Two novel missense mutations in the CETP gene in Japanese hyperalphalipoproteinemic subjects: high-throughput assay by Invader® assay

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Abstract **Cholesteryl ester transfer protein (CETP) deficiency is one of the most important and common causes of hyperalphalipoproteinemia (HALP) in the Japanese. CETP deficiency is thought to be a state of impaired reverse cholesterol transport, which may possibly lead to the development of atherosclerotic cardiovascular disease despite high HDL-cholesterol (HDL-C) levels. Thus, it is important to investigate whether HALP is caused by CETP deficiency. In the present study, we identified two novel missense mutations in the CETP gene among 196 subjects with a marked HALP (HDL-C ⩾ 2.59 mmol/l = 100 mg/dl). The two missense mutations, L151P (CTC→CCC in exon 5) and R282C (CGC→TGC in exon 9), were found in compound heterozygous subjects with D442G mutation, whose plasma CETP levels were significantly lower when compared with those in D442G heterozygous subjects. In COS-7 cells expressing the wild type and mutant CETP, these two mutant CETP showed a marked reduction in the secretion of CETP protein into media (0% and 39% of wild type for L151P and R282C, respectively). These results suggested that two novel missense mutations cause the decreased secretion of CETP protein into circulation leading to HALP. By using the Invader® assay for seven mutations, including two novel mutations of the CETP gene, we investigated their frequency among 466 unrelated subjects with HALP (HDL-C ⩾ 2.07 mmol/l = 80 mg/dl). Two novel mutations were rare, but L151P mutation was found in unrelated subjects with a marked HALP. Furthermore, we demonstrated that CETP deficiency contributes to 61.7% and 31.4% of marked HALP and moderate HALP in the Japanese, respectively.**—Nagano, M., S. Yamashita, K-i. Hirano, M. Ito, T. Maruyama, M. Ishihara, Y. Sagehashi, T. Oka, T. Kujiraoka, H. Hattori, N. Nakajima, T. Egashira, M. Kondo, N. Sakai, and Y. Matsuzawa. **Two novel missense mutations in the CETP gene in Japanese hyperalphalipoproteinemic subjects: high-throughput assay by Invader® assay.** ***J. Lipid Res.*** **2002.** 43: **1011–1018.**

Supplementary key words cholesteryl ester transfer protein deficiency • HDL-cholesterol • genotyping

Reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis, in which small HDL or free apolipoprotein (apo)A-I removes cholesterol from the peripheral cells and delivers it to the liver (1, 2). We have investigated the molecular mechanisms of RCT by analyzing the pathophysiology of subjects with abnormal HDL metabolism and clarified the physiological significance of plasma cholesteryl ester transfer protein (CETP) and hepatic triglyceride lipase in human RCT (3, 4). In this system, CETP is known to facilitate the transfer of cholesteryl ester (CE) from HDL to apoB-containing lipoproteins, and is one of the major determinants of plasma HDL-cholesterol (HDL-C) levels in humans (5–8). This is true especially in the hypertriglyceridemic subjects (9). Furthermore, CETP affects not only plasma HDL-C levels but also the amount of small HDL particles, which may be more important for RCT (8, 10).

Genetic CETP deficiency is the most important and frequent cause of hyperalphalipoproteinemia (HALP) in the Japanese (11–21). Seven mutations have been demonstrated to cause HALP, including two common mutations: a G-to-A substitution at the 5′ splicing donor site of the intron 14 (Int14 +1 G→A) and a missense mutation of exon

Abbreviations: BPI, bactericidal permeability increasing protein; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FRET, fluorescent resonance energy transfer; HALP, hyperalphalipoproteinemia; MAb, monoclonal antibodies; RCT, reverse cholesterol transport.

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15 (D442G). The subjects with CETP deficiency show a variety of abnormalities in the concentration, composition, and function of both HDL and LDL (22–25). CETP deficiency is thought to be physiologically a state of impaired RCT, which may possibly lead to the development of atherosclerosis despite high HDL-C levels. However, the results of the current studies are somewhat contradictory (26–28). Several epidemiological studies have investigated the atherogenicity of CETP. The Omagari study (Akita Prefecture, Japan), where HALP subjects with the Int14 +1 G→A mutation of CETP gene are markedly frequent, has demonstrated that CETP deficiency was not associated with longevity, but instead may be atherogenic (26). Two other epidemiological studies were reported in a population with D442G mutation. One of them was performed in Japanese-American men living in Hawaii (27), the other in the Japanese population living in Kochi Prefecture, Japan (28). These two studies demonstrated similar results in that the prevalence of coronary heart disease was higher in subjects with normal HDL-C. However, the subjects with HALP, irrespective of the presence or absence of the CETP gene mutation, appear to be protected against atherosclerosis. Therefore, further analysis is essential for the complete understanding about atherogenicity of CETP deficiency.

The aim of our study is to investigate how much of the HALP is caused by CETP mutations, what these mutations are, and how they affect CETP function. Our previous study demonstrated that 120 subjects (61.2%) had at least one of the known mutations of the CETP gene among 196 unrelated Japanese subjects with a marked HALP (21). In the present study, we identified two novel missense mutations of the CETP gene among 196 subjects with a marked HALP. Furthermore, we applied the high-throughput assay, Invader® assay, for detection of the CETP gene mutations and investigated their frequency among 466 HALP subjects.

MATERIALS AND METHODS

Study subjects

The subjects in the current study were 466 unrelated Japanese subjects with HALP (HDL-C ≥ 2.07 mmol/l = 80 mg/dl) who were referred to the lipid clinic in Osaka University Hospital and Nakajima Clinic in Omagari City, Akita Prefecture, Japan (21). Among these subjects, 196 had a marked HALP (HDL-C ≥ 2.59 mmol/l = 100 mg/dl). Our previous study demonstrated that 120 subjects (61.2%) had at least one of the known mutations of the CETP gene among 196 unrelated Japanese subjects with a marked HALP (21). This study was approved by the ethical committee of Osaka University. Informed consent was obtained from all subjects.

Measurement of CETP mass and activity

The CETP mass concentration was measured by a sandwich ELISA with two monoclonal antibodies (MAb) specific to human CETP, JHC1, and JHC2, as previously described (21). The CETP activity was determined according to the method described by Kato et al. (29) with minor modifications (21).

Sequence analysis

Genomic DNA was isolated from whole blood with a QIAamp Blood Kit (Qiagen, Valencia, CA). The promoter region and each exon of the CETP gene were individually amplified by PCR (21). PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen). Gel-purified products were sequenced by an ABI Prism 377 DNA sequencer (Perkin-Elmer, Foster City, CA) using a BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer).

Site-directed mutagenesis and transfection of COS-7 cells

Mutagenesis of the CETP cDNA was carried out by using the Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The pCMV4/CETP expression vector was used as the template for PCR (16). The PCR reactions were set up according to the instructions of the manufacturer using the following thermal cycling conditions: 95°C for 1 min, 55°C for 1 min, and 68°C for 12 min. After PCR and *Dpn*I digestion of the parental dam-methylated template (2 h at 37°C), the mutant plasmids were transformed into XL1 blue *Escherichia coli* cells and resulting colonies were screened by sequencing analysis. The plasmids were digested with *Hind*III and *Sma*I and then ligated to the *Hind*III/*EcoR*V site of the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA). COS-7 cells were cultured in DMEM (Gibco BRL, Rockville, MD), supplemented with 10% FCS, 100 U/ml penicillin, and 100 μl/ml streptomycin in 5% CO_2 at 37°C. One day before the transfection procedure, COS-7 cells (2×10^5 cells/well) were plated onto 6-well plates (Iwaki Glass Tokyo, Japan). After 24 h of incubation, 1 μg of each plasmid was transfected into the cells by use of the Fugine 6 Transfect Reagent (Boehringer Mannheim, Germany) and then grown in the same medium for 48 h. The media were stored at −80°C after 10 min centrifugation at 800 *g*. Whole cell lysates were prepared by sonication with 150 μl of PBS, pH 7.4 containing 100 μg/ml Leupeptin, 1 μmol/l PMSF, and 4 mmol/l EDTA. The transfection experiment was carried out in duplicate and three times for each experiment.

Western blot analysis

The medium (1 ml) was incubated for 18 h at 4°C with anti-human CETP polyclonal antibodies (20 μg protein). Protein A Sepharose (20 μl) (Pharmacia, North Peapack, NJ) was then added to the mixture to form immune complex and incubated for 18 h at 4°C. After centrifugation, the precipitates were washed three times with 0.1 M sodium phosphate (pH 7.0), resuspended in SDS-PAGE sample buffer, heated to 95°C for 5 min, and then subjected to SDS-PAGE followed by Western blotting. The whole cell lysates (20 μg each) were also subjected to 5–20% SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) and Western blotting was performed by using horseradish peroxidase-labeled anti-rabbit CETP MAb, 14-8F, which cross-reacted with the peptide 451-473 of human CETP (30, 31). Detection was carried out by the chemiluminescence reagent (NEN Life Science Products, Inc., Boston, MA) according to manufacturer's protocol.

Detection of CETP gene mutations by Invader® assay

We applied the Invader® assay for screening two novel and five known mutations of the CETP gene. The Invader® assay combines structure-specific cleavage enzymes and a universal fluorescent resonance energy transfer (FRET) system (32–35). Primary probes and Invader oligonucleotide for each mutation were designed with Invader® Creator software to have theoretic annealing temperatures of 63°C and 77°C, respectively, using a nearest-neighbor algorithm on the basis of final probe and target concentrations. The primary probes and Invader oligonucleotides used to detect CETP gene mutations by Invader® assay are shown

TABLE 1. The oligonucleotide sequence of wild type, mutant, and Invader® probes with Invader® assay to detect mutations of the CETP gene

Mutation	Position	Nucleotide	Target	Probe	Sequence	Dye
−69 G→A	Promoter	G→A	Anti-sense	Wild	CGCGCCGAGGGGAAGAGCCTCATGTTC	RED
				Mutant	ATGACGTGGCAGACAGAAGAGCCTCATGTTCC	FAM
				Invader	GGCCAGGAAGACCCTGCTGCCCT	
L151P	Exon 5	T→C	Sense	Wild	CGCGCCGAGGAGCAGCTTATGGAAAGAC	RED
				Mutant	ATGACGTGGCAGACGGCAGCTTATGGAAAGAC	FAM
				Invader	ACTTACTCTCGCTCCCCTTGGAGATGCAGGT	
G181X	Exon 6	G→T	Anti-sense	Wild	CGCGCCGAGGGGACAGGTGAGTGAGG	RED
				Mutant	ATGACGTGGCAGACTGACAGGTGAGTGAGG	FAM
				Invader	TTCATCTCCTTCACCCTGAAGCTGGTCCTGAAGC	
R282C	Exon 9	C→T	Sense	Wild	CGCGCCGAGGGGCCATCCTGGAAAG	RED
				Mutant	ATGACGTGGCAGACAGCCATCCTGGAAAGC	FAM
				Invader	CGTCTCCCATCAGGCTGAGCATGAGGCT	
Int14 +1 G→A	Intron 14	G→A	Anti-sense	Wild	ATGACGTGGCAGACGTAAGTGTGGGCTGGA	FAM
				Mutant	CGCGCCGAGGATAAGTGTGGGCTGGA	RED
				Invader	GCTGTGGGCATCCCTGAGGTCATGTCTCT	
Int14 +3 T ins	Intron 14	T insertion	Anti-sense	Wild	CGCGCCGAGGTAAGTGTGGGCTGGAG	RED
				Mutant	ATGACGTGGCAGACTTAAGTGTGGGCTGGA	FAM
				Invader	CTGTGGGCATCCCTGAGGTCATGTCTCGC	
D442G	Exon 15	A→G	Anti-sense	Wild	CGCGCCGAGGACATCATCAACCCTGAGA	RED
				Mutant	ATGACGTGGCAGACGCATCATCAACCCTGAG	FAM
				Invader	TCATGAACAGCAAAGGCGTGAGCCTCTTCGT	

The underline indicates the flap sequences of primary probes.

in **Table 1**. The Invader® assay FRET detection plates were provided from Third Wave Technologies, Inc (Madison, WI), in which almost all the generic components of an Invader assay (Cleavase® enzyme, FRET probes, MOPS buffer, and polyethylene glycol) were dried together. The biplex format of the Invader® assay enables simultaneous detection of two DNA sequences in a single well. In brief, the probe/Invader®/$MgCl_2$ mixture was prepared by combining 3 μl of primary probe/Invader® mix and 5 μl of 22.5 mM $MgCl_2$ per reaction. The primary probes/Invader® mixture contained 3.5 μmol/l wild primary probe, 3.5 μmol/l mutant primary probe, 0.35 μmol/l Invader® oligonucleotide, and 10 mmol/l MOPS. Eight microliter of primary probe/Invader®/$MgCl_2$ mixture was added into 96 well plate. Seven microliters of 5 fmol/l synthetic target oligonucleotides, 10 μg/ml yeast tRNA (no target blank), and genomic DNA samples (≥15 ng/μl) were added, which were denatured by incubation at 95°C for 10 min. After 15 μl of mineral oil (Sigma, St. Louis, MO) were overlayed into all reaction wells, the plate was incubated isothermally at 63°C for 4 h in the DNA thermalcycler (PTC-200; MJ Research, Watertown, MA) and then kept at 4°C until fluorescence measurements. The fluorescent intensities were measured by the fluorescence microtiter plate reader (Cytoflour 4000; Applied Biosystems) with excitation, 485 nm/20 nm (Wavelength/Bandwidth) and emission, 530 nm/25 nm for FAM; excitation, 560 nm/20 nm and emission, 620 nm/40 nm for RED. The genotyping was analyzed by calculation with the ratios of net counts with wild primary probe to net counts with mutant primary probe. The genotypes based on the Invader® assay were compared with the previously obtained results using PCR-restriction fragment length polymorphism analysis with five known mutations among 196 marked HALP subjects (21). The concordance rate of these experiments was 100% for all mutations (980 genotypes). A typical result in the Invader® assay analyzed by the fluorescence signals with Fluore image analyzer (FLA-3000G; Fuji Photo Film, Co. Ltd., Tokyo, Japan) is shown in **Fig. 1**.

Analytical methods

Total cholesterol, triglyceride, and HDL-C in plasma were measured with an automated analyzer (Hitachi 7450) and commercial kits (Daiichi Pure Chemical Industries, Co. Ltd., Tokyo, Japan). LDL-C concentration was calculated according to the equation of Friedewald et al. (36). Plasma concentrations of apoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE were mea-

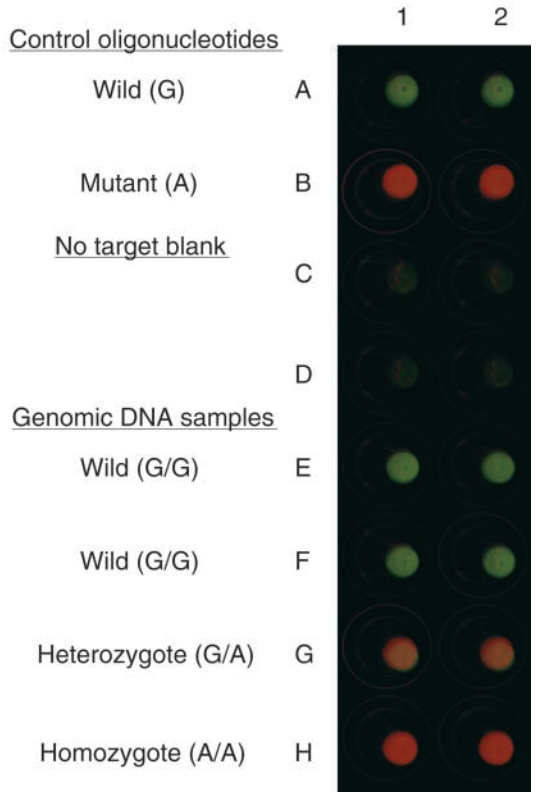

Fig. 1. Detection of CETP gene mutation by Invader® assay. A part of 96-well plate shows the results of the fluorescent signal with Image analyzer when the Int14 +1 G→A mutation was detected by Invader® assay. The wild type in green; mutant homozygote in red; mutant heterozygote in orange.

TABLE 2. Lipid profiles and plasma CETP levels in the HALP subjects with the novel mutations of the CETP gene

Mutation	L151P HE/ D442G HE (A)	L151P HE	R282C HE/ D442G HE (B)	D442G HE n = 84	D442G HO n = 12	Int 14 +1 G→A HE/ D442G HE n = 24	Controls n = 90
Sex	M	F	M	M/F 25/59	M/F 3/9	M/F 10/14	M/F 53/37
Age, years	50	47	42	57 ± 11[b]	55 ± 16[b]	52 ± 10[b]	32 ± 6
Total cholesterol, mmol/l	5.82	7.84	5.46	6.15 ± 1.16[b]	5.74 ± 0.57[b]	5.92 ± 1.03[b]	4.42 ± 0.59
HDL cholesterol, mmol/l	2.74	2.97	3.52	2.51 ± 0.36[b]	2.74 ± 0.49[b]	3.21 ± 0.80[b]	1.55 ± 0.34
Triglyceride, mmol/l	0.86	0.45	0.52	0.89 ± 0.51	0.82 ± 0.37	0.94 ± 0.45	1.03 ± 0.67
LDL cholesterol, mmol/l	2.69	4.65	1.71	3.23 ± 1.14[b]	2.66 ± 0.70	2.56 ± 0.98	2.48 ± 0.62
Apo A-I, mg/dl	218	ND	260	190 ± 37[b]	205 ± 26[b]	206 ± 52[b]	123 ± 16
Apo A-II, mg/dl	35.2	ND	46.7	35.4 ± 7.3	38.0 ± 9.8[a]	40.7 ± 10.4[b]	33.6 ± 5.1
Apo B, mg/dl	79	ND	60	93 ± 27[b]	77 ± 18	81 ± 25	79 ± 17
Apo C-II, mg/dl	3.9	ND	5.4	4.6 ± 1.3[b]	4.5 ± 1.3[a]	5.4 ± 1.6[b]	3.7 ± 1.2
Apo C-III, mg/dl	13.1	ND	15.1	12.0 ± 3.2[b]	11.5 ± 2.1[a]	15.0 ± 5.8[b]	8.9 ± 4.0
Apo E, mg/dl	4.5	ND	4.1	5.9 ± 2.2[b]	6.6 ± 2.2[b]	7.5 ± 2.9[b]	4.6 ± 0.9
CETP mass, μg/ml	0.4	1.2	0.9	2.0 ± 0.6[b]	1.2 ± 0.3[b]	0.6 ± 0.1[b]	2.4 ± 0.6
CETP activity, %	31	ND	40	86 ± 19[b]	61 ± 8[b]	29 ± 3[b]	100 ± 19

Values are expressed as mean ±SD. HE, heterozygote; HO, homozygote; L151P HE/D442G HE, compound heterozygote for L151P and D442G; R282C HE/D442G HE, compound heterozygote for R282C and D442G; Int 14 +1 G→A HE/D442G HE, compound heterozygote for Int14 +1 G→A and D442G;

ND, not determined.

[a] $P < 0.01$.

[b] $P < 0.001$ compared with control subjects.

sured by immunoturbidimetry method (Daiichi Pure Chemical Industries, Co. Ltd.).

RESULTS

Among 120 marked HALP subjects with known CETP gene mutations, the mean plasma CETP mass of 29 subjects (10 men and 19 women) with heterozygous D442G mutation were 2.1 ± 0.6 μg/ml (mean ± SD). Two heterozygous subjects with the D442G mutation, subject A and B, had a significantly lower plasma CETP mass level compared with the other D442G heterozygous subjects (0.4 μg/ml and 0.9 μg/ml, respectively). Their plasma CETP levels were similar to homozygotes with D442G or compound heterozygotes with Int14 +1 G→A and D442G (**Table 2**). This result indicated that these two subjects might have another unknown mutation in the CETP gene. Subsequently, 17 exons and the promoter region of the CETP gene were analyzed by direct sequencing. Sequence analysis of the CETP gene in the subject A had an additional novel substitution of T-to-C at codon 151 in exon 5, resulting in the amino acid change of Proline (CCC) for Leucine (CTC) (L151P) (**Fig. 2A**). Furthermore, the subject B had a novel substitution of C-to-T at codon 282 in exon 9, resulting in the amino acid change of Cysteine (TGC) for Arginine (CGC) (R282C) (Fig. 2B). No other sequence alteration except for I405V polymorphism was found in the coding regions and promoter regions of the CETP gene. Subjects A and B had the genotype of I/V and I/I for I405V polymorphism, respectively.

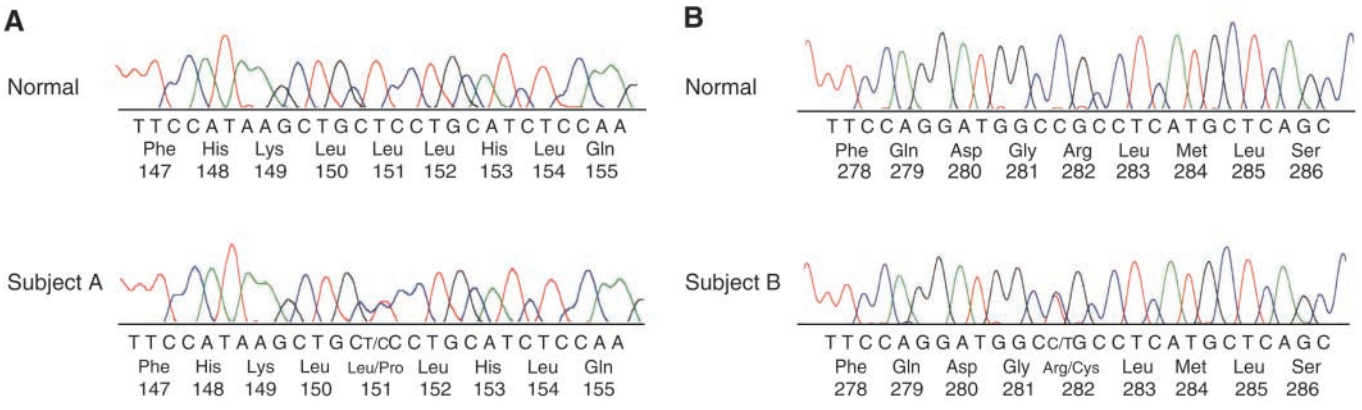

Fig. 2. Partial DNA sequence of the CETP gene from the subjects A and B and a normal control. A: In subject A, the sequence analysis revealed a heterozygous T-to-C substitution at codon 151 in exon 5, which replaced a codon for Leucine (CTC) with that for Proline (CCC). B: In subject B, the sequence analysis revealed a heterozygous C-to-T substitution at codon 282 in exon 9, which replaced a codon for Arginine (CGC) with that for Cysteine (TGC).

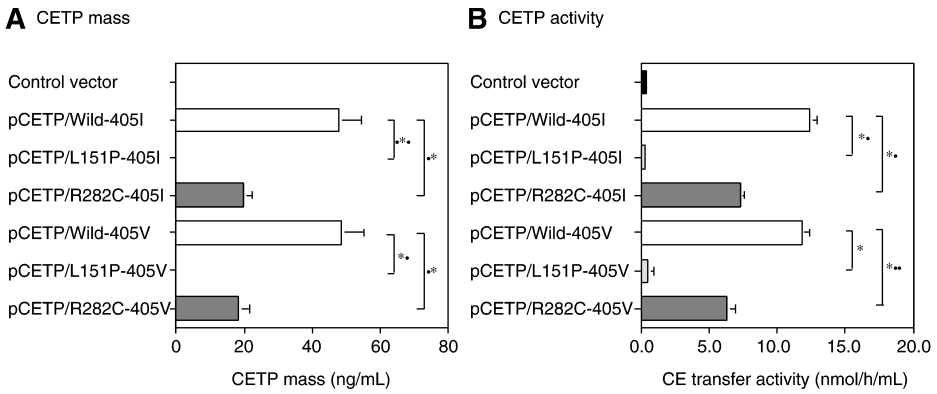

Fig. 3. CETP levels in culture media of COS-7 cells transfected with the CETP-gene constructs. The CETP plasmids as indicated were transfected into COS-7 cells by Fugine 6 transfect reagent. CETP mass (A) and CE transfer activity (B) were measured in the cell medium after 48 h transfection. Values represent the mean ± SD of three separate experiments. *$P < 0.001$ by unpaired t-test.

To ascertain how these two missense mutations influence CETP function, we constructed mutant CETP plasmids consisting of L151P and R282C mutants with each genotype for I405V polymorphism, and measured CETP mass and activity in the media from COS-7 cells transiently transfected with each mutant CETP (**Fig. 3**). In the medium in which each construct was transfected, CETP mass determined by ELISA showed undetectable CETP mass (less than 0.25 ng/ml) in the L151P mutant (Fig. 3A). In the R282C mutant, CETP mass was 19.6 ± 3.0 and 18.2 ± 3.1 ng/ml in the R282C with 405I genotype and with 405V genotype, respectively, and was significantly lower (41% and 38%, $P < 0.001$) than those of the wild type. CETP mass concentration was also confirmed by a commercial kit (CETP ELISA-DAIICHI, Daiichi Pure Chemical Industries, Co. Ltd.) (30) (data not shown). The CE transfer activity in the R282C mutant was significantly lower compared with wild type (7.3 ± 0.3 vs. 12.4 ± 0.5 nmol/h/ml and 6.4 ± 0.6 vs. 11.8 ± 0.6 nmol/h/ml for 405I genotype and 405V genotype, respectively, $P < 0.001$) (Fig. 3B).

We analyzed the expression levels of CETP protein in both media and whole cell lysates from the transfected COS-7 cells by Western blotting with anti-rabbit CETP MAb, 14-8F, which cross-reacted with the peptide 451-473 of human CETP (31). No CETP protein was detected in the media from the transfected cells with the L151P mutant, although a comparable expression of CETP protein was detected in the cells (**Fig. 4**). The expression level of CETP protein from the media with the R282C mutant was lower than that with the wild type. In the CETP expression in the media, similar results were obtained between CETP mass by ELISA and Western blot.

We screened the L151P and R282C mutations by the Invader® assay in 466 unrelated HALP (HDL-C ⩾ 2.07 mmol/l = 80 mg/dl) subjects, including 196 marked HALP subjects. We found a marked HALP subject with L151P mutation, but not R282C except for the probands (Table 2). The allele frequencies of L151P and R282C among 466 HALP subjects were 0.002 and 0.001, respectively. The CETP mass level in the heterozygous subject with L151P mutation was 1.2 μg/ml, and was similar to that with the heterozygous Int14 +1 G→A mutation, indicating this mutation was dominant negative (21). Furthermore, we screened five known mutations among 270 moderate HALP subjects. Eighty-five of 270 moderate HALP subjects had at least one of the known mutations. The allelic frequency of Int14 +1 G→A and D442G among these moderate HALP subjects was 0.063 and 0.135, respectively. Our present study demonstrated that 31.4% of moderate HALP would be caused by the mutation of the CETP gene.

DISCUSSION

Seven mutations in the CETP gene have so far been identified in the Japanese subjects with CETP deficiency, which include three splicing site, two nonsense, one pro-

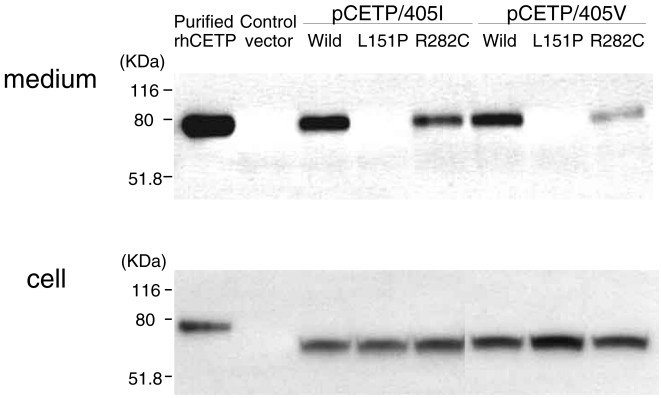

Fig. 4. Expression of transfected CETP-gene constructs in COS-7 cells. The media after immunoprecipitation and the whole cell lysates were subjected to Western blot analysis. The expressed CETP was detected by anti-rabbit CETP monoclonal antibody, 14-8F, which cross-reacted with the peptide 451–473 of human CETP.

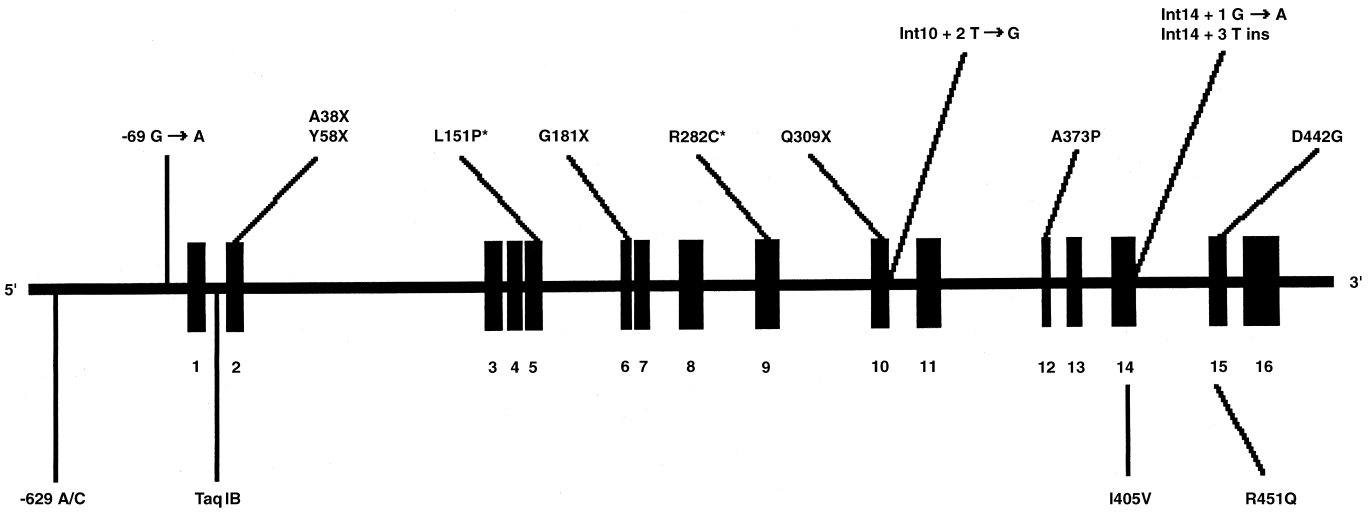

Fig. 5. Mutations and polymorphisms in the human CETP gene. *These mutations were identified in this study. The upper portion shows the mutations identified among hyperalphalipoproteinemic subjects. The lower shows the nucleotide substitutions and amino acid change reported to be functional polymorphism, which affected plasma CETP and/or HDL-cholesterol levels.

moter, and one missense mutation (**Fig. 5**) (11–21). In the present study, we identified two novel missense mutations, L151P and R282C, of the CETP gene in 196 unrelated Japanese subjects with a marked HALP (HDL-C ⩾ 2.59 mmol/l = 100 mg/dl). The transfection experiment demonstrated that these two missense mutations of the CETP gene cause the reduction of CETP protein to plasma leading to HALP. The L151P mutation is the first missense mutation of the CETP gene, which causes no secretion of CETP protein to the circulation.

The plasma CETP belongs to the lipid transfer/lipopolysaccharide binding protein family (LT/LBP), together with the phospholipids transfer protein (PLTP), the LBP, and the bactericidal permeability increasing protein (BPI) (37). Although these four proteins have different physiological functions, they have marked biochemical similarities. The three-dimensional model for CETP and PLTP has been built using the crystallographic data available for BPI (38, 39). They have an elongated, boomerang-shaped molecule with two lipid-binding pockets opening to its concave side. The Leucine at codon 151 is likely to be structurally important because that amino acid is a conserved homologous residue among LT/LBP family members, and located within one of the β-sheets (βB7) in the NH2-terminal domain (38, 39). The A373P mutation of the CETP gene has been identified in the HALP subjects from German (40). The Alanine at codon 373 is also located within the homologous residue and β-sheet (βE3), but is not within the binding pockets or on the concave face of the protein (38, 39). Arginine at codon 282, a positively charged residue, is thought to be in the vicinity of the C-terminal pocket and interacts with the phosphate group of a bound phospholipid (38). Arginine at codon 282 is likely to be structurally rather than functionally important because the specific activity of the R282C mutant was similar to that of the wild type in this study.

A common polymorphism of CETP protein at codon 405, I405V, is thought to be associated with altered levels of the protein and HDL-C (41, 42). Our results indicated that this amino acid change had no affect on the CETP protein level and its specific activity in the transfected cells, in agreement with other data that this polymorphism is in linkage dysequilibrium with a functional one (42). Its association between CETP mass and genotype may be due to a genetically linked polymorphism in the regulatory region, such as the −629 A/C polymorphism, which is located within the nuclear factor Sp1 and/or Sp3 binding site of the promoter region (43).

In the present study, we applied the high-throughput assay, Invader® assay, for detection of the CETP gene mutations, including two novel mutations. The Invader® assay could offer a simple diagnostic platform to detect mutations and single nucleotide polymorphisms (SNPs) with high specificity and high sensitivity, directly from unamplified genomic DNA in a homogeneous, isothermal, FRET-based format (32–35). The Invader assay is a useful and powerful tool to identify HALP with CETP deficiency. Together with our previous report (21), 206 of 466 HALP subjects had at least one of the known mutations and novel mutations in this study, suggesting that 44.2% of HALP would be caused by the mutations of the CETP gene in the Japanese. The frequency of two common mutations, Int14 +1 G→A and D442G, in the Japanese HALP, was 0.139 and 0.138, respectively. The elucidation of the molecular basis and pathophysiology of HALP other than CETP deficiency is an important issue for the future investigation.

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