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Association between rs1049673 polymorphism in CD36 and premature coronary heart disease

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ABSTRACT. Risk factors for premature coronary heart disease in China can be multiple; we investigated Chinese Han patients with premature coronary heart disease and a possible association with CD36 polymorphism at rs1049673, rs7755, and rs321159 sites. Outpatients were recruited according to chest X-ray coronary arteriography results; they were divided into two groups: early coronary artery lesions (premature coronary heart disease group, test group) and a control group. Coronary arteriography and laboratory blood examinations were conducted to analyze risk factors for coronary heart disease and CD36 polymorphisms. Seventy nine test and 56 control group patients were recruited. Compared with the control, the test groups had a significantly higher proportion of male patients, smoking, diabetes and metabolic syndromes, significantly higher levels of TG, LDL-C, ox-LDL, WBC, UA, FBG, and significantly lower levels of HDL-C. For rs1049673, rs7755, and rs321159 sites, patients with premature coronary heart disease have family genetic predisposition at high LDL-C level with GA, AA, and TT genotypes. Unconditional logistic regression analysis showed that gender, diabetes, high TG, LDL-C level and C carriers

of rs1049673 significantly affected risk for premature coronary heart disease.

Key word: Coronary heart disease; Risk factors; CD36; rs1049673; rs7755; rs321159

INTRODUCTION

Coronary heart disease (CHD) is an epidemic characterized by premature onset and high mortality. The World Health Organization (WHO) reports that more than 70% of coronary deaths occur in subjects older than 70 in North America and Western Europe and that the mortality of the young has increased in recent years (Ford and Capewell 2007; O'Flaherty et al., 2008; Bertuccio et al., 2011). Risk factors of premature CHD could be multiple, ranging from social, economic, psychological, lifestyle (such as smoking, sedentary lifestyle, improper diet), and biological (abnormal lipids, hypertension, diabetes, and obesity), and genetic factors such as mutations at specific chromosomal locations and single nucleotide polymorphisms (SNPs) have also been implicated.

Many risk factors of coronary heart disease (CHD) have been reported (Grammer et al., 2011; Rai et al., 2008; Waterworth et al., 2011), while the association between the polymorphism of scavenger receptors (SR) and CHD has been rarely reported. Recent studies showed that SR constitutes the main part of the foam cell, an important factor indicating the beginning and development of atherosclerosis (Glass and Witztum 2001; Libby, 2002). CD36, a member of the class B scavenger receptor family, is a receptor of natural lipoproteins, including low-density lipoproteins (LDL), high-density lipoproteins (HDL) and very low-density lipoproteins (VLDL). Besides, when combined with denatured lipids, it can lead to the formation of foam cells (Calvo et al., 1998; Thorne et al., 2007). A number of studies have indicated that the overexpression or polymorphism of CD36 is associated with insulin resistance, metabolic syndrome, and serum triglyceride and free fatty acid levels (Ma et al., 2004; Corpeleijn et al., 2006; Love-Gregory et al., 2008).

CD36 on the platelet surface is the bridge to lipid metabolism disorders and thrombosis (Nergiz-Unal et al., 2011). Our early studies showed that metabolic syndrome plays a pivotal role in premature CHD among Chinese patients (Che et al., 2011). In this study, we recruited Chinese Han patients with premature CHD and studied the association with CD36 polymorphism at the rs1049673, rs7755, and rs321159 sites.

MATERIAL AND METHODS

Patients

All patients were recruited from the Department of Cardiology, the Second Hospital of Tianjin Medical University between March 2009 and March 2011. These patients had symptoms of chest pain or suspected coronary artery disease, and underwent a coronary angiography after being evaluated by their attending physician.

These patients were divided into two groups according to their coronary angiography result, the criteria of the recruited patients are shown in Figure 1. The study was approved and registered by our hospital's ethics committee; all subjects signed a written informed consent form. All works were undertaken following the provisions of the Declaration of Helsinki.

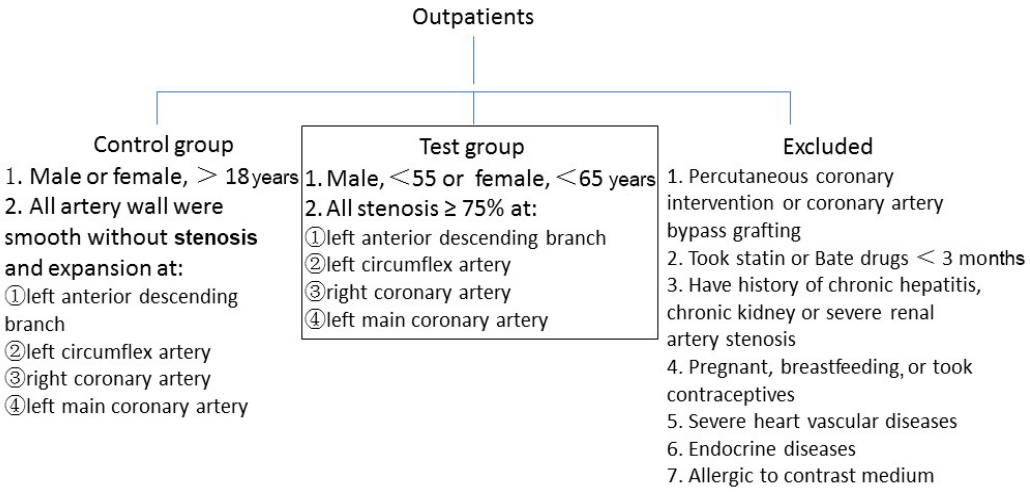

Figure 1. Patient screening process.

Coronary arteriography

Coronary angiography was performed using the standard Judkins technique (Judkins, 1967). Coronary angiograms were visually evaluated by two experienced observers according to the clinical review process. Localization and percent luminal diameter reduction were documented for the left main coronary artery branch, left anterior descending branch, left circumflex artery, and right coronary artery with stenosis. The main branches such as diagonal branch and obtuse marginal branch were classified as related main artery. Coronary angiograms were analyzed by computer-assisted quantitative coronary angiography (QCA). CHD was defined as more than 1 (≥) atherosclerotic plaque in a major coronary artery (≥1.5 mm lumen diameter) causing ≥50% luminal diameter stenosis by the QCA test.

Clinical data collection and diagnostic criteria

All participants' body mass index (BMI) was calculated. The detail record included gender, age, history of high blood pressure, diabetes mellitus (DM), smoking history, premature CHD family history. The standard diagnosis is shown in Table 1.

Laboratory examination

Peripheral venous blood samples were obtained from participants in the morning, where they were asked to fast at least 8 h. Routine blood tests were performed, including hepatorenal function, fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), LDL-C, HDL-C, VLDL-C, apoA1, apoB and plasma fibrinogen. An enzyme-linked immunosorbent assay (ELISA) kit (Calbiochem, San Diego, CA, USA) was used to determine oxidized LDL (ox-LDL) level in plasma according to manufacturer instructions.

Table 1. Primary diagnosis of premature coronary heart disease (CHD) and control group patients.

Definition	Evaluation index	Threshold value
Overweight	BMI	≥25.0 kg/m^2
Diabetes mellitus	Related symptom plus plasma glucose	≥11.1 mM
	Fasting blood-glucose	≥7.0 mM
	Blood sugar at sugar tolerance test (2 h)	≥11.1 mM
	Clear DM history	
Smoking	Smoke at least once/day	1/day
	Continuous smoking	>1 year
Premature CHD family history	Direct relative has premature CHD	Male ≤ 55
		Female ≤ 65
Drinking	Alcohol intaking	≥25 g/day
	Continuous drinking	≥100 g/week
		≥1 year
High TC level	TC	>5.18 mM
High TG level	TG	>1.70 mM
Low HDL-C level	HDL-C	<1.04 mM
High LDL-C level	LDL-C	>3.37 mM

TC = total cholesterol; TG = triglycerides; HDL-C = high-density lipoproteins cholesterol; LDL-C = low-density lipoproteins cholesterol; BMI = body mass index; DM = diabetes mellitus.

Measurement of CD36 gene polymorphism

After 10 mL blood were drawn from the cubital vein and anti-coagulated, the white blood cells were separated and immediately stored at -80°C. Extraction of genomic DNA and PCR amplification were conducted according to the instructions of the related reagent suppliers. The template contained three SNPs (rs1049673, rs7755, rs321159) of CD36 DNA. After the extension and purification, the products were analyzed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOFMS).

Statistical method

Data were analyzed by the SPSS11.5 software. Quantitative data are expressed as means ± SE. Comparison between two groups with normal distribution was done by an independent sample *t* test, and with non-normal distribution by the Mann-Whitney U test. Risk factors were analyzed by multi-factor logistic regression analysis. $P < 0.05$ was considered to be statistically significant.

RESULTS

Seventy-nine premature CHD patients and 56 control patients were recruited in our study. The basic clinical characteristics and routine blood test results (Table 2) showed no statistical significance in premature family history, hypertension rate, BMI and TC, VLDL-C, apoprotein A1, platelets, fibrinogen, blood urea nitrogen, serum albumin, total bilirubin, and conjugated bilirubin levels in the test and control groups.

The test group had a higher proportion of male patients (68 *vs* 41%), smoking (59 *vs* 39%), diabetes (39 *vs* 13%) and metabolic syndrome (apoprotein B: 0.86 ± 0.15 *vs* 0.76 ± 0.09 mM) compared with controls ($P < 0.05$). The levels of TG (2.36 ± 1.88 *vs* 1.66 ± 1.19 mM), LDL-C (3.20 ± 0.80 *vs* 2.83 ± 0.58 mM), ox-LDL (634 *vs* 561µg/L), UA (376.6 ± 116.9 *vs* 312.0 ± 100.2 mM), FBG (7.2 ± 2.8 *vs* 6.3 ± 3.1 mM) were remarkably higher in the test group ($P < 0.05$), while the level of HDL-C (1.08 ± 0.18 *vs* 1.31 ± 0.28 mM) was lower in the test group ($P < 0.05$).

Table 2. Basic clinical characters and blood routine examination comparison result in two groups.

Variant	Control (N = 56)	Test group (N = 79)	P value
Age, years	61.2 ± 10.4	53.6 ± 6.9	0.001
Male (N, %)	23 (41)	54 (68)	0.002
Premature FH (N, %)	3 (5)	6 (8)	0.608
Hypertension (N, %)	30 (54)	54 (68)	0.081
Diabetes (N, %)	7 (13)	31 (39)	0.009
Smoker (N, %)	22 (39)	47 (59)	0.021
Body mass index (kg/m^2)	24.1 ± 3.1	25.1 ± 3.1	0.073
Triglyceride (mM)	1.66 ± 1.19	2.36 ± 1.88	0.016
Total cholesterol (mM)	4.91 ± 0.80	5.10 ± 1.07	0.272
LDL cholesterol (mM)	2.83 ± 0.58	3.20 ± 0.80	0.005
HDL cholesterol (mM)	1.31 ± 0.28	1.08 ± 0.18	<0.001
VLDL cholesterol (mM)	0.78 ± 0.41	0.88 ± 0.41	0.401
Apoprotein A1 (mM)	1.24 ± 0.17	1.20 ± 0.16	0.429
Apoprotein B (mM)	0.76 ± 0.09	0.86 ± 0.15	0.008
Oxidized LDL (μg/L)	561 (440-660)	634 (512-844)	0.002*
Platelet (x 10^{12}/L)	220 ± 57	226 ± 48	0.558*
Fibrinogen (g/L)	2.9 ± 0.6	2.9 ± 0.5	0.858
Blood urea nitrogen (mM)	5.6 ± 2.0	6.6 ± 5.8	0.217
Serum creatinine (μM)	69.9 ± 20.1	80.0 ± 37.7	0.003*
Uric acid (μM)	312.0 ± 100.2	376.6 ± 116.9	0.001*
Serum albumin (g/L)	40.0 ± 3.0	39.0 ± 3.2	0.079
Total bilirubin (μM)	11.5 ± 4.0	12.5 ± 5.4	0.255
Conjugated bilirubin (μM)	2.6 ± 1.3	2.8 ± 1.6	0.402
Fasting blood glucose (mM)	6.3 ± 3.1	7.2 ± 2.8	0.042

*Result of nonparametric Mann-Whitney test, other quantitative data was compared by independent sample *t* test.

rs1049673, rs7755, and rs321159 polymorphism analysis

For rs1049673, G carriers had a higher proportion of premature CHD (60.7% in control group and 51.3% in test group) than did C carriers (39.3% in control group and 48.7% in test group, P = 0.030). For rs7755, A carriers had a higher proportion of premature CHD (60.9% in controls and 51.3% in test group) than did G carriers (39.1% in controls and 48.7% in test group, P = 0.032). For rs321159, T carriers had a higher proportion of premature CHD (66.7% in controls and 72.8% in test group) than did G carriers (33.3% in control and 27.2% in test group), but the difference was not statistically significant (P = 0.065). All these SNP results are shown in Table 3.

As we can see from Table 4 (detailed data not shown), for rs1049673, CC carriers had a higher level of LDL-C than GG carriers, which showed family genetic predisposition with statistical significance (P = 0.049), which means patients with premature CHD all had the CC or GC genotype. For rs7755, AA carriers had a higher level of LDL-C than GG carriers with statistical significance (P = 0.05). It also showed a family genetic predisposition, which means patients with premature CHD all had the CC or GC genotype. For rs321159, TT carriers had the highest level of LDL-C, while GG carriers had the lowest level (P = 0.010), and the other genotype showed no significant difference.

Independent predictors of premature CHD

To evaluate the level of the mutation of rs1049673 in the CD36 gene among CHD risk factors, binary logistic regression analysis was used. The three main coronary artery vessel lesions were considered as dependent variables, and gender, hypertension, DM, hyperuricemia, high level of TG and LDL-C, high and low level of HDL-C, positive C carriers

at rs1049673, and positive G carriers at rs7755 were considered independent variables. After unconditional logistic regression analysis, the results (Table 5) showed gender, DM, high TG symptom, high LDL-C symptom and C carriers of rs1049673 all could predic premature CHD remarkably.

Table 3. Composition of genotypes at rs1049673, rs7755, and rs3211956 sites.

Site	Genotype	Control (N = 56)	Test group (N = 79)	P value
rs1049673	GG	37.5% (21)	25.3% (20)	0.281
	GC	46.4% (26)	51.9% (41)	
	CC	16.1% (9)	22.8% (18)	
	G	60.7% (68)	51.3% (81)	0.030
	C	39.3% (44)	48.7% (77)	
rs7755	AA	38.2% (21)	25.3% (20)	0.260
	GA	45.5% (25)	51.9% (41)	
	GG	16.3% (9)	22.8% (18)	
	A	60.9% (67)	51.3% (81)	0.032
	G	39.1% (43)	48.7% (77)	
rs3211956	TT	40.7% (22)	55.7% (44)	0.127
	GT	51.9% (28)	34.2% (27)	
	GG	7.4% (4)	10.1% (8)	
	T	66.7% (72)	72.8% (115)	0.065
	G	33.3% (36)	27.2% (43)	

Table 4. Distribution and clinical character of rs1049673, rs7755, and rs3211956 polymorphism in CD36.

	N	PFH (%)	P	LDL cholesterol (mM)	P
rs1049673					
GG	41	0	0.03	2.9 ± 0.7	0.049
GC	67	11		3.0 ± 0.8	
CC	27	7		3.3 ± 0.7	
rs7755					
GG	41	0	0.03	2.89 ± 0.69	0.05
GA	66	11		3.03 ± 0.75	
AA	27	7		3.34 ± 0.73	
rs3211956					
GG	12	0	0.102	2.69 ± 0.70	0.01
GT	55	4		2.91 ± 0.62	
TT	66	11		3.23 ± 0.78	

PFH = Premature family history of coronary heart disease; LDL = low-density lipoproteins. Quantitative data is compared by ANOVA test.

Table 5. Binary gradually logistic regression analysis of independent predictor in premature coronary heart disease.

Variant	B	SE	Wald	P
Gender	1.595	0.469	11.557	0.001
rs1049673 dominant inheritance*	1.117	0.468	6.314	0.012
Diabetes	2.082	0.612	11.584	0.001
High TG level	0.996	0.440	5.130	0.024
High LDL-C level	1.201	0.519	5.357	0.021
Constant	4.607	0.903	26.034	<0.001

The following factors were excluded from logistic regression equation: positive G carriers at rs7755, hypertension, hyperuricemia, low-high density cholesterol symptom. The value of enumeration data is as follows: gender "0" represents female, "1" represents male. The condition of coronary "0" represents normal, "1" represents lesion. Diabetes, high TG symptom, high LDL-C symptom, smoking history "0" represents no, "1" represents existence. *According to the genetic model of rs1049673 (GG or GC genotype is 0, CC genotype is 1). According to the genetic model of rs7755 (AA genotype is 0, AG or GG genotype is 1).

DISCUSSION

Studies have shown that conventional cardiovascular risk factors differ markedly between premature and non-premature CAD (Genest et al., 1991; Reibis et al., 2012). Reibis et al. (2012) reported dyslipidemia, as well as family smoking history, were dominant risk factors in a young group of premature CAD. Hence, we excluded the patients receiving lipid-lowering statins or other lipid-lowering drugs and patients who received percutaneous coronary intervention and bypass surgery. The relationship between the standard LDL-C and coronary events were confirmed (LaRosa et al., 2005; Reiner et al., 2011; Robinson and Stone, 2006), whereas the relation between hypertriglyceridemia and coronary heart disease (CHD) is still controversial (Cullen, 2000). Previous experience in young post-infarction patients indicates that young patients are characterized by mixed lipid disorders, particularly those resulting from disturbances in the metabolism of triglyceride-rich lipoproteins (Tornvall et al., 1991). In our study, hypertriglyceridemia and DM were proven to be an essential risk factor of premature CAD in Chinese Han people. Especially at an age less than 65, males are more likely to have CAD compared with females (Vaidya et al., 2007; Hoffmann et al., 2008). Our study confirmed this result.

As for rs1049673 in the CD 36 gene, Vaidya et al. (2007) demonstrated that the Framingham risk equation underestimated total incident 10-year CAD events in individuals with a sibling history of premature CAD, and the excess risk observed could be attributed to genetic and other susceptibility factors. The polymorphism of CD36 has been reported to be associated with glucose metabolism disorders as well as triglyceride and free fatty acid levels. Ma et al. (2004) investigated the polymorphism of 21 allele sites in CD36 in 585 native non-diabetic Caucasian patients. As shown by the results, there was a strong relation between the polymorphism of rs1049673 as well as two other sites and free fatty acid level ($P = 0.02$). More specifically, the rs1049673 polymorphism revealed a stronger association with male patients ($P = 0.008$). G homozygous male patients had a higher free fatty acid level than males who were C homozygous, while they were similar in triglyceride and HDL levels. In addition, although for rs1049673, the level of serum LDL was higher in the CC genotype carriers than those with the GG genotype, the gap was not statistically significant (Ma et al., 2004). Noel et al. (2010) investigated 1178 Puerto Ricans living in Boston. They confirmed that SNPs of CD36 were remarkably associated with metabolic syndrome in diabetes and systolic hypertension in the high incidence group. In detection of six alleles in the site, the SNP rs1049673 showed the strongest link with metabolic syndrome, in which the possibility of metabolic syndrome among minimum allele carriers increased (odds ratio = 1.89). We found that CC carriers of rs1049673 had a higher level of serum LDL than did GG carriers in Chinese Han people, while the association between SNPs in this gene and the level of serum TG in diabetes is still unknown. More importantly, we initially proved that the C carriers of rs1049673 had a higher opportunity to have premature CHD than did G carriers. This may be due to the high level of serum LDL in C carriers, which is also the main characteristic of blood fat in premature CHD. Thus, we believe that the SNP rs1049673 in CD36 is an independent predictor of premature CHD.

As for rs7755, this CD36 SNPs was studied previously, and phenotypes were shown to be related to lipid metabolism (Corpeleijn et al., 2006; Ma et al., 2004). We initially proved that different genotypes at rs7755 site had a strong link with the level of LDL, and that A carriers had a higher proportion of premature CHD as well as a higher level of LDL than did G carriers. However, the proportion of diabetes and BMI, FBG and triglyceride levels between

different genotypes of rs7755 did not differ. This indicates that these SNPs influence mainly LDL metabolism rather than the level of blood glucose and triglycerides at the incidence of premature CHD. Heni et al. (2011) demonstrated that the CD36 SNP rs3211956 significantly is associated with BMI. Another study showed the SNP in CD36 was significantly ($P = 0.04$) more common in patients with acute MI (minor allele frequency 10.5%) than patients with stable exertional angina (minor allele frequency 8.0%) (Knowles et al., 2007). Our study confirmed that different genotypes of rs3211956 are associated with the level of LDL-C and that T carriers have a higher risk of premature CHD but with no statistical significance.

In general, the polymorphisms of rs1049673, rs7755, and rs3211956 are all associated with the level of LDL-C, but the related mechanism is still unknown. CD36 is not only an essential receptor combining the long-chain fatty acid and denatured LDL, but also participates in the reverse transport of HDL to the liver. Cholesterol is reverse transported to the liver from the surrounding tissues by HDL, which combines with related receptors on liver cell surface and is then transferred into the liver cell by endocytosis. Finally it becomes the bile acid and is expelled outside. Brundert et al. (2011) confirmed that compared with wild-type mice, knockdown CD36 gene mice absorb less [^{3}H] and [^{125}I] markers in the liver. Besides, cultured primary hepatocytes totally unable to absorb HDL *in vitro*. On the contrary, CD36 expressed in adenovirus-mediated cells show recovery of HDL uptake ability. Thus, the polymorphism of CD36 could influence the level of plasma LDL-C by the reverse transport of cholesterol. Among the SNPS studied, rs1049673 and rs7755 show little relevance to premature CHD, and rs1049673 is an independent risk factor of premature CHD. The polymorphism of rs1049673 and rs7755 in CD36 could affect other factors besides plasma LDL-C.

Meanwhile, effects of the three polymorphisms on plasma ox-LDL were involved in our study. Studies showed that normal LDL is not able to induce foam cell formation. But modified LDLs, especially the oxidized LDLs, can induce foam cell formation from macrophages. Atherosclerotic lesions are characterized by the accumulation of ox-LDL and the infiltration of macrophages and T cells (Binder et al., 2002; Hansson and Hermansson, 2011). The course of LDL oxidization mainly happens on the vessel wall, but recently ox-LDLs were reported to be detected in peripheral blood (Johnston et al., 2006). We also observed that the level of serum ox-LDL in premature CHD patients was higher than in controls. The main way to eliminate ox-LDL is phagocytosis of macrophages by SR, whose main member is CD36 (Park et al., 2012; Picard et al., 2010). So, the polymorphism of the CD36 gene could influence the level of ox-LDL. The polymorphisms of rs1049673, rs7755, and rs3211956 in CD36 significantly impact the level of LDL-C in peripheral blood, but cannot affect the level of ox-LDL. The possible reasons may be: 1) the difference between different levels of ox-LDL in patients from the same group is very large, making it hard to assess the impact of ox-LDL in different genotypes and thereby needing a larger sample size; 2) the polymorphism of CD36 may influence the content of ox-LDL in the walls of blood vessels rather than in peripheral blood.

Our data were only based on a small-scale case-control study. We hope that larger prospective studies in the future might explore the role of CD36 variants in the development of this condition.

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