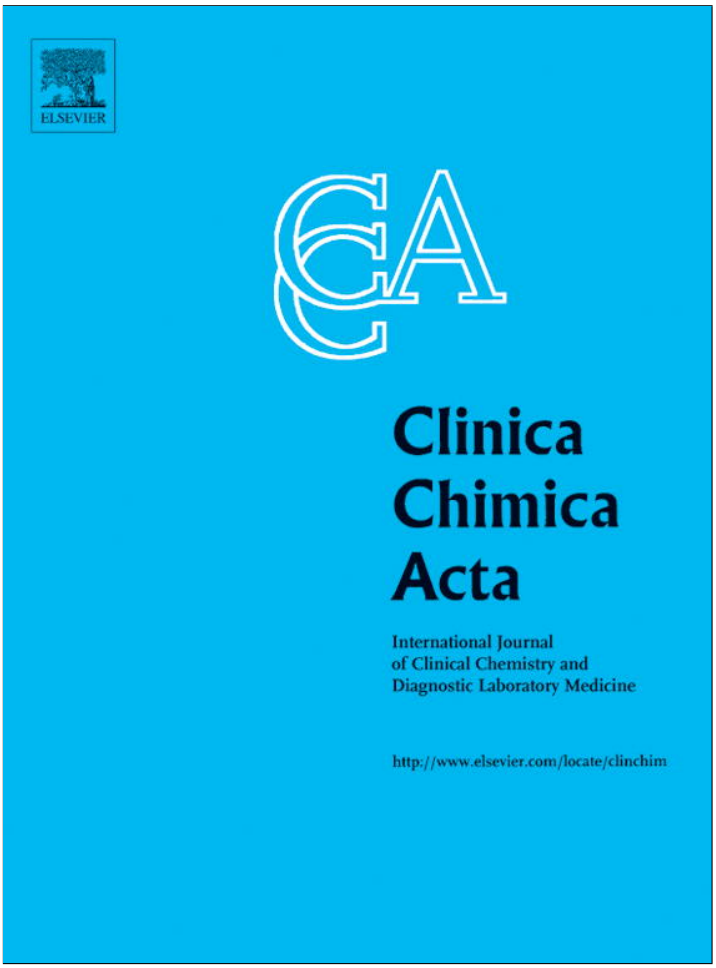

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Functional characterization of novel variants in the *CETP* promoter and the *LIPC* gene in subjects with hyperalphalipoproteinemia

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ABSTRACT

Background: Variants in the *CETP* and the *LIPC* genes, encoding cholesteryl ester transfer protein and hepatic lipase, respectively, are associated with high levels of HDL-cholesterol or hyperalphalipoproteinemia (HALP). Recently, we have identified three novel variants in the *CETP* promoter and two novel variants in *LIPC* in Thai subjects with HALP. In this study, we investigated the functions of these 5 variants in vitro.
Methods: For *CETP* promoter variants, we used site-directed mutagenesis, transient expression in HepG2 cells and luciferase reporter assay. For *LIPC* variants, cDNA was cloned and mutagenesis for missense variants was performed before expression in HepG2 cells.
Results: The transcriptional activities of −49G>T,−70C>T, and −372C>T *CETP* promoter variants were markedly reduced (5%, 8% and 30%, respectively, compared to that of the wild-type, $P<0.001$). For *LIPC* variants, hepatic lipase activities in the lysates of cells transfected with c.421A>G (p.G141S) and c.517G>A (p.V173M) variants were 41% and 46%, respectively, compared to that of the wild-type ($P<0.05$).
Conclusions: The recently-identified variants in the *CETP* promoter and in the *LIPC* gene may contribute to HALP. Our result may have a diagnostic application in the genetic evaluation of subjects with high HDL-cholesterol levels.

1. Introduction

Plasma levels of high-density lipoprotein-cholesterol (HDL-C) are inversely correlated with the risk of cardiovascular disease, a major cause of mortality worldwide [1]. Levels of HDL-C are modulated by several genetic and environmental factors. In Japan, very high levels of HDL-C, also known as hyperalphalipoproteinemia (HALP), are associated with deficiency of cholesteryl ester transfer protein (CETP) due to genetic variants in the *CETP* gene [2]. Previously, we have shown that Thai subjects with HALP had lower plasma activities of CETP and hepatic lipase [3]. Both CETP and hepatic lipase are important proteins in HDL metabolism. Subsequently, we identified a number of rare and common variants in *CETP* and *LIPC* genes, which encode for CETP and hepatic lipase (or lipase member C), respectively, in these subjects [4,5].

Among the rare variants we recently identified, several of them were novel [5] and the functional consequences of these variants were unclear. In the *CETP* gene promoter, 3 point mutations, −49G>T,−70C>T, and −372C>T, were found, whereas 2 novel missense variants, c.421A>G (p.G141S) and c.517G>A (p.V173M), were discovered in the *LIPC* gene [5]. Although sequence examination and bioinformatic studies indirectly suggested that these variants probably affected the functions, definite proof requires further experiments. In the present study, we performed experiments to functionally characterize these 5 mutations in vitro.

2. Materials and methods

2.1. Subjects and biochemical measurements

Among the cohort of 64 ambulatory Thai subjects with severe HALP (HDL-C levels ≥2.59 mmol/L or ≥100 mg/dL on at least 2 occasions), we previously identified 3 novel heterozygous point variants in the *CETP* promoter, −49G>T,−70C>T, and −372C>T, in 4 subjects and 2 heterozygous missense variants in the *LIPC* gene, c.421A>G (p.G141S) and c.517G>A (p.V173M), in 2 subjects [5]. None of these variants were found in 113 normolipidemic subjects. Lipid levels were measured using enzymatic methods in an automated system by Roche. Plasma

Abbreviations: CETP, cholesteryl ester transfer protein; HALP, hyperalphalipoproteinemia; HDL-C, high-density lipoprotein-cholesterol; LIPC, lipase member C or hepatic lipase.

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CETP activity and hepatic lipase activity were determined as previously described [3]. All of the studied subjects gave written informed consent and the study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. The study was performed in accordance to the Declaration of Helsinki for experiments involving humans.

2.2. Transcriptional activity of variants in the CETP promoter

The promoter region of the human *CETP* gene was cloned into pGL3 basic luciferase expression vector (Promega, Madison, WI) as previously described [6]. pRL-TK vector (Promega) was used as an internal control. Site-directed mutagenesis causing point mutations at position −49G>T,−70C>T, and −372C>T of the promoter region of the *CETP* gene was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The primers used for mutagenesis are shown in Table 1.

HepG2 human hepatocellular carcinoma cells were cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum in 12-well plates at 2×10^5 cells per well and transfected using Lipofectamine reagent as previously described [4]. Two μg of each *CETP* promoter constructs was cotransfected with 0.2 μg of pRL-TK to account for variable transfection efficiency. Cells were incubated for 20 h and luciferase activity was measured on the supernatant of the cell lysates. Relative luciferase activity was calculated from the ratio of luminescence from the experimental reporter to that from the control reporter. Data were averaged from 4 independent experiments performed in duplicate.

2.3. Hepatic lipase activity of HepG2 cells expressing variants of the LIPC cDNAs

Total RNA was extracted from human liver tissues and the full length *LIPC* cDNA was amplified using reverse transcriptase-polymerase chain reaction. Site-directed mutagenesis resulting in missense mutations of the *LIPC* gene, c.421A>G (p.G141S) and c.517G>A (p.V173M), was performed using the QuikChange mutagenesis kit. Wild-type and mutant human *LIPC* cDNAs were then inserted into the expression vector pcDNA3.1. HepG2 cells were seeded 1 day prior to transfection at a confluence of 1.5×10^6 cells/6-cm petri dish. The cells were transfected with 12 μg of the pcDNA3.1 plasmid constructs using Lipofectamine. pcDNA.3.1/*lacZ* was used as a positive control and transfection efficiency was determined as previously reported. For hepatic lipase activity, culture media containing heparin (20 U/mL) and cells were collected at 48 h after transfection. Cells were washed in PBS, solubilized in 1 mL of 50 mM NH_3/NH_4Cl (pH 8.1) containing heparin, and sonicated. Media and cell lysates were stored at −70 °C until assayed for hepatic lipase activity as previously described [3]. Experiments were performed in triplicate.

Table 1
Primers used for site-directed mutagenesis for the *CETP* promoter and the *LIPC* gene.

Sequence name	Sequence (5′ to 3′)
CETP −49G>T F	CAT GTT CCG TTG GGG CTG GGC
CETP −49G>T R	GCC CAG CCC CAA CGG AAC ATG
CETP −70C>T F	AGA CCC TGC TGC CTG GAA GAG CCT CA
CETP −70C>T R	TGA GGC TCT TCC AGG CAG CAG GGT CT
CETP −372C>T F	CAA CAG TAT CTG GTA AGA ATT CAA TGT
CETP −372C>T R	ACA TTG AAT TCT TAC CAG ATA CTG TTG
LIPC G141S F	CAC CCG CCT TGT GAG CAA GGA GGT CGC
LIPC G141S R	GCG ACC TCC TTG CTC ACA AGG CGG GTG
LIPC V173M F	CCT GGG TGC ACA CAT GTC AGG ATT TGC C
LIPC V173M R	GGC AAA TCC TGA CAT GTG TGC ACC CAG G

2.4. Statistical analysis

Data are presented as mean ± SEM unless indicated otherwise. Statistical significance was evaluated by use of the Student's *t*-test for comparison of unpaired data. One-way ANOVA with posthoc analyses was used to compare data among multiple groups. *P* value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software program (version 12, Chicago, IL).

3. Results

3.1. Functional analysis of three novel point variants in the CETP promoter

Three different novel point variants of the *CETP* promoter, −49G>T, −70C>T, and −372C>T, were found in 4 HALP subjects (Fig. 1). Clinical characteristics and laboratory results of these subjects are shown in Table 2. Patient No. 3 harbored both a novel −70C>T promoter variant and a common p.D459G variant (rs2303790) in exon 15 of the *CETP* gene, known to be associated with reduced CETP activity and HALP [7]. A study of this proband's family suggested that Patient No. 3 was a compound heterozygote for both variants. All 4 subjects with these point variants had low plasma CETP activity compared to that of the control group (Table 2).

Since binding sites for various transcription factors are found in the *CETP* promoter [8], we investigated whether these point variations would affect the promoter activity. Site-directed mutagenesis was performed and the variant constructs were transfected into HepG2 cells. The transcriptional activity was assessed using a luciferase reporter gene assay. We found that the transcriptional activities of these 3 point variants, −49G>T,−70C>T, and −372C>T, were markedly reduced (0.32 ± 0.06, 0.46 ± 0.07, and 1.77 ± 0.05 arbitrary units, respectively) compared to that of the wild-type (5.93 ± 0.21, $P<0.001$ in all) (Fig. 2). When the level of *CETP* expression in the cells transfected with the wild-type construct was set at 100%, those of the three variants were 5.4%, 7.8%, and 29.8%, respectively.

3.2. Functional analysis of two novel missense variants in the LIPC gene

Two different novel missense variants of the *LIPC* gene, c.421A>G (p.G141S) and c.517G>A (p.V173M), were also found in 2 HALP subjects (Table 2). Both of the two probands had low plasma hepatic lipase activities. In order to confirm the functional changes of these two variants, we expressed them in HepG2 cells and analyzed hepatic lipase activities in both the media and the cell lysates. The results are shown in Fig 3. Cells transfected with the wild-type *LIPC* cDNA contained significant amount of hepatic lipase activity both intracellularly and in the culture media. However, hepatic lipase activity in the lysates of cells transfected with the G141S cDNA was 41.4% of that of the wild-type

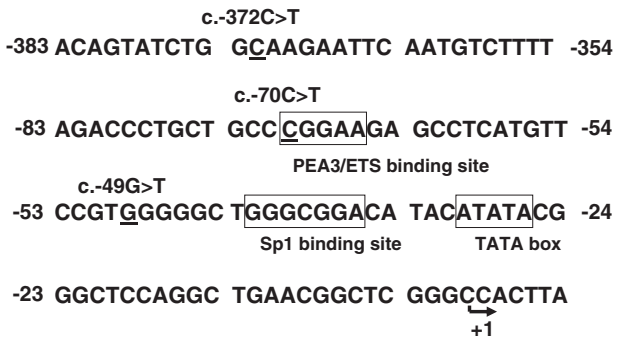

Fig. 1. Partial nucleotide sequence of the promoter region of the human *CETP* gene. +1 indicates the transcriptional start site. The three novel variants are indicated by the bold underline.

Table 2
Clinical characteristics of subjects who had rare variants in the *CETP* promoter and the *LIPC* gene.

Subject	Age (yr)	Sex	Gene	Variants	HDL-C level (mg/dL)	CETP activity (pmol/μL/h)	Hepatic lipase activity (nmol/mL/min)
1	60	Female	*CETP*	−49G>T	122	33	NA
2	66	Female	*CETP*	−70C>T	108	32	NA
3	67	Male	*CETP*	−70C>T, p.D459G[a]	111	14	NA
4	56	Female	*CETP*	−372C>T	117	12	NA
5	73	Female	*LIPC*	c.421A>G (p.G141S)	137	NA	25
6	38	Female	*LIPC*	c.517G>A (p.V173M)	151	NA	131
Control [b]	57±2	–	–	–	65±3	44±3	227±16

NA: not applicable.
[a] Subject 3 is a compound heterozygote for −70C>T and p.D459G (rs2303790).
[b] n=38 [3].

LIPC cDNA (0.75±0.09 vs. 1.81±0.12 mU/plate, $P<0.05$). Similarly, hepatic lipase activity in the media of cells transfected with the G141S cDNA was only 15.6% of that of the wild-type *LIPC* cDNA (1.22±0.09 vs. 7.84±0.58 mU/plate, $P<0.05$).

For the V173M variant, hepatic lipase activity in the lysates of cells transfected with the V173M cDNA was 46.4% of that of the wild-type *LIPC* cDNA (0.84±0.05 vs. 1.81±0.12 mU/plate, $P=0.05$). In addition, hepatic lipase activity in the media of cells transfected with the V173M cDNA was only 15.1% of that of the wild-type *LIPC* cDNA (1.18±0.02 vs. 7.84±0.58 mU/plate, $P<0.05$).

4. Discussion

HALP is a heterogeneous condition resulting from interactions between various genetic and environmental factors. In Asians, genetic variants in *CETP* and *LIPC*, which encode CETP and hepatic lipase, are frequently associated with HALP [5,9,10]. In contrast, variants in *CETP* are quite rare in Caucasians with HALP [11] and variants in *LIPG*, which encodes endothelial lipase, are more common [12]. High levels of angiopoietin-like protein 3, a known inhibitor of endothelial lipase [13], are also found in Asians with HALP [14].

CETP is an important protein in HDL metabolism and genetic variants in the *CETP* gene have been associated with HDL-C levels [15,16]. A number of variants in the coding region of the *CETP* gene have been found in subjects with HALP [9,16], but only a few reports have documented genetic variants in the *CETP* promoter associated with HALP [4,17]. Nagano et al. first reported a proband who had a point variant at position −69 (−69>A) along with reduced CETP activity and marked HALP [17]. Our group recently identified a deletion variant in the *CETP* promoter in a proband who had CETP deficiency and HALP [4]. Using a resequencing approach in a larger group of subjects with HALP, three novel point variants, −49G>T,−70C>T, and −372C>T, were subsequently found in the *CETP* promoter but their significance was unclear [5]. In the present study, we provided evidence that each of the three point variants identified was associated with a marked reduction in transcriptional activity, a reduced plasma CETP activity, and HALP.

Binding sites for a number of transcription factors have been identified in the promoter region of the human *CETP* gene [6,18,19], therefore, point variations at the *CETP* promoter might affect binding to various transcription factors (Fig. 1). For example, position −49 is located a few nucleotides upstream from the Sp1 binding site, therefore, a point variant at position −49 might affect binding of Sp1 at this site. It has been shown that both Sp1 and Sp3 act as coactivators at the -39 site [6]. A binding site for PEA3/ETS transcription factor is located further upstream in the -66 to -70 segment of the *CETP* gene promoter. A point variant at position −69 has been associated with a marked reduction in transcriptional activity in vitro [17]. Our experiment with a variant construct at position −70 showed a remarkably similar result, suggesting that a point variant at position −70 might affect binding to PEA3/ETS transcription factors as well. For the −372 point variant, it is still not apparent which transcription factor is involved. ARP-1 is a nuclear hormone receptor that binds to positions −93 to −118, but deletion of nucleotides -636 to -300 abolished transcriptional induction by ARP-1, suggesting that ARP-1 might interact with other factors upstream from position -300 to activate transcription [18]. Collectively, our result suggests that different point variants in the human *CETP* promoter affect binding to various transcription factors/nuclear hormone receptors, resulting in different degrees of reduction in the transcriptional activity.

Hepatic lipase is another key enzyme in lipid metabolism. Low levels of plasma hepatic lipase activity are found in subjects with HALP

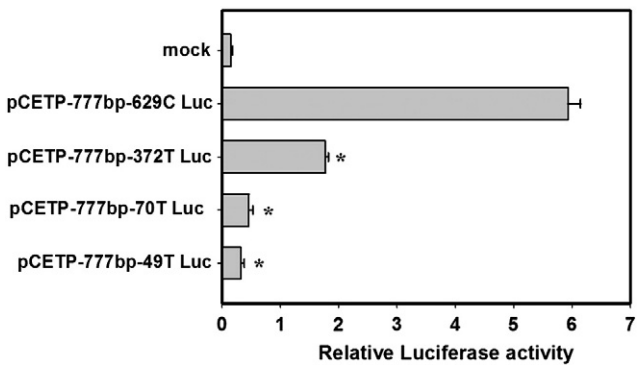

Fig. 2. Transcriptional activity in HepG2 cells transfected with *CETP* promoter/reporter gene constructs. HepG2 cells were transiently transfected with wild-type or variant constructs. Luciferase activity was measured in the cell lysates and was normalized to pRL-TK luciferase. Each value represents the mean±SEM of four separate transfections, *: $P<0.001$ vs. −629C.

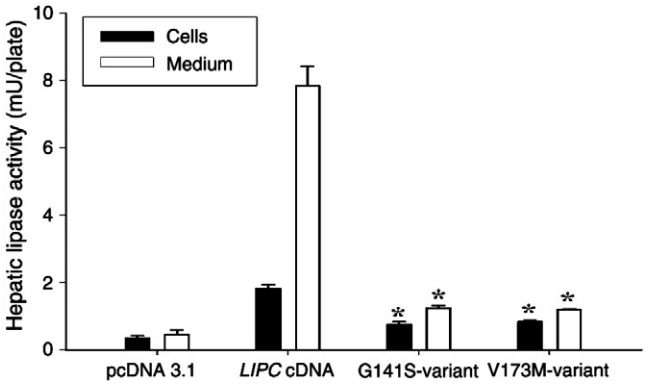

Fig. 3. Hepatic lipase activity in the cell lysates and in the media after expression of the G141S variant and the V173M variant in the HepG2 cells. Hepatic lipase activity (mU/plate) was measured in the cell lysates (black columns) and in culture medium (white columns). Each value represents the mean±SEM of three measurements. *: $P<0.05$ vs. the wild-type *LIPC* DNA.

[3,20,21] and mutations in the *LIPC* gene have been observed in these subjects [3,22,23]. We previously identified two novel missense variants in the *LIPC* gene, c.421A>G (p.G141S) and c.517G>A (p.V173M), in subjects with HALP and both of them were predicted to be functionally damaging by several bioinformatic programs [5]. Our current study provided further proof that these two missense variants were associated with a reduction in hepatic lipase activity when transiently expressed in vitro. Both a glycine residue at position 141 and a valine residue at position 173 are strictly conserved across different animal species and among related proteins in the lipase superfamily, and they are located adjacent to the serine residue at position 168, which is part of the Ser-Asp-His catalytic triad [24]. Although hepatic lipase activities in the cell lysates and media were markedly reduced to the similar extent in cells expressing these two missense variants (Fig. 3), the reduction in plasma hepatic lipase activity of the proband harboring the c.421A>G (p.G141S) variant was much more pronounced compared to that of the c.517G>A (p.V173M) variant (Table 1). This result suggests that other factors, either genetic or environmental, may play an additional role in determining hepatic lipase activity in the circulation [25]. For example, abnormally high levels of angiopoietin-like protein 3 have been found in subjects with HALP [14] and angiopoietin-like protein 3 is known to be a strong inhibitor of hepatic lipase in vivo [26].

In conclusion, our present work provides evidence that the recently-identified variants in the *CETP* promoter and in the *LIPC* gene are associated with low *CETP* promoter and hepatic lipase activities in vitro and suggest that these variants may be responsible for HALP in our subjects.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgments

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