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Coronary Artery Disease

Hsa-mir-499 rs3746444 T/C Polymorphism is Associated with Increased Risk of Coronary Artery Disease in a Chinese Population

Weiqiang Chen,[1#] Donghua Shao,[2#] Haiyong Gu,[3] Jie Gong[4] and Jian Zhang[1]

Background: Coronary artery disease (CAD) is a complex disease resulting from a combination of environmental and genetic factors. We hypothesized that polymorphisms *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G might contribute to CAD susceptibility.

Methods: We studied the association between the three polymorphisms and the risk of CAD in a Chinese population using 435 CAD patients and 480 controls. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to perform the genotyping, and the differences were analysed.

Results: When the *hsa-mir-499* rs3746444 TT homozygote genotype was used as the reference group, the TC, CC or TC/CC genotypes were associated with a significantly increased risk of CAD [TC vs. TT: adjusted odds ratio (OR) 1.41, 95% confidence interval (CI) 1.02-1.94, p = 0.04; CC vs. TT: adjusted OR 3.14, 95% CI 1.77-5.56, p < 0.001; CC/TC vs. TT: adjusted OR 1.68, 95% CI 1.25-2.26, p < 0.001). In the recessive model, when the *hsa-mir-499* rs3746444 TT/TC genotypes were used as the reference group, the CC homozygote genotype was associated with a significantly increased risk of CAD (adjusted OR 2.87, 95% CI 1.63-5.04, p < 0.001). Risk factors such as diabetes mellitus (DM), hypertension, smoking and low high-density ipoprotein cholesterol (HDL-c) were also associated with a significantly increased risk for CAD. Logistic regression analyses revealed that *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G polymorphisms were not associated with risk of CAD.

Conclusions: These findings suggested that the functional polymorphism *hsa-mir-499* rs3746444 T/C is associated with CAD susceptibility.

Key Words: Cardiovascular disease • *Hsa-mir-499* • Molecular epidemiology • Polymorphisms

INTRODUCTION

Micro-Ribonucleic Acids (miRNAs) are an abundant class of small non-coding, single-stranded ribonucleic acid (RNA) that form base-pairs with target mRNAs to negatively regulate their translational efficiency and stability.[1] MiRNAs are involved in various biological processes, such as cell proliferation, cell death, stress resistance and fat metabolism.[2] The single nucleotide polymorphism (SNP) rs3746444 T/C is located at the pre-miRNA region of *hsa-miR-499*. Previous studies have demonstrated that *hsa-mir-499* rs3746444 T/C is related to many diseases, including cancers,[3] autoimmune disease,[4] inflamatory arthritis,[5] and coronary artery disease (CAD).[6-8]

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[1]Department of Cardiovascular Medicine, Cardiovascular Clinical College of Tianjin Medical University & TEDA International Cardiovascular Hospital, Tianjin 300457; [2]Department of Anesthesiology; [3]Department of Cardiothoracic Surgery, Affiliated People's Hospital of Jiangsu University, Zhenjiang 212002; [4]Department of Cardiolgy, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi 214023, China. Corresponding author: Jie Gong, Department of Cardiolgy, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi 214023, China. E-mail: gongjnjmu@hotmail.com & Jian Zhang, Department of Cardiovascular Medicine, Cardiovascular Clinical College of Tianjin Medical University & TEDA International Cardiovascular Hospital, Tianjin 300457, China; E-mail: zj5082@yahoo.com.cn
[#] Weiqiang Chen and Donghua Shao contributed equally to this work.

Previous studies revealed that the rs 3746444 T/C polymorphism conferred a risk for CAD, although others found no association between rs3746444 and CAD.[9] Interleukin-1-receptor-associated kinase 1 (IRAK1), the target of another miRNA, *hsa-miR-146a*, is a signaling molecule transducing Toll/interleukin-1 receptor (TIR) activation.[10] IRAK1 is a key intracellular signaling protein that is involved in pathogen-mediated inflammation, and its target genes include inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10) and IL-12] and costimulatory molecules.[11,12] IRAK1 is constitutively activated in atherosclerosis patients.[12] Complementary deoxyribonucleic acid (cDNA) microarray analysis revealed that IRAK1 is expressed at high levels in human coronary arteries.[13] Genetic variants of *IRAK1* were found to be associated with the plasma concentration of C-reactive protein (CRP) in Caucasian women.[14] The *IRAK1* rs3027898 A>C genotype was also reported to be associated with atherothrombotic cerebral infarction.[15]

The activation of nuclear factor-kappa B (NF-κB) is the most prominent event of the inflammatory response.[16] Stimulation through the interleukin-1 receptor (IL-1R) and some Toll-like receptors (TLRs) induces ubiquitination of tumour-necrosis factor receptor-associated factor 6 (TRAF6) and IRAK1, which are the signaling components required for NF-κB and mitogen-activated protein kinase activation.[17] Another NF-κB pathway signal, the receptor activation of the NF-κB ligand (RANKL), has been shown to be involved in disease pathogenesis.[18] The C allele of *RANKL* polymorphism rs7984870 C/G might promote interactions between activated T cells and dendritic cells by increasing transcription levels upon stimulation.[16] A significant association between *RANKL* polymorphisms and the occurrence of acute coronary syndrome (ACS) was previously observed.[19]

Few studies have focused on the influences of polymorphisms *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G on the susceptibility to CAD. Therefore, we performed a hospital-based case-control study to genotype a cohort of 435 CAD patients and 480 controls in a Chinese population.

MATERIAL AND METHODS

Study population

This case-control study included 435 consecutive patients with CAD from Tianjin TEDA International Cardiovascular Hospital between November 2011 and July 2012. The study also utilized 480 CAD-free controls who were inpatients used to exclude CAD by quantitative coronary angiography (QCA), also from Tianjin TEDA International Cardiovascular Hospital. All 435 CAD patients and 480 inpatients received QCA with a Cardiovascular Measurement System (Philips Integris and Philips Allura Xper) shortly after being admitted to the hospital, and coronary angiograms were analyzed by two experienced interventional cardiologists. CAD patients were defined as having angiographic coronary stenosis of at least 50% lumen reduction in at least one major epicardial coronary artery. Exclusion criteria were patients who did not meet the CAD diagnosis standard after QCA, and an inability to give written informed consent. The 480 CAD-free inpatients were considered as controls, whose pathologies included luminal narrowing < 50% and no obvious clinical symptom such as ST-elevation, and no myocardial infarction, percutaneous coronary intervention or coronary artery bypass grafting history. We have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals. Each subject was interviewed; after written informed consent was obtained, an approximate 2-ml venous blood sample was collected from each subject. Individuals who smoked once a day for over 1 year were defined as smokers. Subjects who consumed ≥ 3 alcoholic drinks a week for > 6 months were considered to be alcohol drinkers. Diabetes mellitus was defined as self-reported diabetes mellitus or nonfasting glucose levels ≥ 11.1 mmol/L (200 mg/dL). Hypertension was defined as blood pressure level exceeding 140/90 mmHg or use of antihypertensive therapy. The study was approved by the Ethical Committee of Tianjin Medical University.

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

Blood samples were collected using vacutainers and transferred to test tubes containing ethylenediamine tetra-acetic acid (EDTA). Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Germany). Genotyping was done by MALDI-

TOF MS using the MassARRAY system (Sequenom, San Diego, CA, USA) as previously described.[20] Completed genotyping reactions were spotted onto a 384-well spectroCHIP (Sequenom) using a MassARRAY Nanodispenser (Sequenom), and were analyzed by MALDI-TOF-MS. Genotype calling was done in real time with MassARRAY RT software (version 3.1; Sequenom), and was analyzed using MassARRAY Typer software (version 4.0; Sequenom). For quality control, repeated analyses were undertaken on 10% of the randomly selected samples.

Statistical analyses

Differences in demographics, variables, and genotypes of the *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G polymorphisms were evaluated by using a chi-squared test. The associations between *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G genotypes and risk of CAD were estimated by computing the odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression analyses, and by using crude ORs. The Hardy-Weinberg equilibrium (HWE) was tested by a goodness-of-fit chi-squared test to compare the observed genotype frequencies to the expected frequencies among controls. All statistical analyses were done with SAS software (version 9.1.3; SAS Institute, Cary, NC, USA).

RESULTS

Characteristics of the study population

Subjects were adequately matched for age (p = 0.38) and sex (p = 0.40) for CAD cases and controls in Tianjin. The average body mass index (BMI) was not significantly different between CAD cases and the controls (p = 0.22). For the family disease history, 98 (22.5%) cases had a family history of CAD which was significantly higher than that of the controls (13.1%) (p < 0.001). There were more hypertension and diabetes mellitus in CAD cases than in controls (p = 0.009 and p = 0.009, respectively). The demographic and clinical characteristics of all subjects are summarized in Table 1. No significant differences occurred for hyperlipidemia between CAD cases and controls (p = 0.29). There were more smokers in CAD cases (56.8%) than those in the controls (44.0%) (p < 0.001). The level of fasting blood glucose, uric acid, serum creatinine, triglyceride and fibrinogen in CAD cases were higher than those of the controls, while the level of high density lipoprotein of CAD cases was significantly lower than that of the controls. Among 915 DNA samples (435 CAD patients and 480 controls in Tianjin), *hsa-mir-499* rs3746444 T/C polymorphism was successful in 421 (96.8%) CAD cases and 464 (96.7%) controls. Genotyping was successful in 432 (99.3%) CAD patients and 476 (99.2%) controls for *IRAK1* rs3027898 C/A and in 418 (96.1%) CAD patients and 468 (97.5%) controls for *RANKL* rs7984870 C/G (Table 2). The concordance rates of repeated analyses were 100%.

Associations between *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G polymorphisms and risk of CAD

The genotype frequencies of the *hsa-mir-499* rs 3746444 T/C polymorphism were 62.7% (TT), 26.1% (TC) and 11.2% (CC) in CAD patients, and 73.7% (TT), 22.2% (TC) and 4.1% (CC) in the controls (p < 0.001) (Table 3). When the *hsa-mir-499* rs3746444 TT homozygote genotype was used as the reference group, the TC, CC or TC/CC genotypes were associated with a significantly increased risk for CAD (TC vs. TT: adjusted OR 1.41, 95% CI 1.02-1.94, p = 0.04; CC vs. TT: adjusted OR 3.14, 95% CI 1.77-5.56, p < 0.001; CC/TC vs. TT: adjusted OR 1.68, 95% CI 1.25-2.26, p < 0.001). In the recessive model, when the *hsa-mir-499* rs3746444 TT/TC genotypes were used as the reference group, the CC homozygote genotype was associated with a significantly increased risk for CAD (adjusted OR 2.87, 95% CI 1.63-5.04, p < 0.001).

None of the *IRAK1* rs3027898 C/A and *RANKL* rs 7984870 C/G polymorphisms achieved a significant difference in the genotype distributions between cases and controls. Logistic regression analyses revealed that *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G polymorphisms were not associated with the risk of CAD (Table 3). In our research, we also found some risk factors such as diabetes mellitus, hypertension, smoking and low HDLc were associated with a significantly increased risk for CAD (DM: OR 1.62, 95% CI 1.11-2.19, p = 0.009, hypertension: OR 1.40, 95% CI 1.04-1.88, p = 0.009, smoking: OR 1.67, 95% CI 1.31-2.46, p < 0.001, HDL-c: OR 2.08, 95% CI 1.44-3.01, p < 0.001).

Table 1. The distributions of the selected variables in the CAD cases and controls

Variable	CAD (n = 435)	Controls (n = 480)	p
Mean age, y	61.30 (±9.71)	60.79 (±7.54)	0.38
Woman, %	155 (35.6)	184 (38.3)	0.40
Mean BMI, kg/m^2	25.99 (±3.28)	26.24 (±2.75)	0.22
Family history of CAD, %	98 (22.5)	63 (13.1)	< 0.001
Previous smoker, %	247 (56.8)	211 (44.0)	< 0.001
Previous drinker, %	82 (18.9)	81 (16.9)	0.44
Hypertension, %	305 (70.1)	297 (61.9)	0.009
Hyperlipidemia, %	274 (63.0)	286 (59.6)	0.29
Diabetes mellitus, %	113 (26.0)	90 (18.8)	0.009
Previous stroke, %	22 (5.1)	11 (2.3)	0.03
Previous myocardial infarction, %	100 (23.0)	—	—
Previous percutaneous coronary intervention, %	221 (50.8)	—	—
Previous coronary artery bypass grafting, %	41 (9.4)	—	—
Fasting blood glucose (mmol/l)	5.92 (±2.25)	5.41 (±1.07)	< 0.001
Uric acid (μmol/l)	325.82 (±85.40)	312.60 (±79.60)	0.02
Creatinine (μmol/l)	68.69 (±18.02)	62.84 (±21.45)	< 0.001
Total cholesterol (mmol/l)	4.70 (±1.07)	4.86 (±2.85)	0.28
Triglyceride (mmol/l)	1.89 (±1.67)	1.64 (±0.95)	0.006
High density lipoprotein (mmol/l)	1.03 (±0.24)	1.16 (±0.33)	< 0.001
Low density lipoprotein (mmol/l)	3.02 (±0.93)	3.07 (±0.83)	0.40
Fibrinogen (g/l)	2.74 (±0.64)	2.61 (±0.62)	0.002
Aspirin, %	427 (98.2)	414 (88.5)	< 0.001
Clopidogrel, %	312 (71.7)	15 (3.2)	< 0.001
Nitroglycerin, %	350 (80.6)	61 (13.0)	< 0.001
Angiotensin converting enzyme inhibitor, %	148 (34.1)	126 (27.0)	0.02
Angiotensin II receptor blockers, %	97 (22.4)	74 (15.8)	0.01
β-blocker, %	333 (76.6)	230 (49.1)	< 0.001
Ca^{2+} antagonist, %	171 (39.4)	156 (33.3)	0.06
Statins, %	418 (96.1)	310 (66.2)	< 0.001
Diuretics, %	41 (9.5)	21 (4.5)	0.003
Insulin, %	37 (8.5)	10 (2.1)	< 0.001
Oral hypoglycemic drugs, %	102 (23.5)	55 (11.8)	< 0.001

BMI, body mass index; CAD, coronary artery disease; Ca^{2+}, calcium channels. Bold values are statistically significant (p < 0.05); The medical information was available in 433-435 CAD cases and 465-468 non-CAD controls.

Table 2. Primary information for *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G polymorphisms

Genotyped SNPs	Chromosomes	Location	Regulome DB score[a]	TFBS[b]	Splicing (ESE or ESS)[c]	MAF[d] for Chinese in database	MAF in our controls (n = 480)	p value for HWE[e] test in our controls	% Genotyping value
hsa-mir-499 rs3746444 T/C	20	Intron	5	Y	Y	0.167	0.152	< 0.05	96.7
IRAK1 rs3027898 C/A	X	3′ near gene	4	—	—	0.165	0.181	< 0.05	99.2
RANKL rs7984870 C/G	13	Intron	No data	Y	—	0.522	0.493	0.647	96.8

[a] http://www.regulomedb.org/; [b] TFBS, Transcription Factor Binding Site; [c] http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm; [d] MAF, minor allele frequency; [e] HWE, Hardy-Weinberg equilibrium.

Table 3. Logistic regression analysis of associations between *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G polymorphisms and risk of CAD

Genotype	CAD cases (n = 435)		Controls (n = 480)		OR (95% CI)	p	Adjusted OR[a] (95% CI)	p
	n	%	n	%				
hsa-mir-499 rs3746444 T/C								
TT	264	62.7	342	73.7	1.00	—	1.00	—
TC	110	26.1	103	22.2	1.38 (1.01-1.89)	0.04	1.41 (1.02-1.94)	0.04
CC	47	11.2	19	4.1	3.20 (1.84-5.59)	< 0.001	3.14 (1.77-5.56)	< 0.001
TC+CC	157	37.3	122	26.3	1.67 (1.25-2.22)	< 0.001	1.68 (1.25-2.26)	< 0.001
TT+TC	374	88.8	445	95.9	1.00	—	1.00	—
CC	47	11.2	19	4.1	2.94 (1.70-5.10)	< 0.001	2.87 (1.63-5.04)	< 0.001
C allele	204	24.2	141	15.2				
IRAK1 rs3027898 C/A								
CC	328	75.9	348	73.1	1.00	—	1.00	—
CA	59	13.7	84	17.6	0.75 (0.52-1.07)	0.12	0.76 (0.52-1.11)	0.15
AA	45	10.4	44	9.2	1.09 (0.70-1.69)	0.72	1.01 (0.64-1.59)	0.97
CA+AA	104	24.1	128	26.9	0.86 (0.64-1.16)	0.33	0.85 (0.62-1.16)	0.30
CC+CA	387	89.6	432	90.8	1.00	—	1.00	—
AA	45	10.4	44	9.2	1.14 (0.74-1.77)	0.55	1.06 (0.68-1.66)	0.80
A allele	149	17.2	172	18.1				
RANKL rs7984870 C/G								
CC	95	22.7	123	26.3	1.00	—	1.00	—
CG	209	50.0	229	48.9	1.18 (0.85-1.64)	0.32	1.15 (0.82-1.61)	0.43
GG	114	27.3	116	24.8	1.27 (0.88-1.85)	0.21	1.16 (0.79-1.71)	0.44
CG+GG	323	77.3	345	73.7	1.21 (0.89-1.65)	0.22	1.15 (0.84-1.59)	0.38
CC+CG	304	72.7	352	75.2	1.00	—	1.00	—
GG	114	27.3	116	24.8	1.14 (0.84-1.54)	0.40	1.06 (0.78-1.45)	0.71
G allele	437	52.3	461	49.3				

[a] Adjusted for age, sex, smoking, drinking, hypertension, diabetes mellitus, family history of CAD; Bold values are statistically significant (p < 0.05). CI, confidence interval; OR, odds ratio.

DISCUSSION

We determined the association between polymorphisms *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G and the risk of CAD in a Chinese population. We found that the *hsa-mir-499* rs3746444 T/C polymorphisms might be associated with the increased risk of CAD.

MiRNAs have been investigated as potential diagnostic biomarkers in patients with acute myocardial infarction.[21,22] An miRNA array analysis indicated that miR-499 was produced almost exclusively in the heart.[23] MiR-499 levels were significantly elevated in patients with myocardial infarction; miR-499 levels also reflected myocardial damage in cardiovascular disease.[24]

MiR-499 is located in the intron 20 of human cardiac β-myosin heavy chain 7B gene (MYH7B), and is particularly expressed in skeletal muscle, cardiac cells and brain tissue.[25] It has been shown that miR-499 can protect cardiomyocyte apoptosis by inhibiting mitochondrial apoptosis pathway,[26] and the knock-down of miR-499 induces myocardial apoptosis and increases the infarct size.[27] In addition, miR-499 regulates the expression of inflammatory cytokines, including TNF-α, annexin A1, CRP, IL-2, IL-2R, IL-6, IL-17Rβ, IL-18R, and IL-23α.[5] Rs 3746444 is located in the stem region of the miR-499, and may damage the secondary structure stability and thus affect the miRNA maturation process and binding affinities to its target genes.[28] It is reasonable that rs 3746444 may contribute to the susceptibility to various diseases by regulating distinct sets of downstream genes.

Many studies have shown that *hsa-mir-499* polymorphism rs3746444 T/C was associated with the increased risk of breast cancer, dilated cardiomyopathy,

inflammatory arthritis, autoimmune diseases and coronary artery disease.[28,29] *Hsa-mir-499* polymorphism rs3746444 T/C polymorphism was also investigated in lung cancer and coal workers' pneumoconiosis, but no association was identified.[18,30] Meta-analysis from Song et al. indicated associations between the *miR-499* rs374644 and inflammatory arthritis.[31] A few studies have suggested that *miR-499* rs374644 might be involved in the pathogenesis of autoimmune diseases, but the results remain conflicting.[32] Wang et al. found that the *hsa-mir-499* mutated homozygote was associated with a 3.23-fold significantly increased risk of CAD, which is consistent with our results.[8] Potential mechanism for such an association might be due to impaired ability to inhibit apoptosis and inflammation.

Genetic polymorphisms often vary between ethnic groups. In the present study, with 480 controls, we reported that the allele frequency of *hsa-mir-499* rs3746444 T/C (0.152) was similar to that reported in Chinese populations (0.167). We have identified DNA features and regulatory elements that contain the coordinates of the SNP (http://www.regulomedb.org/), indicating that *hsa-mir-499* SNP rs3746444 T/C SNP is functional (Table 2).

Considering *hsa-mir-499* rs3746444 T/C mutant alleles in the control group, ORs, CAD samples and control samples, the power of our analysis (α = 0.05) was 0.864 in 435 CAD cases and 480 controls with an OR = 1.68 for *hsa-mir-499* rs3746444 T/C.

However, several limitations of the present study also need to be addressed. First, this was a hospital-based case-control study, and the subjects were not fully representative of the general population; thus, selection bias was unavoidable. Second, the polymorphisms investigated, based on their functional considerations, may not offer a comprehensive view of the genetic variability of *hsa-mir-499*, *IRAK1* and *RANKL*. Third, because of the relatively moderate number of patients evaluated, a single case-control study is not sufficient to fully interpret the relationship between polymorphisms *hsa-mir-499* rs3746444 T/C, *IRAK1* rs3027898 C/A and *RANKL* rs7984870 C/G and susceptibility to CAD. Studies with larger numbers of subjects are necessary to confirm our findings. Finally, environmental factors differ between Chinese and other populations. CAD risk is likely to be influenced by gene-gene and gene-environment interactions; therefore, the *hsa-mir-499*, *IRAK1* and *RANKL* genes may be associated with different degrees of genetic risk in different ethnic groups and under different environmental exposures.

CONCLUSIONS

In conclusion, the present study provided strong evidence that the *hsa-mir-499* rs3746444 T/C polymorphism might be associated with increased risk of CAD. However, our results were obtained from a moderate-sized sample and further studies are needed to confirm the results.

CONFLICT OF INTEREST

None of the authors has any potential financial conflict of interest related to this manuscript.

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