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Congenital Heart Disease

MiR-196a2 rs11614913 T>C Polymorphism is Associated with an Increased Risk of Tetralogy of Fallot in a Chinese Population

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Background: MicroRNAs (miRNAs) are a family of endogenous, small, noncoding single-stranded RNAs that act as post-transcriptional gene regulatory elements. MiRNA polymorphisms may be associated with susceptibility to congenital heart disease (CHD). The aim of this study was to evaluate the impact of miRNA single nucleotide polymorphisms (SNPs) on CHD susceptibility.

Methods: We genotyped two functional SNPs, miR-196a2 rs11614913 and miR-146a rs2910164, in a case-control cohort of 173 Chinese patients with tetralogy of Fallot (TOF) and 207 non-CHD controls.

Results: When the miR-196a2 rs11614913 TT homozygote genotype was used as the reference group, the TC genotype was not associated with an increased risk of TOF. The CC genotype was associated with a borderline significantly increased risk for TOF. In the recessive model, when the miR-196a2 rs11614913 TT/TC genotypes were used as the reference group, the CC homozygote genotype was associated with a significantly increased risk of TOF (OR = 1.96, 95% CI = 1.18-3.25, p = 0.01). The miR-146a rs2910164 C>G polymorphism was not associated with developing TOF.

Conclusions: Our findings suggested that the miR-196a2 rs11614913 T>C polymorphism may play a role in the development of TOF. Future larger studies that include populations of other ethnicities are required to confirm these findings.

Key Words: Congenital heart disease • MiRNA • Molecular epidemiology • Polymorphisms • Tetralogy of Fallot

INTRODUCTION

MicroRNAs (miRNAs) are a family of endogenous, small, noncoding single-stranded RNAs of approximately 22 nucleotides that act as post-transcriptional gene regulatory elements for up to 30% of all human genes.[1-3] MiRNAs act by binding to the 3'-untranslated region of specific messenger RNAs (mRNAs) and target them for degradation or translational repression.[4]

Congenital heart disease (CHD) is the leading non-infectious cause of death in children and the most common type of structural malformation of the heart and large blood vessels, with a prevalence of 4-10 per 1000 live births.[5,6] In China, the prevalence of CHD is 73.2 per 10,000 births in areas with a high prevalence. MiRNAs have been shown to be involved in cardiac development.[7]

Recently, miR-196a was identified as an upstream regulator of Hoxb8 and Sonic hedgehog (Shh) in vivo in the context of limb development.[8] Yekta et al. first

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reported that miR-196a is expressed from *Hox* gene clusters in mammals, and that *Hox* genes are targets of miR-196a.[9] MiR-196a-directed Hoxb8 RNA cleavage products have been detected in the total RNA extracted from E11.5 (ventricular septation) mouse embryos using a sensor transgene for *Hox* complex-embedded miR-196a.[10]

In a previous study of CHD in a Chinese population, Xu et al. genotyped the miR-196a2 rs11614913 single nucleotide polymorphism (SNP) and three other pre-miRNA SNPs (miR-146a rs2910164, miR-149 rs2292832 and miR-499 rs3746444) in 1324 cases of CHD and 1783 non-CHD controls. They found that miR-196a2 rs11614913 CC was associated with a significantly increased risk of CHD in all three stages combined.[11] In a genotype-phenotype correlation analysis of 29 cardiac tissue samples of CHD, the rs11614913 CC genotype was associated with significantly increased levels of mature miR-196a expression. In vitro binding assays further revealed that the rs11614913 variant affects HOXB8 binding to mature miR-196a.[11] MiR-146a rs2910164 is also involved in NF-κB signaling and apoptosis.[12]

To evaluate the impact of the miR-196a2 rs11614913 and miR-146a rs2910164 SNPs on CHD susceptibility, we performed genotyping analyses for the two SNPs in a hospital based case-control study of 173 cases of tetralogy of Fallot (TOF) and 207 healthy controls in a Chinese population.

MATERIALS AND METHODS

Study population

The study population comprised 173 patients with TOF and 207 non-CHD controls. Patients were consecutively recruited from the Xinhua Hospital, Shanghai, China, between March 2009 and October 2011. All patients were diagnosed with non-syndromic CHDs by echocardiography, and the diagnosis of TOF was confirmed during surgery. The controls were non-CHD patients recruited from the same hospital during the same time period and most of them had trauma or infectious diseases. Each control was matched to each case by age (±3 years) and sex.

All subjects were genetically unrelated ethnic Han Chinese. After informed consent was obtained from their parents, each subject and her/his parents were questioned in person by trained interviewers using a structured questionnaire to obtain information about their medical history. Cases with structural malformations that involved another organ system or those with known chromosomal abnormalities were excluded. Exclusion criteria also included a positive family history of CHD in a first-degree relative (parent or sibling), maternal diabetes mellitus (DM), phenylketonuria, maternal exposure to teratogens (e.g., pesticides, organic solvents) during pregnancy, as well as maternal infection with rubella, influenza or febrile illness during pregnancy. Control individuals with congenital anomalies were also excluded.

This study was conducted in accordance with the declaration of Helsinki and with the approval of the Institutional Review Board and Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China. Written informed consent was obtained from the guardians of all participants.

Isolation of DNA and genotyping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Samples of venous blood (2 ml) were collected from patients using Vacutainer tubes. These were transferred to tubes containing ethylenediamine tetra-acetic acid (EDTA). Genomic DNA was isolated from whole blood with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotyping was undertaken by MALDI-ToF-MS as described previously.[13,14] SNP genotyping was done using the MassArray system (Sequenom, San Diego, California, USA) by the MALDI-TOF-MS method according to manufacturer's instructions. Completed genotyping reactions were spotted onto a 384-well spectroCHIP system (Sequenom, San Diego, California) using a MassArray Nanodispenser (Sequenom, San Diego, California), and the genotype determined by MALDI-TOF-MS. Genotype calling was done in real time with MassArray RT software version 3.1 (Sequenom, San Diego, California), and analyzed using MassArray Typer software version 4.0 (Sequenom, San Diego, California).[15]

Statistical analysis

Differences in the distributions of demographic characteristics, selected variables, the variant alleles

and genotypes of *miR-196a2* rs11614913 and *miR-146a* rs2910164 SNPs between the cases and controls were evaluated using the Student t-test (for continuous variables) and χ^2 test (for categorical variables). The associations between miR-196a2 rs11614913 and miR-146a rs2910164 genotypes and risk of CHD were estimated by computing the odds ratios (ORs) and their 95% confidence internals (CIs) from logistic regression analyses. All of the statistical analyses were performed with Statistical Analysis System software (v.9.1.3e; SAS Institute, Cary, North Carolina, USA).

RESULTS

Characteristics of the study population

The demographic and clinical characteristics of all subjects are summarized in Table 1. The ages were 2.54 ± 2.04 yrs for the cases and 2.70 ± 2.71 yrs for the controls (p = 0.52), and 56.1% of the cases and 57.5% of the controls were males and the difference was not significant (p = 0.78), indicating that frequency-matching by age and sex was adequate. Of the 173 TOF patients, 114 (65.9%) were isolated TOF, 27 (15.6%) were TOF with atrial septal defect, 11 (6.4%) were TOF with patent ductus arteriosus, 3 (1.7%) were TOF with absent pulmonary valve, 6 (3.5%) were TOF with pulmonary atresia, 5 (2.9%) were TOF with muscular ventricular septal defect. The primary information for two genotyped SNPs was in Table 2. The genotyping success rate was 98.95% for miR-196a2 rs11614913 C>T and miR-146a rs2910164 C>G in all 380 samples. Minor allele frequency (MAF) in our controls was similar to MAF for Chinese in the database for all two SNPs (Table 2). The observed genotype frequencies for miR-146a rs2910164 C>G polymorphism in the controls were in Hardy-Weinberg equilibrium (HWE) (p = 0.86) instead of miR-196a2 rs11614913 C>T (p = 0.01) (Table 2).

MiR-196a2 rs11614913 T>C and miR-146a rs2910164 C>G polymorphisms and TOF susceptibility

The miR-196a2 rs11614913 T>C and miR-146a rs2910164 C>G genotype distributions in the cases and controls were shown in Table 3. The observed genotype frequencies for miR-146a rs2910164 C>G polymorphism were in HWE in the controls instead of miR-196a2 rs11614913 T>C. In single-locus analyses, the genotype frequencies of miR-196a2 rs11614913 T>C were 25.9% (TT), 47.6% (TC), and 26.5% (CC) in TOF patients and 26.2% (TT), 58.3% (TC), and 15.5% (CC) in control subjects, and the difference was significant (p = 0.03). When the miR-196a2 rs11614913 TT homozygote genotype was used as the reference group, the TC genotype was not associated with the risk for TOF (TC vs. TT: OR = 0.83, 95% CI = 0.51-1.35, p = 0.45); the CC genotype was associated with a borderline significantly increased risk for TOF (CC vs. TT: OR = 1.73; 95% CI = 0.94-3.16; p = 0.08). In the recessive model, when the miR-196a2 rs11614913 TT/TC genotypes were used as the reference group, the CC homozygote genotype was associated with a significantly increased risk for TOF (OR = 1.96, 95% CI = 1.18-3.25, p = 0.01). In the dominant model, the miR-196a2 rs11614913 TC/CC variants were not associated with the risk of TOF, compared with the miR-196a2 rs11614913 TT genotype (OR = 1.02, 95% CI = 0.64-1.62, p = 0.94) (Table 3).

Table 1. Comparison of tetralogy of Fallot (TOF) patients and controls by selective characteristics

Variable	TOF (n = 173)	Controls (n = 207)	p
Age, years (mean ± SD)	2.54 ± 2.04	2.70 ± 2.71	0.52
Male/female	97/76	119/88	0.78
Isolated TOF, no (%)	114 (65.9%)	–	–
TOF with ASD*, no (%)	27 (15.6%)	–	–
TOF with PDA#, no (%)	11 (6.4%)	–	–
TOF with ASD and PDA, no (%)	7 (4.0%)	–	–
TOF with absent pulmonary valve, no (%)	3 (1.7%)	–	–
TOF with pulmonary atresia, no (%)	6 (3.5%)	–	–
TOF with muscular VSD†, no (%)	5 (2.9%)	–	–

* ASD, atrial septal defect; # PDA, patent ductus arteriosus; † VSD, ventricular septal defect.

Table 2. Primary information for miR-196a2 rs11614913 T>C and miR-146a rs2910164 C>G

Genotyped SNPs	miR-196a2: rs11614913 T>C	miR-146a: rs2910164 C>G
Chromosome	12	5
Regulome DB Score	5	No data
TFBS	Y	Y
Splicing (ESE or ESS)	Y	Y
MAF for Chinese in database	0.48	0.40
MAF in our controls (n = 207)	0.45	0.43
p value for HWE test in our controls	0.01	0.86
% Genotyping value	98.95%	98.95%
1st-PCRP	ACGTTGGATGTCGACGAAAACCGACTGATG	ACGTTGGATGCAGAGATATCCCAGCTGAAG
2nd-PCRP	ACGTTGGATGCTGATCTGTGGCTTAGGTAG	ACGTTGGATGAAGCCGATGTGTATCCTCAG
AMP_LEN	115	115
Tm (NN)	49	46.7
UEP_MASS	6971.6	6058.9
UEP_SEQ	cccacCTCGGCAACAAGAAACTG	ccttTGTCAGTGTCAGACCT
EXT1_CALL	C	C
EXT1_MASS	7218.7	6306.1
EXT1_SEQ	cccacCTCGGCAACAAGAAACTGC	ccttTGTCAGTGTCAGACCTC
EXT2_CALL	T	G
EXT2_MASS	7298.7	6346.2
EXT2_SEQ	cccacCTCGGCAACAAGAAACTGT	ccttTGTCAGTGTCAGACCTG

AMP_LEN, amplicon length; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; EXT1_CALL, name given to the analyte 1 mass peak in the mass spectrum; EXT2_CALL, name given to the analyte 2 mass peak in the mass spectrum; EXT1_MASS, mass of analyte 1; EXT2_MASS, mass of analyte 2; EXT1_SEQ, sequence of analyte 1; EXT2_SEQ, sequence of analyte 2; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; Regulome DB Score, http://www.regulomedb.org/; TFBS, transcription factor binding site (http://snpinfo.niehs.nih.gov/snpinfo/ snpfunc.htm); Tm (NN), extend primer melting temperature, calculated by nearest neighbor method; UEP_MASS, extend primer mass; UEP_SEQ, extend primer sequence; 1st-PCRP, primary amplification primer; 2nd-PCRP, secondary amplification primer.

Table 3. Logistic regression analysis of associations between miR-196a2 rs11614913 T>C and miR-146a rs2910164 C>G polymorphisms and risk of CHDs

Genotype	Cases (n = 173)		Controls (n = 207)		OR (95% CI)	p
	n	%	n	%		
miR-196a2: rs11614913 T>C						
TT	44	25.9	54	26.2	1.00	
TC	81	47.6	120	58.3	0.83 (0.51-1.35)	0.45
CC	45	26.5	32	15.5	1.73 (0.94-3.16)	0.08
CC vs. TC vs. TT						**0.03**
TC+CC	126	74.1	152	73.8	1.02 (0.64-1.62)	0.94
TT+TC	125	73.5	174	84.5	1.00	
CC	45	26.5	32	15.5	**1.96 (1.18-3.25)**	**0.01**
T allele	169	49.7	228	55.3	1.00	
C allele	171	50.3	184	44.7	1.25 (0.94-1.67)	0.12
miR-146a: rs2910164 C>G						
CC	57	33.3	65	31.7	1.00	
CG	77	45.0	102	49.8	0.86 (0.54-1.37)	0.53
GG	37	21.6	38	18.5	1.11 (0.62-1.98)	0.72
GG vs. CG vs. CC						0.62
CG+GG	114	66.7	140	68.3	0.93 (0.60-1.43)	0.74
CC+CG	134	78.4	167	81.5	1.00	
GG	37	21.6	38	18.5	1.21 (0.73-2.01)	0.45
C allele	191	55.8	232	56.6	1.00	
G allele	151	44.2	178	43.4	1.03 (0.77-1.38)	0.84

The genotyping was successful in 170 cases and 206 controls (3 cases and 1 control not genotyped) for miR-196a2 rs11614913 T>C, successful in 171 cases and 205 controls (2 cases and 2 controls not genotyped) for miR-146a rs2910164 C>G; bold values are statistically significant (p < 0.05). CI, confidence interval; OR, odds ratio.

MiR-146a rs2910164 C>G polymorphism didn't achieve significant difference in the genotype distributions between the cases and the controls (p = 0.62). Logistic regression analyses revealed that miR-146a rs2910164 C>G polymorphism was not associated with the risk of TOF (Table 3).

DISCUSSION

In this case-control study, we investigated the association of miR-196a2 rs11614913 T>C and miR-146a rs2910164 C>G SNPs with the risk of developing TOF in a Chinese population. We found that the miR-196a2 rs11614913 T>C SNP was positively correlated with the risk for TOF, while the miR-146a rs2910164 C>G SNP was not.

MiR-196a2 rs11614913 CC was associated with significantly increased levels of mature miR-196a expression. In vitro binding assays further revealed that the rs11614913 variant affects HOXB8 binding to mature miR-196a.[11] The miR-196a2 rs11614913 CC or CC/CT genotypes are also associated with an increased risk of cancer development,[16,17] Moyamoya disease,[18] severe coronary artery disease,[19] and chronic obstructive pulmonary disease.[20] Our results were in accordance with previous study by Xu et al., who found this miR-196a2 polymorphism was associated with a significantly increased risk of CHD.[11]

Genetic polymorphisms often vary between ethnic groups. In this study of 207 non-CHD controls, we determined that the allele frequency of miR-196a2 rs11614913 T>C (0.45) was similar to those reported previously in Chinese (0.48) and Japanese (0.40) populations. However, the mutant homozygote genotype among controls had a lower frequency than previously reported in European (0.56) and Sub-Saharan African (0.85) control populations (http://www.ncbi.nlm.nih.gov/SNP).

Using the Power and Sample Size Calculation software (PS, version 3.0, 2009, http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize), the power of our analysis (α = 0.05) was 0.901 in the 173 TOF cases and 207 controls (OR = 1.96) for the miR-196a2 rs11614913 T>C SNP.

Several limitations of the study need to be addressed. First, we did not obtain blood samples from the mothers to evaluate the etiological role of miR-196a2 rs11614913 T>C and miR-146a rs2910164 C>G SNPs in CHDs. Second, as our study was a hospital-based case-control study, selection bias could not be fully excluded. Further large population-based studies are therefore warranted to confirm the role of miR-196a2 rs11614913 T>C and miR-146a rs2910164 C>G SNPs in TOF susceptibility. Thirdly, the sample size of this study was insufficiently large to evaluate the low penetrance of these SNPs.

CONCLUSIONS

In conclusion, our findings suggest that the miR-196a2 rs11614913 T>C SNP may play a role in the susceptibility of children to developing TOF. To confirm these current findings, future larger studies that include other ethnic groups will be required.

CONFLICT OF INTERESTS

All authors have no disclosures or conflict of interest.

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