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Metallothionein 2A genetic polymorphism and its correlation to coronary heart disease

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Abstract. – OBJECTIVE: To investigate the gene polymorphism of metallothionein 2A (MT2A)-838G/C and its correlation to coronary heart disease in the Han population of Jiangsu, China.

PATIENTS AND METHODS: The MT2A-838G/C was examined in 287 patients with coronary heart disease (CHD group) and 226 healthy controls (control group) by using mono-labeled fluorescent probes. Meanwhile, relevant variables of all subjects were measured, including blood lipid and glucose profiles and body mass index (BMI). The extent of the coronary artery disease was evaluated based on Gensini's coronary artery scoring method.

RESULTS: Three distinct genotypes were identified. The highest frequency was observed for genotype GG, followed by genotype GC and CC. There were statistically significant differences in the genotype and allele frequency distribution of the MT2A gene-838G/C polymorphism between the CHD and the control group ($p < 0.05$). The allele frequency of MT2A-838C in the CHD patients were higher than that in the healthy controls (31.4% vs 24.6%, $p = 0.016$). The CHD risk in C allele carriers (including genotype GC+CC) was 1.562 folds as high as in GG allele carriers (OR = 1.562, 95% confidence intervals (CI): 1.099-2.218, $p = 0.013$). According to the results of logistic regression analysis, the C allele was an independent risk factor for CHD ($p < 0.05$). Gensini's coronary artery disease score were higher in C allele carriers than in non-C allele carriers ($p < 0.05$).

CONCLUSIONS: The gene polymorphism of MT2A-838G/C is correlated to CHD. The C allele might be a CHD-susceptible gene and might also have an effect on the extent of coronary artery disease.

Key Words:
Coronary heart disease, Metallothionein proteins, Polymorphism, Single nucleotide.

Introduction

Atherosclerosis is the main pathological basis of coronary heart disease (CHD), of which, oxidative stress plays an important role[1,2]. Oxidative stress is the imbalance between the production and clearance of oxygen free radicals in the body, resulting in the accumulation of reactive oxygen species (ROS) and oxidative damage process. ROS could induce endothelial cell apoptosis. Exposure to O_2-and H_2O_2 might lead to functional loss of endothelial cells. ROS could promote growth and migration of the vascular smooth muscle cells, and also mediate platelet-derived growth factor (PDGF) and thrombin-induced proliferation of vascular smooth muscle cells. Cumulative evidence demonstrated that oxidative stress and inflammation were two key steps for atherosclerosis development. Moreover, oxidative stress is also the initiation factor for atherosclerotic inflammation. Antioxidant therapy might decrease oxidative stress, reduce the generation of lipid peroxidation modified products[3], thereby prevent or delay the key step for atherosclerosis formation from the genesis.

Metallothionein (MT) is a low-molecular weight protein which is sulfhydryl-rich and could form chelates with metal ions. Metallothionein could scavenge extensive ROS species, including superoxide anion, hydrogen peroxide and hydroxyl (-HO), and is an endogenous antioxidant[4]. The thiol groups in MT could interact with H_2O_2 directly[5], and its ability to scavenge-HO was found to be 100 folds higher than that of reduced glutathione[6]. Meanwhile, MT oxidation leads to zinc release which regulates gene transcription, activates antioxidant enzymes and superoxide dismutase[7], and assists with the anti-oxidative process[8]. Göbel et al[8] demonstrated a high MT concentration in lipid-rich plaques of smooth muscle cells during the investigation of MT distribution in human atherosclerotic plaques, suggesting an oxidant-dependent mechanism for the increase of MT concentration. MT might exert its role in cardiovascular protection through radical scavenging activities and suppression of lipid peroxidation[4,10].

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MT2A is a major subtype of the MT family[11]. Its gene polymorphisms are associated with atherosclerosis. Giacconi et al[12] described the correlation between the 838C/G polymorphism of MT2A gene and carotid artery stenosis. Moreover, the 838C/G polymorphism of MT2A gene is correlated to diabetic atherosclerosis[13], and the risk to develop atherosclerosis increases in patients carrying the AA allele. So, the single nucleotide polymorphism of MT2A might have an effect on the anti-oxidative role of MT protein, thereby, influencing the occurrence and development of atherosclerosis. Currently, there are few genetic studies investigating the correlation between MT2A and CHD.

In this study, the correlation between the MT2A-838G/C and CHD was investigated in the Han population of Jiangsu, China, using molecular biology techniques. In addition, the effects of the distinct genotypes on blood lipid and glucose profiles, as well as the extent of coronary artery disease, were analyzed to investigate the possible roles of the genetic variation in the pathogenesis of CHD.

Patients and Methods

Subjects

The CHD group included 287 subjects, 234 men and 53 women, with an average age of (62.05±9.45) years old, who underwent coronary angiography and reported positive findings (≥ 50% diameter stenosis for at least one branch of the coronary artery). All the subjects met the 1979 WHO diagnostic criteria for CHD. The control group included 226 healthy outpatients; 170 men and 56 women, aged (61.69±8.20) years old. All subjects were screened for CHD, hypertension, and diabetes, and reported negative findings. They were ruled out for cerebrovascular disease, severe hepatic and renal dysfunction, infection, malignancy and autoimmune diseases. They did not receive any lipid-lowering therapies within one month before their enrollment. All subjects from both groups were from Jiangsu, China, and no phylogenetic relationship was observed.

Methods

Collection of Clinical Data

The height and body weight were measured by specialized personnel, and the BMI (= body/height^2, kg/m^2) was estimated. The life style data (e.g., smoking status) was obtained by questionnaire. After fasting for 12 hours, all subjects provided 2 mL of venous blood which was used to profile fasting plasma glucose (FPG), total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) using an automatic biochemical analyzer.

Preparation of DNA of Peripheral Blood Leukocytes

After fasting for 12 hours, 2 mL of blood was drawn from the cubital vein and mixed with the EDTA-K2 anticoagulant. Relevant genomic DNA was extracted using the blood genomic DNA extraction kit (UIIQ-10 column) (Sangon Biotech, Shanghai, China). DNA concentrations were estimated according to the corresponding optical densities (OD260/OD280), and extracted genomic DNA was dissolved in TE and stored in a –20°C freezer.

Genotype Identification of MT2A Gene 838G/C Locus

Single nucleotide polymorphism was examined using the ShineRoar probe technology[14] modified mono-labeled fluorescent probes based on the polymerase chain reaction (PCR) assays. Primers and probes were analyzed and designed on Primer 5.0 platform. Upstream primer: 5'-CACCATCCTCAGGGGAATTAAAGCA-3', downstream primer: CCAGAGACAGAATCAAGTCAAAGCTGTT-3', probe: 5'-FAM-CCTGACCGTGA CCGT-P-3'. These primers and probes were synthesized by Sangon Biotech, Shanghai. The total volume of the PCR reaction system was 25 μL, containing template DNA 2 μL; 10× buffer 2.5 μL, 25 μmol/L$MgCl_2$ 2.5 μL, 4×dNTPs 0.5 μL, 5 U/μL TaqDNA polymerase 0.25 μL, 0.1 μL (100 μmol/L) upstream and downstream primers, respectively, 10 μmol/L probe 0.2 μL and ddH_2O 16.85 μL. PCR amplification and melt curves were analyzed by Roche Light Cycler (Version: 3.5) for gene amplification detection. Cycling reaction conditions: denaturation at 95°C for 1 min, 95°C for 5s and 65°C for 40 s, with a temperature conversion rate of 20°C/s, for total of 40 cycles; Melt curve analysis program for amplification products: 95°C for 30s, 40°C for 4 min, gradual increase to 80°C, with a temperature conversion rate of 0.1°C/s. Fluorescence data were collected continuously. For experimental quality control, repeated genotype analysis was conducted for 10% of the randomly selected samples with the template

replaced by double-distilled water as a negative control for each batch of PCR reaction.

Coronary Angiography and Integral Quantification of Coronary Stenosis Grading

All patients with CHD underwent selective coronary angiography using the Judkins technique. Two experienced cardiologists were responsible for reviewing the angiographic results, and coronary scores were estimated using the Gensini's method[15] based on the percentage of the narrowest stenosis diameter within the diameter of normal vessels.

Statistical Analysis

Data analyses were implemented using the SPSS13.0 statistical software (SPSS Inc., Chicago, IL, USA), with quantitative data represented as mean ± standard deviation. For averages, a pairwise comparison was conducted by *t*-test. Frequencies of the genotype and alleles were estimated using the gene counting method. The conformity of study subjects to the Hardy-Weinberg balances as well as intergroup comparisons between genotype and allele groups were analyzed by chi-square test. The correlation between the genotype and CHD occurrence was analyzed by logistic regression, and the odds' ratio (OR) and 95% CI were used to report the degree of correlation. $p < 0.05$ was statistically significant for any difference.

Results

Comparison of the General Clinical Data Between the CHD Group and the Control Group

There were no statistical differences in age, gender, BMI, TC, LDL-C between the two groups ($p > 0.05$). The percentage of patients with a smoking history was higher in the CHD group than the control group ($p < 0.05$). The profiles of FPG and TG were higher in the CHD group than the control group, but, the HDL-C profile were lower in the CHD group than the control group ($p < 0.001$) (Table I).

Discrimination of Gene Polymorphism for the 838G/C of MT2A Gene

The difference between the gene sequences could be determined by the melting temperature (Tm) changes of the sensitive probe[14]. Based on the different TM profiles, three different genotypes were proposed for this locus: Type GG, type CC and type GC. Among these genotypes, a higher singular melting trough was observed at about 57.63°C. However, there was a nucleotide mismatch between genotype CC and the probe, and the TM value was drifted to 49.96°C, with the appearance of a singular melting trough. But for genotype GC, the double melting troughs were observed with the TM values of 57.63°C and 49.96°C respectively (Figure 1).

Genotype and Allele Frequency Distribution of MT2A Gene 838G/C Polymorphism

In this study, the genotype distribution of the CHD group and the control group were analyzed by balancing test. This suggested that both groups met the Hardy-Weinberg equilibrium criteria ($p > 0.05$) and were representative of their corresponding populations. The allele frequencies of 838G/C polymorphism were 46.0%, 45.3% and 8.7% for allele-GG, GC and CC respectively in the CHD group, corresponding to 57.1%, 36.7% and 6.2% in the control group. There were statistical differences in the allele frequency distribution between these two groups (p

Table I. Comparison of general clinical data between the CHD group and the Control group.

Item	CHD group (n=287)	Control group (n=226)	*p* value
Age (years)	62.05 ± 9.45	61.69 ± 8.20	0.647
Gender (male/female)	234/53	170/56	0.083
Current smoking (%)	49.5	40.3	0.037
BMI (kg/m^2)	23.71 ± 1.79	23.45 ± 1.98	0.120
FPG (mmol/L)	6.61 ± 2.41	5.63 ± 0.64	0.000
TC (mmol/L)	4.87 ± 1.60	4.94 ± 1.10	0.616
TG (mmol/L)	2.18 ± 1.34	1.75 ± 1.04	0.000
LDL-C (mmol/L)	2.44 ± 0.95	2.54 ± 0.80	0.251
HDL-C (mmol/L)	1.13 ± 0.27	1.35 ± 0.47	0.000

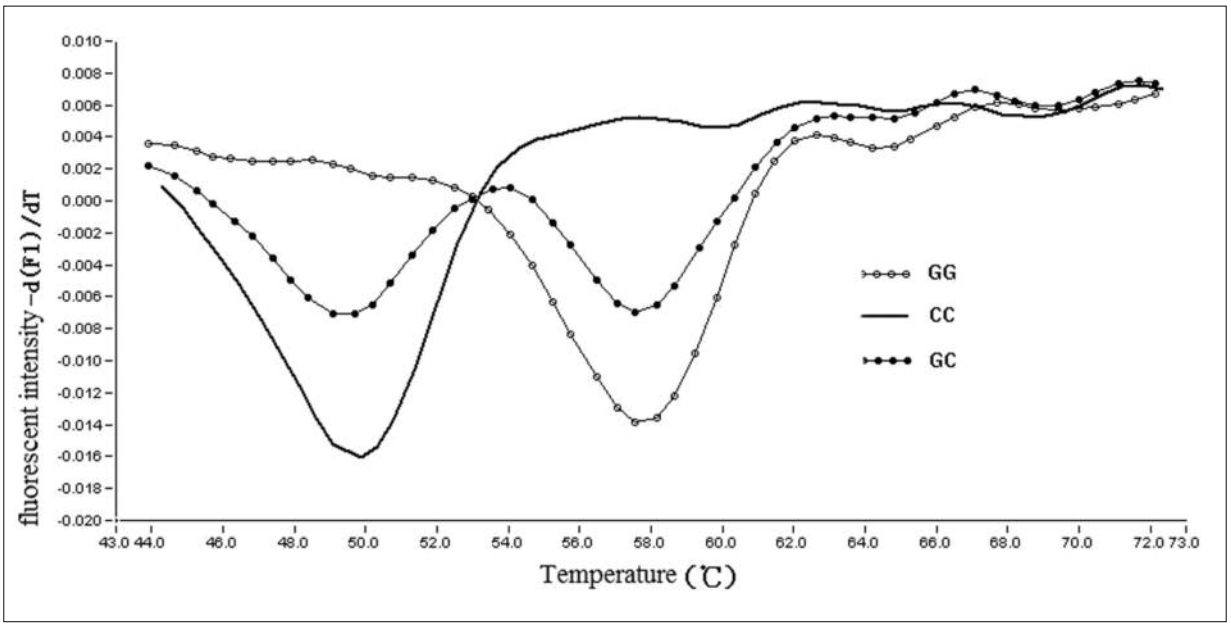

Figure 1. Melting curve profiles for the polymorphism loci/C of MT gene. Notes: The profile of fluorescence intensity versus the first order of negative reciprocal temperature, denoting melting troughs of distinct TM values.

< 0.05). As the relative risk of genotype frequency demonstrated, the CHD risk of C allele carriers (genotype GC + CC) were 1.562 folds higher than GG allele carriers (OR = 1.562, 95% CI: 1.099-2.218, p = 0.013). There were statistically significant differences in the allele frequency distribution between both groups (p < 0.05), with the frequency of C allele higher in the CHD group compared with the control group (31.4% vs 24.6%, p = 0.016) (Table II).

Logistic Regression Analysis of Risk Factors for CHD

With CHD as the dependent variable and the C allele (genotype GC+CC), gender, age, smoking history, BMI, FPG, plasma TC, TG, LDL-C and HDL-C as the independent variables, multivariate logistic regression analysis was implemented. According to the results, C allele was an independent variable for CHD (OR = 1.507, 95% CI: 1.022-2.223, p = 0.038). Moreover, FPG, TG, HDL-C were also identified to be the risk factors for CHD (Table III).

Analysis of Correlation Between the Genotype Versus Clinical Parameters or the Extent of Coronary Artery Disease for the Group of CHD

According to the results, subjects carrying C allele (genotype GC + CC) and subjects not car-

Table II. Frequencies of the 838G/C polymorphism genotypes and alleles in the CHD group and the control group.

Gene	CHD group (n = 287) (n, %)	Control group (n = 226) (n, %)	OR	95% CI	*p* value
Genotype*					
GG	132 (46.0)	129 (57.1)	1.000		
GC	130 (45.3)	83 (36.7)	1.531	1.060-2.209	0.023
CC	25 (8.7)	14 (6.2)	1.745	0.869-3.507	0.115
GC+CC	155 (54.0)	97 (42.9)	1.562	1.099-2.218	0.013
Allele#					
G	394 (68.6)	341 (75.4)	1.000		
C	180 (31.4)	111 (24.6)	1.403	1.063-1.852	0.016

Note: *χ^2 = 6.344; p = 0.042; #χ^2 = 5.757; p = 0.016.

Table III. Logistic regression analysis on the risk factors of coronary heart disease.

Variables	Partial regression coefficient	Standard errors	*p* value	OR	95% CI	
Carrying c allele	0.410	0.198	0.038	1.507	1.022	2.223
Sex	0.147	0.246	0.550	0.863	0.533	1.398
Age	0.013	0.011	0.268	1.013	0.990	1.036
TC	-0.254	0.138	0.066	0.776	0.591	1.017
TG	0.335	0.118	0.005	1.398	1.109	1.763
LDL-C	0.159	0.165	0.333	1.173	0.849	1.620
HDL-C	-1.537	0.351	0.000	0.215	0.108	0.428
Fasting blood sugar	0.407	0.085	0.000	1.502	1.271	1.776
Smoking	0.276	0.199	0.166	1.317	0.892	1.945
Body mass index	0.064	0.054	0.230	1.066	0.960	1.185

rying C allele (genotype GG), showed no statistical differences in age, BMI, hypertension history, FPG, TC, TG, LDL-C and HDL-C ($p > 0.05$), and with the Gensini scores of patients carrying C allele higher than those not carrying C allele ($p < 0.05$).

Discussion

The pathogenesis of CHD is very complicated, to which multiple genetic and environmental factors contribute. CHD genesis is related to oxidative stress, which might be induced by CHD risk factors such as hypertension, dyslipidemia, diabetes, high hyperhomocysteinemia and obesity[1,2]. Many signal transduction pathways are very sensitive to oxidative stress and play important roles in the pathogenesis of atherosclerosis. Oxidative stress induced production of Ox-LDL in large amounts, is the core step in the early stages of atherosclerotic plaque formation. The formation of atherosclerosis could be blocked or delayed through antioxidant induced reduction of oxidative stress.

As one of the vital endogenous antioxidants, MT could suppress free-radial induced oxidative damages to tissues and cells. The biological effects of MT are related to its physiochemical properties. It has nucleophilic thiol groups susceptible to interactions with electrophilic species, free radicals, and has potent antioxidant activity. As well, MT could regulate zinc release under oxidation conditions[16]. During the pathological process of LDL oxidization, zinc release could help to suppress oxidative modification, prevent lipid peroxidation products enter into macrophages, and is associated with suppression of lipid peroxidation[17], antioxidant activities[18,19], activity amplification of antioxidant enzymes, and reduction of oxidant damages to cells[20]. Zinc also plays an important role in the protection of endothelial cell integrity and the prevention of inflammatory response[21]. According to the molecular conformation of MT, the reductive thiol groups contained in an MT molecule could bind to seven zinc ions[22]. If there were insufficient intracellular zinc storage, MT could release the bonded zinc ions[23]. Interaction with MT is of great im-

Table IV. Comparison of the clinical parameters and the severity of coronary artery lesions between different genotypes in the CHD group.

Items	GG (n=132)	GC + CC (n=155)	*p* value
Age (years)	60.98 ± 11.10	62.95 ± 7.70	0.087
Body mass index (kg/m^2)	23.51 ± 1.66	23.88 ± 1.89	0.084
History of hypertension (%)	60.6	67.7	0.208
Fasting blood sugar (mmol/L)	6.56 ± 2.38	6.65 ± 2.45	0.769
TC (mmol/L)	4.91 ± 1.40	4.84 ± 1.75	0.702
TG (mmol/L)	2.27 ± 1.54	2.10 ± 1.15	0.299
LDL-C (mmol/L)	2.50 ± 0.94	2.40 ± 0.96	0.365
HDL-C (mmol/L)	1.15 ± 0.26	1.12 ± 0.28	0.408
Coronary Gensini score	34.98 ± 20.00	41.17 ± 22.35	0.015

portance to maintain normal physiological functions. MT dysfunction might be associated with blockage of zinc release and the reduction of intracellular zinc concentration[24], resulting in an increase of oxidative damage risk, genesis and development of atherosclerosis.

Single nucleotide polymorphism of MT might be associated with functional changes. This phenomenon might also have an effect on zinc usage. As demonstrated in the correlation study between the 838C/G polymorphism of MT2A gene and carotid artery stenosis[12], significant decreases of zinc concentration in erythrocyte ($p < 0.01$) and of zinc use ($p < 0.001$) were observed in GG allele carriers compared with those in GC and CC allele carriers. For the 647A/C locus of MT1A gene, in T2DM (Type II diabetes) patients concomitant with CHD, C allele carriers (genotype CC + AC) showed a decrease of zinc release from MT ($p = 0.003$). MT2A is one of the major subgroups of the MT family, and there is still no definite conclusion on the correlation between the 838G/C polymorphism of MT2A gene and CHD.

A case-control study was implemented to analyze the correlation between MT2A 838G/C polymorphism and CHD in Han habitants of Jiangsu, China. The single nucleotide polymorphism of the target gene was examined by combining the ShineRoar probe technique and melting curve method. In the study population, for the genotype distribution of MT2A gene 838G/C polymorphism, the highest frequency was observed for genotype GG, followed by genotype GC and CC. To make sure the disease relevance of the population, an analysis using the Hardy-Weinberg balance method was conducted. These subjects were found to be representative of their corresponding populations.

In this study, the distribution differences in the genotypes of MT2A gene 838G/C polymorphism locus and allele frequencies between the CHD and the control group were statistically significant ($p < 0.05$). The frequencies of GC+CC genotype and C allele were higher in the CHD group than in the control group, and the CHD risk of C allele carriers (genotype GC + CC) was 1,562 folds higher than GG allele carriers. Moreover, according to logistic regression analysis, C allele was an independent variable for CHD. As results suggest, MT2A gene 838G/C polymorphism is correlated to CHD in Han habitants of Jiangsu, China. C allele might be a CHD-susceptible gene; and the presence of G/C exchange in this locus might interfere with normal MT2A role and intracellular zinc concentration, and then contribute to atherosclerosis.

MT could change lipid metabolism through prevention of lipid peroxidation[26]. Thus, MT gene polymorphism might also have an effect on lipid profile[13]. Thus, as for CHD group, there were no statistically significant differences in TC, TG, LDL-C and HDL-C profiles between C allele and non-C allele carriers ($p > 0.05$). The correlation between the gene polymorphism of this locus and CHD might not be dependent on its effects on blood lipid profiles. During the investigation on the correlation between gene polymorphism and coronary artery disease, the severity of coronary artery disease was evaluated based on Gensini scoring system. In this study, the coronary scores of C allele carriers were higher than those of non-C allele carriers ($p < 0.05$), which suggested the gene polymorphism of this locus was related not only to the genesis of CHD, but also to the severity of the coronary artery disease.

Giacconi et al[12] proposed 838G allele was a susceptible gene for carotid stenosis, suggesting the correlation between G allele and atherosclerosis. However, this finding was inconsistent with our results. Theoretically, atherosclerosis is concomitant with systemic arterial system. The following points were considered: (1) the occurrence of both diseases is not completely in parallel. (2) In studies of carotid stenosis, including patients with ischemic attack and stroke, the existence of special MT3 subtypes in the nervous system and interactions among different subtypes might be possible[27]. (3) The differences in the study results might be related to the sample size, age of subjects, gene mutations, particular environments and ethnicity.

Conclusions

As the results of this study show, the 838G/C polymorphism is correlated to CHD in the Han population of Jiangsu, China. The distribution of polymorphism in this group has provided the rationale and data for the correlation between MT2A versus the development of CHD. The roles of MT2A and the genetic variation in the molecular mechanism of the pathogenesis need to be further elucidated. Moreover, the occurrence of CHD is up to the interactions of genetic

and environmental factors. So the correlation between the 838G/C polymorphism of MT2A gene and CHD need to be validated through more comprehensive and extensive investigations.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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