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Original Research

Association of a MiR-499 SNP and risk of congenital heart disease in a Chinese population

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Abstract: MicroRNAs (miRNAs) play an important role in heart development. Single nucleotide polymorphisms (SNPs) in miRNAs have been shown to associate with congenital heart disease (CHD). Methionine synthase (MTR), a key enzyme of folate metabolism, is involved in the early embryonic development. In this study, we aimed to test whether MTR is a direct target of miR-499, and to estimate the associations between miR-499 polymorphisms and the risk of CHD in Chinese population. Gene polymorphisms were analyzed in 1615 subjects including 792 healthy controls and 823 CHD patients. The miR-499 SNP were genotyped and the associations between the SNP frequencies and CHD were assessed by computing odds ratios (ORs) and 95% confidence intervals (95% CIs), as well as by applying Chi-square tests. Dual reporter assay was carried out to test whether MTR is a direct target gene of miR-499. The miR-499 rs374644 AG genotype was not associated with the CHD risk (AG vs. AA. OR=1.27, 95%CI=0.85-1.81, p=0.20). The GG genotype was associated with a significantly increased CHD risk (GG vs. AA. OR=5.33, 95%CI=1.80-15.83, p=0.001). The AG/GG variants were associated with a significantly increased CHD risk, compared with the AA genotype (OR=1.56, 95%CI=1.16-2.10, p=0.003). MiR-499 mimics inhibits the expression of *MTR*. MiR-499 directly targeted on MTR. Thus, our study suggested that miR-499 directly targets on MTR and the polymorphisms of rs3746444 may be associated with CHD risk in Chinese individuals.

Key words: miR-499; MTR/MS; SNP; Congenital heart disease.

Introduction

Congenital heart disease (CHD) refers to the dysfunction of heart that arise during cardiac embryogenesis. CHD is among the most common congenital defects in new born and about 1.35 million infants are born with CHD each year worldwide (1, 2). Although the improved treatment and prognosis of CHD patients, the etiology of CHD still unclear. Genetic and environmental factors play important role in etiology of CHD. About 20% of CHDs can be attributed to the Mendelian syndromes, chromosomal aberrations, environmental risk factors or single gene defects, while the majority of CHD arise via various multiple genetic contributors (3). The heart was the first forming organ during development of mammalian embryonic. A population of cells in the mesoderm that initiate the cardiogenesis committed to a cardiogenic fate. The development of heart is controlled by an evolutionarily conserved network of transcription factors that connects signaling pathways with genes for muscle growth and contractility (5). Mutations in cardiac regulatory genes frequently cause CHD typically characterized by lesions in humans (6). It was demonstrated that the mutations in GATA4, Nkx2.5 and Tbx5 are associated with a wide spectrum of developmental heart defects (7, 8).

Methionine synthase (MTR or MS) is one of the key enzymes in folate metabolism, which catalyzed the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine to remove homocysteine. It was demonstrated that a defective MTR enzyme could lead to the folate deficiency-related birth defects (9-11). MTR activity is essential for the early embryonic development. Homozygous MTR knockout mice are embryonically lethal (12). Due to the lack of activity of compensatory pathways during the early embryonic development, homocysteine removal is due exclusively to MTR activity in the embryo (13, 14). MTR deficiency caused by the −186T>G and +905G>A SNPs could affect the folate-methionine metabolic pathway and lead to a high homocysteine level in the early embryonic development (15). Methionine synthase reductase (MTRR) are another major regulatory enzyme involved in homocysteine metabolic pathway. MTRR is responsible for the activation of methionine synthase. A meta-analysis demonstrated a suggestive result that the A66G variant in *MTRR* (rs1801394), but not the A2756G in *MTR* (rs1805087), might associated with the increase of CHD risks (16). These contradictions imply that many of the folate variants in the association studies cannot satisfactorily explain the relationship between the folate metabolism and the CHD risk (15).

MicroRNAs (miRNAs) are a family of endogenous small non-coding RNAs involved in various processes of development and physiology through suppressing the gene expression (17-19). It was demonstrated that miRNAs play crucial roles in the heart development by conditional deletion of enzymes that are essential for miRNA processing such as Dicer or Drosha, which led to the embryonic lethality with abnormal cardiac morphogenesis (17, 18, 20). MiR-1 and miR-133 are two cardiac specific miRNAs, regulate the muscle develop-

ment and function by targeting the key regulators of heart development (21, 22). Accumulating evidences have demonstrated that the sequence variations in miRNA genes are associated with human diseases, including cancer and CHD (23). It was demonstrated that a genetic variant of rs11614913 in miR-196a2 sequence was associated with a significantly increased risk of CHD. The homozygous CC variant of miR-196a2 (rs11614913) was negatively associated with the atrial septal defect compared with the wild-type TT variant (23). The miR-196a2 rs11614913 T>C polymorphism may play a role in the development of tetralogy of Fallot (24). The miR-27a (rs11671784, rs895819) and miR-499 (rs3746444) showed association with the atrial septal defect. However, the association of the miR-499 with MTR, and the genetic polymorphism of miR-499 with the risk of congenital heart disease are unclear. In this study, we investigated the contribution of genotype rs3746444 variant in miR-499 to the risk of CHDs. Our data showed that miR-499 targets on MTR and its rs3746444 variant is functional and represent a risk factor to congenital heart disease.

Materials and Methods

Study population

A total of 823 non-syndromic CHD patients (Table 1) and 792 control subjects with no reported cardiac phenotypes were recruited for this study from September 2015 to March 2017 from Qilu Hospital of Shandong University and Qilu Children Hospital of Shandong University. Informed consent was obtained from patients or guardians. Controls were non-CHD outpatients from the same geographic area. The study was approved by the ethics committee of Shandong University and adhered to the tenets of the Declaration of Helsinki. The patients were routinely screened by clinical examination, chest X-rays, electrocardiography and ultrasonic echocardiogram, and the diagnosis of pathological types was confirmed by open heart surgery. 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 (24). Control individuals with congenital anomalies were also excluded. All subjects were genetically unrelated ethnic Han Chinese.

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 the Vacutainer tubes. These were transferred to tubes containing ethylenediamine tetra-acetic acid (EDTA). Genomic DNA was isolated from the whole blood with the QIAamp DNA Blood Mini Kit (Qiagen, Germany). Genotyping was undertaken by the MALDI-ToF-MS as described previously (25, 26). SNP genotyping was done using the MassArray system (Sequenom, USA) by the MALDI-TOF-MS method according to manufacturer's instructions. Completed genotyping reactions were spotted onto a 384-well spectroCHIP system (Sequenom, USA) using a MassArray Nanodispenser (Sequenom, USA), and the genotype determined by the MALDI-TOF-MS. Genotype calling was done in real-time with MassArray RT software (version 3.1, Sequenom, USA), and analyzed using the MassArray Typer software (version 4.0, Sequenom, USA)(25). Primary amplification primmer for rs3746444 is forward primer 5′-ACGTTGGATGACGGGAAGCAGCACAGACTT-3′ and reverse primer 5′-ACGTTGGATGGGCTGTTAAGACTTGCAGTG-3′.

Cell culture and transfection

Human HEK293ET cells and human cardiac myocyte (ATCC, USA) were grown in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Plasmids were transfected into cells by Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from transfected cells using Trizol (Invitrogen, USA). The reverse transcription kit was purchased from Takara, Japan. qRT-PCR was conducted by an Applied Biosystems 7500 PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. The primer sequences used for qRT-PCR are showed as follow: *MTR*, forward, 5'-GAGAGCACGTCTTCTCTGCC-3', and reverse, 5'-TCTTCAGACCTTCGGGTTGC-3'; *GAPDH*, forward, 5'-GTCGGTGTGAACGGAT TT-3', and reverse, 5'-ACTCCACGACGTACTCAGC-3'. The mRNA expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

Dual reporter assay

The miR-499 mimics (Genepharma, China) and *MTR* 3′-UTR reporter plasmids were cotransfected.

Table 1. Comparison of CHD patients and controls by selective characteristics.

Variable	CHD (n=823)	Controls (n=792)	*p*
Age, year (mean±SD)	2.49±2.01	2.81±2.56	0.57
Male/female	413/410	390/402	0.82
Septation defect (%)	495 (60.1%)	--	
Cyanotic heart disease (%)	142 (17.3%)	--	
Aortic deformity (%)	85 (10.3%)		
other complex cardiac malformations (%)	101 (12.3%)	--	

Note: Differences were evaluated using χ^2 test.

After 24 h, the cells were collected and analyzed for luciferase activity with the dual-luciferase reporter assay system (Promega, USA). Human *MTR* 3′UTR (wide type, position 3891-3897 of *MTR* 3'UTR 5′-GCCCCUGACCCCUCCAGUCUUAG-3′, has-miR-499, 3′-UUUGUAGUGACGUUCAGAAUU-5′; MUT: position 3891-3897 of *MTR* 3'UTR 5′-GCCCCUGACCCCUCCGCGGAACG-3′, has-miR-499, 3′- UUUGUAGUGACGUUCAGAAUU-5′; the binding sites were underlined.) was amplified using primers from a genomic DNA sample and cloned into the psi-check2 vector (Promega, USA) with NotI and XhoI digestion.

Statistical analysis

Differences in the distributions of demographic characteristics, selected variables, the variant alleles and genotypes of miR-499 (rs3746444) SNP between the cases and controls were evaluated using the paired Student t-test (for continuous variables with a normal distribution, data was presented as mean ± standard deviation) and $\chi 2$ test (for categorical variables). The associations between the miR-499 rs3746444 genotypes and the risk of CHD were estimated by computing the odds ratios (ORs) and their 95% confidence internals (CIs) from the logistic regression analyses. All of the statistical analyses were performed with the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 18.0) using P<0.05 as the significant criteria.

Results

Characteristics of the study population

The demographic and clinical characteristics of all subjects are summarized in Table 1. The ages were 2.49±2.01 years for the CHD patients and 2.81±2.56 years for the controls (p=0.57), and 50.2% of the CHD patients and 49.2% of controls were males. The differences were not significant (p=0.82), indicating the adequate frequency-matching by age and sex. Of the 823 CHD patients, 495 (60.1%) were septation defect, 142 (17.3%) were cyanotic heart disease, 85 (10.3%) were aortic deformity, and 101 (12.3%) were other complex cardiac malformations.

miR-499 rs3746444 A>G polymorphisms and CHD susceptibility

The genotype distribution of miR-499 rs3746444 A>G in the cases and controls were described in Table 2. In single-locus analyses, the genotype frequencies of miR-499 rs3746444 A>G were 59% (AA), 34% (AG), and 7% (GG) in CHD patients and 67% (AA), 31% (AG), and 2% (GG) in control subjects, and the difference was significant (p=0.04). When the miR-499 rs3746444 AA homozygote genotype was used as the reference group, the AG genotype was not associated with the risk for CHD (AG vs. AA. OR=1.27, 95%CI=0.85-1.81, p=0.20); the GG genotype was associate with a borderline significantly increased risk for CHD (GG vs. AA. OR=5.33, 95%CI=1.80-15.83, p=0.001). The miR-499 rs3746444 AG/GG variants were associated with the risk of CHD, compared with the miR-499 rs3746444 AA genotype (OR=1.56, 95%CI=1.16-2.10, p=0.003).

miR-499 directly targets on MTR

To investigate whether MTR is the target of miR-499, binding site was predicted and the luciferase reporter test was performed (Figure 1). By using luciferase reporter system, the relative luciferase activity in *MTR*-3'UTR-WT (wild type) was decreased by miR-499 mimics, but not changed in *MTR*-3'UTR-MUT (mutant type). Then, we detected the level of *MTR* transcript in the miR-499 mimics transfected human cardiac myocyte cells (Figure 2). Results confirmed that miR-499 inhibited the expression of *MTR* mRNA. Thus, miR-499 directly targeted on MTR.

Discussion

In this case-control study, we investigated the association of miR-499 rs3746444 A>G SNP with the risk of developing CHD in a Chinese population. We found that the miR-499 rs3746444 A>G SNP was positively correlated with the risk for CHD. In vitro assay further revealed that the MTR as a target of miR-499.

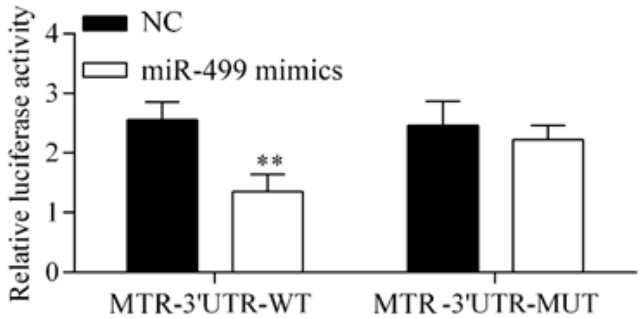

Figure 1. *MTR* is the target of miR-499. Human *MTR* 3′UTR (wide type, position 3891-3897 of *MTR* 3′-UTR 5′-GCCCCUGACCCCUCCAGUCUUAG-3′, has-miR-499, 3′- UUUGUAGUGACGUUCAGAAUU-5′; MUT: position 3891-3897 of *MTR* 3′-UTR 5′-GCCCCUGACCCCUCCGCGGAACG-3′) was amplified using primers from a genomic DNA sample and cloned into the psi-check2 vector (Promega, USA) with NotI and XhoI digestion. The underline indicates the binding sites. The miR-499 mimics (Genepharma, China) and *MTR* 3′-UTR reporter plasmids were cotransfected. After 24h, the luciferase reporter test showed that miR-499 directly targeted *MTR*. **p<0.01 vs. NC.

Table 2. Logistic regression analysis of association between miR-499 rs3746444 A>G polymorphisms and risk of CHDs.

Genotypes	Cases (N = 823) N (%)	Controls (N = 792) N (%)	OR (95% CI)	*P*
AA	485 (0.59)	531 (0.67)	1	
AG	280 (0.34)	246 (0.31)	1.27(0.85-1.81)	0.20
GG	58 (0.07)	16 (0.02)	5.33 (1.80-15.83)	0.001
G allele	198 (0.24)	135 (0.17)	1.56 (1.16-2.10)	0.003
AG/GG	338 (0.41)	262 (0.33)		

Note: Differences were evaluated using χ^2 test.

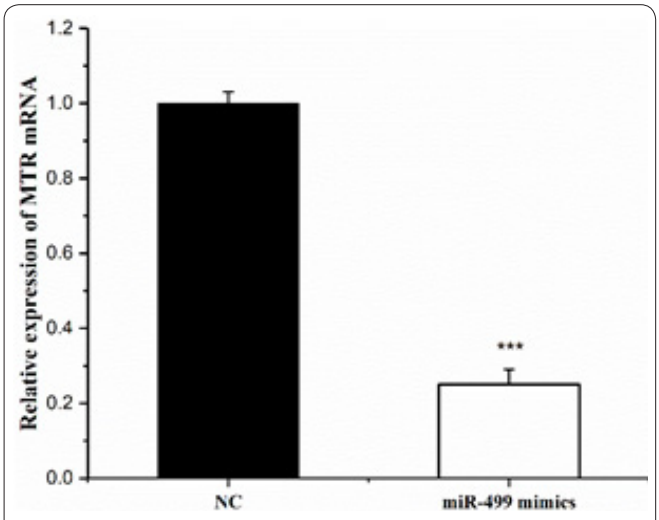

Figure 2. Expression of *MTR* mRNA in miR-499 mimics transfected- human cardiac myocytes. After transfection with miR-499. The *MTR* mRNA was detected by qRT-PCR. *** $p<0.001$ vs. NC.

MTR is a key enzyme involved in the folate metabolism by catalyzing the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine to remove homocysteine. MTR activity is essential for the early embryonic development. A defective MTR enzyme could lead to the folate deficiency-related birth defects (9-11). Due to the lack of activity of compensatory pathways during the early embryonic development, homocysteine removal is due exclusively to the MTR activity in the embryo (13, 14). Homozygous *MTR* knockout mice are embryonically lethal (12). *MTR* deficiency caused by the −186T>G and +905G>A SNPs could affect the folate-methionine metabolic pathway and lead to a high homocysteine level in the early embryonic development (15). A meta-analysis demonstrated a suggestive result that the A66G variant in *MTRR* (rs1801394), but not the A2756G in *MTR* (rs1805087), might associated with the increase of CHD risk (16). It's important to clear whether miR-499 SNPs regulated *MTR* and thus associates with the increase of CHD risk in the future.

miR-499 rs3746444 (T>C) were found to be associated with the ulcerative colitis patients of north Indian population (27). The miR-499 (rs3746444) AG/GG genotypes were associated with an increased risk of breast cancer in the Chinese individuals (28). A meta-analysis suggested that the hsa-mir-499 rs3746444 T > C polymorphism is associated with the risk of cancer in Asians, mainly in Iranian and Chinese population (29). However, rs3746444 T > C polymorphism is negatively associated with the risk of esophageal cancer (29). miR-499 AG was significantly associated with the decreased risk for hepatocellular carcinoma when compared with miR-499 AA genotype (30). The miR-27a (rs11671784, rs895819) and miR-499 (rs3746444) showed association with the atrial septal defect. It was consistent with our data (23). In this study, we found that the AG genotype was not associated with the risk for CHD, the GG genotype was associate with a borderline significantly increased risk for CHD. The miR-499 rs3746444 AG/GG variants were associated with the risk of CHD. The miR-499 directly targets on MTR, and the association of miR-499 rs3746444 A>G SNP with the risk of developing CHD might explain the relationship between folate metabolism and CHD risk. It was also demonstrated that MiR-499 rs374644 A/G was statistically associated with the risk of dilated cardiomyopathy (31). MiR-499 rs3746444 A>G polymorphisms were diverse association with three phenotypes of isolated CHD: atrial septal defect (ASD), ventricular septal defect (VSD), and patent ductus arteriosus (PDA) (23). The association between miR-499 SNP and other specific cardiac diseases should be carried out in the future.

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-499 rs3746444 A>G SNP 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-499 rs3746444 A>G SNP in CHD susceptibility. Thirdly, the sample size of this study might insufficiently large to evaluate the low penetrance of these SNPs. In addition, although we found that miR-499 targets on MTR using HEK392ET cells, it's not clear their association in risk of CHD. It is also unclear whether miR-499 SNP regulates MTR to lead CHD. A combination analysis study should be taken out using cardiomyocytes in the future. Moreover, other possible confounders might be investigated in the future.

In summary, the miR-499 rs3746444 A>G SNP were associated with the risk of CHD. The miR-499 directly targets on *MTR*, and the association of miR-499 rs3746444 A>G SNP with the risk of developing CHD might explain the association between the folate metabolism and the CHD risk.

Conflict of interest

None.

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