Original Article

Apolipoprotein C-Ⅱ Deficiency with No Rare Variant in the *APOC2* Gene

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Aim: **Familial apolipoprotein C-Ⅱ (apoC-Ⅱ) deficiency is a rare autosomal recessive disorder with marked hypertriglyceridemia resulting from impaired activation of lipoprotein lipase. In most cases of apoC-Ⅱ deficiency, causative mutations have been found in the protein-coding region of *APOC2*; however, several atypical cases of apoC-Ⅱ deficiency were reported to have markedly reduced, but detectable levels of plasma apoC-Ⅱ protein (hereafter referred to as hypoapoC-Ⅱ), which resulted from decreased promoter activity or improper splicing of apoC-Ⅱ mRNA due to homozygous mutations in *APOC2*. Here we aim to dissect the molecular bases of a new case of hypoapoC-Ⅱ.**
Methods: **We performed detailed biochemical/genetic analyses of our new case of hypoapoC-Ⅱ, manifesting severe hypertriglyceridemia (plasma triglycerides, 3235 mg·dL^{-1}) with markedly reduced levels of plasma apoC-Ⅱ (0.6 mg·dL^{-1}).**
Results: **We took advantage of a monocyte/macrophage culture system to prove that transcription of apoC-Ⅱ mRNA was decreased in the patient's cells, which is compatible with the reported features of hypoapoC-Ⅱ. Concomitantly, transcriptional activity of the minigene reporter construct of the patient's *APOC2* gene was decreased; however, no rare variant was detected in the patient's *APOC2* gene. Fifty single nucleotide variants were detected in the patient's *APOC2*, but all were common variants (allele frequencies >35%) that are supposedly not causative.**
Conclusions: **A case of apoC-Ⅱ deficiency was found that is phenotypically identical to hypoapoC-Ⅱ but with no causative mutations in *APOC2*, implying that other genes regulate apoC-Ⅱ levels. The clinical entity of hypoapoC-Ⅱ is discussed.**

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Key words; Chylomicronemia, Apolipoprotein C-Ⅱ deficiency, Single nucleotide polymorphism, *cis*-regulatory region, Rare variant

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Introduction

Hypertriglyceridemia is of clinical importance not only as a sign of metabolic syndrome, but also as an independent risk factor for cardiovascular events[1-3]. Elucidation of the mechanisms of hypertriglyceridemia is an emerging need with the worldwide preva-

lence of metabolic syndrome. Triglycerides (TG) are carried in the form of TG-rich lipoproteins (TGRL) in plasma. TGRLs are secreted either as chylomicrons from the intestine or as very low density lipoproteins (VLDL) from the liver, followed by catabolism by lipoprotein lipase (LPL) that hydrolyzes TGs in TGRL with subsequent uptake of remnant lipoproteins primarily by the liver. A defect in one of these pathways results in hypertriglyceridemia[4].

ApoC-II, a member of the apoE/apoC-I/apoC-IV/apoC-II gene cluster, is a 79-amino-acid polypeptide primarily biosynthesized in the liver and intestine, and is present on plasma chylomicrons, VLDL, and high-density lipoproteins (HDL). ApoC-II plays an important role as a co-factor of LPL[5, 6]. The importance of apoC-II in regulating plasma TG levels is evidenced by human apoC-II deficiency[7], which is a rare autosomal recessive disorder characterized by extreme hypertriglyceridemia, as seen in LPL deficiency[8, 9].

The molecular defects of apoC-II deficiency are heterogeneous. Most cases had causative mutations in the protein-coding region of the apoC-II gene (*APOC2*)[10], resulting in null apoC-II (an initiation codon mutation[11]), or nonfunctional apoC-II protein (nonsense mutations[12]; frameshift mutations[13]; missense mutations[14] (reviewed in[15, 16])). Notably, several cases were reported to have markedly reduced, but detectable levels of apoC-II protein with apparently normal electrophoretic patterns (for simplicity, hereafter referred to as hypoapoC-II)[17-19]. These are all characterized by a reduction in apoC-II mRNA transcription due to i) decreased promoter activity of *APOC2* (apoC-II$_{Köln}$[19]), or ii) a donor splice-site mutation of intron 2 of *APOC2* that results in markedly reduced levels of apoC-II mRNA (ApoC-II$_{Hamburg}$[17], ApoC-II$_{Tokyo}$[18]).

We report a patient with apoC-II deficiency in which no causative mutations were detected in the apoC-II gene. Our patient is a typical case of hypoapoC-II manifesting hypertriglyceridemia, coincided with markedly reduced, but detectable levels of apoC-II in plasma. As in other reported cases of hypoapoC-II, apoC-II mRNA transcription was decreased by 50%, at least partially due to reduced transcriptional activity of the *cis*-regulatory region of the patient's *APOC2* gene, as shown by minigene reporter experiments. However, no rare variant was identified in any regions of *APOC2*, suggesting the involvement of additional genetic defect(s) that regulate apoC-II protein levels in plasma. To our knowledge, this is the first report of apoC-II deficiency with no rare variant found in the *APOC2* gene. Together with other cases[17-19], the clinical entity of hypoapoC-II is discussed.

Table 1. Biochemical characteristics of the patient at the time of presentation

Total cholesterol (mg·dL^{-1})	249
HDL-cholesterol (mg·dL^{-1})	39.8
Triglycerides (mg·dL^{-1})	1435
Apolipoprotein A-I (mg·dL^{-1})	120
Apolipoprotein A-II (mg·dL^{-1})	28.3
Apolipoprotein B (mg·dL^{-1})	121
Apolipoprotein C-II (mg·dL^{-1})	ND
Apolipoprotein C-III (mg·dL^{-1})	28.0
Apolipoprotein E (mg·dL^{-1})	19.9
Apolipoprotein E genotype	E3/E3

At the time of evaluation of these laboratory data, the patient was treated with fenofibrate (600 mg per day) and ethyl icosapentate (1800 mg per day). Apolipoprotein E genotype was determined by restriction fragment length polymorphism analysis using PCR primers (5'-TCCAAGGAGCTGCAGGCGGCGCA-3' and 5'- GCCCCGGCCTGGTACACTGCCA-3') and restriction endonucleases (AflIII and HaeII) as described previously (Zivelin A et al. Clin Chem 1997; 43: 1657-1659). ND: not detected.

Methods

Subjects

The proband was a 47-year-old Japanese man manifesting type I hyperlipidemia (plasma level of triglycerides, 3235 mg·dL^{-1}; total cholesterol, 385 mg·dL^{-1}) with recurrent episodes of pancreatitis. This patient was previously characterized and reported as having apoC-II deficiency[20]. The diagnosis of apoC-II deficiency was made based on undetectable levels of apoC-II protein by the immunodiffusion assay. On referral to our hospital, he had been treated with fenofibrate and ethyl icosapentate. He also had a past medical history of conversion disorder with several episodes of cataplexy starting at age 36 years. Detailed clinical characteristics are available in **Table 1** as well as in the previous report[20].

In addition to the proband, his normolipidemic parent (mother) was investigated. Unrelated control subjects without hypertriglyceridemia were also investigated. His father's biological sample was unavailable since he had died at age 42 years with the diagnosis of cerebral vascular disease. Although his parents were not related, we found a case of consanguinity in his family (the patient's uncle and aunt were related), and consanguinity was rather common in the local community where both of his parents were raised.

Informed consent was obtained from the subjects, and all procedures were subjected to approval by the human genome, gene analysis research ethics committee of the University of Tokyo.

Table 2. Sequences of PCR primers for cloning human cDNA probes

cDNA probe	Sequences of forward and reverse primers	PCR product *bp*
apoC-II	5'-AGGACAGCCTGCCAGAGTCTG-3' 5'-AGAATTCAGGCTAGAGTTGGGAG-3'	462
apoC-IV	5'-AGAAATGTCCCTCCTCAGAAACAG-3' 5'-AACATTTTAACCCTGGTCCTTGTC-3'	393
CLPTM1	5'-CATGTGTACATCTCAGAGCACG-3' 5'-GATGGGGTAGTAGTCCTTCTGC-3'	566
MCSF	5'-TCGGAGTACTGTAGCCACATGA-3' 5'-ACTGCCTGGATCCACTGTGT-3'	638

Biochemical Analyses of Plasma Apolipoproteins

Blood was obtained from the patient, his mother, and normal control subjects. Fractions of plasma, buffy coat and total blood cells were separated by low-speed centrifugation. The buffy coat was stored at −80℃ until processed later to obtain genomic DNA as described in the following section. One microliter of plasma was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot analysis using anti-human apoC-II polyclonal antibody (Calbiochem, San Diego, CA, USA). Apolipoprotein control serum- Daiichi High (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), was used as a standard to assess plasma concentrations of apoC-II.

For isoelectric focusing (IEF) of plasma apolipoproteins, TGRLs, isolated by ultracentrifugation, were delipidated with ethanol:diethylether (3:1) to obtain apolipoproteins, which were then subjected to electrophoresis using Immobiline DryStrip (pH 4-7; GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions; proteins were visualized by Coomassie brilliant blue staining. For two-dimensional PAGE (2D-PAGE), TGRL apolipoproteins were separated by IEF, then by SDS-PAGE, and apoC-II protein was detected by immunoblotting using the same anti-apoC-II antibody as described above.

Lipase Activity Assay

Post-heparin plasma (PHP) was obtained 15 min after an intravenous injection of heparin (30 units$\cdot$kg^{-1}) to overnight fasted subjects. Lipase activity in plasma (6.7 μL/reaction) was determined by measuring the amount of FFA released from ^{3}H-triolein emulsions (Amersham Biosciences, Tokyo, Japan) as described previously[21, 22], and expressed as μmol$^{-1}\cdot$h$^{-1}\cdot$mL^{-1}. LPL activity was calculated by subtracting the activity in the presence of 1 mol$\cdot$L^{-1} NaCl (representing hepatic triglyceride lipase (HTGL) activity) from the activity in the absence of 1 mol$\cdot$L^{-1} NaCl (total lipase activity). Various amounts of plasma from normolipidemic subjects were added to the assay mixture to supply an excess amount of apoC-II proteins.

Northern Blot Analysis of Primary Monocytes/ Macrophages Derived from Peripheral Blood

Human monocytes/macrophages were isolated from peripheral blood by Lymphoprep (Nycomed, Roskiled, Denmark), and cultured as described previously[23]. On day 7, cells were incubated with or without 10 μmol$\cdot$L^{-1} of Liver X receptor (LXR) agonist (T0901317) for 24 h, and harvested. In a separate experiment, actinomycin D (Sigma-Aldrich, Tokyo, Japan) was added to the incubation medium at 10 μg$\cdot$L^{-1} to inhibit RNA synthesis.

Total RNA was isolated from cells by TRIzol reagent (Invitrogen Corp., Tokyo, Japan), and equal amounts of RNA were subjected to Northern blot analysis as described[23]. Probes for apoC-II, apoC-IV, cleft lip and palate associated transmembrane protein 1 (CLPTM1), and monocyte colony stimulating factor (MCSF) were constructed from cDNA fragments amplified by RT-PCR using cDNA obtained from monocytes/macrophages as a template. Probes for apoE and ribosomal protein, large, P0 (RPLP0) were previously described[23, 24]. Primer sequences used for each probe construction are listed in **Table 2**. Signals were detected and analyzed by Phosphor-Imager Screen and BASTATION software (FUJIFILM Corp., Tokyo, Japan). Intensity of each band was measured by ImageJ 1.39 software (NIH).

Southern Blot Analysis and Genomic DNA Copy Number Analysis

Genomic DNA (10 μg/reaction) were digested with 5 units of XbaI, SacI, PstI, EcoRI and BamHI (New England Biolabs, Beverly, MA, USA) at 37℃

Table 3. Sequences of PCR primers for DNA copy number analysis

Target gene	Sequences of forward and reverse primers	PCR product *bp*
APOC2	5'-GGGCTCTCCTGACACACTCT-3' 5'-TGGATGCAGTCGGTGGTAT-3'	243
RPLPO	5'-GCTCCTCTTAGGCCCGGGAC-3' 5'-ACCCTGCACTTACGATGATCTTAAGGA-3'	185

overnight, and subjected to Southern blot analysis as described previously[22].

For DNA copy number analysis, genomic DNA (25 ng/reaction) was subjected to real-time PCR by LightCycler 480 system II (Roche Applied Science, Tokyo, Japan) to amplify the *APOC2* gene and *RPLP0* gene (as a control). Primer pairs (**Table 3**) were designed to amplify genomic DNA, but not mRNA. Cycle numbers for a given threshold were determined for both *APOC2* and *RPLP0*, and the relative DNA copy number (*APOC2*/*RPLP0*) was calculated.

Sequencing the *APOC2* and Other Genes in Plasma TG Metabolism

Genomic DNA was extracted from the buffy coat obtained from peripheral blood as described above, using the Blood & Cell Culture DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The *APOC2* gene, spanning from the 3' end of the last exon of the *APOC4* to the 5' end of the first exon of *CLPTM1*, was amplified by PCR using genomic DNA (0.1 μg/reaction), PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Otsu, Japan), and a set of primer pairs as listed in **Table 4**. The PCR condition was as follows: 30 cycles of denaturation (98℃ for 10s) and extension (68℃ for 1 min/kb). The PCR fragment was purified by the MinElute Gel Extraction Kit (Qiagen), and sequenced using DNA sequencer CEQ 2000 (Beckman Coulter, Inc., Tokyo, Japan) with primers described in **Table 5**. Alternatively, 3 to 7 subclones were sequenced to compensate for possible errors during extension with GXL polymerase. Other genes involved in plasma TG metabolism (*LPL*, apolipoprotein A-V (*APOA5*), apolipoprotein C-III (*APOC3*), lipase maturation factor 1 (*LMF1*), GPI-anchored high-density lipoprotein (HDL)-binding protein 1 (*GPIHBP1*)) were sequenced as described[25, 26].

Dual Luciferase Reporter Gene Assays

Reporter constructs containing the *APOC2* minigene were constructed as follows: The PCR prod-

Table 4. Sequences of PCR primers for DNA amplification of the *APOC2* gene

Direction	Primer sequences
forward	5'-TCTAGAGGATCCTTCCCCAGTGTGGC-3'
reverse	5'-AGGACAAGACTCTCAGAGGCT-3'

uct of the *APOC2* gene as described above was cloned into pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) using the In-Fusion PCR Cloning Kit (Clontech Laboratories, Inc., Mountain View, CA, USA); thereafter, the firefly luciferase gene was inserted just before the apoC-II stop codon to generate an *APOC2* minigene reporter construct. HEK293 cells and HepG2 cells were plated in a 48-well plate in triplicate, followed by transient transfection with 300 ng of the reporter construct along with 20 ng Renilla expression vector (pRL-SV40 (Promega), as a transfection control) using Attractene transfection reagent (Qiagen). Cells were lysed 48 h after transfection and cell lysates were used for the measurement of firefly and Renilla luciferase activities according to the supplier's instructions (Promega for firefly luciferase; Toyo B-Net Co., Ltd (Tokyo, Japan) for Renilla luciferase). The firefly luciferase activities were normalized against control Renilla luciferase activities.

Genotyping of Single Nucleotide Polymorphisms (SNPs) in *APOC2*

We included 384 persons who participated in an annual health check conducted by the Hiroshima Atomic Bomb Casualty Council Health Management Center (Hiroshima, Japan). Written informed consent was obtained from all the participants. We genotyped variants by direct sequencing, performed with the BigDye terminator (Life Technologies, Carlsbad, CA) and resolved by an ABI 3700 automated DNA sequencer (Life Technologies). The results were integrated using a Sequencher (Gene Codes, Ann Arbor, MI). Variants were genotyped manually.

Table 5. Primers for sequencing the *APOC2* gene and the intergenic region between *APOC2* and *CLPTM1*

Position in gene	Direction	Primer sequences
Promoter, Exon 1	fw	5'-AAGCTCTGACTCCATTCCCA-3'
Promoter, Exon 1	rv	5'-ACACGGGCTTCTAATA-3'
Intron 1	fw	5'-CGGAGCTGGTGAGGACA-3'
Intron 1	fw	5'-TGAGCTTCTGCTTAGAGTTAGGGT-3'
Intron 1	rv	5'-CACCTCAGCCACCCTAACTCTA-3'
Intron 1	fw	5'-AACTGTAGGCTGGGCGTG-3'
Intron 1	fw	5'-AATTGTAGACCATTTGCTTGTGTTC-3'
Intron 1	fw	5'-ATTAGCAGCTGGGCATGGT-3'
Intron 1, Exon 2	rv	5'-TGGGCTGGGAAGATGCT-3'
Exon 2, Intron 2	fw	5'-GCTGTGTCCAAGTCCATGC-3'
Intron 2, Exon 3	rv	5'-TGGATGCAGTCGGTGGTAT-3'
Exon 3, Intron 3	fw	5'-GGGCTCTCCTGACACACTCT-3'
Exon 4	fw	5'-CTCCCTCTAACCATCTGTGCTT-3'
Exon 4	rv	5'-GCTGAGGCACACAGAATCG-3'
Intergenic region	fw	5'-CAATCTCGGCTCACTGC-3'
Intergenic region	fw	5'-CATAGACACAGCTAGTCCACAGTG-3'
Intergenic region	fw	5'-CCTAGGCTAGTCTCCAATTCCTG-3'
Intergenic region	fw	5'-GCCAAGATTCCTGTATCCTGAG-3'
Intergenic region	fw	5'-GTGGGGAGGGGAAGACCCTG-3'
Intergenic region	fw	5'-TCAGAGTAGCTGGACCACAGG-3'
Intergenic region	fw	5'-AGTGCGATGGCATGATCT-3'
Intergenic region	fw	5'-GAATTCAGTGGCATGATCTT-3'
Intergenic region	rv	5'-GTCTGTAACTCCAGCTACTCG-3'
Intergenic region	fw	5'-ATACAGCAGTGACCACAACA-3'
Intergenic region	rv	5'-TGTTGTGGTCACTGCTGTAT-3'
Intergenic region	fw	5'-CCATCTAACTACGTCTTCCCA-3'

fw, forward; rv, reverse.

Statistical Analyses

Results are presented as the means ± SE. Student's *t*-test was employed to compare the means between two groups. All calculations were performed with Stat View version 5.0 for Windows (SAS Institute Japan Ltd., Tokyo, Japan).

Results

Electrophoretic Analysis of Plasma ApoC-II Protein

First, we performed immunoblot analysis of the patient's serum using anti-human apoC-II polyclonal antibody to assess if the patient's serum was deficient in apoC-II even with this highly sensitive method. Unexpectedly, a trace amount of apoC-II protein was identified in the patient's plasma (**Fig. 1A**). The concentration of apoC-II in the patient's plasma was estimated to be 0.6 $mg \cdot dL^{-1}$, which was well below the average plasma apoC-II concentration of healthy Japanese subjects (2.9 ± 1.3 $mg \cdot dL^{-1}$)[27], and that of heterozygotic cases of apoC-II deficiency (1.8 ± 0.5 $mg \cdot dL^{-1}$)[28]. Notably, the molecular weight of the patient's apoC-II was identical to that of the normal subject. The electrophoretic patterns of the patient's residual apoC-II were also indistinguishable from that of the normal subject as demonstrated by IEF (**Fig. 1B**) or by 2D-PAGE (**Fig. 1C**), suggesting that the patient's plasma contains apoC-II protein, albeit at markedly reduced levels. These phenotypes are compatible with those of the reported cases of hypoapoC-II[17-19].

Lipoprotein Lipase Activity in Post-Heparin Plasma (PHP)

Next, we assessed if LPL activity was impaired in the patient's plasma. Without the addition of normal plasma, LPL activity of the patient's PHP was reduced by 48% compared with that of the control subject (control vs. the patient: 8.6 vs. 4.5 $\mu mol \cdot h^{-1} \cdot mL^{-1}$). Addition of normal plasma dose-dependently increased the patient's LPL activity by 1.5-fold (4.5,

5.8, and 6.6 $\mu mol \cdot h^{-1} \cdot mL^{-1}$, by the addition of 0, 0.5, and 1 μL of normal plasma, respectively), indicating that the patient's plasma lacked a co-factor for LPL, but not LPL *per se*. HTGL activity was found to be reduced in the patient's PHP (control vs. the patient: 10.3 vs. 2.4 $\mu mol \cdot h^{-1} \cdot mL^{-1}$), as often seen in homozygous apoC-II deficiency for unknown reasons[6].

Northern Blot Analysis of Primary Monocytes/ Macrophages Derived from Peripheral Blood

One possible explanation for the decreased levels of plasma apoC-II protein might be some defects in apoC-II mRNA transcription, as reported in other cases of hypoapoC-II. To rule out this possibility, we first measured mRNA levels of apoC-II in monocytes/ macrophages isolated from peripheral blood, known cell types that express apoC-II endogenously[29]. With only 2 μg of total RNA loaded, we successfully detected abundant mRNA expression of apoC-II in the monocytes/macrophages of normal control subjects (**Fig. 2A**). In contrast, the patient's monocytes/macrophages showed 60% reduction in apoC-II mRNA compared with normal control subjects, whereas the mRNA levels remained unchanged for all other genes tested: apoC-IV and apoE, genes in the same gene cluster; CLPTM1, a gene located 3' downstream of apoC-II; MCSF and RPLP0, control genes (**Fig. 2A, left**). Importantly, the size of apoC-II mRNA of the patient was identical to that of normal subjects. Incubation with LXR agonist (T0901317), which is known to upregulate the transcription of genes in the apoE/C-I/ C-II/C-IV gene cluster, increased apoC-II mRNA levels in the cells of both the patient and controls to similar extents (controls, 1.7- and 1.4-fold; patient, 2-fold), indicating that the response to LXR agonist is well preserved in the patient's cells (**Fig. 2A, right**). ApoC-II mRNA levels in the patient's cells were decreased by 53% compared with control cells even in the presence of LXR agonist. As demonstrated by actinomycin D experiment (**Fig. 2B**), the stability of apoC-II mRNA was not different between the patient and normal subject, suggesting that transcription of apoC-II mRNA was impaired in the patient's cells.

Southern Blot and Genomic DNA Copy Number Analyses of the *APOC2* Gene

To rule out the possibility that reduced levels of apoC-II mRNA resulted from a major gene rearrangement, we performed Southern blot analysis (**Fig. 3A**). No difference was observed between the patient and control samples for all enzymes tested except for SacI, which is due to SNP rs5120 in intron 1 (**Table 6**), resulting in SacI digestion in the DNA of the control subject but not that of the patient. To exclude the possibility that one of the *APOC2* alleles was missing from the patient's genome, the DNA copy number of the *APOC2* gene relative to the control gene, *RPLP0*, was examined by real-time PCR, revealing no major

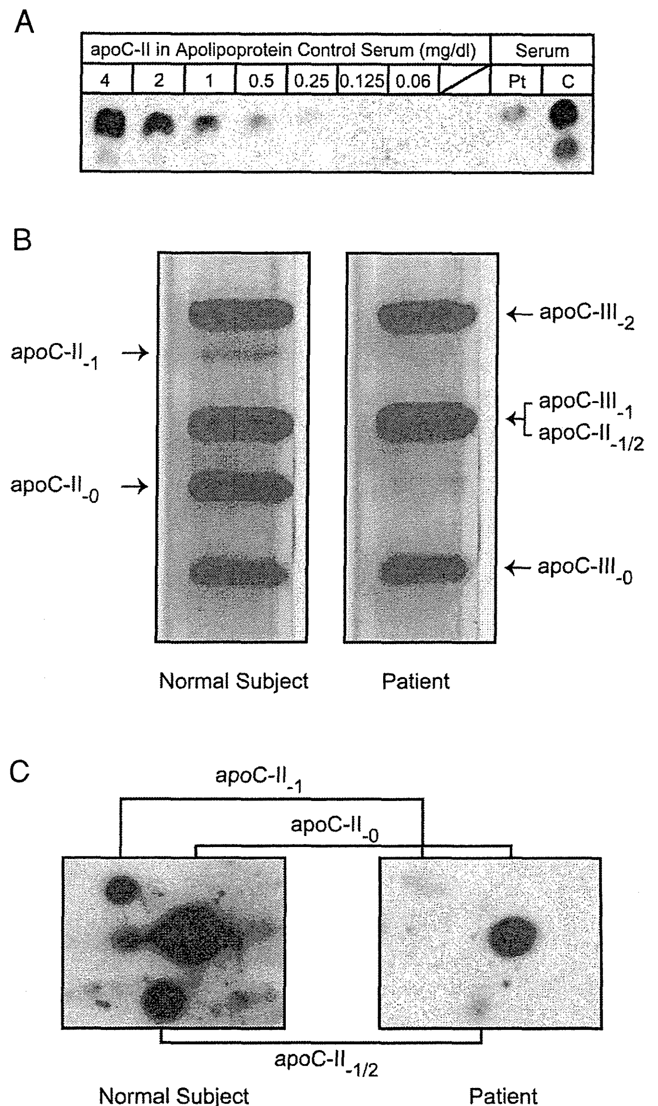

Fig. 1. Plasma apolipoprotein analysis.

(A) Immunoblot analysis of apoC-II. One μL of plasma from the patient (Pt), control subject (C), or apolipoprotein control serum (Daiichi High; Daiichi Pure Chemicals Co., Ltd.) with various concentrations of apoC-II, was subjected to SDS-PAGE followed by immunoblotting using anti-human apoC-II polyclonal antibody. (B) For isoelectric focusing of apolipoproteins, 30 μg of delipidated TGRL apolipoproteins was subjected to electrophoresis using Immobiline DryStrip (pH4-7), followed by Coomassie Brilliant Blue staining. (C) For two-dimensional PAGE of apolipoproteins, 2 μg of delipidated TGRL apolipoproteins was electrophoresed as described in Methods. ApoC-II protein was detected by immunoblotting as described above.

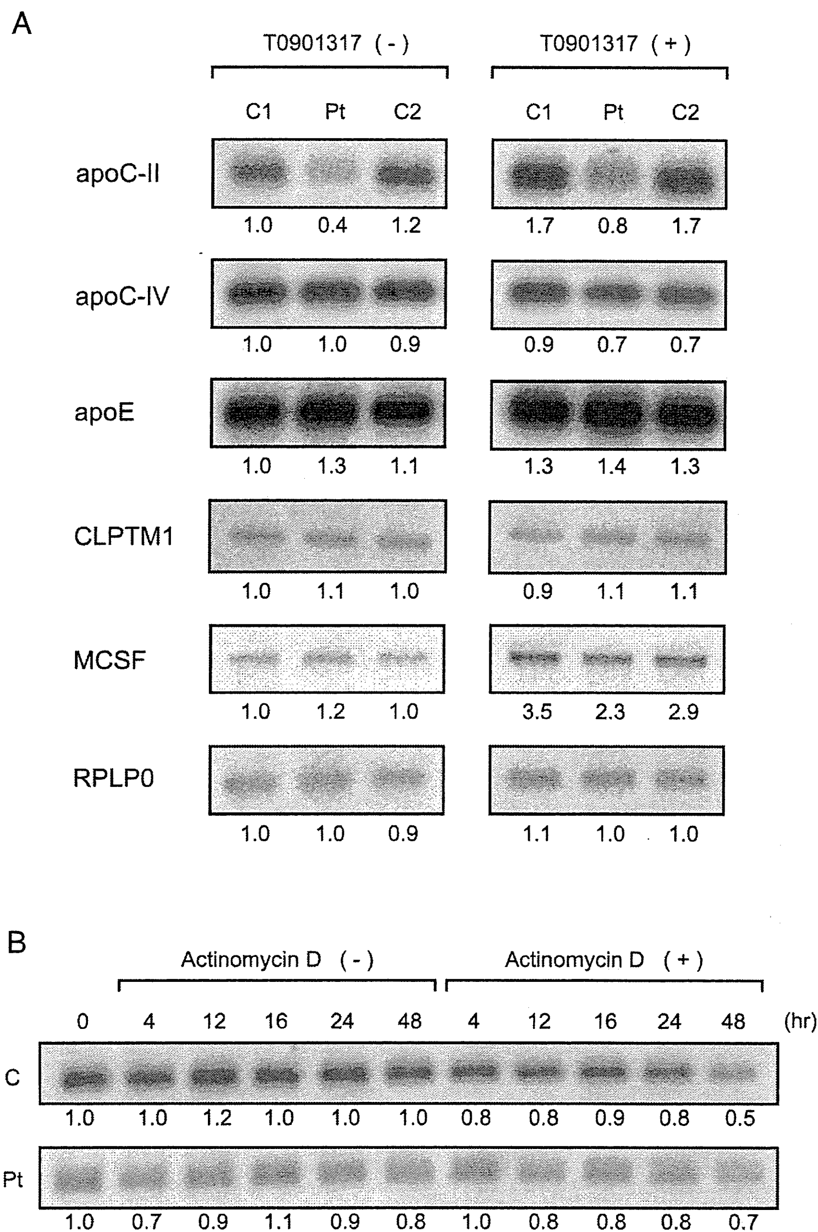

Fig. 2. Northern blot analyses of apolipoproteins in monocytes/macrophages.

(A) Monocytes isolated from peripheral blood of the patient (Pt) and two control subjects (C1 and C2) were plated and differentiated to macrophages. On day 7, cells were incubated with (right panel) or without (left panel) 10 $\mu mol \cdot L^{-1}$ T0901317 (LXR agonist). Cells were harvested the next day to collect mRNA for Northern blot analyses. (B) In a separate experiment, 10 $\mu g \cdot mL^{-1}$ of actinomycin D was added to the incubation medium on day 8 (without T0901317 treatment) to test the stability of apoC-II mRNA. Cells were harvested at the indicated time points. Intensity of each band was quantified by ImageJ 1.39 software (NIH). C, control subject.

difference between the patient and two control subjects (**Fig. 3B**).

APOC2 Minigene Reporter Assay

To determine if decreased apoC-II mRNA transcription in the patient's cells is due to decreased transcriptional activity of the *cis*-regulatory regions of the

patient's *APOC2* gene, we constructed reporter constructs of the *APOC2* minigene amplified from genomic DNA of the patient and control subject; subsequently, the luciferase gene was cloned just before the stop codon of the *APOC2* gene (**Fig. 4A**). Transient transfection of these constructs to HEK293 cells or HepG2 cells revealed that transcriptional activity of the patient's minigene was significantly decreased by 39% and 51%, respectively, compared with that of the control construct (**Fig. 4B**). These results demonstrate that transcriptional repression of the patient's *APOC2* gene is at least partially due to a functional defect in the *cis*-regulatory region of the *APOC2* gene.

Sequencing of the *APOC2* and Other Genes in Plasma TG Metabolism

Next, we sought to identify if there are any sequence variants in the *APOC2* gene of the patient that account(s) for the extremely low levels of apoC-II proteins in the patient's plasma. Sequencing of *APOC2* of the patient and control subject, followed by comparison with the reference sequence (**NC_000019.9**), revealed 50 SNPs (**Table 6**). We also sequenced *APOC2* of the patient's mother and found 42 SNPs that were heterozygous for the patient's mother and homozygous for the patient (data not shown); however, all of these SNPs were common variants with allele frequencies higher than 0.35 (as reported in the dbSNP database, or as identified by direct sequencing of Japanese individuals ($n=384$) for the SNPs where allele frequencies were not reported in the dbSNP database). As apoC-II deficiency is less frequent than LPL deficiency, which occurs in one in a million[30], these common variants are unlikely to be the causative mutations.

Sequencing of other genes involved in plasma TG metabolism (*LPL, APOA5, APOC3, LMF1* and *GPIHBP1*) revealed no rare variant in the patient's gene (data not shown). These results indicate that other yet-to-be-identified genes, which regulate plasma apoC-II protein levels, are responsible for type I hyperlipidemia associated with apoC-II deficiency in this patient.

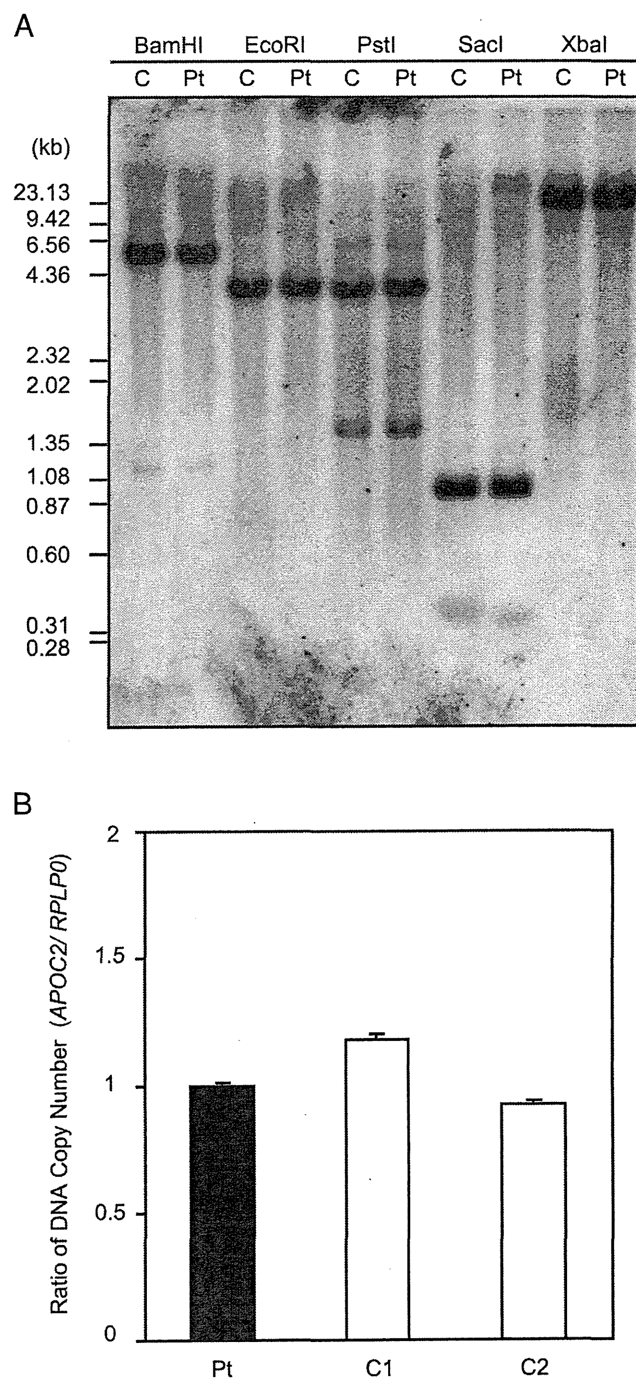

Fig. 3. Southern blot and genomic DNA copy number analyses.

(A) Ten μg of genomic DNA from the patient (Pt) and normal control subject (C) were digested by restriction enzymes overnight and then subjected to Southern blot analysis using apoC-II cDNA probe. (B) DNA copy number analysis. DNA from the patient (Pt) and two normal control subjects (C1 and C2) were used to amplify the genomic regions of *APOC2*, or *RPLP0*, flanking intron-exon boundaries, by real-time PCR. The DNA copy number of the *APOC2* gene was estimated by the relative amount of *APOC2* to *RPLP0*, and normalized to the value of the patient.

Discussion

ApoC-II deficiency has been well characterized since its first description in 1978[7]. In most cases, homozygous mutations were identified in the protein-coding region, resulting in null, truncated, or non-functional apoC-II protein[15, 16]; however, several atypical cases of apoC-II deficiency have been described, where apoC-II is severely reduced in plasma, but still

Table 6. Overview of the 50 SNPs identified by sequencing the patient's *APOC2* gene and comparison to the reference sequence (**NC_000019.9**)

Nucleotide number	GenBank base no.	Position in gene	RefSNP ID	Minor allele Frequency[a]	Alleles	
					Ref.	Pt.
c.-147C>G	45449199	Promoter	rs2288912	0.35 (C)	C	G
c.-62T>G	45449284	Exon 1	rs2288911	0.35 (T)	T	G
c.-14+157T>A	45449489	Intron 1	rs111268759	NA[b]	T	A
c.-14+159T>A	45449491	Intron 1	rs7249138	0.49 (T)	T	A
c.-14+162_-14+163delGA	45449494	Intron 1	rs10605424	NA[b]	GA	-
c.-14+701C>T	45450033	Intron 1	rs9304644	0.44 (C)	C	T
c.-14+1076T>C	45450408	Intron 1	rs9304646	0.43 (T)	T	C
c.-14+1090_-14+1091insAA	45450422	Intron 1	rs34457201	NA[c]	-	AA
c.-13-851A>G	45450872	Intron 1	rs4803774	0.43 (A)	A	G
c.-13-795T>C	45450928	Intron 1	rs4803775	0.35 (T)	T	C
c.-13-281A>G	45451442	Intron 1	rs7256684	0.43 (A)	A	G
c.-13-103T>A	45451620	Intron 1	rs5120	0.35 (T)	T	A
c.216-145G>C	45452271	Intron 3	rs3745152	0.45 (C)	G	C
c.216-143_216-142insTTC	45452273	Intron 3	rs3833214	0.48 (TTC)[d]	-	TTC
c.216-81T>C	45452335	Intron 3	rs4803776	0.36 (T)	T	C
c.*188_*189insT	45452694	3'-UTR	rs150448996	0.42 (T)[d]	-	T
c.*306C>T	45452812	3'-UTR	rs1130742	0.36 (T)	C	T
c.*638C>T	45453144	i.r.	rs7257468	0.44 (C)	C	T
c.*645T>G	45453151	i.r.	rs7258345	0.44 (T)	T	G
c.*659C>T	45453165	i.r.	rs7257476	0.44 (C)	C	T
c.*733G>A	45453239	i.r.	rs12709889	0.36 (A)	G	A
c.*1150G>A	45453656	i.r.	rs10423208	0.44 (G)	G	A
c.*1372G>A	45453878	i.r.	rs10402642	0.44 (G)	G	A
c.*1730G>A	45454236	i.r.	rs7246900	0.44 (G)	G	A
c.*1857G>A	45454363	i.r.	rs11083752	0.44 (G)	G	A
c.*2180T>C	45454686	i.r.	rs7248162	0.44 (T)	T	C
c.*2253A>G	45454759	i.r.	rs7247227	0.44 (A)	A	G
c.*2260G>A	45454766	i.r.	rs7247551	0.35 (G)	G	A
c.*2952G>A	45455458	i.r.	rs892101	0.44 (G)	G	A
c.*3200C>T	45455706	i.r.	rs7251501	0.44 (C)	C	T
c.*3209C>G	45455715	i.r.	rs7251503	0.44 (C)	C	G
c.*3395C>G	45455901	i.r.	rs12460346	0.46 (C)	C	G
c.*3399C>T	45455905	i.r.	rs12460347	0.50 (T)	C	T
c.*3415A>G	45455921	i.r.	rs7254723	0.47 (A)	A	G
c.*3460C>T	45455966	i.r.	rs12460352	0.44 (C)	C	T
c.*3479A>C	45455985	i.r.	rs35670684	NA[e]	A	C
c.*3481delT	45455987	i.r.	rs34356599	0.47 (T)	T	-
c.*3595G>A	45456101	i.r.	rs4803777	0.45 (G)	G	A
c.*3991T>C	45456497	i.r.	rs4803779	0.44 (T)	T	C
c.*4122C>T	45456628	i.r.	rs4803780	0.44 (C)	C	T
c.*4599A>G	45457105	i.r.	rs3760626	0.44 (A)	A	G
c.*4674T>C	45457180	i.r.	rs3760627	0.44 (T)	T	C
c.*4719G>A	45457225	i.r.	rs3760628	0.44 (G)	G	A
c.*4787C>T	45457293	i.r.	rs66867801	0.44 (C)	C	T
c.*4800C>T	45457306	i.r.	rs7259679	0.44 (C)	C	T

(Cont Table 6)

Nucleotide number	GenBank base no.	Position in gene	RefSNP ID	Minor allele Frequency[a]	Alleles	
					Ref.	Pt.
c.*4813G>A	45457319	i.r.	rs66771331	0.44 (G)	G	A
c.*4871G>A	45457377	i.r.	rs73047641	0.44 (G)	G	A
c.*4887T>C	45457393	i.r.	rs73047643	0.36 (C)	T	C
c.*5061T>C	45457567	i.r.	rs7245611	0.44 (T)	T	C
c.*5178G>C	45457684	i.r.	rs10426750	0.44 (G)	G	C

Standardized nomenclature according to the Human Genome Variation Society is given in column 1, with the correlating GenBank number in column 2 (ref no: **NC_000019.9**). Reference SNP IDs (RefSNP ID) and minor allele frequencies as reported in dbSNP database are given in column 4 and 5, respectively. The alleles of the reference (Ref.: **NC_000019.9**) and patient (Pt.) are given in column 6 and 7, respectively. UTR, untranslated region; i.r., intergenic region between *APOC2* and *CLPTM1*. [a]minor allele is given in parenthesis. [b]SNPs located in a $(TG)_n(AG)_m$ microsatellite; the patient has $(TG)_{20}(AG)_8$, which is reportedly a common variant (Fornage M et al. *Genomics* 1992; 12: 63-68). [c]The allele frequency not determined even by direct sequencing due to polyA repeat. [d]The allele frequency determined by direct sequencing of Japanese individuals (n=384). [e]The frequency of C allele (the patient type) was 15.2% in 112 individuals, for whom PCR was successful in the control cohort (n=384).

detectable (named hypoapoC-II). In these cases, homozygous mutations were found in the promoter region[19] or in the donor splice site[17, 18] of *APOC2*, resulting in decreased transcription or defective processing of apoC-II mRNA, respectively. Here we describe a new case of hypoapoC-II with no causative mutations found in the *APOC2* gene. Involvement of other genetic defects affecting plasma apoC-II levels is clearly implied.

Our case was not due to mutations in the protein-coding region of *APOC2* (**Fig. 3**, **Table 6**). Markedly reduced levels of plasma apoC-II protein (**Fig. 1**) could result from decreased apoC-II mRNA transcription, as reported in other cases of hypoapoC-II[17-19]. In fact, we found about 50% reduction in endogenous apoC-II mRNA levels in monocytes/macrophages of the patient (**Fig. 2A**) with no apparent change in the rate of mRNA degradation (**Fig. 2B**). We further detected about a 50% decrease in the transcriptional activity of the *cis*-regulatory region of the patient's *APOC2* gene (**Fig. 4B**); however, all the SNPs contained in the *cis*-regulatory regions are common variants. By scrutinizing every type of apoC-II deficiency reported so far, we conclude that this is a new case of apoC-II deficiency without any rare variants in the *APOC2* gene.

The major question here is what other genes cause extremely low levels of apoC-II proteins in the patient's plasma, and exactly what molecular mechanism is involved. Currently, we do not have the answer to these questions. We sequenced other genes involved in plasma TG metabolism (*LPL*, *APOA5*, *APOC3*, *LMF1* and *GPIHBP1*) and found no mutations or rare variants. The causative genetic defects in our patient would most likely involve other unknown genes that regulate plasma apoC-II concentration. The search for a defect (in genes, miRNAs, etc.) by whole-exome or whole-genome sequencing is of particular interest, which could reveal the yet-to-be-identified regulatory mechanisms of the apoC-II protein.

While our data clearly showed that the transcriptional activity of the patient's *APOC2* minigene was reduced by 50% compared with the normal subject (**Fig. 4B**) with an accompanying 50% reduction in apoC-II mRNA levels (**Fig. 2A**), apoC-II protein levels are more profoundly reduced in the patient's plasma (0.6 $mg \cdot dL^{-1}$; **Fig. 1A**): Compared with the apoC-II protein levels in normolipidemic Japanese population (2.9 $mg \cdot dL^{-1}$)[27], the patient has only 21% of the normal amount of plasma apoC-II protein. This discrepancy between apoC-II protein and apoC-II mRNA levels in the patient implies the yet-to-be-identified causative genes that regulate apoC-II protein levels at translational or post-translational levels. Alternatively, the causative genes could be some transcriptional factors that affect apoC-II mRNA transcription, as the discrepancy may result from 1) the difference between the *in vivo* and *in vitro* milieu of transcriptional machinery, or 2) from the decreased transcription of apoC-II mRNA in the liver or intestine *in vivo*.

Another important question is if this level of reduction in plasma apoC-II (0.6 $mg \cdot dL^{-1}$) could explain the observed massive hypertriglyceridemia in this patient. In the seminal paper by Breckenridge *et al.*, it was implied that only 5-10% of the normal levels of apoC-II would be sufficient to keep plasma triglycerides levels within the normal range[7]. Considering that plasma apoC-II concentration of the patient is only 21% of that of normolipidemic Japanese popu-

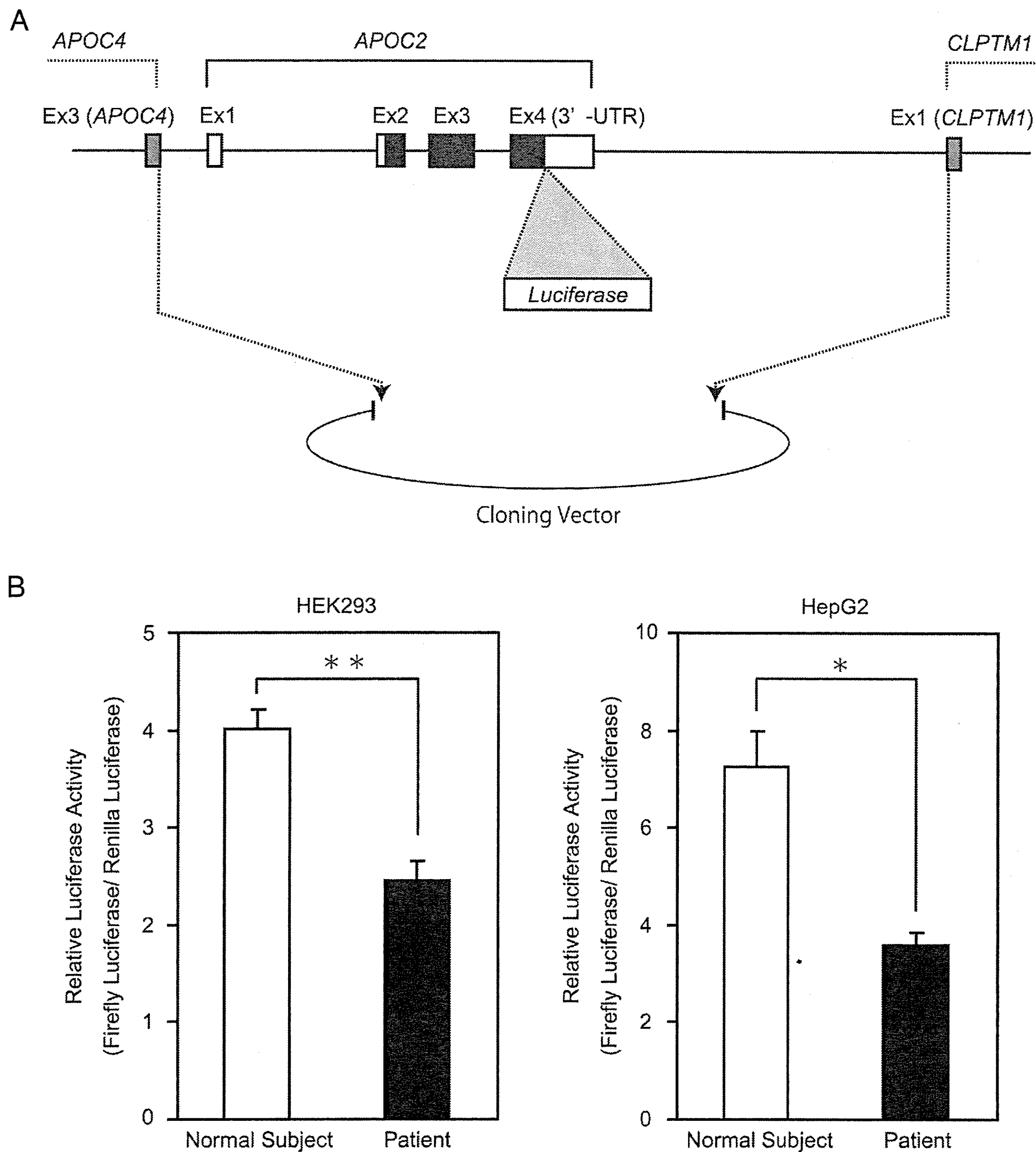

Fig. 4. Minigene reporter assay.

(A) The *APOC2* minigene, spanning from the 3' end of the last exon of *APOC4* to the 5' end of the first exon of *CLPTM1*, were cloned to pGEM-T easy vector, followed by insertion of the firefly luciferase gene just before the stop codon of *APOC2*. (B) HEK293 and HepG2 cells were transiently transfected with these minigene reporter constructs along with Renilla luciferase plasmid as a transfection control. Relative luciferase activity (RLU) was calculated by dividing firefly luciferase activity by Renilla luciferase activity. Data are expressed as the average ± SE of triplicate wells ($^*p<0.01$; $^{**}p<0.005$).

lation as calculated above[27]), this level of hypoapoC-II would not be sufficient to explain hypertriglyceridemia in this patient *per se*. Indeed, another case of hypoapoC-II with similar plasma apoC-II concentration (0.5 mg·dL^{-1}) was reported by Kawano *et al.*, where the patient only had moderate postprandial hypertriglyceridemia (1.46 mmol·L^{-1} (129 mg·dL^{-1}) and 6.14 mmol·L^{-1} (543 mg·dL^{-1}), in the fasted and fed states, respectively)[31]). This phenotypic heterogeneity may result from additional nutritional, environmental, or other genetic factors such as apoE genotype, as mentioned previously[18, 32]). In other words, hypoapoC-II might be a common genetic predisposition to hypertriglyceridemia requiring additional

genetic/environmental/nutritional factors to develop severe hypertriglyceridemia. Further research on additional genetic defects in hypoapoC-II patients may address this issue.

A key feature of hypoapoC-II reported so far is the decreased rate of transcription of apoC-II mRNA[17-19], which should accompany a decrease in apoC-II mRNA levels in endogenous tissues; however, only one study directly demonstrated the reduction in apoC-II mRNA, where they utilized a liver biopsy specimen[17]. Since the measurement of endogenous levels of apoC-II is essential for the diagnosis of hypoapoC-II, our method to detect endogenous levels of apoC-II mRNA using monocytes/macrophages derived from peripheral blood provides a useful diagnostic tool for this subtype of apoC-II deficiency. The culture system is also useful to explore the mechanism of decreased apoC-II mRNA levels (**Fig. 2**).

Conclusion

We report a new case of apoC-II deficiency that is phenotypically identical to hypoapoC-II, but with no causative mutations detected in the *APOC2* gene. Our results clearly suggest the involvement of other genes that regulate plasma apoC-II protein levels. Further studies on hypoapoC-II may uncover the yet-to-be-identified molecular mechanisms that regulate apoC-II and plasma TG metabolism.

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Conflict of Interest

None.

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Committee Report 9

Familial Hypercholesterolemia

Executive Summary of the Japan Atherosclerosis Society (JAS) Guidelines for the Diagnosis and Prevention of Atherosclerotic Cardiovascular Diseases in Japan — 2012 Version

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This is a collaborative work to describe the guidelines for familial hypercholesterolemia issued by the Committee for Epidemiology and the Clinical Management of Atherosclerosis, the Committee for the Diagnosis and Treatment of Familial Hypercholesterolemia and the Research Committee for Primary Hyperlipidemia, Research on Measures against Intractable Diseases.

Heterozygous Familial Hypercholesterolemia

1. Condition and Clinical Picture of FH

Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by abnormal LDL receptors or LDL receptor-related genes, characterized by the triad of (1) hyper-LDL cholesterolemia, (2) premature coronary artery disease (CAD) and (3) tendon/cutaneous xanthoma. Arcus corneae is also characteristic of FH; however, the rate is approximately 30%.

FH by itself is a very high-risk condition for CAD. Untreated men 30 to 50 years of age and women 50 to 70 years of age are likely to develop CAD, such as myocardial infarction and angina pectoris[1]. Early diagnosis and appropriate treatment result in the prevention of premature death. Heterozygous FH exists in approximately one in 500 people, and it is estimated that there are approximately 300,000 patients in Japan. Therefore, heterozygous FH is one of the genetic diseases most frequently encountered by general practitioners.

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2. Diagnosis of Heterozygous FH

1) LDL-C Cutoff Value

Table 1 shows the diagnostic criteria. Using data obtained from a total of 1,397 untreated dyslipidemic patients, including 439 patients with FH and 958 patients without FH, an analysis was performed of

Table 1. Diagnostic Criteria for Heterozygous FH in Adults (15 Years of Age or Older)

1. Hyper-LDL cholesterolemia (an untreated LDL-C level of ≥180 mg/dL)
2. Tendon xanthoma (tendon xanthoma on the backs of the hands, elbows, knees, etc. or Achilles tendon hypertrophy) or xanthoma tuberosum
3. Family history of FH or premature CAD (within the patient's second-degree relatives)

• The diagnosis should be made after excluding secondary hyperlipidemia
• If a patient meets two or more of the above-mentioned criteria, the condition should be diagnosed as FH. In cases of suspected FH, obtaining a diagnosis using genetic testing is desirable.
• Xanthoma palpebrarum is not included in xanthoma tuberosum.
• Achilles tendon hypertrophy is diagnosed if the Achilles tendon thickness is ≥9 mm on soft X-ray imaging.
• An LDL-C level of ≥250 mg/dL strongly suggests FH.
• If a patient is already receiving drug therapy, the lipid level that led to treatment should be used as the reference for diagnosis.
• Premature CAD is defined as the occurrence of CAD in men <55 years of age or women <65 years of age.
• If FH is diagnosed, it is preferable to also examine the patient's family members.

major items, including an LDL-C level of ≥180 mg/dL, the presence of Achilles tendon hypertrophy or cutaneous xanthoma and a history of FH or premature CAD in relatives within the second degree. The results showed a sensitivity of 94.3% and a specificity of 99.1%. In cases involving an LDL-C level of ≥190 mg/dL, the sensitivity was 92.1% and the specificity was 99.1%. Therefore, 180 mg/dL, the level at which the specificity was the same and the sensitivity was higher than that observed at 190 mg/dL, was adopted as the LDL-C cutoff value[2]. Because this analysis showed that 5% of patients with an LDL-C level of ≥250 mg/dL do not have FH, a diagnosis of FH is thus strongly suspected in the presence of an LDL-C level of ≥250 mg/dL alone[3].

2) Soft X-Ray Radiography of the Achilles Tendon

Achilles tendon hypertrophy should be evaluated using soft X-ray radiography. Positioning is performed so that the lower leg bones and sole of the foot form a 90-degree angle, and radiation is administered so that the X-ray enters the center of the lateral malleolus from the side of the foot. The imaging distance should be 120 cm, and the imaging conditions should be 50 kV and 5.0 mA. When the greatest dimension is ≥9 mm, hypertrophy is diagnosed. Conducting the evaluations using ultrasonography is possible, although it has not yet been standardized.

3) Differential Diagnosis

Diseases that must be distinguished from FH include conditions that cause secondary hyperlipidemia (e.g., diabetes mellitus, hypothyroidism and nephrotic syndrome) and a similar disease, familial combined hyperlipidemia (FCHL). FCHL is distinguished by the absence of tendon xanthoma, the presence of small, dense LDL, the presence of other types of dyslipidemia (types IIa, IIb and IV) in the patient's family and, in children, a lower degree of increase in the LDL-C level compared with that observed in FH.

3. Management Targets for LDL-C in Heterozygous FH

Because FH is a disease associated with a very high risk of CAD, FH should be considered to correspond to secondary prevention, and it is desirable to set a management target for the LDL-C level at <100 mg/dL. However, in many cases, it is difficult to achieve a management target for an LDL-C level of <100 mg/dL in FH patients in clinical practice. Therefore, it is acceptable to aim for <50% of the pretreatment level if the management target for LDL-C is not achieved. The achievement of the management target does not always assure the absence of future cardiovascular events. In the treatment of FH, risk assessment cannot be applied using the risk charts provided in these guidelines. This management target should be applied to patients with FH ≥30 years of age, and it is desirable to administer the treatment under the direction of a specialist, in principle. Treatment for FH in patients 15-29 years of age must be administered under the direction of a specialist.

4. Treatment of Heterozygous FH

1) Lifestyle Modification

Lifestyle modification should be performed in FH patients after diagnosis and continued as described in committee report 7A[4]. However, due to the high risk of cardiovascular disease (CVD), screening for CVD before administering exercise therapy is essential. CVD should be evaluated using patient interviews to determine the presence or absence of effort angina, and exercise electrocardiography and echocardiography should be performed. If the existence of ischemic heart disease is suspected, administering treatment for ischemic heart disease before initiating exercise therapy is thus preferred. Smoking cessation and obesity management are also important.

2) Drug Therapy

Statins are the first-line drugs for FH treatment. A retrospective analysis of 329 patients with heterozy-

gous FH conducted in Japan revealed that the use of statins delayed the onset of CAD[5]. If the patient does not respond to monotherapy with statins, other lipid-lowering drugs should be concomitantly used. Such concomitant drugs include ezetimibe, bile acid-binding resins (cholestyramine and colestimide), probucol, fibrates and nicotinic acid derivatives. Although there is no evidence that these combination therapies inhibit cardiovascular events in patients with FH more effectively than statin monotherapy, strict management of the LDL-C level is recommended in patients with FH. A retrospective investigation suggested that probucol delays the recurrence of CAD in patients with heterozygous FH[6].

3) Indications for LDL Apheresis

In heterozygous FH patients, LDL apheresis should be considered if the total cholesterol (TC) level does not decrease to ≤250 mg/dL following intensive drug treatment in the presence of CAD. If LDL apheresis is indicated, it is desirable to consult a specialist.

5. FH in Children

1) Diagnosis of Heterozygous FH in Children

The initial finding of heterozygous FH is hyper-LDL cholesterolemia. In childhood, many patients do not develop physical signs associated with hyper-LDL cholesterolemia, such as Achilles tendon xanthoma and arcus corneae. Therefore, FH in children is primarily diagnosed based on the presence of hyper-LDL cholesterolemia and family history. In the diagnosis of FH in children, if the parent(s) has/have hyper-LDL cholesterolemia, a diagnosis of FH in the parent(s) should be established. The diagnostic criteria for heterozygous FH in children are shown in **Table 2**. Because 95% of healthy children have an LDL-C level of ≤140 mg/dL[7], the cutoff value for screening is defined as 140 mg/dL.

Table 2. Diagnostic Criteria for Heterozygous FH in Children

1. Hypercholesterolemia: an untreated LDL-C level of ≥140 mg/dL (measure the LDL-C level if the TC level is ≥220 mg/dL)
2. Family history of FH or premature CAD within the patient's second-degree relatives

• Pediatric patients exhibit few symptoms, such as tendon xanthoma. Therefore, diagnosing FH in the patient's family members is important.
• The LDL-C level may vary during development. Providing careful follow-up is necessary.
• Premature CAD is defined as the occurrence of CAD in men <55 years of age or women <65 years of age.

2) Treatment for Heterozygous FH in Children

• Nutritional Guidance and Lifestyle Modification

If heterozygous FH is diagnosed, the affected child and their guardians should be directed to modify their lifestyle as soon as possible. Affected children with a smoking habit should be directed to stop smoking. In addition, they should be directed to avoid smoking throughout their life and receive an explanation of the risk of passive smoking; their family members should also be directed to stop smoking.

• Drug Therapy

Evidence pertaining to the age from which treatment should be administered in patients with heterozygous FH has not yet been established in Japan. Because atherosclerotic changes in the coronary arteries are observed from an earlier age in heterozygous FH patients, appropriate LDL-C management is recommended at an earlier age. According to the proposal of the American Academy of Pediatrics, if a patient has an "LDL-C level of ≥190 mg/dL" or an "LDL-C level of ≥160 mg/dL and a family history of premature CAD or at least two risk factors," lipid-lowering treatment should be initiated, even in children, and if lifestyle modification is inadequate, drug therapy should also be considered in boys aged 8 to 10 years or older and in girls after menarche[8]. Among patients who are at a very high risk, such as patients with tendon xanthoma or aortic stenosis or those with a family history of remarkable atherosclerosis, a differential diagnosis of heterozygous FH should be performed. With respect to drug therapy, in terms of safety for growth and development, bile acid-binding resins, which are not absorbed from the gastrointestinal tract, are typically used and are the first-line drugs. Drug therapy for children should be administered under the direction of a specialist.

6. Heterozygous FH in Women

Drug therapy, other than bile acid-binding resins, during pregnancy should be carefully considered due to concerns regarding the risk of fetal malformations. According to the National Institute for Health and Clinical Excellence[9], if pregnancy is diagnosed during drug therapy, lipid-lowering drugs other than bile acid-binding resins should be immediately discontinued, and, if there is a possibility of pregnancy, pregnancy after the discontinuation of drug treatment for three months should be recommended.

Homozygous Familial Hypercholesterolemia

1. Diagnosis of Homozygous FH

Homozygous FH is characterized by the presence of a TC level of ≥600 mg/dL, xanthoma and CVD from childhood, with both parents being heterozygous for FH. Therefore, making a clinical diagnosis is possible. If homozygous FH is suspected even when the TC level is <600 mg/dL, obtaining the diagnosis and therapeutic decisions from a specialist is essential.

2. Drug Therapy for Homozygous FH

Similar to that recommended for patients with heterozygous FH, lifestyle modification, including diet therapy, exercise therapy, smoking cessation and obesity management, provides the basis for treatment in patients with homozygous FH, although intensive LDL-C-lowering treatment is required at an earlier age because patients with homozygous FH face a considerable risk with respect to the development and progression of CAD. However, homozygous FH is much less responsive to drug treatment than heterozygous FH. Therefore, the administration of LDL apheresis once every one to two weeks is necessary. Probucol exerts LDL-C-lowering effects on homozygous FH and may cause the regression or disappearance of xanthoma in the skin or Achilles tendon. For patients with homozygous FH who wish to have children, screening for CAD and the presence of aortic stenosis and supravalvular stenosis should be performed, and appropriate measures should be taken as required to ensure the safe continuation of pregnancy and delivery[10].

3. LDL Apheresis for Homozygous FH

In patients with homozygous FH, it is difficult to decrease the LDL-C level sufficiently using existing drug therapies, and many patients require continued LDL apheresis with extracorporeal circulation starting in childhood. Considering the inhibition of the progression of CVD, the earlier LDL apheresis is initiated, the better; however, it is difficult to perform LDL apheresis until the affected child can be kept in bed during apheresis. Realistically, the timing of treatment initiation is 4 to 6 years of age, when children can lie in bed and extracorporeal circulation can be performed; however, it is recommended that the treatment be initiated as early as possible.

4. Pregnancy and Delivery of Patients with Homozygous FH

It is important to permit patients with homozygous FH to become pregnant as planned. Before pregnancy, screening for atherosclerosis should be performed using carotid ultrasonography, echocardiography and exercise tolerance tests to assess the status of atherosclerosis. By three months before the planned pregnancy, treatment with lipid-lowering drugs other than bile acid-binding resins should be discontinued. Because the cardiovascular system is greatly stressed during late pregnancy, particularly at delivery, performing LDL apheresis during pregnancy is desirable. LDL apheresis can also be safely administered during pregnancy.

5. Homozygous FH Designated as a Specified Disease

In October 2009, homozygous FH was designated as a specified disease in the Specified Disease Treatment Research Program. The criteria for designation are as follows: patients with homozygous FH definitively diagnosed using a genetic analysis of genes involved in the LDL metabolic pathway or measurement of the LDL receptor activity are definitively designated, and patients with remarkable hypercholesterolemia and those with cutaneous xanthoma starting in childhood who are refractory to drug treatment should be designated.

Footnotes

This is an English version of the guidelines of the Japan Atherosclerosis Society (chapter 9) published in Japanese in June, 2012. The details of this Committee Report 9 on Familial Hypercholesterolemia have been previously published as an original manuscript[11]; this is a brief summary.

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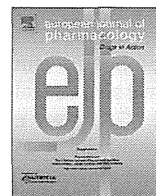

Cardiovascular Pharmacology

Locked nucleic acid antisense inhibitor targeting apolipoprotein C-III efficiently and preferentially removes triglyceride from large very low-density lipoprotein particles in murine plasma

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ABSTRACT

A 20-mer phosphorothioate antisense oligodeoxyribonucleotide having locked nucleic acids (LNA–AON) was used to reduce elevated serum triglyceride levels in mice. We repeatedly administered LNA–AON, which targets murine apolipoprotein C-III mRNA, to high-fat-fed C57Bl/6J male mice for 2 weeks. The LNA–AON showed efficient dose-dependent reductions in hepatic apolipoprotein C-III mRNA and decreased serum apolipoprotein C-III protein concentrations, along with efficient dose-dependent reductions in serum triglyceride concentrations and attenuation of fat accumulation in the liver. Through precise lipoprotein profiling analysis of sera, we found that serum reductions in triglyceride and cholesterol levels were largely a result of decreased serum very low-density lipoprotein (VLDL)-triglycerides and -cholesterol. It is noteworthy that larger VLDL particles were more susceptible to removal from blood than smaller particles, resulting in a shift in particle size distribution to smaller diameters. Histopathologically, fatty changes were markedly reduced in antisense-treated mice, while moderate granular degeneration was frequently seen the highest dose of LNA–AON. The observed granular degeneration of hepatocytes may be associated with moderate elevation in the levels of serum transaminases. In conclusion, we developed an LNA-based selective inhibitor of apolipoprotein C-III. Although it remains necessary to eliminate its potential hepatotoxicity, the present LNA–AON will be helpful for further elucidating the molecular biology of apolipoprotein C-III.

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1. Introduction

Apolipoprotein C-III (apoC-III) is synthesized mainly in the liver and circulates in plasma (Bruns et al., 1984). The mechanism of apoC-III action is primarily thought to be the attenuation of hydrolysis of triglycerides in lipoproteins, principally by inhibiting capillary endothelial lipoprotein lipase activity. Thus, serum accumulation of apoC-III would cause reduced clearance of triglyceride-rich lipoprotein particles from blood, resulting in the blood accumulation of triglyceride-rich lipoproteins (Havel et al., 1973; Wang et al., 1985). ApoC-III is also known to reduce the clearance of triglyceride-rich lipoproteins and their remnants by blocking apolipoprotein B- or apolipoprotein E-mediated uptake of these lipoproteins to low-density lipoprotein (LDL) receptor (Clavey et al., 1995; Sehayek and Eisenberg, 1991). As growing evidence has shown that elevated plasma triglyceride levels are major risk factors for metabolic syndrome, type 2 diabetes and cardiovascular diseases, apoC-III is a potential therapeutic target for these diseases (Goldberg, 2001; Grundy et al., 2004; Hokanson and Austin, 1996; Sarwar et al., 2007). This notion is also supported by the observation that humans with a null mutation in *APOC3* gene show lower fasting and postprandial serum triglycerides and LDL cholesterol and higher high-density lipoprotein (HDL) cholesterol levels, as well as reduced coronary artery calcification, as compared to humans with normal apoC-III activity (Pollin et al., 2008), while some specific single-nucleotide polymorphism carriers in *APOC3* show increased plasma triglyceride levels and evidence of non-alcoholic fatty liver, in addition to

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elevated cardiovascular disease risk (Petersen et al., 2010). A number of studies using genetically engineered mouse models have also revealed the dyslipidemic or atherogenic effects of apoC-III (Gerritsen et al., 2005; Ito et al., 1990; Jong et al., 2001; Takahashi et al., 2003). In addition, attenuation of apoC-III has been shown to be beneficial for type I diabetes (Holmberg et al., 2011; Juntti-Berggren et al., 1993, 2004). Thus, the privation of apoC-III would lead to significant benefits, both indirectly and directly, in the reduction of cardiovascular disease risk (Ooi et al., 2008; Pollin et al., 2008).

There are currently several state-of-the-art gene silencing approaches available for target-specific disruption, such as antisense oligonucleotides (AONs), monoclonal antibodies and small interfering RNAs (siRNAs), which are showing promising results, particularly in dyslipidemia therapy (Norata et al., 2013). Graham et al. (2013) recently reported successful attenuation of apoC-III mRNA and plasma triglyceride levels in preclinical models and humans by using antisense oligonucleotides chemically modified with 2′-*O*-methoxyethyl RNAs, which are known to preferentially distribute to the liver, where apoC-III is synthesized (Graham et al., 2013). Our group has developed a series of conformationally constrained nucleic acids including 2′,4′-bridged nucleic acids (2′,4′-BNAs), which are also known as locked nucleic acids (LNAs) (Mitsuoka et al., 2009; Miyashita et al., 2007; Obika et al., 1997; Yahara et al., 2012). This class of modified nucleotides has been found to have superior potential for antisense therapeutics on account of their extraordinarily high mRNA binding, as well as systemic effects over 2′-*O*-methoxyethyl RNAs (Gupta et al., 2010; Lanford et al., 2010; Lindholm et al., 2012; Prakash et al., 2010; Seth et al., 2009; Yamamoto et al., 2012). Specifically, the in vivo potencies of LNA-based AONs are generally 5 to 10-fold greater than their 2′-*O*-methoxyethyl RNA-containing counterparts (Prakash et al., 2010; Seth et al., 2009). Thus, LNA-based anti-apoC-III AONs are expected to be better alternatives to 2′-*O*-methoxyethyl RNA-containing congeners. We here demonstrated the effective reduction in elevated serum triglyceride levels in mice using LNA-based AONs targeting hepatic apoC-III mRNA.

2. Materials and methods

2.1. Antisense oligonucleotides

LNA was partially incorporated into a 20-mer phosphorothioated oligodeoxyribonucleotide. We prepared two potential AONs, **A301S** (5′-tcttatccagctttattagg-3′) and **A301SL** (5′-TCtTaTCcagcttTaTTaGg-3′), in which lowercase and uppercase letters represent DNA and LNA, respectively. These AONs have an identical sequence targeting murine apoC-III mRNA, a sequence patented by ISIS pharmaceuticals as being highly potent (Crooke et al., 2009). These modified AONs were synthesized and provided by Gene Design (Osaka, Japan). Syntheses were conducted using standard phosphoramidite procedures, and products were carefully processed under aseptic conditions and purified. All products were endotoxin-free and contained low levels of residual salts for in vivo usage.

2.2. In vivo pharmacological experiments

All animal procedures were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan). All animal studies were approved by the Institutional Review Board. C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). All mice were male, and studies were initiated when animals were aged 6–8 weeks. Mice were maintained on a 12-h light/12-h dark cycle and fed ad libitum. Mice were fed normal chow (CE-2; CLEA Japan) or Western diet (F2WTD; Oriental Yeast, Tokyo, Japan) for 2 weeks before the first treatment and throughout the experimental period. Mice received multiple treatments with AONs administered intraperitoneally at doses of 10 and 20 mg/kg/injection. Peripheral blood was collected from the tail vein in BD Microtainers (BD, Franklin Lakes, NJ) for separation of serum. At the time of sacrifice, livers were harvested and snap frozen until subsequent analysis. Collected blood was subjected to serum separation for subsequent analysis.

2.3. High performance liquid chromatography analysis of serum

The cholesterol and triglyceride profiles of serum lipoproteins were analyzed using a dual detection high performance liquid chromatography (HPLC) system with two tandem connected TSKgel LipopropakXL columns (300 mm × 7.8 mm; Tosoh, Tokyo, Japan), in accordance with the methods provided by Skylight Biotech (Akita, Japan). Individual subfractions were quantified by best curve fitting analysis, assuming that the particle sizes of all subfractions followed a Gaussian distribution. Particle sizes for individual subfractions were previously determined as 44.5–64 nm (large VLDL), 36.8 nm (medium VLDL), 31.3 nm (small VLDL), 28.6 nm (large LDL), 25.5 nm (medium LDL), 23 nm (small LDL), 16.7–20.7 nm (very small LDL), 13.5–15 nm (very large HDL), 12.1 nm (large HDL), 10.9 nm (medium HDL), 9.8 nm (small HDL) and 7.6–8.8 nm (very small HDL) (Okazaki et al., 2005; Usui et al., 2002).

2.4. mRNA quantification

Total RNA was isolated from cultured cells or mouse liver tissues using TRIzol Reagent (Life Technologies Japan, Tokyo, Japan) according to the manufacturer's protocols. Gene expression was evaluated by 2-step quantitative reverse transcription PCR (RT-PCR). Reverse-transcription of RNA samples was performed using a High Capacity cDNA Reverse-Transcription Kit (Life Technologies Japan, Tokyo, Japan), and quantitative PCR was performed by TaqMan Gene Expression Assay (Life Technologies Japan, Tokyo, Japan). mRNA levels of target genes were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The following primer sets were used for quantitative PCR: for assay ID, Mm00445670_m1 (apoc3) and Mm99999915_m1 (gapdh).

2.5. Western blotting analysis

Serum was diluted with buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 20 × Complete Mini protease inhibitor cocktail 1:20 (Roche, Indianapolis, IN)) and total protein concentrations were measured with a detergent compatible assay kit (Bio-Rad, Hercules, CA). Solutions were subjected to electrophoresis on 16% Tris-glycine gels (Life Technologies Japan, Tokyo, Japan) at 180 V for 30 min, and were transferred to a PVDF membrane (Bio-Rad). Apo-CIII Western blotting was performed at room temperature for 1 h with an anti-apo-CIII antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 200 mV for 120 min. Membranes were washed three times with PBS containing 0.3% Tween20. Blots were labeled with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescent detection was performed using an ECL prime Western blot detection kit (Amersham Biosciences, Buckinghamshire, UK), and bands were visualized using an LAS-4000 mini image analyzer (Fuji Film, Tokyo, Japan).