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Effects of CETP gene polymorphisms on atherogenic lipoprotein phenotypes in Koreans

Hong Keun Cho[1,2], Yangsoo Jang[1,2], Eun Young Cho[2], Hyun Young Park[2], Hakbae Lee[3] and Jong Eun Lee[§]

[1]Yonsei Univ. Research Institute of Science for Aging, Yonsei University, Seoul, Korea
[2]Division of Cardiology, Cardiovascular Genome Center, Yonsei Medical Institute
[3]Department of Applied Statistics, Yonsei University

Small dense LDL (LDL III) is emerging as a major risk factor for coronary artery disease. LDL III generation is associated with high triglyceride concentration, high hepatic lipase activity, and high cholesterol ester transfer protein (CETP) levels. CETP polymorphisms have been reported to be associated with coronary artery disease. In this study, we investigated the relationship between CETP polymorphism and LDL III generation. VLDL1, VLDL2, IDL and LDL subfractions were measured in 87 normal healthy Korean subjects who had been SNP genotyped for Taq1B, I405V and A629C. We found no differences in LDL subfractions and lipoprotein composition between homozygotes for Taq1B2B2, and those for Taq1B1B1 and Taq1B1B2. There were no differences in LDL subfractions and lipoprotein composition between homozygotes for 629AA, and those for 629AC and -629CC. However, homozygotes for 405VV had a significantly lower LDL III concentration and proportion than those for 405II and 405IV. We concluded that, among the Taq1B, I405V and A629C polymorphisms, only the I405V polymorphism was associated with the concentration and proportion of LDL III.

Key words : LDL III, CETP, polymorphism, I405V, A629C, Taq1B

INTRODUCTION

In the past, people in Asia suffered lower levels of coronary heart disease (CHD) than their Western counterparts. As Asian peoples adopted Western ways, traditional agricultural society was abruptly transformed into an industrialized culture, resulting in great changes in dietary habits and behavioural patterns. These changes in dietary habits led to a shift in Asians' disease patterns. For example, Korea has seen substantial growth in the incidence of CHD during the last twenty years.

High LDL cholesterol (LDLc) is a strong risk factor for CHD. The results of large clinical trials, however, revealed that LDLc could explain only approximately 35 % of the CHD incidence. An additional risk factor, the atherogenic lipoprotein phenotype (ALP), is characterized by a normal or moderately elevated LDLc, together with increased triglyceride and decreased HDLc concentrations in the plasma[1]. Researchers have reported that about two-thirds of CHD patients had ALP characterization. High triglyceride concentrations cause the generation of small dense LDL. Low HDLc concentrations generally reflect decreased anti-atherogenic, anti-oxidant and reverse cholesterol transport capabilities of HDL particles. Interestingly, Koreans generally have lower LDLc, higher triglyceride, and lower HDLc concentrations than Western people. Recently we reported that healthy Korean men and women had lower LDLc, lower HDLc, but higher triglyceride (ALP), than their age- and BMI-matched Scottish counterparts[2]. The principal finding was that Koreans had higher small dense LDL concentrations than Scots, and we hypothesized that this was mainly due to a relatively high cholesteryl ester transfer protein (CETP) activity in Koreans. Several CETP gene polymorphisms have been reported. In the context of atherosclerosis, three polymorphisms have been extensively investigated I405V, A629C and Taq1B. This paper addresses the association between these CETP gene polymorphisms and ALP, especially small dense LDL, in the Korean population.

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§ DNA Link, Milk Building, Yonsei University, Seoul 120-110, Korea

SUBJECTS AND METHODS

Subjects

Fifty-seven women and forty-nine men of Korean descent, and currently living in Korea, were screened for this study. Subjects taking drugs affecting lipid meta-

bolism or those with diabetes mellitus (DM) were excluded, as were estrogen users and subjects with levels of triglyceride greater than 240 mg/dl. After applying these criteria, 48 women and 39 men were eligible and participated in the study. Subjects, who ranged in age from 24 to 60 years, had no history of CVD and were taking no drugs at the time of sampling. Subjects' body weights, heights, waist circumferences and blood pressure were measured. Percent body fat was measured by the leg to leg Bioelectrical impedance (BIA) system (TBF 105, Tanita Corporation of America, Inc., Arlington Heights, IL, USA). Measurements were taken when subjects were in a fasting state, without previous physical exercise; after an overnight fast, blood samples were drawn into K2EDTA at the Yonsei University Cardiovascular Research Center, Seoul. Buffy coats were separated and sent for gene analysis to the Cardiovascular Genome Center, Yonsei University, Seoul, Korea. Plasma was immediately separated and sent on ice by courier to the Pathological Biochemistry Department, Royal Infirmary, Glasgow, within 50 hours.

Lipid assays

Total cholesterol, LDL cholesterol, VLDL cholesterol and HDL cholesterol were measured by the standard Lipid Research Clinics Program. Triglyceride was analyzed using an enzymatic colorimetric test kit (Roche Diagnostic GmbH, Germany).

LDL subfraction analysis by density gradient ultracentrifugation

A discontinuous salt gradient was constructed which permitted isolation of LDL subfractions directly from plasma according to the method of Griffin et al[3]. Three LDL subfractions (LDL I: d 1.025-1.034 g/ml; LDL II: d 1.034-1.044 g/ml; and LDL III: d 1.044-1.063 g/ml) were quantified by non-equilibrium density ultracentrifugation. Total lipoprotein mass and the subfraction distribution profile were used to calculate concentrations of each individual subfraction in terms of mg lipoprotein/100 ml plasma.

Isolation of VLDL1, VLDL2, IDL and LDL

VLDL1 (Sf 60-400), VLDL2 (Sf 20-60), IDL (Sf 12-20), and LDL (Sf 0-12) were isolated from plasma by a modification of the cumulative ultracentrifugation density gradient technique by Lindgren et al[4].

Gradient gel electrophoresis

The particle size distribution of LDL (d: 1.019-1.063g/ml), isolated by sequential floatation ultracentrifugation, was examined by non-denaturing gradient gel electrophoresis as described by Nichols et al[5]. The gels were scanned by computer assisted video densitometry in a Bio-Rad Model 620 Video Densitometer (Bio-Rad Laboratories). LDL size was calculated with reference to the Rf values of the standards.

DNA analysis

Genomic DNA was extracted from 5ml of whole blood, using a commercially available DNA isolation kit (Gentra Genomic DNA purification kit, Minneapolis, USA.) according to the manufacturer's protocol.

SNP genotyping was performed by SNP-IT™ assays using the single base extension method of the SNPstream 25K™ System (Orchid Biosciences, NJ, USA). Briefly, the genomic DNA region spanning the polymorphic site is PCR amplified using one phosphothiolated primer and one regular PCR primer. The amplified PCR products were digested with exonuclease. The 5' phosphothioates protect one strand of the PCR product from exonuclease digestion, resulting in the generation of a single-stranded PCR template. The single-stranded PCR template is overlaid onto a 384 well plate that contains covalently attached SNP-IT™ primer extension primer designed to hybridize immediately adjacent to the polymorphic site. The SNP-IT™ primer is extended for a single base with DNA polymerase and a mixture of an appropriate acycloterminator, which is labeled with either FITC or biotin and is complementary to the polymorphic nucleotide. The identity of the incorporated nucleotide is determined through serial colorimetric reactions with anti-FITC-AP and streptavidin-HRP, respectively. The results of yellow and/or blue color developments were analyzed with the ELISA reader and the final genotype calls were made with the QCReview™ program.

Reference data

For evaluating the validity of CETP gene polymorphisms frequencies in this study, we used reference data acquired from 1800 subjects (including normal healthy subjects and CHD patients), which was kindly shared from the Cardiovascular Genome Center, Yonsei University.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 for Windows (SPSS Inc. IL, USA). For comparisons between the groups, we used Student's t test for continuous variables and chi-square test for categorical variables. All values are described as mean standard deviation. For determining the independent factors for LDL subfractions, we used multiple regression analysis and binomial logistic regression analysis.

RESULTS

Clinical and biochemical characteristics

The mean age of the study population was 39.1 ± 10.2 years. Twenty-three were smokers. Subjects had normal ranges of waist circumference, BMI, and fat mass, as well as normal systolic and diastolic blood pressures. Total cholesterol, triglyceride, LDLc and HDLc concentrations were normal (Table 1).

Table 1. Demographic characteristics, anthropometrical data, blood pressure and lipid profiles of the subjects

	N=87
Age	39.1 ± 10.2
Male/Female	39/48
Menopause	12
Smoker	23
Waist(cm)	79.9 ± 10.1
Body mass index(kg/m2)	23.1 ± 3.3
Fat mass(%)	26.3 ± 6.8
Systolic BP(mmHg)	117 ± 13.1
Diastolic BP(mmHg)	75.3 ± 10.4
Total cholesterol (mg/dl)	175 ± 30.7
Triglyceride (mg/dl)	103 ± 48.1
VLDL cholesterol (mg/dl)	13.4 ± 9.2
LDL cholesterol (mg/dl)	117 ± 28.1
HDL cholesterol (mg/dl)	45.0 ± 11.6

Values are mean ± SD
VLDL : very low density lipoprotein, LDL : low density lipoprotein
HDL : highdensity lipoprotein

Alleles and genotype frequencies

Subjects had relative frequencies of 0.52 and 0.48 for the A and G alleles of the I405V polymorphism. These frequencies do not differ from those reported from Japan and from our reference data. Subjects had relative frequencies of 0.53 and 0.47 for the A and C alleles of the A629C polymorphism, which again were not different from our reference data. Subjects had relative frequencies of 0.64 and 0.36 for the B1 and B2 alleles of TaqIB, and these also showed no difference from our reference data.

Effects of CETP gene polymorphisms on lipids and lipoproteins

The subjects were separated according to I405V genotype (AA + AG vs. GG) and compared by the Student's t-test (Table 2). The age, sex ratio, proportion of smokers and menopausal status did not differ between subjects with the GG genotype and those with AA or AG genotypes. Total cholesterol, triglyceride, LDLc and HDLc concentrations did not differ. Those with the GG genotype had significantly lower VLDLc than those with AA or AG genotypes (10.2 ± 6.6 vs. 14.3 ± 9.8, $p<0.05$). VLDL2, IDL and LDL concentrations were not different between the two groups. Subjects with the GG genotype had significantly lower VLDL1 concentrations than those with the AA or AG genotypes (31.5 ± 21.8 vs 49.5 ± 46.6, $p=0.05$).

When the study population was classified according to the A629C genotype (CC+AC vs. AA), twenty-six subjects were identified as having the AA genotype. The age, sex ratio, proportion of smokers and menopausal status did not differ between subjects with the AA genotype and those with CC or AC genotypes. There were no differences in lipid and lipoprotein profiles (Table 2).

No differences in lipid and lipoprotein profiles were found when subjects having the TaqIB2B2 genotype were compared with those having the TaqB1B1 or B1B2 genotypes (Table 2).

Table 2. Effects of I405V, A629C and TaqIB gene polymorphisms on lipids and lipoproteins

	I405V		A629C		TaqIB	
	AA + AG	GG	CC+AC	AA	B1B1 + B1B2	B2B2
mg/dl	N=66	N=21	N=60	N=26	N=77	N=10
TC	178 ± 30.0	170 ± 32.6	177 ± 32.6	169 ± 23.2	175 ± 30.9	171 ± 30.1
TG	106 ± 51.6	88.4 ± 34.3	107 ± 49.0	94.4 ± 47.0	102 ± 47.7	107 ± 57.8
VLDLc	14.3 ± 9.8	10.2 ± 6.6*	14.3 ± 9.3	11.9 ± 9.0	13.4 ± 9.0	14.4 ± 11.4
LDLc	119 ± 27.1	113 ± 30.1	119 ± 30.4	111 ± 20.2	118 ± 28.5	115 ± 30.9
HDLc	44.8 ± 11.9	46.4 ± 11.3	44.1 ± 11.7	46.2 ± 11.7	44.4 ± 11.9	45.0 ± 10.8
VLDL1	49.5 ± 46.6	31.5 ± 21.8*	48.7 ± 44.5	40.2 ± 39.2	45.3 ± 43.0	53.5 ± 47.1
VLDL2	29.7 ± 17.8	28.9 ± 16.1	32.0 ± 18.1	25.0 ± 14.1	29.7 ± 17.4	28.0 ± 17.6
IDL	39.1 ± 16.7	41.9 ± 17.3	40.8 ± 18.5	38.5 ± 12.4	39.2 ± 16.5	38.3 ± 14.3
LDL	203 ± 56.1	197 ± 58.3	203 ± 57.7	192 ± 52.3	201 ± 55.4	192 ± 66.8

*$p<0.05$
Values are mean ± SD
TC : total cholesterol, TG : triglyceride, VLDL : very low density lipoprotein, IDL : intermediate density lipoprotein, LDL : low density lipoprotein, HDL : high density lipoprotein

Table 3. Effects of I405V, A629C and Taq1B gene polymorphism on LDL subfraction and LDL peak particle diameter

mg/dl	I405V		A629C		Taq1B	
	AA + AG	GG	CC+AC	AA	B1B1 +B1B2	B2B2
	N=66	N=21	N=60	N=26	N=77	N=10
LDL I (mg/dl)	41.4 ± 36.7	39.6 ± 23.3	39.3 ± 34.2	42.6 ± 35.0	40.3 ± 35.2	36.6 ± 26.5
LDL II (mg/dl)	113 ± 61.1	128.6 ± 54.5	118 ± 64.5	103 ± 44.6	117 ± 62.3	93.0 ± 42.2
LDL III (mg/dl)	81.1 ± 74.8	56.1 ± 49.2	77.3 ± 70.8	71 ± 70.1	75.5 ± 71.0	83.1 ± 69.7
% of LDL I	18.9 ± 17.6	18.9 ± 12.3	18.0 ± 16.9	20.4 ± 16.1	18.4 ± 16.9	18.8 ± 14.5
% of LDL II	48.2 ± 20.4	57.4 ± 14.2†	50.2 ± 20.0	48.8 ± 20.0	50.4 ± 20.0	45.0 ± 20.6
% of LDL III	32.9 ± 26.5	23.7 ± 16.5*	31.8 ± 25.1	30.8 ± 26.1	31.2 ± 25.0	36.2 ± 28.3
l (m)	25.9 ± 0.9	26.1 ± 0.5	25.9 ± 0.8	26.0 ± 0.9	26.0 ± 0.8	26.0 ± 0.7

† p<0.05, *p=0.06
Values are mean ρ SD

Table 4. Distribution of CETP gene polymorphism separated by LDL particle size

	≤25.4nm	>25.4nm	
I405V genotype			
AA	10	15	
AG	11	32	
GG	2	20	P=0.04
A629C genotype			
AA	8	19	
AC	7	34	
CC	9	12	
Taq I B genotype			
B1B1	12	24	
B1B2	9	34	
B2B2	3	8	

Effects of CETP gene polymorphisms on LDL subfractions and LDL particle diameters

LDL I, LDL II and LDL III concentrations did not differ between subjects with the GG genotype and those with the AA or AG genotypes for I405V polymorphism (Table 3). Compared to subjects with AA or AG genotypes, however, those with the GG genotype had a significantly higher percentage of LDL II (57.4 ± 14.2 vs 48.2 ± 20.4, p<0.05) and a lower percentage of LDL III (borderline significant at 23.7 ± 16.5 vs 32.9 ± 26.5, p= 0.06). No difference in LDL peak particle diameter was found between the two groups.

Concentrations and relative distributions of the LDL subfraction were not different between subjects with AA or AC genotypes, and those with the CC genotype, for A629C (Table 3). LDL peak particle diameter did not differ between these two groups, either.

No differences in the concentrations and relative distribution of LDL subfractions (as well as LDL peak particle diameter) were found when subjects having the Taq1B2B2 genotype were compared with those having TaqB1B1 or B1B2 genotypes (Table 3).

When the study population was separated according to the 75 percentile of LDL peak particle diameter (25.4nm), among the three polymorphisms, only the GG genotype for the I405V polymorphism was negatively associated with small, dense, LDL incidence (Table 4).

Multiple regression analysis revealed that triglyceride concentration and I405 V polymorphism (AA vs. GG) were the main elements determining the percentage of LDL III subfraction, independent of waist circumference, BMI and Taq1B polymorphism (Table 5). By logistic regression analysis, it was revealed that triglyceride concentration and I405V polymorphism were associated with higher *LDL III* concentrations *(LDL III > 100* mg/dl), (Table 6).

Table 5. Multiple regression analysis for parameters influencing relative distribution of LDLIII (% of LDLIII)

	B	Beta	p	R
Triglyceride	0.28	0.68	<0.001	
I405V(GG vs AA)	18.1	0.30	<0.05	
Model summary for % of LDL III			<0.001	0.75

Table 6. Multiple logistic regression analysis for determinants for probability of higher LDLIII concentration (LDL III > 100 mg/dl)

	Exp(B)	Odds ratio	p	Rsquare
Triglyceride	0.952	1.05	<0.001	
I405V				
I405V(GG vs AA)	0.047	21.2	<0.05	
I405V(AG vs AA)	0.027	36.8	<0.05	
A629C				
A629C(CC vs AA)	0.32	3.1	0.05	
Model summary for LDL III > 100 mg/dl			<0.001	0.66

DISCUSSION

Plasma CETP is a glycoprotein and its main function is to transfer esterified cholesterol from HDL to apo B-containing lipoproteins, in exchange for triglycerides[6]. The net effect of CETP is to promote the transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins and LDL., and the transfer of triglyceride from triglyceride-rich lipoproteins to LDL and HDL[7] ; this process results in increases in the amount of cholesteryl ester in VLDL and increases in the amount of triglyceride in HDL, resulting in reductions of HDL cholesterol. Through the same mechanism, triglyceride rich, cholesterol depleted, LDL are transformed into small dense LDL by hepatic lipase. Therefore, decreases in CETP activity are associated with increases in HDLc and decreases in small dense LDL.

About thirteen human CETP gene polymorphisms have been reported. I405V polymorphism is reported to affect CETP concentration, CETP activity, and HDLc concentration in European populations[8),9)]. In Asian populations, results have proved to be confusing and perplexing. Okumura et al. reported that those of the GG genotype in the Japanese population were associated with low CETP and high HDLc concentrations, along with smaller LDL particle size[10)]. Wu et al., however, reported that this polymorphism was not associated with any change of lipid profile in Taiwanese people[11)]. In our study, I405V CETP polymorphism was not associated with changes in HDLc concentration, and this was consistent with our reference data. LDL subfraction analysis, however, revealed that the GG genotype was associated with higher percentages of LDL II and lower percentages of the LDL III subfraction. Our study has shown that shifting from the GG genotype to the AA genotype was responsible for a 16% increase in LDL III percentage. Compared to subjects with the GG genotype, those with the AA genotype had a 23 fold risk of having higher LDL III concentrations. And subjects with the GG genotype had a low risk for having small dense LDL. The results from our study are opposite to those of Okumura et al. which reported that the GG genotype was associated with smaller LDL particle diameters[7)]. In the Okumura et al. study, subjects with the GG phenotype had lower CETP activity, which usually retards small, dense, LDL generation.

A629C polymorphism is located in the promoter area of the CETP gene. The 629A allele has been reported to be associated with low CETP mass and a higher HDLc concentration[12)]. It was reported that homozygotes for the 629A allele had a 14% higher HDLc concentration than those for the 629C allele[13)]. In our study, A629C polymorphism was not associated with changes in HDLc concentration. Paradoxically, our data showed that homozygotes for the -629A allele had a 3.1 fold risk for having higher LDL III concentrations than those for the 629 C allele. Because we did not measure the CETP activity or mass, we can't explain the putative mechanism. Our reference data also did not show any association between HDLc concentration and A629C polymorphism.

TaqIB polymorphism is one of the most extensively investigated polymorphisms in relation to atherosclerosis. The B2 allele is associated with low CETP activity and high HDLc concentrations in Western people[14)]. In our study, the TaqIB polymorphism was not associated with changes in HDLc concentration or LDL subfraction. Reference data, however, showed that healthy homozygotes for B2B2 alleles had significantly higher HDLc than those for B1B1 or B1B2. So, we cannot exclude the probability that our findings for TaqIB polymorphism may be unrepresentative.

In conclusion, we report that, of the three polymorphisms studied, the I405V polymorphism was consistently associated with higher LDL III con- centrations, higher percentages of LDL III, and smaller LDL particle diameters. Because we did not perform CETP enzyme assays, it is not known if this finding is mainly due to CETP effects or other unknown mechanisms.

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