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Novel mutations of cholesteryl ester transfer protein (CETP) gene in Japanese hyperalphalipoproteinemic subjects

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Abbreviations: CETP, cholesteryl ester transfer protein; HALP, hyperalphalipoproteinemia;

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Abstract

Background: The half of hyperalphalipoproteinemia (HALP) in Japan is caused by CETP gene mutations. Other than two prevalent mutations (D442G and Intron 14 splicing donor site +1 G>A), some rare CETP mutations are found in Japanese HALP subjects.

Methods: CETP gene analysis of genomic DNA from subjects was performed by restriction fragment length polymorphism (RFLP) and sequencing analysis. Mutations which were suspected to cause a splicing defect or a protein secretion defect were investigated in COS-1 cells transfected with a CETP minigene construct or a cDNA expression vector.

Results: Each of three subjects was identified as a carrier of CETP gene mutation of a compound heterozygote of c.653_654delGGinsAAAC and Intron 14 splicing donor site +1 G>A, a heterozygote of c.658G>A or a homozygote of L261R. The c.658G>A mutation was located at the last nucleotide of exon 7, and it was confirmed to cause splicing abnormality revealed by the CETP minigene analysis. The L261R CETP was not secreted to conditioned media of the cells.

Conclusions: Three novel CETP gene mutations are responsible for HALP by CETP deficiency. It is predicted that there are more rare CETP gene mutations in Japanese, and these multiple rare mutations alone or a combination with each of prevalent mutations responsible for mild-to-moderate or marked HALP, respectively.

1. Introduction

Plasma CETP is a major modulator of high density lipoprotein cholesterol (HDL-C) concentration in human. CETP has the role of exchange neutral lipids between lipoproteins. CETP transfers cholesterol ester (CE) from HDL to apolipoprotein B (apo B) containing lipoprotein such as chylomicron or very low density lipoprotein (VLDL), and transfers triglyceride in an opposite direction. HDL-TG which transferred by CETP is hydrolyzed by hepatic lipase and HDL particle size reduces, thereby, it promotes the catabolic rate of apolipoprotein A-I in HDL [1].

CETP is purified from human plasma as a 74 kDa glycoprotein [2]. It forms the boomerang shape and has a long tunnel with two openings, and phospholipids are plugging as sides of the tunnel openings and two of CE or TG bind in the tunnel [3]. The level of CETP activity varies in animal species, and mice, rats and dogs have almost no CETP activity because functional CETP gene is lost in their evolutionary process [4]. While rabbits and monkeys have high CETP activity, humans, hamsters and chickens have intermediate CETP activity [5].

CETP deficiency in human was firstly reported in Japanese hyperalphalipoproteinemia (HALP) [6]. Complete CETP deficiency presents extremely high HDL-cholesterol level and relatively low low density lipoprotein cholesterol (LDL-C) level [7]. About a half of HALP in Japan are caused by CETP gene mutations, and two prevalent mutations of D442G (allele frequency, 3.4% in Japanese population) and intron 14 splice donor site +1 G>A (Int14+1A) (0.8%) are well characterized [8, 9]. The homozygote of D442G causes partial CETP deficiency by -54% and a moderate increase in HDL-C (mean, 96 mg/dL), while the homozygote of Int14+1A causes complete CETP deficiency and extremely high level of HDL-C (mean, 167 mg/dL) [10].

In the present study, we identified novel CETP mutations in marked HALP Japanese subjects, and we add more information on a heterogeneity of CETP gene mutations in Japanese.

2. Materials and methods

2.1. Subjects

Two Japanese subjects with high HDL-C levels and undetectable CETP mass were investigated. The subject 1 was a 65 year-old female. She was aware of extremely high HDL-C level (224 mg/dl) at health check-up at her age of 46. Subject 1 had a history of hypertension and her brother is

HALP and had hypertension treated with amlodipine 5 mg daily since 61 years-old (146/100 mmHg). He had a regular alcohol intake (50-75 g/day). Both subjects had mild carotid atherosclerosis (IMT 0.9 mm), but they do not have atherosclerotic vascular disease. Subject 2 was a 50 year-old female and she was HALP otherwise healthy.

About 45 unrelated subjects from outpatients were referred to the lipid clinic of Kanazawa university hospital or its affiliated hospitals in 1996-2009, and we choose cutoff value with serum CETP mass levels of <1.8 µg/ml and HDL-C levels of 60 mg/dl. The 41 individuals were excluded from the mutation screening because they had a D442G or Int14+1A mutation in CETP gene by PCR-based RFLP screening method (D442G heterozygotes: n=10, D442G homozygotes: n=3, Int14+1A heterozygotes: n=20, Int14+1A homozygotes: n=4, D442G/Int14+1A compound heterozygotes: n=4). The 4 individuals were selected as candidates for further CETP gene mutation analysis. We identified a heterozygote of novel mutation in CETP gene in subject 3 by this analysis. Subject 3 was a 72 year-old female with manic depressive psychosis. She had both bladder and colon cancer, and she received operations at her age of 55 and 70, respectively. Her HDL-C levels varied from 79 to 169 mg/dL in 5 year clinical record. None has died of cardiac cause in her family. All investigated subjects gave their informed consent. The study protocol was approved by the Ethical Committee of Kanazawa University Graduate School of Medical Science.

2.2. Biochemical analyses

Blood samples were collected after a 12-h fast, unless otherwise noted. Serum TC and triglyceride (TG) were determined by enzymatic methods. Cholestest®N HDL (Sekisui Medical, Tokyo, Japan) was used for measurement of HDL-C, and Friedewald calculation was used for LDL-C estimation because a direct method for LDL-C has not been validated in CETP deficiency [7, 11]. Serum apolipoprotein concentrations were determined by immunoassay. CETP mass in serum was measured by enzyme immunoturbidimetry [12].

2.3. Genetic analyses

Genomic DNA was prepared from peripheral white blood cells using a Genomic Purification Kit (Gentra Systems, Minneapolis, MN). The promoter region, 16 exons and intronic junctions of

CETP gene were amplified by PCR using specific primers. Specific primers were designed using Primer 3 plus online software (<http://www.bioinformatics.nl/primer3plus>.) [13]. Screening analysis was performed for two prevalent mutations of CETP gene, Int14+1A and D442G, using RFLP method as previously described [9]. PCR was performed under the following conditions: 95°C for 2 min; 30 cycles of 95°C for 10 s; each optimized annealing temperature for 10 s; 72°C for 30 s. We select 55–60°C of annealing temperature on each primer. DNA direct sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied biosystems, Foster City, CA) on the ABI PRISM 310 Genetic Analyzer (Applied biosystems). The purified PCR production of exon 7 was ligated into pMD-20 vector (Takara Bio, Tokyo, Japan) by Mighty TA-cloning kit (Takara Bio) and then transformed to *E. coli* HST premium 08 (Takara Bio). Subclones were sequenced by ABI 3700 Genetic Analyzer.

2.4. Construction of CETP minigene and cDNA plasmids

To investigate effects of novel mutations for CETP gene splicing and expression, wild and mutant types of CETP minigene plasmid and cDNA plasmid were constructed. For a minigene plasmid, from exon 5 to 8 region of lambda CG5 CETP genomic clone [14] was amplified by PCR, and the product was digested by RsrII and SacII restriction enzymes. The pCU plasmid [15], in which exons and introns of 7-10 of CETP genomic region was replaced in the cDNA, was a kind gift from Dr. Alan Tall (Columbia University, NY, USA). The pCU was similarly digested by RsrII and Sac II, and PCR product which included CETP exon 5-8 was ligated to pCU plasmid. This plasmid was named to pCU5-11 and used as a wild type CETP minigene plasmid. Plasmid construct of CETP gene region was shown in Fig.4. For CETP cDNA expression vector, pCU plasmid and CETP cDNA region of pLAY WT [16] were digested by EcoRV, and they were ligated. L261R mutation in CETP cDNA expression plasmid was generated using PCR-based site-directed mutagenesis. Mutations were confirmed by sequencing. Plasmid preparations were made using alkali lysis with SDS and ethanol precipitation.

2.5. Cell culture and transfections

Monkey kidney COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (Invitrogen Corporation, Carlsbad, CA). Cells were split approximately 16 h prior to transfection in 6-wells plate (2×10^5 cells per well). Transient expressions of plasmid DNA for cells were performed by the polyethylenimine (PEI) method [17]. The plasmid DNA was mixed with PEI and OptiMEM (Invitrogen) at nitrogen of PEI /phosphate of DNA ratio 11.5, and incubated for 30 min at room temperature. Cells were washed twice with PBS(-), and transfection solution was added. The cells were incubated for 8 h at 37°C, 5 % CO₂. Then, the same volume of complete medium to transfection solution was added without removing the transfection solution, and cells were incubated for 48 h. From each plate, cells and medium were collected and stored at -70°C for subsequent determination of CETP immunoreactive mass and mRNA.

2.6. Analysis of CETP mass and RNA

CETP mass in conditioned medium was determined by ELISA (Bio Medical Laboratories, Saitama, Japan). Total RNA from transfected cells was isolated by ISOGEN (WAKO Pure Chemicals, Osaka, Japan). The reaction of reverse transcription was performed using RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham MA) with 1 µg of total RNA according to the manufacturer's instructions. The cDNA was amplified using primers CETP E2A322 (5'-cag ata tca cgg gcg aga ag-3') and CETP E9B997 (5'-cga gtg gaa gac tgc ctc a-3'). PCR product was separated on 3 % NuSieve3:1 agarose (Lonza, Basel, Switzerland) gel and semi-quantified analysis of RT-PCR products amounts were performed by using Typhoon 9400 imaging analyzer (GE Healthcare UK, Little Chalfont, England). Sequences of RT-PCR products were confirmed with direct sequencing.

2.7. Mutation nomenclature

Mutations for changes at DNA levels are described according to CETP cDNA sequence Genbank ID: NT_000078. For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon. Amino acid numbers are described according to CETP protein sequence PDB ID: 2OBD_A

as CETP mature protein.

3. Results

3.1. Clinical and biochemical characteristics of CETP gene mutation

The pedigree of subject 1 is shown in Fig.1. The plasma lipid profile, CETP concentration and other parameters in the subject 1, her family members, subject 2 and subject 3 are shown in Table 1. Subject 1, her brother and subject 2 were the complete deficiency of CETP.

Subject 1 and her brother had extremely high levels of HDL-C (subject 1: 223 mg/dL, II-4: 237 mg/dL). Her daughter had high HDL-C level (III-1: 105 mg/dL), and her son had mild elevated HDL-C level (II-3: 63 mg/dL). Subjects 2 and 3 had high HDL-C levels (147 mg/dL and 162 mg/dL, each). Although she had highly elevated HDL-C levels, subject 3 had partial CETP deficiency and her plasma CETP mass was 1.1 µg/mL being a half of reference value. All probands had no clinical sign of cardiovascular disease.

3.2. Analysis of CETP gene

Subject 1, her brother and daughter were found to have the Int14+1A heterozygote by RFLP screening although subject 1 and her brother had complete CETP deficiency, so we further analyzed CETP genes of her family by sequencing method. As a result, they were found to be the compound heterozygotes for Int14+1A and a deletion of GG and an insertion of AAAC in c.653_654 sites (c.653_654delGGinsAAAC) in exon 7 (Fig.2). The c.653_654delGGinsAAAC mutation is a novel mutation in CETP gene, and it is predicted to cause a frame shift leading to a premature stop codon in exon 8 (amino acid position 218). By sequencing, her daughter did not have any other mutation except Int14+1A, and her son was found to be the c.653_654delGGinsAAAC heterozygote (Fig.1).

Subject 2 was the homozygote for substitution of nucleotide T to G at c.725 position. This substitution causes amino acid change at codon 261 from leucine (CTG) to arginine (CGG) (Fig.3). Subject 3 was found to be the heterozygote of c.658G>A at the last nucleotide in exon 7 (Fig.2). She had I405V (rs5882) polymorphism as the heterozygote.

3.3. The effect of c.658 G>A mutation for CETP pre-mRNA splicing

The c.658G>A mutation identified in subject 3 is located at the last nucleotide of exon 7, which is likely to affect 5' splicing consensus sequence. Therefore, we examined its effect for CETP pre-mRNA splicing. We constructed CETP wild type and c.658 G>A mutated minigene plasmid, and examined whether exon skipping is caused in mutated minigene construct transfected COS-1 cells. RT-PCR product of CETP cDNA region from wild type transfected cells had 688 bp length fragment, while products from the c.658G>A plasmid transfected cells appeared as two bands, a minor band of 628 bp and a major band of 558 bp. As a result of sequencing analysis, fragment of 628 bp was skipping exon 7 (70 bp), and fragment of 558 bp skipped both exon 6 (60 bp) and 7 (Fig.4). The skipping of exon 7 or exon 6-7 is thought to cause frame shift leading a premature stop codon. Semi-quantification of RT-PCR product from the c.658 G>A plasmid transfected cells revealed relative amounts of exon 7 skipping transcripts and both exon 6 and 7 skipping transcripts were each 32.0% and 68.0% in total product amounts from the c.658 G>A plasmid transfected cells (Fig.4).

3.4. The influence of missense mutations for CETP secretion

To assess the influences of L261R mutation for CETP secretion, we measured CETP protein mass in conditioned media from COS-1 cells transiently transfected with the wild type L261R mutant CETP. CETP mass in the medium of wild type was 54.4 ± 21.2 ng/mL, while that from L261R was less than the detective limit (<0.25 ng/mL) (Table 2). The position of L261R was highly conserved in CETP genes of various species (Fig.5)

4. Discussion

In the present study, three novel CETP gene mutations (c.653_654delGGinsAAAC, c.658 G>A and L261R) were identified in Japanese HALP subjects. The c.653_654delGGinsAAAC mutation which was identified in subject 1 showed complete CETP deficiency as the compound heterozygote with Int14+1A mutation. The c.653_654delGGinsAAAC was predicted to cause frame shift leading to a premature stop codon. The truncated mRNA by the mutation is thought to be eliminated in nonsense-mediated mRNA decay system, and the mutated CETP protein would be hardly produced. The Int 14+1G>A mutation is also known to cause exon skipping leading to a premature stop co-

don, and the amounts of its aberrant mRNA in cell are about 1/3 of that of wild type [18]. The extended 3' UTR length by the premature stop codon is longer, and the mRNA is more degraded [19]. Thus, the mRNA having c.653_654delGGinsAAAC mutation would be degraded more rapidly than that of Int14+1G>A.

The c.658G>A mutation was identified as the heterozygote in subject 3 whose plasma CETP level was half as that of controls, and it located at the last nucleotide of exon 7 within 5' splicing donor consensus sequence. We showed this mutation caused two forms of exon skipping in COS-1 cells, both of exon 6 and 7 skipping or only exon 7 skipping. The major transcripts skipped both of exon 6 and 7. The transcripts skipping exon 6 and 7 or only exon 7 both cause frame shift leading to a premature stop codon, and these aberrant mRNA would be degraded rapidly. Intron 7+1 G>T mutation, which was located at the next to c.658 position, which was identified in Caucasians, and the transcripts from peripheral leukocytes of the mutation carrier subject skipped only exon 7 [20]. The reason why patterns of exon skipping between Intron 7+1 G>T mutation and c.658G>A mutation were different was unknown. However, we used overexpressed CETP minigene construct in COS-1 cells because RNA from subject 3 was not available, and distinct kinds of cells or differences between natural or overexpressed expression might account for a different exon skipping pattern.

We also thought the possibility that c.658G>A did not affect splicing and it caused amino acid substitution, A203T, so we constructed c.658G>A mutated CETP cDNA plasmid and performed CETP protein expression analysis. As the result, CETP protein mass in the medium and the transcripts from transfected cell had not significantly changed compared to those of wild-type cell (supplemental fig.1), and it suggests that c.658G>A mutation caused abnormal splicing but it does not have a missense effect.

Subject 3 had also I405V polymorphism as the heterozygote, but it was reported that the effect of I405V as 405VV homozygote for plasma CETP protein level by meta-analysis was $-0.19 \mu\text{g/mL}$ less [21]. I405V polymorphism would decrease CETP mass in subject 3 slightly, but c.658G>A would cause CETP deficiency ($1.1 \mu\text{g/mL}$) by a single mutant allele causing a splicing defect.

The L261R mutation was identified in subject 2 who resulted in the complete CETP deficiency, and it located in exon 9. CETP protein expression analysis showed CETP protein in the medium

from transfected cell was not detected. The transcript levels of L261R cell was not significantly changed compared to the wild type (supplemental fig.1), and L261R mutation is predicted to cause a secretion defect. The position of L261R is predicted to be located at surface of the neck region of the CETP tunnel. Y375S, F265R and F270R are CETP mutagenesis mutants which are not secreted, and these structural positions are located at surfaces in the neck region and they are in the vicinity of L261R position [3]. Probably, these surfaces of the neck region would be essential for CETP protein secretion.

Subjects 1 and 2 were marked HALP with homozygotes of CETP mutation, but subjects with heterozygotes in this study had various HDL-C levels. Other than CETP, LIPC and LPL are responsible for elevated HDL-C level, and T allele of LIPC gene -514C/T polymorphism and 447X allele of LPL gene S447X polymorphism are well known to both cause 3 mg/dL of elevated HDL-C levels in Japanese (LIPC -514T allele: 75%, LPL 447X allele: 22% in Japanese population) [22,23]. If subjects who have a heterozygote of CETP mutation but rather high levels of HDL-C, further analysis involving endothelial lipase [24] and scavenger receptor class B type I (SR-BI) [25,26] activities is needed.

Many CETP gene mutations are reported, such as nonsense mutations (n=8), splice junction mutations (n=6), missense mutations (n=5), small gene deletions and insertions (n=4), promoter mutations (n=2) and others (n=1) (Fig.6) [8, 9, 20, 27-46]. In the present study, we identified c.653_654delGGinsAAAC and c.658G>A from several subjects with HALP, and it suggests that nonsense and splicing defect mutations account for the majority of CETP gene mutations.

In summary, it is predicted that there are more rare CETP gene mutations in Japanese, and it suggests that these multiple rare mutations alone or combinations with each of prevalent mutations such as D442G and Int14+1A responsible for mild-to-moderate or marked HALP, respectively.

Conflict of interest

The authors report no conflicts of interest.

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their technical assistance.

Figure legend

Fig.1 The pedigree of subject 1

The proband is indicated by an arrow. The father of proband had liver dysfunction and he died at his age of 75 of sudden death (I-1). The mother died at her age of 68 of cerebral infarction (I-2). The elder sister died of pneumonia after cerebral infarction at 83 years old (II-1), the second sister died of subarachnoid hemorrhage at 66 years old (II-2), and the younger sister died of renal failure at 52 years old (II-8).

Fig.2 DNA sequences of exon 7 in CETP gene of normal allele.

(A) and mutated allele (c.653_654delGGins AAAC) (B) from subject 1 by subclone sequencing. This mutation causes a frame shift leading to a premature stop codon in exon 8. The sequences (C) from subject 3 by direct sequencing revealed c.658 G>A heterozygote.

Fig. 3 DNA sequences of exon 9 in CETP gene from control subject and subject 2.

In subject 2, the direct sequence shows T to G substitution at codon 261 in exon 9, which means amino acid substitution from Leu (CTG) to Arg (CGG).

Fig. 4 RT-PCR analysis of c.658G>A mutation transfected COS-1 cell

(A) Schema of construct of CETP gene region in pCU 5-11 minigene vector is shown. (B) RT-PCR analysis of CETP cDNA region from a minigene construct transfected COS-1 cells. Mock plasmid, wild type and c.658 G>A mutant type pCU 5-11 minigene plasmid were transfected into COS-1 cells and total RNA was isolated and RT-PCR was performed by using CETP cDNA primer pair spanning from exon 2 to exon 9. (C) Sequencing analysis of RT-PCR product represents 628 bp length product from c.658G>A transfected cell was skipping exon 7 and 558 bp product was skipping both exon 6 and 7.

Fig. 5 Alignment of CETP amino acid sequences

Protein sequences of human, rabbit, chicken, xenopus and zebrafish are shown. The position of L261R is shown by an arrow.

Fig. 6 Summary of CETP gene mutations

Novel mutations revealed in the present study (*)

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Table 1 Plasma lipids profile, CETP concentration and other parameters in subjects with CETP gene novel mutation and its family members

	II-4	II-6 (Subject 1)	II-7	III-1	III-3*	Subject 2	Subject 3	Reference intervals
Age/Sex	74 M	65 F	75 M	44 F	38 M	50 F	72 F	
BMI (kg/m ²)	24.5	20.2	20.5	NA	NA	NA	16.4	
Total cholesterol (mg/dL)	297	319	145	187	192	314	276	128–219
Triglyceride (mg/dL)	52	56	125	28	610	79	66	30–149
LDL-cholesterol (mg/dL) [†]	50	85	80	77	NA	151	101	57–139
HDL-cholesterol (mg/dL)	237	223	40	105	63	147	162	40–99
ApolipoproteinA-I (mg/dL)	289	280	109	211	177	NA	275	122–161
ApolipoproteinA-II (mg/dL)	58.5	32.6	23.3	26.3	38.9	NA	NA	25.1–34.5
Apolipoprotein B (mg/dL)	65	67	75	61	79	NA	76	69–105
Apolipoprotein C-II (mg/dL)	6.8	5.2	5.2	2.2	4.3	NA	NA	1.6–4.2
Apolipoprotein C-III (mg/dL)	17.8	16.7	8.4	7.9	13.8	NA	NA	5.5–9.5
Apolipoprotein E (mg/dL)	6.1	13.3	2.3	4.3	4.9	NA	3.8	2.7–4.5
CETP (µg/mL)	<0.1	<0.1	1.2	1.4	1.0	<0.1	1.1	1.7–2.2

*: after meal, BMI: Body mass index, NA: not available, [†]: by Friedewald calculation

Table 2 CETP mass levels in medium of CETP wild type and L261R transfected COS-1 cells

Genotype	CETP mass in medium (n=6)
Wild type	54.4±21.2 ng/mL
L261R	< 0.25 ng/mL

Fig.1

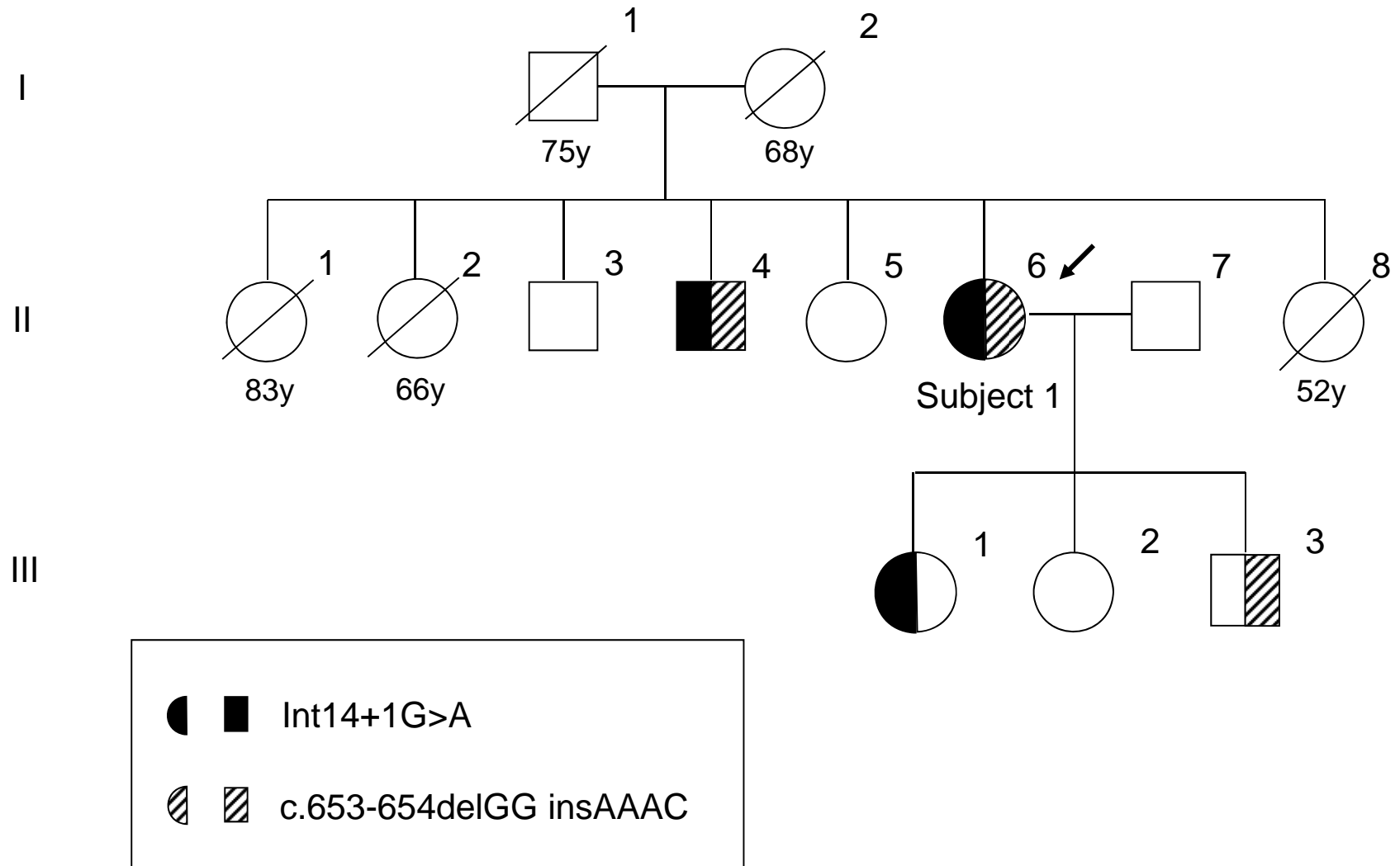
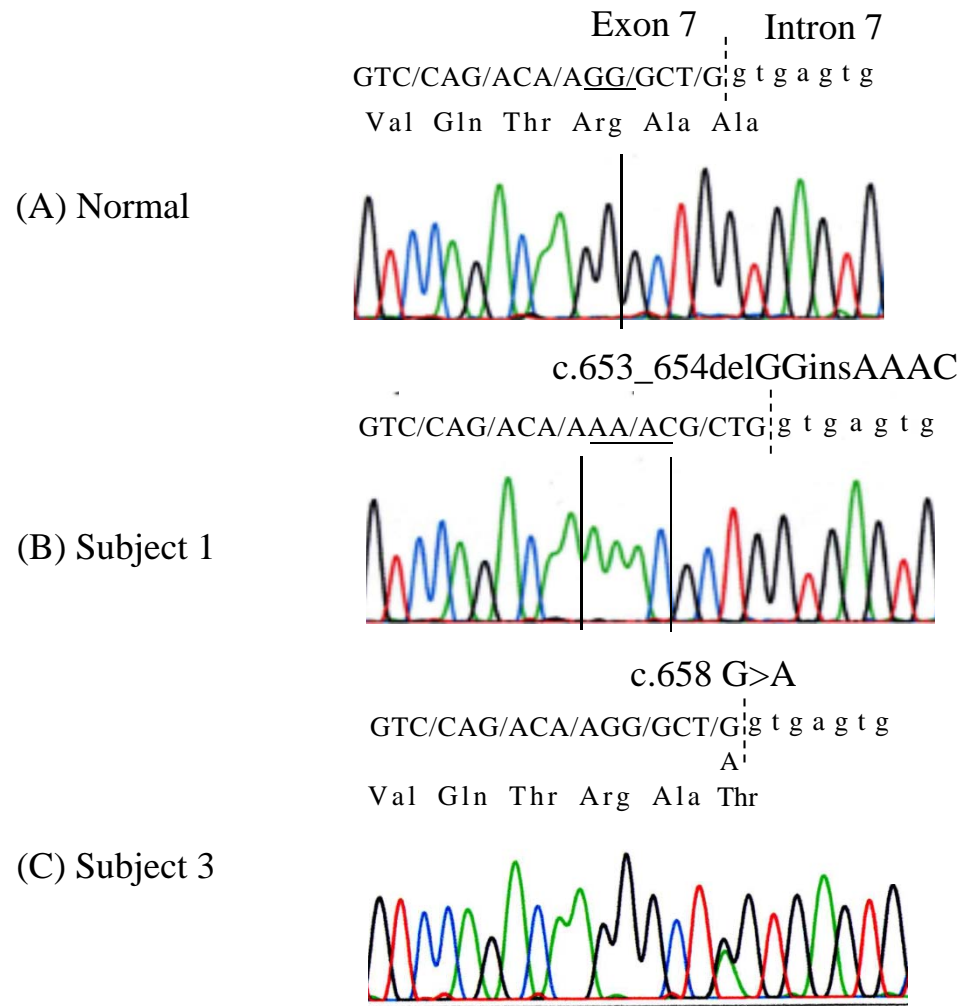
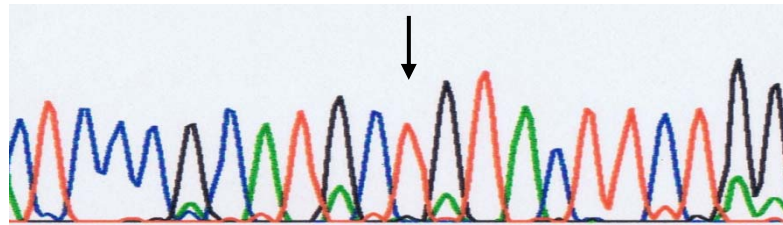


Fig.2



C/TCC/CGC/ATG/CTG/TAC/TTC/TGG/
Ser Arg Met Leu Tyr Phe Trp

Control



C/TCC/CGC/ATG/CGG/TAC/TTC/TGG/
Ser Arg Met Arg Tyr Phe Trp

Subject 2

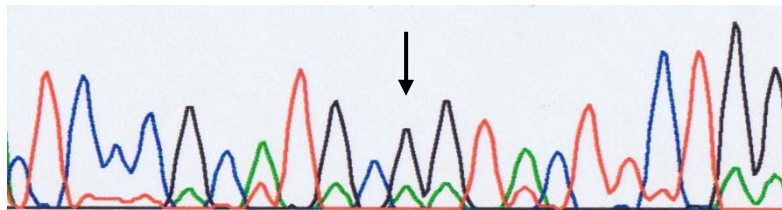


Fig.4

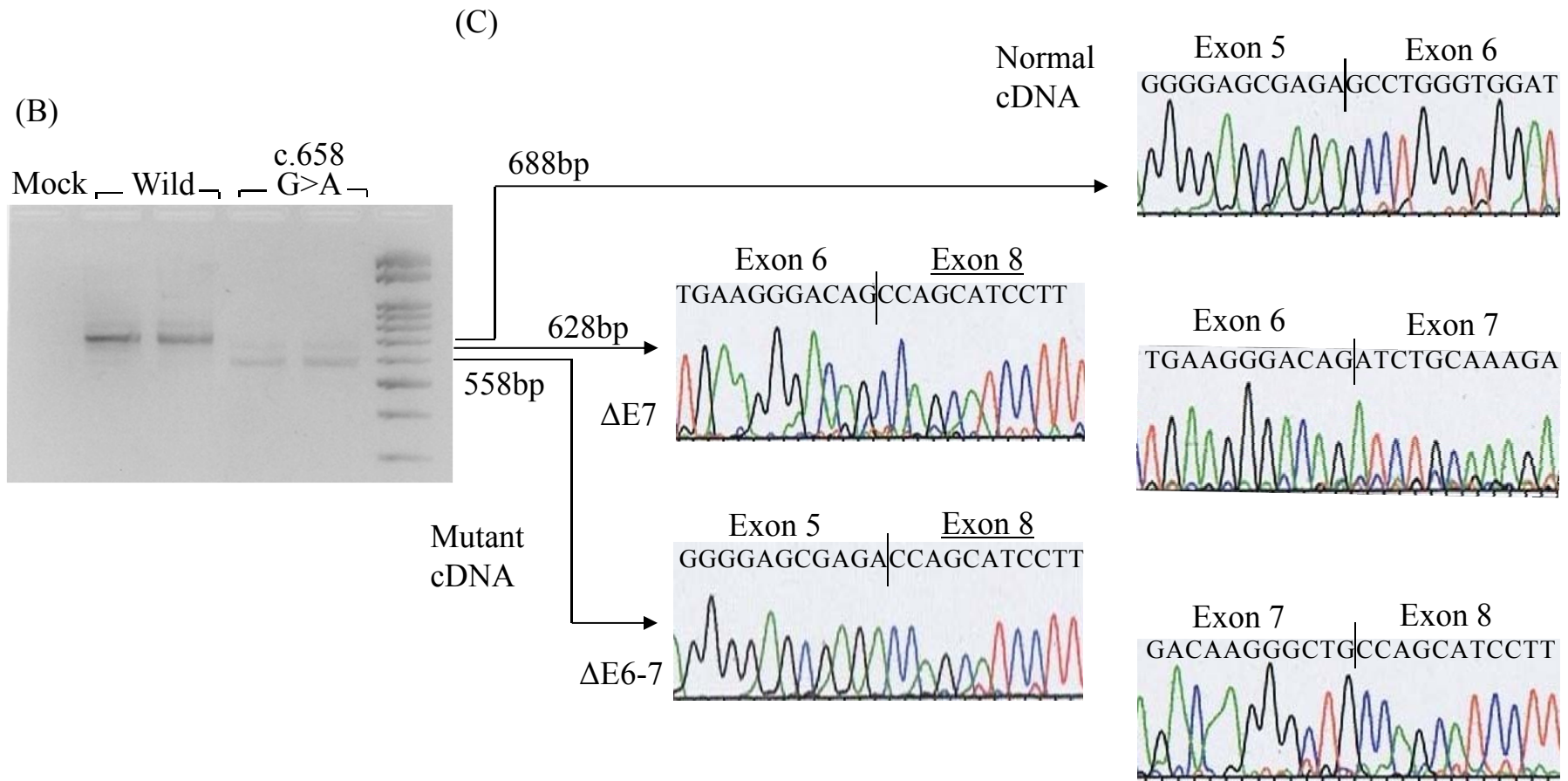
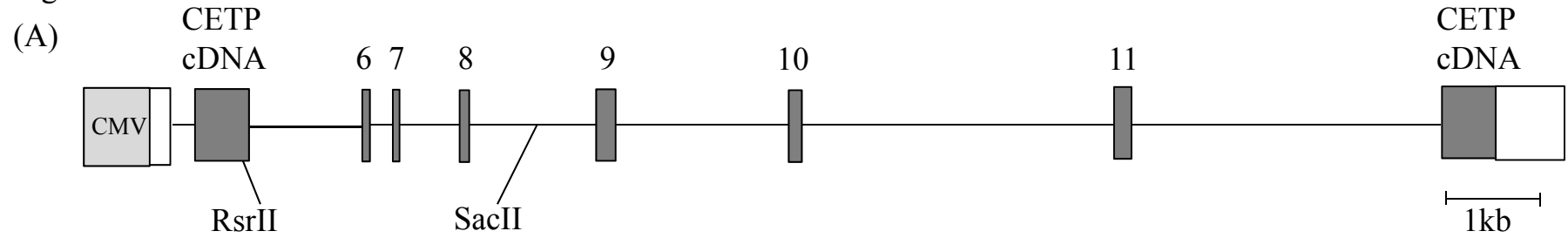
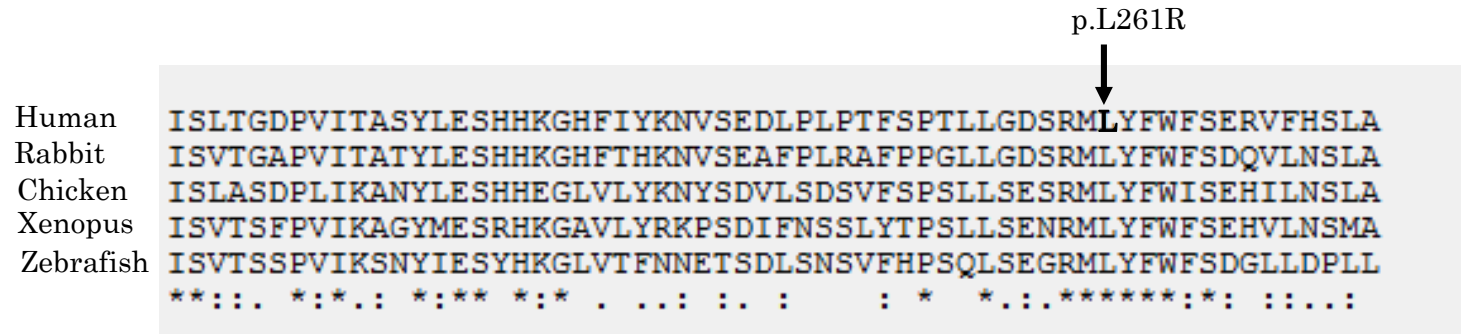
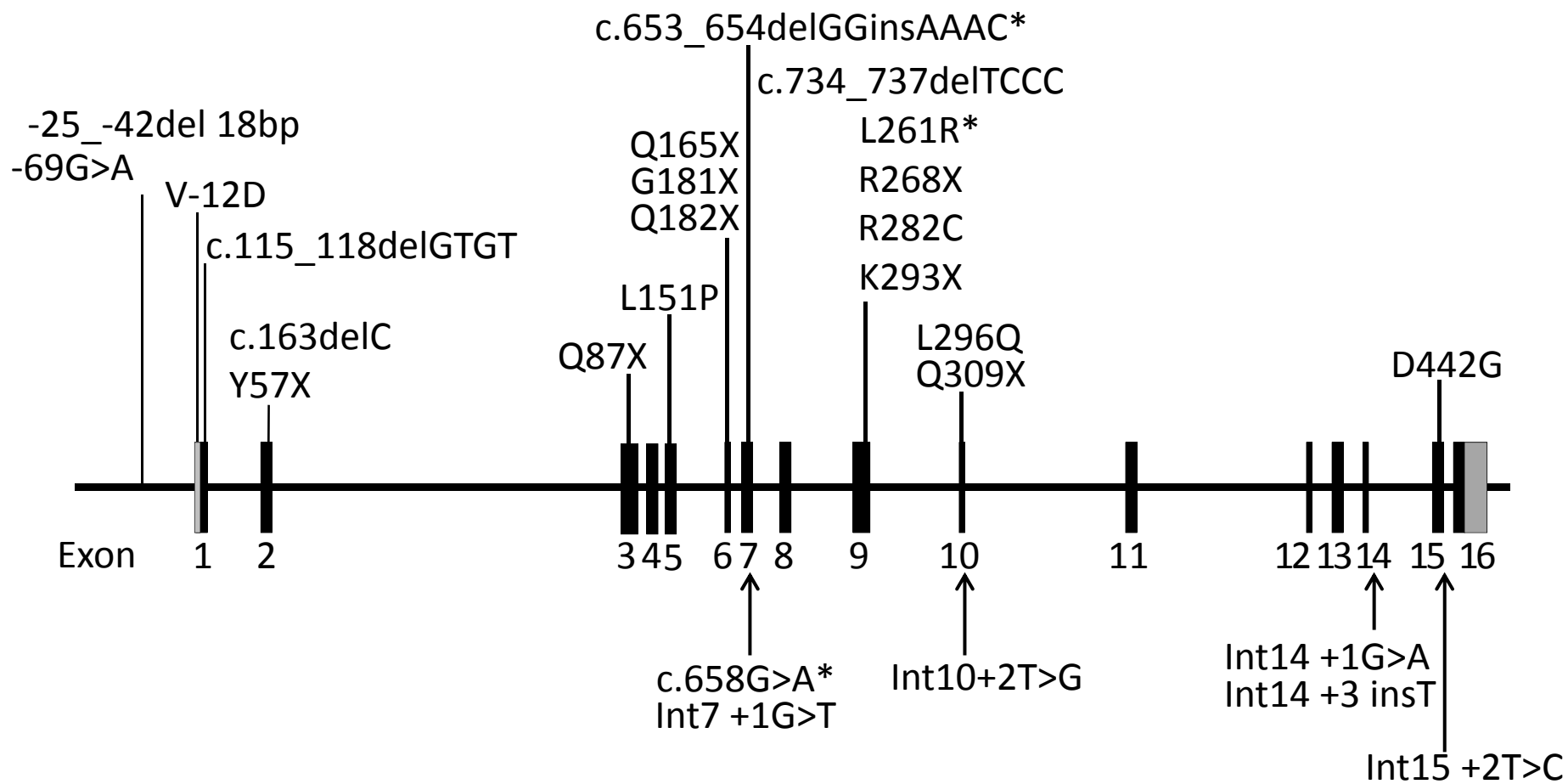


Fig. 5





Supplemental Fig. 1

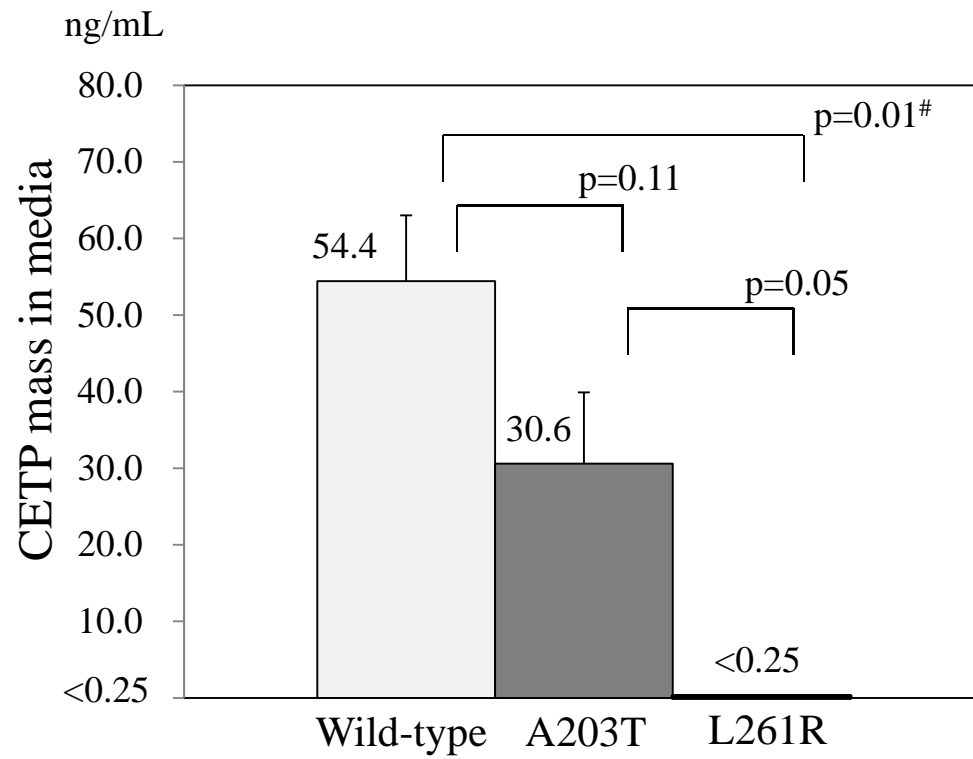
(A) CETP mass in conditioned media from COS-1 cells transiently transfected with wild-type, A203T and L261R mutant CETP cDNA expression vector

Values are shown as mean and SEM (n=6). Statistical analysis was performed by ANOVA and Sheffé's test. The p-value by ANOVA was 0.01. $p < 0.05$ by Sheffé's test was revealed as #.

(B) RT-PCR analysis of CETP and GAPDH genes from wild-type, A203T and L261R CETP transfected COS-1 cells

GAPDH gene was analyzed as an internal control.

(A)



(B)

