was approved by the Institutional Review Board of Nanjing Medical University, Nanjing, China.

CHD was classified into 5 broad categories as previously described (Bruneau, 2008), cyanotic heart disease, left-sided obstruction defects, septation defects, patent ductus arteriosus, and other complex abnormalities, and 2 other CHD classification methods were also used (detailed classifications are shown in Table 1).

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the DNA mini kit (QIAGEN, NED). Genotyping of the pri-miR-34b/c polymorphism (rs4938723) was performed using the MGB-TaqMan SNP genotyping assay from Applied Biosystems Inc. (Foster City, CA, USA). The sequences of primer and probe for SNP (rs4938723) are available on request. The primers for rs4938723 were as follows: forward, 5'-CTC ACC TCC TCT GGG AAC CTT-3', and reverse, 5'-AAG GCC ATA CCA TTC AAG ACA GTA T-3'. Amplifications were performed in the 384-well ABI 7900HT Real Time PCR System (Applied Biosystems). After the completion of the amplification, the fluorescence intensity in each well of the plate was read and analyzed with the SDS 2.4 automated software. Four blank controls were included in each plate to ensure accuracy of the genotyping. About 10% of the samples were randomly selected for repeat assays, and the results were in agreement with the results of the first assays.

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Statistical analyses

SNP allele frequencies were tested against departure from Hardy-Weinberg equilibrium by a goodness-of-fit chi-square test before analysis. Differences in the distributions of demographic characteristics between the cases and controls were determined by the Student *t*-test (for continuous variables) or the chi-square test (for categorical variables). The associations between genotypes of pri-miR-34b/c polymorphisms and susceptibility to CHD were expressed as odds ratios (OR) as risk estimates with 95% confidence intervals (95%CI) adjusted for age and gender by logistic regression analysis. A chi-square test was used to determine the association of category characteristics and pri-miR-34b/c genotype and allele frequencies between CHD patients. All statistical tests were two-sided, and a probability level of $P < 0.05$ was considered to be statistically significant. Data analysis was performed using the Statistical Analysis System software (9.1.3; SAS Institute, Cary, NC, USA).

RESULTS

Characteristics of the subjects studied

A total of 590 CHD patients and 672 CHD-free controls were analyzed in our study. The demographic and selected characteristics of cases and controls are shown in Table 1. Cases and control subjects were well matched for age and gender. All subjects were ethnic Han Chinese.

Table 1. Frequency distribution of selected characteristics between the congenital heart disease (CHD) cases and CHD-free controls.

Variable	Controls (N = 672)		Case (N = 590)		P value
	N	%	N	%	
Age [years (means ± SD)]	2.99 ± 2.74		2.99 ± 2.78		0.985
Gender					0.518
Male	388	57.7	330	55.9	
Female	284	42.3	260	44.1	
CHD classification I					
Cyanotic heart disease			129	21.9	
Left-sided outflow obstruction			15	2.5	
Septation defects			398	67.5	
Patent ductus arteriosus			38	6.4	
Other complex abnormality			10	1.7	
CHD classification II					
Septa and valve abnormalities only			428	72.5	
Other CHD abnormalities			162	27.5	
CHD classification III					
Isolated CHD			365	61.9	
Non-isolated CHD			225	38.1	

With regard to the category characteristics of CHD patients, according to the CHD classification I, 129 patients (21.9%) had cyanotic heart disease, and left-sided outflow obstruction was found in 15 patients (2.5%). A total of 398 (67.5%) and 38 (6.4%) cases involved

septation defects and patent ductus arteriosus in the CHD patients, respectively. Only 10 cases (1.7%) concerned the other complex abnormality. Septa and valve abnormalities occurred in 428 patients (72.5%) in the CHD classification II, and 365 patients (61.9%) had isolated CHD according to the CHD classification III.

pri-miR-34b/c polymorphism in the subjects

The genotype and allele frequencies of pri-miR-34b/c polymorphism (rs4938723) in the subjects are presented in Table 2. All observed genotype frequencies in both cases and controls conformed to Hardy-Weinberg equilibrium (P = 0.932 and 0.899 for cases and controls, respectively). As shown in Table 2, the frequency of CC homozygote was higher in CHD patients than in CHD-free controls (11.4 versus 10.4%), but susceptibility to CHD was not significantly higher with pri-miR-34b/c CC genotype or pri-miR-34b/c allele-containing genotypes (TC and CC) compared with those with the TT genotype, after adjusting for age and gender by logistic regression analysis (P = 0.493, OR = 1.140, 95%CI = 0.784-1.657; P = 0.519, OR = 1.076, 95%CI = 0.861-1.344). The frequency of the C allele in the CHD patients (33.8%) was also not significantly higher than in CHD-free controls (32.4%) (adjusted P = 0.464), indicating that there was no significant association between the pri-miR-34b/c polymorphism and susceptibility to CHD.

Table 2. Genotype and allele frequencies of the pri-miR-34b/c polymorphism (rs4938723) in cases and controls.

Genotype	Cases (N = 590)		Controls (N = 672)		Odds ratio† (95%CI)	P value†
	N	%	N	%		
Genotype						
TT	258	43.7	306	45.5	1 (Reference)	-
TC	265	44.9	296	44.1	1.063 (0.841-1.343)	0.611
CC	67	11.4	70	10.4	1.140 (0.784-1.657)	0.493
TC+CC	332	56.3	366	54.5	1.076 (0.861-1.344)	0.519
Alleles						
T	781	66.2	908	67.6	1 (Reference)	-
C	399	33.8	436	32.4	1.064 (0.901-1.256)	0.464

†Adjusted for age and gender.

pri-miR-34b/c polymorphism and category characteristics in the CHD patients

The pri-miR-34b/c polymorphism has been shown to alter expression of mature miR-34b/c and binding activity of target mRNA, impairing p53-mediated cell cycle arrest and apoptosis. Apoptosis occurs in almost every stage of cardiogenesis (Aikawa and Kawano, 1982; Eisenberg and Markwald, 1995; Kirby and Waldo, 1995; Watanabe et al., 1998; Zhao and Rivkees, 2000), and this polymorphism seems to affect the categories of CHD. However, we observed no association between the pri-miR-34b/c polymorphism and the category characteristics of CHD, including CHD classifications I, II, and III (Table 3), although pri-miR-34b/c allelic C compared with those with the allelic T in CHD classification III was significant (P = 0.041).

Table 3. Category characteristics and pri-miR-34b/c genotype and allele frequencies of congenital heart disease (CHD) patients.

	Genotype			P value		Allele		P value
	TT	TC	CC	CT *vs* TT	CC *vs* TT	T	C	
CHD classification I								
Cyanotic heart disease								
Yes	61 (47.3)	50 (38.7)	18 (14.0)			172 (66.7)	86 (33.3)	
No	197 (42.7)	215 (46.7)	49 (10.6)	0.182	0.584	609 (66.1)	313 (33.9)	0.854
Left-sided outflow obstruction								
Yes	8 (53.3)	6 (40.0)	1 (6.7)			22 (73.3)	8 (26.7)	
None	250 (43.5)	259 (45.0)	66 (11.5)	0.553	0.692	759 (66.0)	391 (34.0)	0.402
Septation defects								
Yes	168 (42.2)	189 (47.5)	41 (10.3)			525 (66.0)	271 (34.0)	
None	90 (46.9)	76 (39.6)	26 (13.5)	0.127	0.550	256 (66.7)	128 (33.3)	0.809
Patent ductus arteriosus								
Yes	18 (47.4)	15 (39.5)	5 (13.1)			51 (67.1)	25 (32.9)	
None	240 (43.5)	250 (45.3)	62 (11.2)	0.536	0.796	730 (66.1)	374 (33.9)	0.861
Other complex abnormality								
Yes	3 (30.0)	5 (50.0)	2 (20.0)			11 (55.0)	9 (45.0)	
No	255 (44.0)	260 (44.8)	65 (11.2)	0.725	0.275	770 (66.4)	390 (33.6)	0.286
CHD classification II								
Septa and valve abnormalities only	182 (42.5)	199 (46.5)	47 (11.0)			563 (65.8)	293 (34.2)	
Other CHD abnormalities	76 (46.9)	66 (40.7)	20 (12.4)	0.242	0.950	218 (67.3)	106 (32.7)	0.624
CHD classification III								
Isolated CHD	148 (40.6)	171 (46.8)	46 (12.6)			467 (64.0)	263 (36.0)	
Non-isolated CHD	110 (48.9)	94 (41.8)	21 (9.3)	0.093	0.093	314 (70.0)	136 (30.0)	0.041

Data are reported as numbers with percent in parentheses.

DISCUSSION

In cardiovascular development, apoptosis, as an important phenomenon, coincides with major developmental processes in specific time windows, where more than 30 locations and time frames have been determined (Pexieder, 1975). Watanabe et al. (1998) suggested that the role of chick outflow tract cardiomyocyte apoptosis is to shorten and rotate the myocardial conus to form the subpulmonic infundibular connection of the right ventricle to the pulmonary artery anteriorly. During cardiac valve formation, cardiac cushions are sculpted to form the fine inflow (mitral, tricuspid) and outflow (aortic and pulmonary) valves and portions of the atrial and ventricular septa (Eisenberg and Markwald, 1995), where it appears that this occurs in part by apoptosis. Significant levels of apoptosis have been observed in the mesenchyme of the bulbar and the atrioventricular cushions of birds and mammals and may contribute to the morphogenesis of these structures (Watanabe et al., 1998; Zhao and Rivkees, 2000). Cells originating in the neural crest migrate extensively throughout the cardiovascular system and are critical to the formation of the aorticopulmonary septum and the media of the great arteries, where they also undergo apoptosis (Kirby and Waldo, 1995). Apoptosis has also been observed in the developing atrial septum, and at the site of formation of the coronary artery orifices (Aikawa and Kawano, 1982).

P53 is a tumor suppressor gene, whose protein is a key transcriptional regulator of a signal transduction pathway that ultimately impacts the decision of a cell to divide, undergo growth arrest, or die; *p53*-regulated genes encode proteins sharing functions in the regulation of cell cycle progression, DNA repair, apoptosis, and angiogenesis (Harris and Levine, 2005). Loss of its function plays a critical role in multistage organogenesis, and *P53* also participates

in the embryonic cardiovascular development process (Abdelwahid et al., 2004; Morgan et al., 2008). The miR-34 family members are direct transcriptional targets of *P53*, and loss of miR-34 function can impair p53-mediated cell cycle arrest and apoptosis (Chang et al., 2007; Corney et al., 2007; He et al., 2007). Some studies have reported that p53 can regulate expression of miRNAs, especially the miR-34 family members (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Tarasov et al., 2007). The miR-34 family members are direct transcriptional targets of p53, and loss of miR-34 function can impair p53-mediated cell cycle arrest and apoptosis.

Although SNPs in miRNA regions have been reported to be rare and unlikely to be functionally important (Saunders et al., 2007), a mutation or a SNP at the miRNA gene region could affect the transcription of pri-miRNA transcripts, the processing of miRNA precursors to mature miRNAs, or miRNA-target interactions (Yang et al., 2006). Recent evidence suggests that rs11614913 SNP in miR-196a2 is associated with susceptibility to CHD (Xu et al., 2009); a potentially functional SNP rs4938723 (T>C) found in the promoter region of pri-miR-34b/c may contribute to susceptibility to primary hepatocellular carcinoma, which may affect transcription factor GATA binding and therefore pri-miR-34b/c expression, impairing p53-mediated cell cycle arrest and apoptosis. On the basis of these previous studies, we predict that this functional variant, the rs4938723 polymorphism in pri-miR-34b/c, is likely involved in susceptibility to CHD.

In our case-control study described here, although the frequency of CC homozygotes or pri-miR-34b/c polymorphism allele-containing genotypes (CT and CC) was higher in CHD patients than in CHD-free controls (11.4 versus 10.4% and 56.3 versus 54.5%, respectively), the frequency of the C allele in the CHD patients (33.8%) was also not significantly higher than in CHD-free controls (32.4%), and no significant association between the pri-miR-34b/c polymorphism and susceptibility to CHD was found.

The pri-miR-34b/c polymorphism has been shown to alter expression of mature miR-34b/c and binding activity of target mRNA, impairing p53-mediated cell cycle arrest and apoptosis. Apoptosis occurs in almost every stage of cardiogenesis, and this polymorphism seems to affect the categories of CHD. In a subsequent analysis of the association between this polymorphism and the category characteristics of CHD, including CHD classifications I, II, and III, we also observed no significant difference in both genotype and allele frequencies, and thus, we conclude that the pri-miR-34b/c polymorphism does not seem to influence the categories of CHD.

In conclusion, our results suggest that the pri-miR-34b/c polymorphism is not associated with susceptibility to CHD in the Han Chinese population. To our knowledge, this is the first report of the association between the pri-miR-34b/c polymorphism and CHD in Chinese samples, and further study from different groups will be necessary to validate our results. Undoubtedly, more detailed characterization of SNPs of miRNAs will improve our understanding of miRNA biogenesis and the potential involvement of these SNPs in human diseases including CHD.

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