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Flotillin-2 Gene Is Associated with Coronary Artery Disease in Chinese Han Population

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Background: Flotillin-2, an important protein of vesicular endocytosis, is commonly used as a marker protein for lipid microdomains. It plays an essential role in cellular cholesterol uptake and biliary cholesterol reabsorption. Excessive cholesterol intake could cause dyslipidemia, which is a major risk factor of coronary artery disease (CAD). *Aims:* To investigate the association between the human *flotillin-2* gene polymorphism and CAD in the Chinese Han population. *Materials and Methods:* Three single-nucleotide polymorphisms (SNPs; rs10205, rs3816848 and rs8081659) of the *flotillin-2* gene were genotyped by real-time polymerase chain reaction in 307 CAD patients and 441 control subjects. *Results:* The genotypic distribution of these three SNPs was significantly different between CAD patients and control subjects (all $p < 0.05$). There were significant differences in the plasma levels of total cholesterol (TC) among different genotypes in the CAD group and control group. For rs3816848, CAD patients with the GG genotype had a higher level of TC than those with an AG or AA genotype ($p < 0.001$). For rs8081659, CAD patients with TT genotype had a higher level of TC than those with a CT or CC genotype ($p < 0.001$). Multiple logistic regression analysis showed that the GG genotype of rs3816848 was an independent risk factor for CAD (odds ratio [OR] = 1.786; 95% CI = 1.099–2.902; $p = 0.019$). *Conclusion:* There was a strong association between polymorphisms of *flotillin-2* gene and CAD in the Chinese Han population. Persons with the GG genotype of rs3816848 may have a higher risk of CAD. Moreover, the plasma levels of TC were significantly different among the different genotypes of the rs3816848 and rs8081659 SNPs in the CAD group as well as the control group.

Introduction

Flotillin-2, a major protein on lipid rafts, is a highly conserved 47-kDa protein, which was initially identified as a protein upregulated during axon regeneration after optic nerve lesion (Schulte *et al.*, 1997; Lang *et al.*, 1998). It localizes in the specific cholesterol-rich microdomains in cellular membranes, which is associated with the cytosolic side of the plasma membrane and presents intracellularly in different vesicular compartments such as endosomes, lysosomes, and phagolysosomes (Solomon *et al.*, 2002; de Gassart *et al.*, 2003; Langui *et al.*, 2004; Langhorst *et al.*, 2005; Banning *et al.*, 2011). Endosome is a compartment of the endocytic membrane transport pathway from the plasma membrane to the lysosome, and the exosome is a small membrane vesicle secreted by cells upon fusion of multivesicular endosomes with the cell surface. Previous studies showed that the endosome and exosome could be the potential source of biomarkers for atherogenesis, and atherothrombosis (VanWijk *et al.,* 2003; Shantsila *et al.*, 2010). Phuyal *et al.* (2014) found that flotillin-2 could affect the composition and release of exosome in PC-3 cells. Flotillin-2 also has been confirmed to be implicated in several cellular processes, such as cellular migration and adhesion, signaling by receptor tyrosine kinases, and mitogen-activated protein kinases (MAPK), as well as membrane trafficking (Banning *et al.*, 2014a; Browman *et al.*, 2007). Ge *et al.* (2011) firstly reported that flotillin-2 was essential for cellular cholesterol uptake, biliary cholesterol reabsorption, and regulation of lipid homeostasis in mice and cultured cells. This finding cast new light on the functional exploration of flotillin-2. In humans, more than 50% of cholesterol absorption is through intestines and excessive cholesterol intake could lead to dyslipidemia, which is a major risk factor of coronary artery disease (CAD) (Wilson *et al.*, 1998; Kreisberg and Oberman, 2002; Ingelsson *et al.*, 2007; Davis and Altmann, 2009; Lloyd-Jones *et al.*, 2010; Roger *et al.*, 2011). Thus, we assume that there may be a certain relationship between flotillin-2 and CAD.

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CAD is the leading cause of death both in the developed and developing countries and is thought to be a complex multifactorial and polygenic disorder resulting from interactions between the genetic makeup and environment (Nelson, 2013). Epidemiological and family studies have repeatedly indicated that genetic predisposition accounts for 40–60% of the risk for CAD (Le, 2006). Prevention and treatment of CAD would be expected to include modification of effects, which are partly genetic and partly environmental. However, the relationship between *flotillin-2* gene and CAD is still unclear. Therefore, we used a case–control study to investigate the association between *flotillin-2* gene and CAD in the Chinese Han population.

Materials and Methods

Ethics statement

A written informed consent was obtained from each participant. The study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University and conducted according to the principles outlined in the Declaration of Helsinki.

Subjects

All CAD patients and control subjects were recruited at the First Affiliated Hospital of Xinjiang Medical University from 2010 to 2013. We used the formula of independent case–control study to calculate sample size. Briefly, we planned the study with one control per case. Earlier data indicated that the probability of exposure among controls is 0.2. If the true odds ratio (OR) for CAD patients relative to controls was 1.7, we needed to study 303 CAD patients and 303 controls to be able to reject the null hypothesis that OR is equaled to 1 with power 0.8. The Type I error probability associated with this test of this null hypothesis was 0.05. Taking the failure of genotyping into account, we enlarged the sample size properly. Finally, 307 CAD patients and 441 control subjects were randomly recruited in this study. All the participants were genetically unrelated. Participants were diagnosed according to their medical history, clinical symptoms, 12-lead electrocardiogram, and laboratory examinations and were confirmed by coronary angiography. The procedures of coronary angiography were undertaken by highly skilled physicians using the Judkins approach. The CAD patients were defined as the presence of at least 50% stenosis in one or more coronary arteries according to current standard guidelines (ACC/AHA guidelines). The control subjects were defined as the absence of stenosis in the coronary artery. Those participants with kidney disease, malignancy, cancer, autoimmune disease, or connective tissue disease were excluded.

DNA extraction and laboratory examination

Blood samples were collected from all participants after fasting for 12 h. DNA was extracted from peripheral vein blood leukocytes using a Whole Blood Genome Extraction Kit (Boiteke Corporation, Beijing, China). The concentrations of blood urea nitrogen (BUN), uric acid (UA), blood glucose, glycated hemoglobin (GH), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using standard methods in the Central Laboratory of First Affiliated Hospital of Xinjiang Medical University.

Single-nucleotide polymorphism selection

The human *flotillin-2* gene is located on chromosome 17q11-q12 and consists of 11 exons separated by 10 introns. This gene consists of $\sim$18.35 kbp. In this study, we screened the data on the International HapMap Project website (http://hapmap.ncbi.nlm.nih. gov/index.html.en) for the TagSNPs of *flotillin-2* gene. We used the minor allele frequency (MAF) $\geq$0.1 and linkage disequilibrium patterns with $r^2 \geq 0.5$ as a cutoff by the Haploview 4.2 software. Then, we achieved 11 tag single-nucleotide polymorphisms (SNPs) of the human *flotillin-2* gene listed in the HapMap phrase II database. SNPs with relatively high MAF have been shown to be useful as genetic markers in genetic association studies (Naganuma *et al.*, 2009). Based on the screening for markers to be used in our genetic association research in Chinese Han subjects, we selected three SNPs (rs10205, rs3816848, and rs8081659) that had a MAF of >0.1. rs10205 located in the 3′ untranslated region (UTR) of the *flotillin-2* gene, whereas rs3816848 and rs8081659 located in the intron. The two SNPs located within the intron would not change the amino acid of *flottin-2* gene.

Genotyping

We used TaqMan® SNP genotyping assays (Applied Biosystems, Foster City, CA). The primers and probes used in the TaqMan SNP Genotyping Assays were chosen based on information available at the ABI website. Briefly, rs10205 was genotyped by assay ID: C_8716662_10. Rs8081659 was genotyped by assay ID: C_191732_20. Rs3816848 was genotyped by assay ID: C_25649124_10. The polymerase chain reaction (PCR) amplification was performed using 2.5 μL of TaqMan Universal Master Mix (40×), 0.15 μL probes, and 1.85 μL ddH_2O in a 6 μL final reaction volume containing 1.5 μL DNA. The PCR amplification was performed using the 7900HT sequence detection system, and thermal cycling conditions were as follows: 95°C for 5 min; 40 cycles of 95°C for 15 s; and 60°C for 1 min. All 96-well plates were read on Sequence Detection Systems (SDS) automation controller software v2.3 (ABI).

Statistical analyses

The continuous variables (e.g., age, body mass index [BMI], pulse rates, BUN, creatinine, cholesterol levels) were expressed as mean ± standard deviation. The difference of variables between CAD patients and control subjects was analyzed by the independent sample *t*-test. Differences in distributions of genotypes and alleles between CAD patients and control subjects were analyzed by the χ^2 test. Multivariate logistic regression analysis was performed to assess the contribution of the major risk factors for CAD. Statistical analyses were performed using SPSS, version 17.0 (SPSS Institute, Chicago, IL). Statistical significance was established at $p < 0.05$.

Results

Characteristics of participants

A total of 748 individuals (307 patients with CAD and 441 healthy controls) participated in this study. The clinical characteristics of the individuals are shown in Table 1. There was no difference in age between patients with CAD and healthy

Table 1. Characteristics of Study Participants

	CAD patients	*Control subjects*	p-*Value*
Age (year)	60.21 (10.22)	59.13 (8.93)	0.125
Man (n, %)	180 (58.6)	237 (53.7)	0.185
Smoking (n, %)	6 (2.0)	13 (2.9)	0.396
Drinking (n, %)	14 (4.6)	13 (2.9)	0.245
BMI ($kg \cdot m^{-2}$)	25.55 (3.07)	26.00 (3.41)	0.065
SBP (mmHg)	125.03 (16.47)	127.56 (16.27)	0.04*
DBP (mmHg)	77.77 (11.26)	78.93 (10.36)	0.152
BUN	5.41 (2.11)	5.23 (1.93)	0.222
Creatinine	77.56 (11.04)	74.69 (10.46)	0.6
UA	332.00 (2.89)	322.81 (6.17)	0.13
Blood glucose	6.27 (2.66)	5.54 (1.51)	<0.001*
GH	2.37 (0.69)	2.23 (0.44)	0.001*
TG ($mmol \cdot L^{-1}$)	2.52 (3.69)	1.90 (1.82)	0.003*
TC ($mmol \cdot L^{-1}$)	4.38 (1.18)	4.12 (0.817)	<0.001*
LDL-C ($mmol \cdot L^{-1}$)	2.54 (0.98)	2.44 (0.89)	0.175
HDL-C ($mmol \cdot L^{-1}$)	1.19 (0.40)	1.16 (0.29)	0.303

Continuous variables are expressed as mean ± SD.
Categorical variables are expressed as percentages.
The *p*-value of the continuous variables and categorical variables were calculated by the independent-sample *t*-test and the χ^2 test, respectively. *$p < 0.05$.
BMI, body mass index; BUN, blood urea nitrogen; CAD, coronary artery disease; DBP, diastolic blood pressure; GH, glycated hemoglobin; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; UA, uric acid.

controls, suggesting that the study was an age-matched case–control study. There were significantly higher concentrations of blood glucose, GH, TC, and TG in patients with CAD than those in healthy controls. However, the systolic blood pressure (SBP) value was significantly lower in the CAD group than that in the control group. No significant differences in sex, smoking, drinking, BMI, DBP, BUN, creatinine, UA, LDL-C, and HDL-C were observed between the two groups.

Allele and genotype distribution of flotillin-2 *gene*

Table 2 shows the distribution of genotypes and alleles for *flotillin-2* gene. There were significant differences in the genotypic and allelic distribution of *flotillin-2* gene between the CAD patient group and healthy control group. For rs10205 and rs8081659, compared to the healthy control group, the prevalence of the mutation genotype (TT) and mutation allele (T) in patients with CAD was significantly increased. For rs3816848, the prevalence of the wild-type genotype (GG) and wild-type allele (G) was significantly higher in patients with CAD than that in the healthy control group.

Plasma concentrations among different genotypes of flotillin-2 *gene*

We analyzed the plasma concentrations (blood glucose, GH, TC, and TG) among different genotypes of *flotillin-2* gene. For rs3816848 and rs8081659, there were significant differences in the plasma levels of TC among different genotypes in the CAD group and total group (Table 3). For rs3816848, CAD patients with GG genotype had a higher level of TC than those with AG or AA genotype ($p < 0.001$). For rs8081659, CAD patients with TT genotype had a higher level of TC than those with CT or CC genotype ($p < 0.001$).

Table 2. Distribution of Genotypes and Alleles in Patients with CAD and Control Subjects

SNP	*Genotype/ allele*	*CAD*	*Control subjects*	*Chi-square*	p-*Value*
rs10205	CC	169 (55.0)	272 (61.7)		
	CT	111 (36.2)	150 (34.0)		
	TT	27 (8.8)	19 (4.3)	7.511	0.023*
	C	449 (73.1)	694 (78.7)		
	T	165 (26.9)	188 (21.3)	6.202	0.013*
rs3816848	GG	79 (25.7)	57 (12.9)		
	AG	111 (36.2)	196 (44.4)		
	AA	117 (38.1)	188 (42.6)	20.266	<0.001*
	G	269 (43.8)	300 (34.4)		
	A	345 (56.2)	572 (65.6)	13.495	<0.001*
rs8081659	CC	141 (45.9)	242 (54.9)		
	CT	111 (36.2)	156 (35.4)		
	TT	55 (17.9)	43 (9.8)	12.07	0.002*
	C	393 (64.0)	640 (72.6)		
	T	221 (36.0)	242 (27.4)	12.4	<0.001*

SNP, single-nucleotide polymorphisms.
*$p < 0.05$.

However, there was no difference in the levels of blood glucose, GH, and TG among various genotypes in each group (the results were not listed).

Logistic regression of CAD risk factors

Multifactor logistic regression analysis revealed three independent risk factors for CAD: SBP, blood glucose, and GG genotype of rs3816848. After adjustments of smoking, TC, TG, LDL-C, and HDL-C, those with the GG genotype in rs3816848, had a significantly higher risk of CAD (OR = 1.786; 95% CI = 1.099–2.902; $p = 0.019$) (Table 4). We also analyzed the interaction of *flotillin-2* gene and nongenetic factors in the risk of CAD. To our disappointment, the result showed that interaction between *flotillin-2* gene and nongenetic factors was irrelevant to the pathogenesis of CAD.

Table 3. Plasma Concentration of TC Among Different Genotypes of *Flotillin-2* Gene

Genotype	*Total*	*CAD*	*Control subjects*
rs10205			
CC	4.19 (0.905)	4.34 (1.044)	4.09 (0.793)
CT	4.29 (1.129)	4.43 (1.412)	4.19 (0.851)
TT	4.18 (0.9810	4.42 (1.007)	3.83 (0.857)
p-value	0.375	0.814	0.149
rs3816848			
GG	4.48 (1.280)	4.84 (1.384)	3.97 (0.909)
AG	4.19 (1.001)	4.28 (1.251)	4.14 (0.825)
AA	4.14 (0.807)	4.16 (0.852)	4.13 (0.780)
p-value	0.004*	<0.001*	0.361
rs8081659			
CC	4.17 (0.986)	4.29 (1.206)	4.11 (0.826)
CT	4.15 (0.810)	4.22 (0.812)	4.11 (0.807)
TT	4.61 (1.337)	4.91 (1.573)	4.22 (0.819)
p-value	<0.001*	0.001*	0.699

*$p < 0.05$.

Table 4. Multiple Logistic Regression Analysis for CAD Patients and Control Subjects of Han Population

Variables	*B*	*Wald*	P-*value*	*OR*	*95% CI for OR*	
					Lower	*Upper*
Age	0.018	3.847	0.050	1.018	1.000	1.036
Sex	–0.226	1.770	0.183	0.798	0.572	1.113
SBP	–0.011	4.464	0.035*	0.989	0.979	0.999
Blood glucose	0.117	5.553	0.018*	1.124	1.020	1.240
GH	0.354	3.712	0.054	1.424	0.994	2.041
TG	0.107	2.166	0.141	1.113	0.965	1.283
TC	0.148	2.197	0.138	1.160	0.953	1.412
SNP1 (CC+CT vs. TT)	0.508	1.937	0.164	1.662	0.813	3.399
SNP2 (AA+AG vs. GG)	0.580	5.480	0.019*	1.786	1.099	2.902
SNP3 (CC+CT vs. TT)	0.233	0.785	0.376	1.262	0.754	2.112

SNP1: rs10205; SNP2: rs3816848; SNP3: rs8081659.
OD, odds ratio.
*$P < 0.05$.

Discussion

Our data showed that there were significant differences in the genotypic and allelic distribution of *flotillin-2* gene between patients with CAD and control subjects in the Chinese Han population. Significant differences were observed in the level of TC among different genotypes, and the CAD patients with GG genotype of rs3816848 had a higher level of TC than those with AG or AA genotype. Similar results were seen in the polymorphism of rs8081659. Eliminating the influences of confounding factors, we found that the subjects with the GG genotype of rs3816848 in *flotillin-2* gene were at a moderately increased risk of CAD, which may be one of the genetic risk factors of CAD in the Chinese Han population. Thus, the present study may provide some new insights into the genetic mechanisms of CAD.

Flotillin-2 is a highly conserved protein that localizes into specific cholesterol-rich microdomains in cellular membranes. It is expressed ubiquitously, and its expression is particularly high in the brain, heart, lung, and placenta, but fairly low in the pancreas and liver (Edgar and Polak, 2001). The function of flotillin-2 has been a research hot spot. Researches have reported that flotillin-2 might be involved in the pathogenesis of a variety of diseases such as tumors and inflammatory diseases. Previous studies reported that flotillin-2 seemed to play an essential role in T-cell activation by controlling the assembly of signaling complexes and cytoskeletal rearrangements necessary for prolonged signaling to occur after T-cell receptor stimulation (Stuermer *et al.*, 2001; Solomon *et al.*, 2002). Overexpression of flotillin-2 resulted in the induction of numerous filopodia-like protrusions in various cell lines, suggesting a role of flotillin-2 as a signaling protein in actin-dependent processes (Carolin *et al.*, 2004). There were close relations between flotillin-2 and the uptake of cholesterol in the process of endocytosis in intestinal cholesterol absorption. However, the dysfunction of flotillin-2 could cause excessive cholesterol intake, which was one of the major risk factors leading to hypercholesterolemia and cardiovascular diseases, especially atherosclerosis (Babuke *et al.*, 2009; Ge *et al.*, 2011). The hypocholesterolemic drug ezetimibe disrupts the association between Niemann-Pick C1-like 1 and flotillin-2, which blocks the formation of the cholesterol-enriched microdomains (Ge *et al.*, 2011). Study of the *flotillin-2* gene knockout mice models showed that the regulators of transcription of growth-associated genes were upregulated in these mice. Those genes include transcription factors such as ATF3, ATF4, JunB, Fos, and Egr1, which are positive regulators of cell proliferation and survival (Banning *et al.*, 2014b). Meanwhile, flotillin-2 might be involved in the pathogenesis of inflammatory bowel disease (IBD). The levels of *flotillin-2* mRNA and protein were reduced in IBD patients compared to the control subjects (Gauss *et al.*, 2013). Hazarika *et al.* (2004) reported that upregulation of flotillin-2 was associated with melanoma progression and modulated expression of the thrombin receptor protease-activated receptor 1. In addition, the expression of flotillin-2 was increased in the patients with gastric cancer and breast cancer (Pust *et al.*, 2013; Zhu *et al.*, 2013). All in all, flotillin-2 has a close relationship with the development and progression of numerous diseases. However, the pathogenesis between flotillin-2 and CAD is still poorly understood. To our knowledge, this is the first study to investigate the common allelic variant in *flotillin-2* gene and its association with CAD in the Chinese Han population. It also demonstrated that the risk of CAD was increased in person with GG genotype of rs3816848 in the Chinese Han population. In the future, more attention should be poured into understanding how genetic variation at this gene affects cholesterol absorption, CAD pathogenesis, and response to lipid-lowering treatments.

In summary, the data presented in our study showed a strong association between the *flotillin-2* gene and CAD in the Chinese Han population. People with GG genotype of rs3816848 may have a higher risk of CAD. Moreover, the plasma levels of TC were significantly different among the different genotypes of rs3816848 and rs8081659 in the CAD group and total group.

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Author Contributions

Conceived and designed the experiments, Y.-T.M., Y.-N.Y.; performed the experiments, Z.-X.Y., B.-D.C.;

analyzed the data, A.M., Y.Z.; contributed reagents/materials/analysis tools, F.L.; and wrote the article, J.-Y.L., Z.-Y.F.

Author Disclosure Statement

No competing financial interests exist.

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