**CETP (cholesteryl ester transfer protein) promoter − 1337 C > T polymorphism protects against coronary atherosclerosis in Japanese patients with heterozygous familial hypercholesterolaemia**

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**ABSTRACT**

CETP (cholesteryl ester transfer protein) and HL (hepatic lipase) play a role in the metabolism of plasma lipoproteins, but the effects of CETP and LIPC (gene encoding HL) genotypes on coronary atherosclerosis may be dependent on LDL (low-density lipoprotein)-receptor activity. Recently, the −1337 C > T polymorphism in the CETP gene has been reported in REGRESS (Regression Growth Evaluation Statin Study) to be a major determinant of promoter activity and plasma CETP concentration. In the present study, we have investigated the effects of the CETP promoter −1337 C > T and LIPC promoter −514 C > T polymorphisms on serum lipid profiles and risk of coronary atherosclerosis in 206 patients (154 males) with heterozygous FH (familial hypercholesterolaemia). To evaluate coronary atherosclerosis, we used CSI (coronary stenosis index) calculated from coronary angiograms. The CETP −1337 T allele was less frequent in subjects with a CSI ≥ 14 (mean value) in the group with coronary artery disease (P = 0.04, as determined by χ² test). ANOVA revealed that HDL-C (high-density lipoprotein-cholesterol) and triacylglycerol (triglyceride) levels were not significantly higher in the presence of the CETP promoter −1337 C > T allele. Combined with LIPC promoter polymorphisms, HDL-C levels were highest and CSI were lowest with CETP −1337 CT + TT and LIPC −514 CC genotypes, but a significant interaction was not shown. A multiple logistic regression analysis revealed that, in patients with coronary atherosclerosis, the CETP −1337 CC genotype was a significant genetic risk factor in FH (odds ratio = 2.022; P = 0.0256). These results indicate that the CETP promoter −1337C > T polymorphism is associated with the progression of coronary atherosclerosis in Japanese patients with FH, independent of HDL-C and triacylglycerol levels.

**Key words:** cholesteryl ester transfer protein (CETP), coronary artery disease, familial hypercholesterolaemia, hepatic lipase, single nucleotide polymorphism.

**Abbreviations:** AP, angina pectoris; Apo, apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CETP, cholesteryl ester transfer protein; CSI, coronary stenosis index; FH, familial hypercholesterolaemia; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; MI, myocardial infarction; NCBI, National Center for Biotechnology Information; REGRESS, Regression Growth Evaluation Statin Study.

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CETP (cholesteryl ester transfer protein) is a key player in the metabolism of major plasma lipoproteins. CETP mediates the transfer of cholesteryl esters from HDL (high-density lipoprotein) to Apo (apolipoprotein) B-containing lipoproteins in exchange for triacylglycerols (triglycerides) [1]. CETP activities are known to be highly affected by genetic factors. For example, individuals with homozygous CETP deficiency have high HDL-C (high-density lipoprotein)-cholesterol levels and low LDL-C (low-density lipoprotein)-cholesterol levels, and have no evidence of premature atherosclerosis [2]. Also, CETP gene polymorphisms, especially the TaqIB polymorphism identified in intron 1, is reported to be highly associated with plasma CETP concentrations and HDL-C levels. Moreover, recent meta-analyses revealed that this polymorphism is associated with the incidence of CAD (coronary artery disease) [3–7]. However, this polymorphism is unlikely to be functional by itself, instead representing a surrogate marker of functional variants of the CETP gene [8]. Indeed, previous studies have shown that the CETP promoter $-629$ A $>$ C polymorphism has almost complete linkage disequilibrium with the TaqIB polymorphism [9,10], and that this polymorphism is associated with CAD [11]. On the other hand, we have reported [8] that the haplotype block consisting of $-2668$ G/A, $-2505$ C/A, $-1337$ C/T and the shortest (gaaa) repeat had a stronger association than TaqIB2 or $-629$ A/C with low plasma CETP concentrations and high HDL-C levels in healthy Japanese males. Moreover, functional interaction between $-629$ C/A, $-971$ G/A and $-1337$ C/T polymorphisms in the CETP gene is a major determinant of promoter activity and plasma CETP concentration in REGRESS (Regression Growth Evaluation Statin Study) [12].

In addition to CETP, HL (hepatic lipase) also plays a crucial role in the metabolism of plasma lipoproteins. HL is involved in the hydrolysis of triacylglycerol and phospholipids in LDL (intermediate-density lipoprotein) and large LDL particles to form smaller and denser LDL particles, and also plays a major role in promoting the conversion of HDL$_2$ into HDL$_3$ particles [13]. The effects of the LIPC genotype (the gene encoding HL) on atherosclerosis have been controversial [14], and may be dependent on LDL-receptor activity.

FH (familial hypercholesterolaemia) is an autosomal-dominant disorder characterized by primary hypercholesterolaemia with tendon xanthomas and premature CAD caused by mutations in the LDL receptor [15,16]. Mortality from CAD is reported to be several times higher in subjects with heterozygous FH than in the general population [15,16]. There are several reports that polymorphisms or mutations in the CETP gene influence the clinical characteristics of FH subjects [17,18]. Carmena-Ramon et al. [17] reported that in FH the TaqIB2 allele was associated with higher HDL-C and ApoAI levels. On the other hand, our previous study [18] showed that increased HDL-C levels caused by a heterozygous CETP deficiency was insufficient to prevent CAD in FH.

With this background, the present study investigated the effects of CETP promoter $-1337$ C $>$ T and LIPC promoter $-514$ C $>$ T polymorphisms on coronary atherosclerosis in Japanese patients with heterozygous FH.

**METHODS**

**Study participants**

We enrolled 206 consecutive Japanese patients with heterozygous FH (26–83 years old; 154 males) who attended our hospital. FH was diagnosed when one of the following two criteria was met: (i) primary hypercholesterolaemia (>$5.96$ mmol/l (>230 mg/dl) in any age group) in a patient with tendon xanthomas, or (ii) primary hypercholesterolaemia with a definitive diagnosis of FH in any first-degree relative [19]. All the females were postmenopausal, as defined by the absence of menstruation for >6 months or having attained an age of ≥60 years. Those with surgical menopause were excluded. For patients with MI (myocardial infarction), the age at the first event was recorded, whereas for patients with AP (angina pectoris), the age at which coronary angiography was performed was recorded. Inclusion criteria for this study were FH patients who were examined by coronary angiography because of chest symptoms and/or a positive exercise test before lipid-lowering therapy was initiated. Individuals who had thyroid disease, levels of triacylglycerol $\geq 4.52$ mmol/l (>400 mg/dl) or who received lipid-lowering agents, corticosteroid or oestrogen hormone replacement therapy were excluded. All patients provided informed consent for participation in the present study, which was approved by the Ethical committee of Kanazawa University Graduate School of Medical Science.

**Assessment of CAD**

For the evaluation of CAD, we used CSI (coronary stenosis index) to quantify the severity of coronary atherosclerosis. The severity of stenotic changes was assessed by a score assigned to each of the 15 segments according to the classification of the American Heart Association Grading Committee. A normal coronary angiogram was graded as 0, stenosis of ≤25 % was graded as 1, 25–50 % stenosis was graded as 2, 50–75 % stenosis was graded as 3, and >75 % stenosis was graded as 4. CSI was defined as the sum of these scores in all 15 segments, producing a maximal value of 60 [15]. In the present study, MI was diagnosed in 56 subjects with...
heterozygous FH (48 male), and AP was diagnosed in 53 subjects with heterozygous FH (all male). The mean CSI was 14.0 ± 11. The mean CSI in subjects who were diagnosed with MI and AP was 20, whereas the mean CSI in those subjects who were without clinical symptoms of CAD was 8. In our previous study [15], we observed that the age of coronary artery stenosis detectable by angiogram occurs after 17–25 years of age in male and female subjects with heterozygous FH. In the present study, 86% of the subjects with MI and AP had a CSI > 14, whereas 80% of subjects without clinical symptoms of CAD had a CSI < 14. Therefore we diagnosed CAD as being present when CSI was > 14.

Assessment of conventional risk factors

Data for BMI (body mass index), smoking history, alcohol drinking, blood pressure, diabetes status and lipid profile were collected. Hypertension was considered to be present if any antihypertensive treatment had been instituted, if systolic blood pressure was > 160 mmHg or diastolic blood pressure > 95 mmHg. Diabetes mellitus was diagnosed if fasting plasma glucose was ≥ 6.70 mmol/l (>120 mg/dl) or ≥ 11.10 mmol/l (>200 mg/dl) at 120 min after 75 g of oral glucose loading, or if HbA1c (glycated haemoglobin) was ≥ 6.5%. For smoking status, we defined subjects who smoked ≤ 10 cigarettes/day as non-smokers, past smokers as ex-smokers and current smokers.

Laboratory analysis

Blood samples were collected from subjects after 12 h of fasting before starting lipid-lowering agents. Total cholesterol, triacylglycerols and HDL-C levels were determined by standard enzymatic methods. LDL-C levels were calculated using the Friedewald formula [20]. Plasma CETP levels were determined by sandwich ELISA, as described previously [21].

Determination of CETP and LIPC promoter polymorphisms

Genomic DNA was isolated and purified from peripheral white blood cells. The CETP promoter – 1337 C > T polymorphism and the LIPC promoter – 514 C > T polymorphism (− 480 in older reports) were analysed by PCR-RFLP (restriction-fragment-length polymorphism) methods, as described previously [8,22]. Accession numbers are as follows: CETP, gene ID 1071 [NCBI (National Center for Biotechnology Information) Entrez Gene database], nucleotide sequence NM_000078 (NCBI Entrez Nucleotide database) and – 1337 C/T SNP rs17231506 (NCBI SNP database); and LIPC, gene ID 3990 (NCBI Entrez Gene database), nucleotide sequence NM_000236 (NCBI Entrez Nucleotide database), – 514 C/T SNP rs1800588 (NCBI SNP database) and – 514 C/ T USF binding site cctttgaca(c/t)gagggtaag.

Statistical analyses

All values are expressed as means ± S.D. unless otherwise noted. The allele frequency was estimated by gene counting. One-way ANOVA was performed, followed by multiple comparisons using Fisher’s protected least significant difference. Serum HDL-C was adjusted by multiple linear regression analysis. The prevalence of patients with hypertension, diabetes mellitus, current and past smoking, and alcohol drinking were compared between different groups using a χ² test. A multiple logistic regression analysis was used to predict CAD from the genotype of polymorphism, with conventional risk factors as covariates. A probability value of P < 0.05 was considered to be significant. All tests were performed with StatView software (version 5.0; SAS Institute).

RESULTS

Characteristics of study subjects

The clinical and biochemical characteristics of the study population either with CAD or without CAD (non-CAD) are summarized in Table 1. A total of 94 the subjects with heterozygotes FH were suffering from CAD. There were significantly more males and subjects with hypertension and diabetes mellitus in the CAD group compared with the non-CAD group.

Association between – 1337 C > T polymorphism and CSI

The frequency of the CETP promoter – 1337 T allele was 0.20 in both males and females; lower than in Caucasians [12]. A few subjects in the present study had
the CETP promoter −1337 TT genotype (11 males and two females), and the T allele was less frequent in subjects with a CSI > 14. The distribution of the CETP promoter −1337 CC genotype differed significantly between those with a CSI > 14 and those with a CSI < 14 (P = 0.0426, as determined by a χ² test).

CETP promoter polymorphism and CETP concentrations

We compared plasma CETP concentrations between the −1337 CC and −1337 CT + TT genotypes in a subset of 44 subjects (25 males; Table 2). The CETP concentration tended to be lower in the presence of the T allele (P = 0.14).

CETP promoter polymorphism, lipid profile and development of CAD

The characteristics of subjects according to CETP promoter polymorphism are summarized in Table 3. As there were only two females with the TT genotype, we analysed men and women combined. HDL-C levels were not significantly higher in TT genotype, and the CSI tended to be lower in patients carrying the T allele (P = 0.19).

Effects of CETP and LIPC promoter polymorphisms on lipid profile and CSI

The frequency of the LIPC promoter −514 T allele was 0.53 in males and 0.50 in females, which is similar to the frequencies previously reported in Japanese subjects, but higher than those in Caucasians [22,23]. To investigate the effects of CETP and LIPC promoter polymorphisms on lipid profile, we compared four subgroups stratified by high CETP genotype CC compared with low CETP CT + TT, and high LIPC genotype CC compared with low LIPC genotype CT + TT. Figure 1 shows that the HDL-C level was significantly higher in −514 CC/−1337 CT + TT than in −514 CC/−1337 CC [1.22 ± 0.36 mmol/l (47 ± 14 mg/dl) compared with 0.98 ± 0.30 mmol/l (38 ± 10 mg/dl) respectively; P < 0.02], and it was significantly higher in −514 CC/−1337 CT + TT than in −514 CT + TT/−1337 CC in both CT + TT (P < 0.05). LDL-C levels did not differ significantly between the four groups. CSI was significantly lower in −514 CC/−1337 CT + TT than in −514 CC/−1337 CC (9.6 compared with 17.2 respectively; P = 0.02), suggesting an interaction between CETP and LIPC genotype on CSI.

Multiple logistic regression analysis

A multiple logistic regression analysis was performed to determine the association of CAD and CETP promoter polymorphism and other conventional risk factors. Gender, hypertension, diabetes mellitus and CETP −1337 CC genotype exhibited significantly higher odds ratios; however, age, smoking, HDL-C and triacylglycerol levels, and the presence of LIPC −514 C > T were not significant variates (Table 4).

DISCUSSION

The present study investigated the effects of CETP and LIPC promoter polymorphisms on serum lipid profiles and risk of coronary atherosclerosis in subjects with heterozygous FH. None of the other coronary risk factors differed significantly between CETP genotypes; however, multiple logistic regression analysis revealed that coronary atherosclerosis was associated with the CETP −1337 CC genotype. An interaction between the CETP and LIPC genotypes for plasma HDL-C and CAD has also been shown.

To our knowledge, this is the first study on the effects of the CETP promoter −1337 C > T polymorphism
in coronary atherosclerosis and, therefore, the first to suggest that the CETP promoter $-1337$ C $>$ T polymorphism is associated with the severity of coronary atherosclerosis in heterozygous FH. In a previous study [8], this polymorphism was associated with low plasma CETP concentrations and high HDL-C levels more strongly than with the TaqIB2 allele in elderly Japanese males and, recently, this polymorphism has been reported to be a major determinant of promoter activity and plasma CETP concentration in REGRESS [12]. Therefore we investigated this $-1337$ site rather than the well-known TaqIB polymorphism. As subjects with FH have a high risk of premature CAD, we determined the existence of early stage coronary atherosclerotic changes by using CSI. Our present data suggest that the association of the CETP genotype with cardiovascular risk is independent of serum HDL-C levels. As indicated in Table 3, there was no significant difference in HDL-C/adjusted HDL-C levels between CETP genotypes. The CETP TaqIB2 allele was associated with HDL-C, especially HDL2-C, in Japanese subjects [24] and, therefore, if we had assessed HDL2-C, this might have revealed a significant difference between the genotypes.

There are conflicting reports as to whether CETP is pro- or anti-atherogenic. Humans with homozygous CETP deficiency have markedly high HDL-C levels and decreased LDL-C levels, with no clear evidence of premature atherosclerosis [2]. A CETP gene mutation (D442G) was shown to be associated with increased LDL particle size [25], suggesting that CETP is pro-atherogenic. In contrast, Hirano et al. [26] have reported that the prevalence of CETP deficiency was lower in individuals older than 80 years of age residing in a district of northern Japan, suggesting that CETP deficiency is not association with longevity, and the same investigators have shown that reduced CETP activity in conjunction with reduced HL activity is associated with an increased risk of CAD [27]. On the other hand, Moriyama et al. [28] found in a cross-sectional analysis that HDL-C elevation ($\geq 80$ mg/dl) was protective against coronary heart disease, regardless of CETP genotype, in 19044 male and 29487 female Japanese subjects. In addition, a recent prospective study in the Honolulu Heart Program has shown the protective effects of heterozygous CETP deficiency against CAD, although the effect was not statistically significant [29].

Table 4 Multivariate adjusted relative prevalence odds ratio of coronary atherosclerosis by multiple logistic regression analysis

<table>
<thead>
<tr>
<th>Variate</th>
<th>Odds ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.021 (0.993–1.050)</td>
<td>0.1431</td>
</tr>
<tr>
<td>Sex</td>
<td>4.283 (1.788–10.259)</td>
<td>0.0011</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2.628 (1.252–5.519)</td>
<td>0.0107</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2.136 (1.081–4.218)</td>
<td>0.0289</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.992 (0.969–1.015)</td>
<td>0.3261</td>
</tr>
<tr>
<td>CETP $-1337$ C $&gt;$ T polymorphism</td>
<td>2.022 (1.090–3.754)</td>
<td>0.0256</td>
</tr>
<tr>
<td>LIPC $-514$ C $&gt;$ T polymorphism</td>
<td>0.856 (0.562–1.305)</td>
<td>0.4698</td>
</tr>
</tbody>
</table>
At lower CETP concentrations, LDL-receptor activity is up-regulated, causing a reduction in serum LDL levels and leading to atheroprotection. Lowering CETP activity may be beneficial in an affluent environment, where high-fat and cholesterol-rich diets increase plasma LDL-C levels and down-regulate hepatic LDL-receptors, such as in FH. We presume that individuals with FH have higher CETP activity or concentration than normolipidaemic controls [30,31], which would be less pro-atherosclerotic when they carry the CETP promoter −1337 T allele. De Grooth et al. [32] reported a significant positive correlation between carotid intima-media thickness and CETP levels in FH, suggesting that plasma CETP would be pro-atherogenic in FH. There are also some reports on the CETP TaqIB polymorphism and impaired glucose tolerance [33], suggesting that CETP could be pro-atherogenic independently of lipid metabolism. In the present study, however, there was no significant difference between CETP promoter −1337 C>T polymorphism and serum glucose levels (5.99 ± 1.94 mmol/l in −1337 CC compared with 5.72 ± 1.33 mmol/l in −1337 CT + TT; P = 0.20), and no difference in diabetes prevalence (results not shown).

In addition to CETP, HL also plays a crucial role in the metabolism of plasma lipoproteins, but the effects of CETP and HL activity on lipid profile and CAD are unclear [14,34]. The present study found no association between the LIPC promoter −514 C>T polymorphism and CAD and HDL-C levels; however, CSI with the CETP −1337 T allele and LIPC −514 CC was lowest in the subgroup. In another study from our laboratory (M. Takata and A. Inazu, unpublished work), HL activity was significant higher in −514 CC than CT + TT (0.282 ± 0.011 compared 0.231 ± 0.005 mmol/l respectively, P < 0.001) in hyperlipidaemic patients (n = 325, of which 183 were male). In human studies, HL activity tends to be elevated in the presence of smoking [35], insulin resistance in Type II diabetes mellitus [36], in females with omental fat mass [37] and males in general. These reports suggest that HL is pro-atherogenic. On the other hand, it has been reported that HL activity is lower in patients with CAD than in those without CAD [38]. Another group found that HL activity did not differ between subjects with and without CAD in REGRESS [39]. In an environment of low HL activity, LDL increases and it may be pro-atherogenic [40]. HL also promotes the formation of small and dense atherogenic LDL particles [13]. Lowering HL activity in hypertriglyceridaemia may decrease the pro-atherogenic risk due to an improved lipid profile, notably an increased LDL size [14]. In conditions where LDL-receptor activity is low, as in FH, HL activity appears to be inversely associated with CAD in subjects with low CETP concentrations (Figure 1), suggesting that the flux of cholesterol through the system of HDL-C transport may be more important in preventing atherosclerosis.

The main limitations of the present study were the relatively small sample size and the absence of data on HDL subclass and LDL particle size.

In conclusion, the CETP promoter −1337 C>T polymorphism is associated with the progression of coronary atherosclerosis in Japanese patients with FH, independent of HDL-C and triacylglycerol levels. We believe that this genetic variant of the CETP gene promoter could be an important determinant of coronary atherosclerosis in FH, and genotype differences between promoter variants and missense mutations need to be clarified in future investigations.

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