# A novel nonsense mutation (G181X) in the human cholesteryl ester transfer protein gene in Japanese hyperalphalipoproteinemic subjects

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**Abstract** Cholesteryl ester transfer protein (CETP) plays an important role in regulating the concentration and composition of high density lipoprotein (HDL) and low density lipoprotein (LDL). Although several genetic abnormalities causing CETP deficiency have been identified in the Japanese subjects with a marked hyperalphalipoproteinemia (HALP), there are many CETP-deficient subjects for whom the genetic abnormalities have not been clarified. In the present study, we analyzed the molecular basis of an HALP subject without CETP activity and mass, and found a novel mutation in the CETP gene. This novel mutation (G181X) was a G-to-T substitution at codon 181 of exon 6 which replaced a codon for glycine (GGA) with a premature stop codon (TGA). The G181X mutation created a new cutting site by restriction enzyme MaeIII. To estimate the frequency of G181X, we investigated unrelated 294 HALP (HDL-cholesterol ≥2.59 mmol/L = 100 mg/dl) subjects by restriction fragment length polymorphism (RFLP) analysis with Mae III. One (0.34%) HALP subject was homozygous and four (1.36%) were heterozygous for this mutation. The allelic frequency of a G-to-T substitution at codon 181 of exon 6 was 0.0102 in HALP subjects. From the lipid analysis of the proband and the homozygote, it was clarified that the G181X mutation had dominant effects on HDL and LDL metabolism, similar to a G-to-A substitution at the 5′ splice donor site of the intron 14 (1451 + 1G → A). In conclusion, the G181X mutation is one of causes of HALP in the Japanese HALP subjects, having dominant effects on lipid metabolism.—**Arai, T., S. Yamashita, N. Sakai, K. Hirano, S. Okada, M. Ishigami, T. Maruyama, M. Yamane, H. Kobayashi, S. Nozaki, T. Funahashi, K. Kameda-Takemura, N. Nakajima, and Y. Matsuzawa.** A novel nonsense mutation (G181X) in the human cholesteryl ester transfer protein gene in Japanese hyperalphalipoproteinemic subjects. *J. Lipid Res.* 1996. **37:** 2145–2154.

**Supplementary key words** atherosclerosis • cholesteryl ester transfer protein deficiency • high density lipoprotein • mutation • reverse cholesterol transport

Cholesteryl ester transfer protein (CETP) is a plasma glycoprotein that catalyzes the transfer of cholesteryl ester (CE) from high density lipoprotein (HDL) to apolipoprotein (apo) B-containing lipoproteins and is one of the major determinants of plasma HDL-cholesterol levels (1, 2). Brown et al. (3) first identified the molecular basis of CETP deficiency in a Japanese subject with a marked hyperalphalipoproteinemia (HALP), and thereafter several laboratories including ours have reported that in Japan most of cases with familial HALP were associated with a genetic deficiency of plasma CETP (3–9). We showed that the lipoprotein abnormalities in CETP-deficient subjects were characterized by the presence of polydisperse low density lipoprotein (LDL) enriched with triglycerides (TG) and CE-rich large HDL particles (6, 7, 9). These results indicated that CETP plays an important role in modulating both quantity and quality of plasma lipoproteins. In addition, this protein is thought to play an important role in "reverse cholesterol transport" (10–12). In this system, HDL removes cholesterol from peripheral tissues followed by esterification of cholesterol by lecithin:cholesterol acyltransferase. CETP transfers the CE from HDL to apoB-containing lipoproteins. The CE transferred is finally removed from the circulation as a result of hepatic uptake via receptor-mediated and receptor-independent pathways.

It has been identified that a G-to-A substitution at the 5′ splice donor site of the intron 14 (1451 + 1G → A) and a missense mutation of exon 15 (D442G) in the

Abbreviations: apo, apolipoprotein; CETP, cholesteryl ester transfer protein; CE, cholesteryl ester; HALP, hyperalphalipoproteinemia; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PCR, polymerase chain reaction; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein.

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CETP gene are common mutations associated with HALP and that they are relatively frequent in the Japanese general population (4, 5, 8, 13). Although HALP caused by decreased CETP activity can be explained for the most part by these two common mutations, there are some CETP-deficient HALP subjects whose CETP gene abnormalities are still unknown. In the current study, we have identified a novel nonsense mutation in an HALP subject without CETP activity and mass. Furthermore, we investigated the effects of this novel mutation on the lipoprotein metabolism and its prevalence in the Japanese HALP subjects.

## METHODS

### Subjects investigated

We investigated an HALP subject whose plasma CETP activity and mass were both undetectable. The proband was a 62-year-old Japanese woman. Her serum total cholesterol (TC) level was moderately elevated (6.32 mmol/L), while her serum HDL-cholesterol level was markedly high (3.96 mmol/L). She had a mild hypertension (systolic blood pressure 162 mmHg and diastolic blood pressure 92 mmHg, respectively). She had corneal arcus, but did not have xanthomas. She had no signs of coronary atherosclerosis. She developed a transient hyperthyroidism at the age of 60, which was well treated with thiamazole (Mercazole®). Her father died of urinary bladder cancer at the age of 80. Her mother died of stroke at the age of 86. Other family members were generally well.

To estimate the prevalence of the novel nonsense mutation, we also investigated 294 (145 women and 149 men) unrelated HALP Japanese subjects including the proband, referred to our hospital with a diagnosis of HALP (HDL-cholesterol ≥2.59 mmol/L = 100 mg/dl). Patients with diseases that may affect HDL-cholesterol levels such as primary biliary cirrhosis, nephrotic syndrome, and thyroid dysfunction were excluded. Subjects who were consuming an excess amount of alcohol (≥80 g/day) or those who were taking corticosteroids were also excluded. We identified one homozygous subject with the novel mutation by this investigation. The homozygous subject was a 67-year-old man who had suffered from cerebral infarction at the age of 65. His serum HDL-cholesterol was markedly elevated at the first examination (4.30 mmol/L). We also analyzed the lipoprotein abnormalities associated with this novel mutation. Thirty normolipidemic healthy control subjects (15 women and 15 men) were investigated for comparison. Informed consent was obtained from the controls and HALP subjects.

### Determination of serum lipids and apolipoproteins

Venous blood was drawn after an overnight fast. Very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), LDL, $HDL_2$, and $HDL_3$ were isolated by sequential preparative ultracentrifugation at densities 1.006, 1.019, 1.063, 1.125, and 1.210 g/mL, respectively (14). The concentrations of TC, free cholesterol, TG, and phospholipid in serum and lipoprotein fractions were measured enzymatically using commercial kits (Determiner® TC5, FC-555, TGS-555, and PL, Kyowa Medex Co., Tokyo, Japan). The concentrations of CE were calculated as (total cholesterol - free cholesterol) × 1.68. HDL-cholesterol was measured by a heparin-$Ca^{2+}$ precipitation method (15). Serum concentrations of apoA-I, A-II, B, C-II, C-III, and E were measured by a single radial immunodiffusion method (16). Protein concentration was determined by the method of Lowry et al. (17).

### Nondenaturing polyacrylamide gradient gel electrophoresis

Ultracentrifugally separated LDLs and HDLs were analyzed by nondenaturing gel electrophoresis on 4% to 30% polyacrylamide gradient gels (Pharmacia-LKB, Uppsala, Sweden) (18). Electrophoresis was performed at 125V for 24 h at 4°C in Tris-borate buffer (0.09 mol/L Tris, 0.08 mol/L boric acid, 0.003 mol/L $Na_2EDTA$, pH 8.3). The gels were stained for 30 min in 50% methanol/9% acetic acid/0.1% Coomassie Brilliant Blue R-250 and destained with 20% methanol/9% acetic acid.

### Measurement of CETP activity and protein mass

Plasma CETP activity was measured according to the method of Kato et al. (19). Briefly, discoidal bilayer particles were used as CE donors, and LDL was used as the acceptor. The transfer of $^{14}C$-labeled cholesteryl oleate in discoidal bilayer particles to LDL was monitored after incubation for 30 min at 37°C with or without the addition of 3 μL plasma as a source of CETP. Discoidal bilayer particles and LDL were separated by sodium dextran sulfate and $MnCl_2$ precipitation, and the radioactivity in the supernatant and precipitates was then determined. The CETP activities were linearly increased within 30 min of incubation. CETP activity was expressed as the percent of control value. CETP mass was measured by an enzyme-linked immunosorbent assay of Sato et al. (20) using monoclonal antibodies raised against synthetic CETP peptide for capture antibody and enzyme-labeled monoclonal antibodies raised against purified human CETP for second antibodies. The assay was performed in the presence of 0.5% sodium dodecyl sulfate (SDS). Serum CETP mass levels correlated well with plasma CETP activity ($r = 0.77$, $P < 0.001$). The intra-assay coefficient of variance was 6–8%

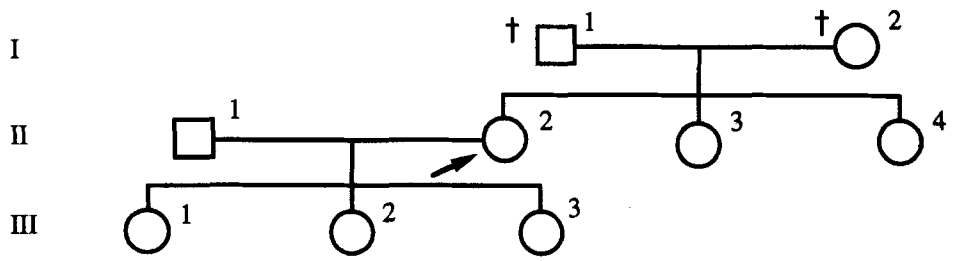
(in three different serum samples, six assays a day) and the inter-assay coefficient of variance was 9–12% (in three different serum samples, over 5 days).

### DNA analysis

Genomic DNA was isolated from peripheral leukocytes by the method of Kunkel et al. (21). Each exon of the CETP gene was amplified using paired synthetic oligonucleotides complementary to the human CETP gene sequence. The paired nucleotide sequences of oligonucleotide primers corresponded to 5′- and 3′-flanking intronic sequence of target exons, respectively. For subcloning procedures, cleavage sites for the restriction enzymes EcoRI and BamHI were incorporated into each primer. The primers used to amplify exon 6 were 6-A (5′-GCGGATCCCTCCATGGATGCACAGGACT-3′), which is located 36 nucleotides 5′ of exon 6 of the CETP gene and 6-B (5′-GCGAATTCGAGTGCCTCC-TTCCAGCTGC-3′), which is located 149 nucleotides 3′ of exon 6, generating a 309-bp fragment. Polymerase chain reaction (PCR) was conducted in an automated DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) with denaturation at 94°C for 2 min, annealing at 64°C for 1 min, and primer extension at 72°C for 2 min for a total of 30 cycles. Amplified DNA was digested with EcoRI and BamHI under the conditions recommended by the manufacturer (TAKARA Co., Kyoto, Japan). Restriction fragments were subjected to 2% Nu-Sieve GTG agarose (FMC Bioproducts, Rockland, ME) gel electrophoresis and the desired fragments were isolated by extraction with Wizard PCR Preps DNA Purification System (Promega Co., Madison, WI). The products were subcloned into PUC 19 vector. Several isolated clones were sequenced on both strands by the dideoxy chain termination method.

### Restriction fragment length polymorphism analysis

Genomic DNA was subjected to amplification by symmetrical PCR, using two primers for detection of a novel nonsense mutation in exon 6: primer 6-A and primer 6-C (5′-CAGGGAGTCAGCCAGCCTCA-3′), which is located 6 nucleotides 3′ of the exon 6 of the CETP gene. The novel nonsense mutation in exon 6 created a new restriction site for Mae III (Boehringer Mannheim Biochemica, Germany) and the PCR-amplified fragments of exon 6 had only one cleavage site. For detection of




| | I-1 | I-2 | II-1 | II-2 | II-3 | II-4 | III-1 | III-2 | III-3 | Controls |
|---|---|---|---|---|---|---|---|---|---|---|
| Sex | M | F | M | F | F | F | F | F | F | M/F 15/15 |
| Age (y) | 80 | 86 | 64 | 62 | 56 | 54 | 35 | 34 | 31 | 39 ± 9 |
| Total cholesterol * | 3.94 | 5.10 | 3.50 | 6.32 | 3.81 | 4.66 | 6.84 | 3.73 | 4.89 | 4.87 ± 0.80 |
| HDL-cholesterol * | 1.55 | 1.58 | 1.09 | 3.96 | 1.76 | 1.91 | 3.19 | 2.28 | 1.94 | 1.34 ± 0.34 |
| Triglyceride * | 0.87 | 1.16 | 1.10 | 0.79 | 0.51 | 0.70 | 0.79 | 0.44 | 0.93 | 0.99 ± 0.45 |
| Apo A-I ** | n.d. | 145 | 146 | 219 | 139 | 173 | 213 | 168 | 173 | 147 ± 13 |
| Apo A-II ** | n.d. | 29 | 29 | 35 | 28 | 37 | 37 | 33 | 35 | 32 ± 5 |
| Apo B ** | n.d. | 94 | 75 | 71 | 47 | 85 | 76 | 48 | 85 | 94 ± 22 |
| Apo C-II ** | n.d. | 2.8 | 3.0 | 3.7 | 1.5 | 3.0 | 4.1 | 2.0 | 4.0 | 3.2 ± 1.4 |
| Apo C-III ** | n.d. | 6.5 | 7.5 | 13.8 | 5.4 | 9.0 | 10.4 | 6.6 | 10.6 | 7.3 ± 2.3 |
| Apo E ** | n.d. | 6.9 | 2.9 | 9.1 | 4.3 | 4.1 | 5.0 | 4.3 | 4.4 | 4.0 ± 0.8 |
| CETP activity, % | n.d. | n.d. | 92 | 4 | 60 | 56 | 52 | 56 | 50 | 100 ± 20 |
| mass, μg/mL | n.d. | n.d. | 1.30 | 0.00 | 0.62 | 0.58 | 0.51 | 0.54 | 0.60 | 1.67 ± 0.68 |

* ; mmol/L, ** ; mg/dL. HDL, high density lipoprotein; Apo, apoprotein; CETP, cholesteryl ester transfer protein; n. d., not determined. The arrow shows the proband (II-2). Lipids and CETP levels of controls are represented as mean ± S.D.

**Fig. 1.** Pedigree of the proband's family and their lipid profiles and CETP levels. The upper panel shows the pedigree of the proband's family (the proband is indicated with an arrowhead). Table in the lower panel presents their lipid profiles and CETP levels.

intron 14 splicing defect, two primers were used as reported previously (5), primer 14-A (5′-CTTCTGTGCCTCCAGGGAGGACTCACCATGG-3′), which is located 15 nucleotides 5′ of exon 14 and primer 14-B (5′-GGCACCCAGTTTCCCCTCCAGCCCACACAT-3′), which is located 3 nucleotides 3′ of exon 14. Primer 14-B was designed to generate an Nde I endonuclease cleavage site, when one base at the 5′ splice donor site of intron 14 in the CETP gene is mutated from guanine (G) to adenine (A). PCRs for the two mutations were conducted with denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and primer extension at 72°C for 2 min for a total of 30 cycles. Amplified PCR products were precipitated and aliquots were digested with Mae III or Nde I, followed by electrophoresis in a gel containing 4% Nu-Sieve GTG-agarose and 1% standard agarose (TAKARA Co., Kyoto, Japan). After staining in ethidium bromide, DNA fragments were visualized on a standard UV transilluminator.

## RESULTS

In the current study, we first investigated a subject with a marked HALP whose CETP activity and mass were totally absent. **Figure 1** shows the pedigree of the proband's family, their lipid profiles, and CETP levels. The subject II-2 in Fig. 1 indicates the proband. As the father of the proband (I-1) had died of urinary bladder cancer, we could obtain only his serum lipid data. The proband and her daughter (III-1) had mild hypercholesterolemia, while their serum HDL-cholesterol was markedly elevated (3.96 and 3.19 mmol/L, respectively). Serum HDL-cholesterol levels of her father (I-1), mother (I-2), sisters (II-3, 4) and daughters (III-2, 3) were moderately elevated (1.55–2.28 mmol/L). CETP activity and mass levels of the proband and her family members were also measured as shown in Fig. 1. Plasma CETP activity and serum CETP mass levels of her sister (II-3, 4) and daughters (III-1, 2, 3) were approximately one-half of controls.

The molecular basis of CETP deficiency in the proband was analyzed by the PCR method. All of the 16 exons and the exon–intron boundaries of the CETP gene were sequenced using PCR-amplified DNA as a template. Sequence analysis of the CETP gene in the proband revealed a G-to-A substitution at the 5′ splice donor site of the intron 14 (1451 + 1G → A, data not shown) and a novel mutation, a G-to-T substitution at codon 181 in exon 6 (G181X) which replaced a codon for glycine (GGA) with a premature stop codon (TGA) (**Fig. 2**). The former mutation is one of the common mutations in the Japanese HALP subjects (4, 8). No other sequence alteration was found in the protein coding regions of the proband's CETP gene.

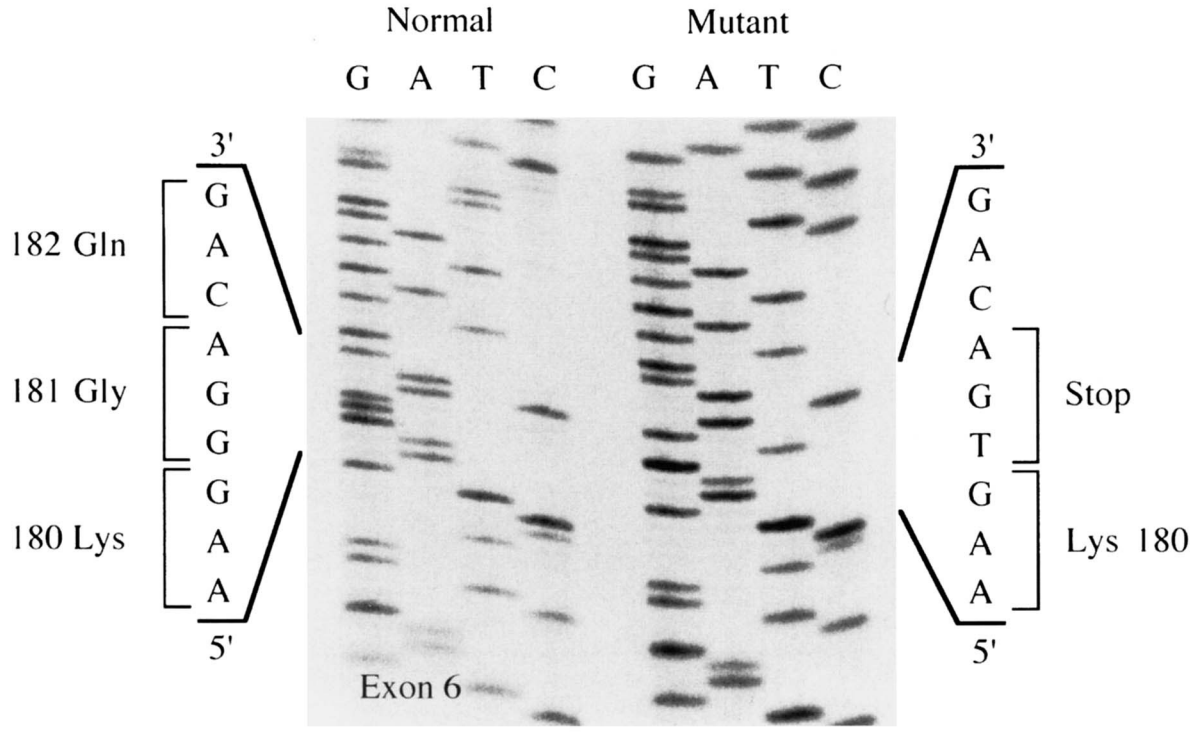



**Fig. 2.** DNA sequence of cloned CETP gene (exon 6) from the proband and a normal control. The sequence analysis revealed a G-to-T substitution at codon 181 in exon 6 which replaced a codon for glycine (GGA) with a premature stop codon (TGA).

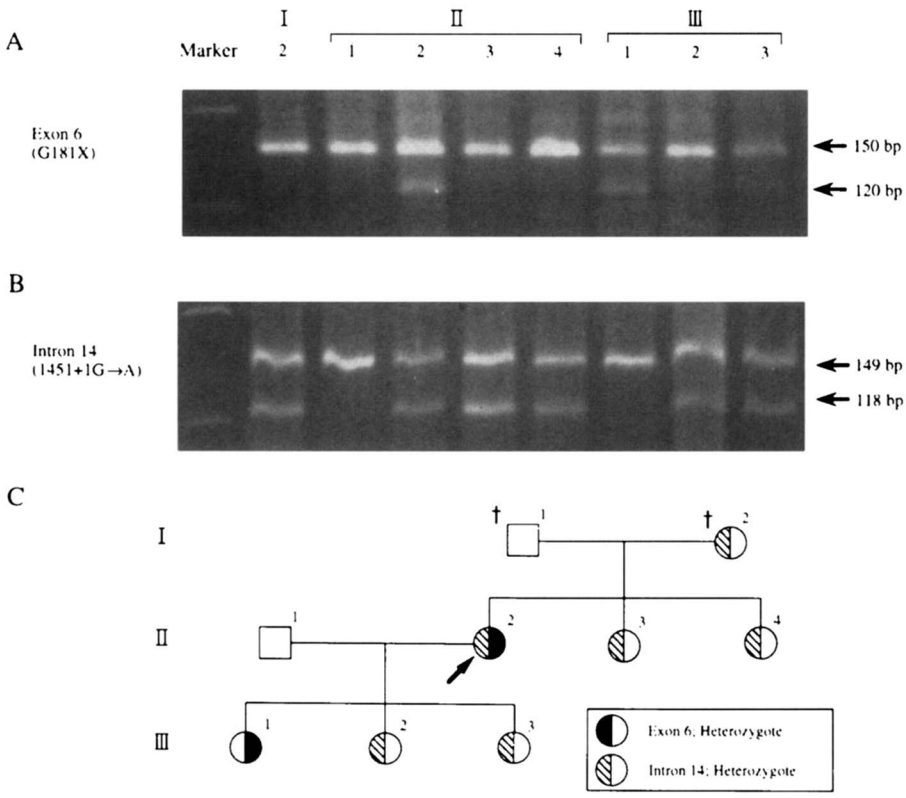



**Fig. 3.** (A) Five percent agarose gel electrophoretic patterns of PCR-amplified DNA in exon 6 and intron boundaries after digestion with Mae III. The nonsense mutation (G181X) created a new cutting site for restriction enzyme Mae III. In heterozygotes (G/T), two bands of 150 bp (normal allele) and 120 bp (mutant allele) were demonstrated. The proband (II-2) and her daughter (III-1) were heterozygotes for the nonsense mutation. (B) Five percent agarose gel electrophoretic patterns of PCR-amplified DNA in exon 14 and boundaries after digestion with Nde I. Normal allele gave a band of 149 bp and mutant allele (a G-to-A substitution at the 5′ splice donor site of intron 14, 1451 + 1G → A) gave a band of 118 bp. (C) A summary of G181X and 1451 + 1G → A mutations in the family. The proband was a compound heterozygote of G181X and 1451 + 1G → A. Her daughter (III-1) was a heterozygote of G181X. Her mother (I-2), sisters (II-3, 4) and the other two daughters (III-2, 3) were heterozygotes for 1451 + 1G → A.

**Figure 3** shows the results of an RFLP analysis of G181X and 1451 + 1G → A and summarizes the gene mutations in the proband's family. Figure 3-A shows the agarose gel electrophoretic patterns of PCR-amplified DNA after digestion with Mae III. The nonsense mutation (G181X) created a new cutting site for restriction enzyme Mae III. In this method, wild type DNA (G/G) from normal subjects gave a band of 150 bp. DNA from homozygotes (T/T) of this mutation gave a band of 120 bp. In heterozygotes (G/T), two bands of 150 bp (normal allele) and 120 bp (mutant allele) were detected. The agarose gel electrophoretic patterns demonstrated that the proband and her daughter (III-1) were heterozygotes of G181X. Figure 3-B shows the agarose gel electrophoretic patterns of PCR-amplified DNA after digestion with Nde I, of which method was previously described for detection of the common mutation at the intron 14 splice donor site (1451 + 1G → A) (3). All subjects in this family except for II-1 and III-1 were heterozygotes of 1451 + 1G → A. These results on the CETP gene mutations are summarized in Fig. 3-C. The proband was a compound heterozygote of G181X and 1451 + 1G → A. The daughter of the proband (III-1) was a heterozygote of G181X. Proband's mother, sisters and two daughters (III-2 and III-3) were heterozygotes of 1451 + 1G → A.

To evaluate the possibility that this novel mutation may be involved in the pathogenesis of HALP, we analyzed 294 unrelated HALP subjects, whose serum HDL-cholesterol levels were more than 2.59 mmol/L (= 100 mg/dL). As shown in **Table 1,** one subject (0.34%) was homozygous and three (1.36%) were heterozygous for G181X. The allelic frequency of G181X was calculated to be 0.0102 in HALP subjects. The homozygous subject was not in consanguineous marriage. **Table 2** shows the frequencies of the mutations in the exon 6, intron 14, and exon 15 (G181X, 1451 + 1G → A, and D442G, respectively) in 294 HALP subjects. The frequency of the intron 14 mutation was 29.6% in 294 HALP subjects (homozygote, 4.1% and heterozygote, 25.5%). The frequency of the exon 15 mutation was 21.1% (homozygote, 2.0% and heterozygote, 19.1%).

TABLE 1. Serum lipid levels and frequency of the exon 6 CETP gene mutation in HALP subjects

| | n | Serum Lipids | | | Frequency of Genotype | | | Allelic Frequency of T |
|---|---|---|---|---|---|---|---|---|
| | | Total Cholesterol | HDL-Cholesterol | Triglyceride | T/T | G/T | G/G | |
| | | | *mmol/L* | | | | | |
| HALP Subjects | 294 | 6.37 ± 1.48 | 3.13 ± 0.73 | 1.12 ± 1.09 | 1 | 4 | 289 | 0.0102 |

The exon 6 CETP gene mutation indicates a G-to-T substitution at codon 181 in exon 6. To evaluate the possibility that this novel mutation may be involved in the pathogenesis of hyperalphalipoproteinemia (HALP), we analyzed 294 unrelated HALP subjects, whose serum HDL-cholesterol level was more than 2.59 mmol/L (= 100 mg/dL). One subject (0.34%) was homozygous and four (1.36%) were heterozygous for G181X. The allelic frequency of G181X was calculated to be 0.0102 in HALP subjects.

The frequency of compound heterozygote for the intron 14 and exon 15 mutations was 5.4%. Two out of four heterozygotes of the exon 6 mutation also had the intron 14 mutation. One was the proband and the other was the Hetero (B) shown in **Table 3.**

For clarifying the influence of G181X mutation upon lipid metabolism, we investigated the lipoprotein profiles of the proband, the homozygous and three heterozygous subjects of G181X. Their serum lipids profiles are shown in Table 3. Serum HDL-cholesterol levels were markedly elevated (the proband, Homo, Hetero (A), (B), and (C) of the exon 6 mutation in Table 3; 3.96, 2.59, 2.59, 2.61, and 3.00 mmol/L, respectively). ApoA-I concentrations were increased in these subjects except for the Homo of exon 6. ApoC-III and E levels were elevated in the proband, Homo and Hetero (A) of exon 6 mutation. Their apoB levels tended to be decreased. The lipoprotein fraction analysis by sequential preparative ultracentrifugation demonstrated that the increases in HDL-cholesterol were attributed to the marked increment in $HDL_2$-cholesterol. CETP activity and mass in the Homo of exon 6 were absent. CETP activity of the Hetero (B) was 62% of control, although the Hetero (B) was detected to have both the exon 6 and intron 14 mutations. We speculate that these two mutations may exist on the same allele in the Hetero (B).

We also analyzed the changes in the particle size of their LDL and HDL as reported previously (6, 7, 9). **Figure 4** shows 4% to 30% polyacrylamide gradient gel electrophoretic patterns of plasma lipoproteins prepared by ultracentrifugation ($d < 1.210$ g/mL) in a homozygous subject of 1451 + 1G → A mutation, the proband (who was a compound heterozygote of G181X and 1451 + 1G → A), and the homozygote of G181X. LDL fractions of the proband and the homozygous subject were markedly polydisperse and consisted of several bands. The particle sizes of their HDL were much larger than those of normolipidemic controls. These data were compatible with the lipid profile in CETP-deficient subjects (1451 + 1G → A homozygotes) as reported previously (22).

## DISCUSSION

CETP deficiency is characterized by a marked HALP and abnormalities of all lipoprotein fractions including VLDL, LDL, and HDL (2–9). We reported that there are

TABLE 2. Frequency of the exon 6, intron 14, and exon 15 CETP gene mutations in HALP subjects

| | n | % | Exon 6 | | | Intron 14 | | | Exon 15 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | T/T (Homo) | G/T (Hetero) | G/G (Normal) | A/A (Homo) | G/A (Hetero) | G/G (Normal) | G/G (Homo) | A/G (Hetero) | A/A (Normal) |
| Exon 6 | | | | | | | | | | | |
| T/T (Homo) | 1 | 0.3 | 1 | | | 0 | 0 | 1 | 0 | 0 | 1 |
| G/T (Hetero) | 4 | 1.4 | | 4 | | 0 | 2 | 2 | 0 | 0 | 4 |
| G/G (Normal) | 289 | 98.3 | | | 289 | 12 | 73 | 209 | 6 | 56 | 227 |
| Intron 14 | | | | | | | | | | | |
| A/A (Homo) | 12 | 4.1 | 0 | 0 | 12 | 12 | | | 0 | 0 | 12 |
| G/A (Hetero) | 75 | 25.5 | 0 | 2 | 73 | | 75 | | 0 | 16 | 59 |
| G/G (Normal) | 207 | 70.4 | 1 | 2 | 204 | | | 207 | 6 | 40 | 161 |
| Exon 15 | | | | | | | | | | | |
| G/G (Homo) | 6 | 2.0 | 0 | 0 | 6 | 0 | 0 | 6 | 6 | | |
| A/G (Hetero) | 56 | 19.1 | 0 | 0 | 56 | 0 | 16 | 40 | | 56 | |
| A/A (Normal) | 232 | 78.9 | 1 | 4 | 227 | 12 | 59 | 161 | | | 232 |

We investigated the frequencies of the mutations in the exon 6, intron 14, and exon 15 (G181X, 1451 + 1G → A, and D442G, respectively) in 294 hyperalphalipoproteinemic (HALP) subjects. The frequency of the intron 14 mutation was 29.6% in 294 HALP subjects (homozygote, 4.1% and heterozygote, 25.5%). The frequency of the exon 15 mutation was 21.1% (homozygote, 2.0% and heterozygote, 19.1%). The frequency of compound heterozygote for the intron 14 and exon 15 mutations was 5.4%. Two out of four heterozygotes of the exon 6 mutation also had the intron 14 mutation.

TABLE 3. Lipid profiles and CETP levels in the proband, homozygote, and heterozygotes for the exon 6 CETP gene mutation and homozygote for the intron 14

| | Exon 6 | | | | | Intron 14 | |
|---|---|---|---|---|---|---|---|
| | Proband | Homo | Hetero (A) | Hetero (B) | Hetero (C) | Homo | Control |
| Sex | F | M | F | F | M | M/F 4/1 | M/F 15/15 |
| Age (y) | 62 | 67 | 58 | 30 | 27 | 47 ± 7 | 39 ± 9 |
| Total cholesterol, mmol/L | 6.32 | 4.81 | 8.21 | 4.79 | 7.07 | 7.66 ± 1.28 | 4.87 ± 0.80 |
| HDL-cholesterol, mmol/L | 3.96 | 2.59 | 2.59 | 2.61 | 3.00 | 6.00 ± 1.30 | 1.34 ± 0.34 |
| Triglyceride, mmol/L | 0.79 | 1.07 | 1.05 | 0.58 | 0.68 | 1.82 ± 1.38 | 0.99 ± 0.45 |
| ApoA-I, mg/dL | 219 | 165 | 180 | 180 | n.d. | 222 ± 31 | 147 ± 13 |
| ApoB, mg/dL | 71 | 50 | 82 | 66 | n.d. | 66 ± 10 | 94 ± 22 |
| ApoC-II, mg/dL | 3.7 | 6.4 | 8.4 | 3.0 | n.d. | 8.2 ± 2.6 | 3.2 ± 1.4 |
| ApoC-III, mg/dL | 13.8 | 14.5 | 16.1 | 8.5 | n.d. | 28.1 ± 12.5 | 7.3 ± 2.3 |
| ApoE, mg/dL | 9.1 | 6.7 | 6.5 | 4.9 | n.d. | 13.5 ± 6.6 | 4.0 ± 0.8 |
| LDL cholesterol, mmol/L | 2.20 | 1.85 | n.d. | n.d. | n.d. | 2.66 ± 1.14 | 2.74 ± 0.73 |
| $HDL_2$ cholesterol, mmol/L | 3.49 | 2.20 | n.d. | n.d. | n.d. | 4.25 ± 1.16 | 1.12 ± 0.38 |
| $HDL_3$ cholesterol, mmol/L | 0.47 | 0.39 | n.d. | n.d. | n.d. | 0.70 ± 0.26 | 0.49 ± 0.13 |
| $HDL_2$ chol./$HDL_3$ Chol. ratio | 7.43 | 5.64 | n.d. | n.d. | n.d. | 6.64 ± 2.39 | 2.29 ± 2.92 |
| CETP activity, % | 4 | 6 | 70 | 62 | n.d. | 5 ± 3 | 100 ± 20 |
| CETP mass, μg/mL | 0.00 | 0.00 | 1.20 | n.d. | n.d. | 0.00 ± 0.00 | 1.67 ± 0.68 |

For clarifying the influence of G181X mutation upon lipid metabolism, we investigated the lipoprotein profiles of the proband, the homozygous and three heterozygous subjects of G181X. Serum HDL-cholesterol levels were markedly elevated in the subjects with exon 6 mutation. ApoA-I concentrations were increased in these subjects except for the Homo of exon 6. ApoC-III and E levels were elevated in the proband, Homo and Hetero (A) of exon 6. Exon 6 indicates a G-to-T substitution at codon 181 in exon 6 (G181X) and Intron 14, a G-to-A substitution at the 5′-splice donor site of intron 14 (1451 + 1G → A). Data are shown as mean ± SD; n.d., not determined.

two common and prevalent mutations in the CETP gene in the Japanese HALP subjects (8, 13). One is a G-to-A substitution at the 5′-splice donor site of intron 14 (1451 + 1G → A) and the other an A-to-G mutation in the exon 15 which substitutes glycine for aspartic acid (D442G) (3, 23). In the current study, a novel mutation in the CETP gene has been identified. This mutation was a G to T substitution at codon 181 in exon 6 (G181X) which replaced a codon for glycine (GGA) with a premature stop codon (TGA). CETP activity and mass were undetectable in a homozygous subject of G181X and a compound heterozygous subject with G181X and 1451 + 1G → A. These subjects had lipid profiles similar to those of homozygous CETP deficiency with 1451 + 1G → A. These results suggested that the G181X mutation caused a total absence of CE transfer from HDL to apoB-containing lipoproteins. We have also examined the frequency of G181X in HALP patients. As the G181X mutation creates a new cutting site for restriction enzyme Mae III, we investigated the frequency of G181X in 294 HALP subjects by RFLP analysis with Mae III. One (0.34%) homozygote and four (1.36%) heterozygotes were detected. The allelic frequency of G181X was 0.0102 in HALP subjects. Therefore, this mutation may also be one of the abnormalities in the CETP gene causing a marked elevation of HDL-cholesterol, although its frequency was rather low compared with 1451 + 1G → A and D442G mutations.

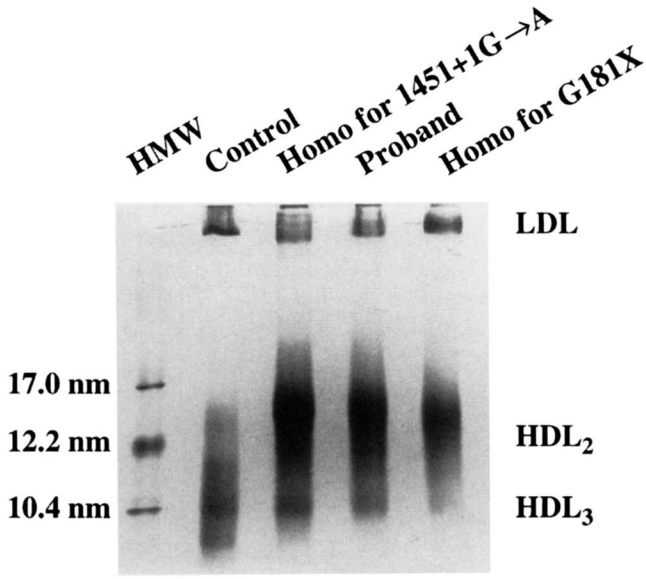



**Fig. 4.** Four percent to 30% nondenaturing polyacrylamide gradient gel electrophoresis patterns of plasma lipoprotein preparated by ultracentrifugation (d < 1.210 g/mL) in a homozygote of 1451 + 1G → A, the proband who was a compound heterozygote of G181X and 1451 + 1G → A, and a homozygote of G181X. Polydisperse LDLs and large HDL, similar to those observed in a homozygote of 1451 + 1G → A, were present in the proband and the homozygote of G181X.

A number of gene mutations have been reported in disorders of lipid metabolism such as familial hypercholesterolemia, lecithin:cholesterol acyltransferase deficiency, and lipoprotein lipase deficiency. However, only a few mutations in the CETP gene have been reported so far. Since Brown et al. (3) first reported in 1989 a CETP-deficient family associated with 1451 + 1G → A, a D442G mutation and a nonsense mutation at codon 309 in exon 10 (G309X) have been reported (23, 24). Inazu et al. (5) reported a family with an intron 14 splice donor site mutation caused by a T insertion at the position +3 from the exon 14/intron 14 boundary (1451

+ 3insT). Hirano et al. (8) reported that the frequency of heterozygotes of 1451 + 1G → A was approximately 0.98% in the Japanese general population and that the allelic frequency of 1451 + 1G → A was estimated to be 0.0049. Recently, Sakai et al. (13) reported that approximately 31% of HALP subjects, who do not possess the 1451 + 1G → A mutation, were heterozygotes or homozygotes for the D442G mutation. In the current study, the allelic frequency of the novel G181X mutation in HALP subjects was 0.0102. To date, CETP deficiency has been found mostly in Japan. However, Funke et al. (25) have recently reported one individual with two missense mutations (A373P; R451Q) and another proband with a premature stop codon due to a cytosine deletion in codon 38 of the CETP gene in 30 German individuals with HDL-cholesterol concentrations above the 95th percentile. These results indicate that CETP deficiency is distributed not only in Japan but in the other areas of the world. The analysis of CETP mutations in different populations may contribute to the identification of anthropogenetic origins of each population.

In the current study, we demonstrated a novel mutation of the CETP gene markedly affecting the lipoprotein profiles. As CETP consists of 476 amino acids, CETP gene with G181X may form CETP polypeptides of only one third in size. Plasma CETP activity and serum CETP mass were undetectable in the proband who was a compound heterozygote of G181X and 1451 + 1G → A and in the homozygous subject. Their serum HDL-cholesterol levels were markedly increased, particularly in the $HDL_2$ fraction. Polydisperse LDLs and large HDL that are observed in homozygous CETP deficiency with 1451 + 1G → A mutation were similarly demonstrated in the proband and the homozygous subject of G181X on polyacrylamide gradient gel electrophoresis (6, 7, 9). CETP polypeptide produced by the CETP gene with D442G mutation had a reduced specific activity (23). Tall (12) reported that the catalytic site involved in CE transfer exists in the C-terminal domain. Therefore, the short polypeptides produced by the G181X mutation are not likely to have CE transfer activity even if it is present in plasma.

The relationship of CETP to atherosclerosis is complex and still controversial. Some investigators have considered that the defect of CETP may be a beneficial state, as plasma lipid profiles in CETP deficiency are characterized by high plasma HDL-cholesterol and relatively low LDL-cholesterol levels (4). Marotti et al. (26) reported an accelerated atherosclerosis in CETP transgenic mice. However, CETP was also thought to play an important role in the "reverse cholesterol transport", which is one of the major protective systems against atherosclerosis (7–12). As we reported, LDL and HDL of CETP-deficient subjects are abnormal in their composition and function (6, 7, 9). Large and CE-rich HDL in homozygous CETP deficiency could not prevent macrophages from accumulating cholesterol induced by acetylated LDL nor remove cholesterol from lipid-laden macrophages (7, 27). Small and polydisperse LDL in CETP deficiency had a reduced binding affinity for LDL receptor in fibroblasts (6, 7, 28). We previously reported two cases associated with corneal opacity, coronary heart disease, and low hepatic triglyceride lipase activity and mass (10, 29). One of these cases has been identified as a homozygote of the D442G mutation (13). Recently, we have reported that CETP-deficient patients with low hepatic triglyceride lipase activity were closely associated with atherosclerotic cardiovascular disease including coronary heart disease (30, 31). CETP and hepatic triglyceride lipase were reported to cooperate to form very small HDL particles which actively removes cholesterol from lipid-laden macrophages (32). Moreover, we reported that the decrease in serum HDL-cholesterol was closely correlated with the reduction in Achilles tendon thickness in patients with familial hypercholesterolemia during the treatment with probucol, which raises plasma CETP levels (10, 33). Furthermore, in the CETP transgenic mice mated with hypertriglyceridemic apoC-III transgenic mice, a reduction in the atherosclerotic lesions in the aorta was observed (34). These results suggest that the lipoprotein changes resulting from the deficiency of CETP activity may not be a desirable state for preventing atherosclerosis. Therefore, further investigations should be made to elucidate the relationship of CETP and atherosclerosis from a view point of the "reverse cholesterol transport".

The authors thank Dr. Sato (Eisai Co., Tsukuba, Japan) for measuring the protein mass of CETP. The skillful technical assistance of Mrs. Mayumi Koyama is gratefully acknowledged. Supported by grants from the Japanese Ministry of Education (No. 4404085 and 3557117), a Research Grant for the Adult Disease and HMG-CoA Reductase Research Fund from Sankyo Pharmaceutical Co., Ltd.

*Manuscript received 7 June 1996 and in revised form 1 July 1996.*

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