J Hum Genet (2001) 46:633–639

ORIGINAL ARTICLE

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A novel apolipoprotein E5 variant with a 24-bp insertion causing hyperlipidemia

Received: July 10, 2001 / Accepted: August 8, 2001

Abstract A tandem 24-bp insertion in the apolipoprotein E (*apo E*) gene was detected in a patient with elevated triglyceride, apolipoprotein (apo) CII, and apo CIII levels. This novel variant, apo E5ss, showed in position apo E5 by isoelectric focusing and was of larger molecular weight than apo E3 during two-dimensional gel electrophoresis. Polymerase chain reaction-single strand conformation polymorphism analysis using the primer pairs that cover all the coding regions was useful for rapid detection of the variant of the apo E allele. Apo E5ss may have a 24-bp insertion caused by slipped mispairing, resulting in a tandem duplication of amino acid residues 135–142 [APOE, 24-BP INS, DUP CODONS 135–142]. The proband was the only person with apo E5ss among the 806 Japanese males that we examined. We inspected six other reported apo E5 variants in the literature.

Key words Apolipoprotein E · Apo E5 · Hyperlipidemia · Insertion · Tandem duplication · PCR-SSCP · Two-dimensional gel electrophoresis · Variant

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[1] The notation of three small letters such as apo E follows the classification of apolipoprotein based on isoelectric focusing. *Apo* ε indicates the apolipoprotein genotype. APOE shows the allelic variant if amino acid substitution can be clarified by sequencing. The allelic variant was coded according to McKusick (1998, OMIM).

Introduction

Apolipoprotein E (apo E[1]) is a constituent of chylomicrons, chylomicron remnants, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL). It plays an important role as a ligand for the apo E receptor-mediated uptake of VLDL in the catabolism of triglyceride-rich lipoproteins. It also functions as a ligand for the cholesterol uptake of IDL and low-density lipoproteins (LDL) through an interaction with LDL receptors (Mahley 1988). Human apo E is composed of 299 amino acid residues and has a molecular weight of 34,200. In plasma, there are three common isoforms of apo E: apo E2, E3, and E4. The classification of the isoforms of apo E has been based on the global charge of the protein per one unit and determined by isoelectric focusing (IEF) (Zannis et al. 1981; Utermann et al. 1980; Mahley 1988). According to the reverse order of the mobility of IEF, other isoforms have been designated by the name "apo E1" to "apo E7". Apo E3 is the most commonly occurring isoform encoded by the *apo ε3* genotype.

Compared with the apo E3 isoform, the apo E4 isoform has a Cys→Arg substitution at position 112, and the apo E2 isoform has an Arg→Cys substitution at position 158. The receptor-binding domain was formerly reported to be in the vicinity of amino acid residues 133–160 of apo E3 (Rall et al. 1982a; Lalazar et al. 1988). Compared with the apo E3 isoform, apo E2 [APOE, R158C] has a decreased functional catabolic rate and exhibits markedly impaired binding to the LDL receptor (Weisgraber et al. 1982). Type III hyperlipoproteinemia (HLP) is caused by homozygosity for apo E2 [APOE, R158C] (Breslow et al. 1982). In patients with type III hyperlipoproteinemia, various mutations of a Cys residue were reported (Havel et al. 1983; Rall et al. 1989). On the other hand, apo E4 [APOE, C112R] is known to be related to elevated levels of plasma total cholesterol (TC) and LDL-cholesterol (LDL-C), even though apo E4 [APOE, C112R] and apo E3 have similar binding activities for the LDL receptor (Weisgraber et al. 1982).

Furthermore, various mutations of the commonly occurring apo E3 as determined by IEF were reported. Thus, it is increasingly important to clarify the genotype of apolipoproteins by sequencing. Moreover, aside from hyperlipidemia, apo E4 [APOE, C112R] is known to be a major risk factor for Alzheimer's disease (Corder et al. 1993; Saunders et al. 1993; Poirier et al. 1993; Noguchi et al. 1993; Yoshizawa et al. 1994).

Apo E5 has been designated by the fact that the isoelectric point (PI) of the main spot of apo E5 shifts two charge units away from that of the apo E3 spot by IEF. Apo E5 [APOE, E3K] was first detected in a Japanese patient with hyperlipidemia. It migrates faster than apo E3 during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Yamamura et al. 1984; Tajima et al. 1988; Maeda et al. 1989). The receptor binding activity of apo E5 [APOE, E3K] is about twice that of apo E3 (Dong et al. 1990; Wardell et al. 1991). There have been several reports of apo E5 mutations such as apo E5 [APOE, E13K], apo E5 [APOE, Q81R AND C112R], apo E5 Frankfurt [APOE, P84R AND C112R], apo E5 [APOE, E212K], and apo E5s (Mailly et al. 1991; Ruzicka et al. 1993; Feussner et al. 1996; Yamanouchi et al. 1994). In addition, the gene frequency of *apo ε5* was about 0.005, which was the highest next to those of the common apo E alleles (*ε3*, *ε4*, and *ε2*) and *apo ε7*. We have already reported apo E5s detected by two-dimensional electrophoresis (2-DE) (Yamanouchi et al. 1994). In this study, we present another novel apo E5 variant, E5ss. We applied the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and the sequencing using our primer pairs to detect all the variants throughout the entire protein-coding sequence of apo E. We describe the characterization of apo E5ss and the mechanism of the 24-bp tandem duplication, and compare this variant with the other six reported apo E5 variants whose nucleotide sequences are in the literature.

Proband and methods

Proband

The proband was a 57-year-old Japanese man (body mass index = 23.6) who had a history of gastric surgery. Blood samples from the proband after overnight fasting were obtained with his informed consent. His lipid levels indicated hyperlipidemia (TC, 181 mg/dl; triglyceride (TG), 394 mg/dl; and HDL cholesterol, 33.7 mg/dl). The uric acid level was slightly high (8.6 mg/dl, normal range 3.4–7.8 mg/dl). The serum γ-GTP level was high (89 mU/ml; normal range, ≦40 mU/ml). Other biochemical examinations showed normal results. Examination of the proband's family members was not possible.

Apo E phenotyping

Plasma apo E phenotypes were determined by 2-DE according to the method of O'Farrell (1975) with slight modifications to detect apo E variants with the basic PI (Yamanouchi et al. 1994). The apo E spots on the slab gels were confirmed by immunoblotting with an anti-human apo E antibody (Cappel Laboratories Inc., Malvern, PA, USA).

PCR amplification and SSCP analysis of the *apo E* gene

As shown in Table 1, the PCR primers that we designed based on the nucleotide sequences of the *apo E* gene (Paik et al. 1985) spanned all of the protein coding sequences. The target DNA was amplified using Taq DNA polymerase (AmpliTaq, Perkin Elmer, Cetus, Norwalk, CT, USA), performed in 36 cycles, each consisting of denaturation at 95°C for 1 min, annealing at 69°–73°C for 2 min, and extension at 73°C for 2 min after preheating at 95°C for 5 min.

SSCP analysis was carried out essentially according to the method described previously (Orita et al. 1989; Bannai et al. 1996). One microliter of the amplified solution was mixed with 7 μl of denaturing solution (95% formamide, 20 mM ethylenediaminetetraacetate (EDTA), 0.05% bromophenol blue, and 0.05% xylene cyanol FF). The mixture was denatured at 95°C for 5 min and immediately cooled on ice, and 1 μl of the mixture was applied to a 10% polyacrylamide gel (acrylamide monomer:bisacrylamide = 49:1, with 5% glycerol). Electrophoresis was carried out in 45 mM Tris-borate (pH 8.0)/ 1 mM EDTA at 37°C or 45°C, and a constant current of 40 mA was applied for 2 h. A minigel electrophoresis apparatus equipped with a constant temperature control system ($90 \times 80 \times 1\,mm^3$, AE-6410 and AE-6730, ATTO, Tokyo, Japan) was used. The electrophoretic temperature is indicated in Table 1. The SSCP patterns were visualized by silver staining (Daiichi, Tokyo, Japan).

DNA sequence analysis

PCR products showing aberrant band patterns by SSCP analysis were ligated into a pCR vector using a TA cloning kit (Invitrogen, San Diego, CA, USA) and their sequences determined following the dideoxy chain termination method. Sequencing was carried out with an automated sequencer (377XL, Applied Biosystems, Foster city, CA, USA) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit.

Measurement of lipoprotein lipid and apolipoprotein levels in the serum

The serum levels of TC, TG, high-density lipoprotein cholesterol (HDL-C), apo B, apo E, apo AI, apo AII, apo CII, and apo CIII were measured according to the method of Yamanouchi et al. (1994).

Plasma lipoproteins were subjected to agarose gel electrophoresis using the Pol-E-Film system (Nihon Syoji, Osaka, Japan) and visualized by diformazan staining (cholesterol staining). Densitometric scanning of the gels was

Table 1. Primers of exons 3 and 4 of apo E gene used for polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP)

Name of primer	Primer sequence	Position	Product size (bp)	PCR		SSCP
				Polymerizing temperature	Reaction mixture	Electrophoretic temperature
APOE-F1	5′-TCA CCC TGC CCA CCA TGG CTC CA-3′	2700–3060 (exon 3)	361	69°C	A	37°C
APOE-R1	5′-GGT ATA GCC GCC CAC CAG GAG G-3′					
APOE-F2	5′-TCC CCC GCC TCC CCA CTG TGC GAC-3′	3546–3825 (exon 4)	280	73°C	B	37°C
APOE-R2	5′-GGC GAG GCG CAC CCG CAG CTC CTC-3′					
APOE-F3	5′-CTG GGC GCG GAC ATG GAG GAC GT-3′	3721–4035 (exon 4)	315	73°C	B	45°C
APOE-R3	5′-CTG GGC CCG CTC CTG TAG CGG CT-3′					
APOE-F4	5′-CTC AGC GCC ATC CGC GAG CGC CT-3′	3931–4220 (exon 4)	290	73°C	B	37°C
APOE-R4	5′-TCC ACC AGG GGC TCG AAC CAG C-3′					
APOE-F5	5′-GGC CCA GCA GAT ACG CCT GCA G-3′	4149–4384 (exon 4)	236	73°C	A	37°C
APOE-R5	5′-CTC CCG CTG CAG GCT GCG CGG A-3′					

The nucleotide numbers of the apo E gene according to Paik et al. (1985)
A, 500 mM KCl, 100 mM Tris-HCl (pH 8.4), 15 mM MgCl, and 200 mM gelatin; B, 500 mM KCl, 100 mM Tris-HCl (pH 8.8), 15 mM MgCl, 1% Triton, and 10% dimethylsulfoxide

performed with a dual-wavelength flying-spot scanner densitometer (CS-9000, Shimazu, Kyoto, Japan).

Results

Apo E5ss phenotype in 2-DE

Figure 1 shows the 2-DE patterns of apo E5ss, together with the data for its identification on the gel by immunoblotting. Apo E5ss was in the position apo E5 by IEF and had a slower migration than that of apo E3 by SDS-PAGE. The spot size of apo E5ss was much smaller than that of apo E3.

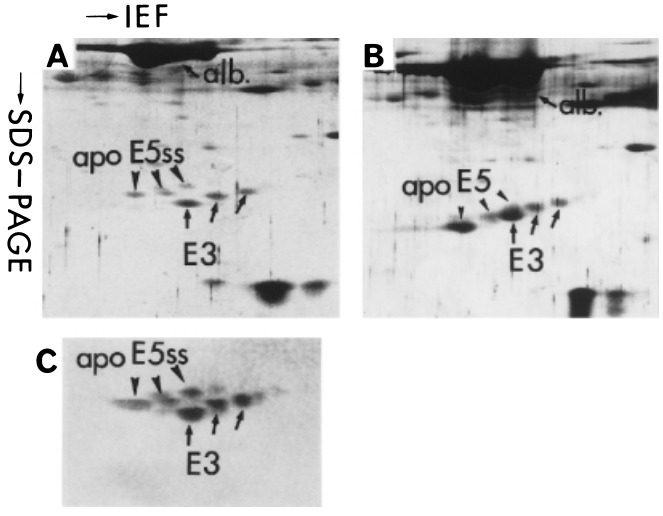

Fig. 1. Two-dimensional gel electrophoresis patterns of apo E5ss. **A** Silver staining of apolipoprotein E3/5ss (*apo E3/5ss*). **B** Silver staining of apo E3/5. **C** Immunoblotting of apo E3/5ss using specific antibody of apo E. Isoelectric focusing (*IEF*) is from *left to right* and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*SDS-PAGE*) is from *top to bottom*. Albumin is shown as *alb*. Apo E5ss is in the position apo E5 by IEF and has a slower migration than that of apo E3 by SDS-PAGE

Detection of the novel apo E variants and nucleotide sequences of apo E5ss

Figure 2 shows the SSCP patterns of the PCR products amplified using the primer pair APOE-F3 and APOE-R3. We clearly distinguished apo E5ss from the common apo E alleles (apo E2, E3 and E4) and apo E5s. The dots indicate the band of the novel variant, E5ss. This indicates that the mutation in nucleotides 3721–4035 (numbering based on the published sequence) (Paik et al. 1985) results in the formation of apo E5ss. The other PCR products (primer pairs: APOE-F1 and APOE-R1, APOE-F2 and APOE-R2, APOE-F4 and APOE-R4, APOE-F5 and APOE-R5) of apo E3/5ss were identical to apo E3 as determined by PCR-SSCP analysis.

DNA sequencing with cloning was carried out for the PCR products amplified using the primer pair APOE-F3 and APOE-R3. Sequencing of the *apo E5ss* gene from the proband revealed a tandem repeat of eight amino acids resulting from the 24-bp insertion, that is, E5ss [APOE, 24-BP INS, DUP CODONS 135–142]. Amino acids encoded by nucleotides 135–142 were the same as those encoded by nucleotides 143–150 (numbering based on the published

sequence) (Paik et al. 1985) (Fig. 3). Half of the clones that we analyzed showed the same sequence as the sequence mentioned earlier, because of the heterozygosity of apo E5ss of the proband. The nucleotide sequence of the other protein-coding region of the *apo E* gene was identical to the published nucleotide sequence (Paik et al. 1985; Rall et al. 1982b).

Lipid levels of proband with apo E5ss

The lipoprotein profiles of the proband with apo E5ss indicated hypertriglyceridemia (serum TG, 394 mg/dl). The serum apo CII and apo CIII levels were markedly elevated at 14.0 mg/dl (normal range, 1.1–5.0 mg/dl) and 33.8 mg/dl (normal range, 4.0–14.0 mg/dl), respectively. The concentrations of the other apolipoproteins in the proband were within the normal range (apo AI, 142 mg/dl; apo AII, 33 mg/dl; apo B, 123 mg/dl; apo E, 4.5 mg/dl). Cholesterol-stained agarose gel electrophoresis also revealed higher levels of cholesterol-rich pre-β lipoprotein (Fig. 4). This pattern was similar to that of samples from patients with type IV hyperlipidemia.

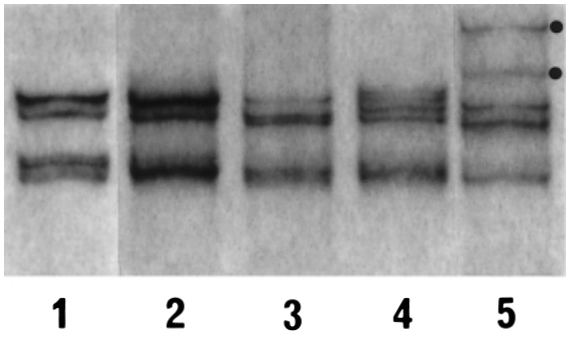

Fig. 2. Polymerase chain reaction-single strand conformation polymorphism analysis of apo E variants. *Lane 1*, apo E2/3; *lane 2*, apo E3/3 (most common type); *lane 3*, apo E3/4; *lane 4*, apo E4/5s (our previous reported case); *lane 5*, apo E3/5ss (proband). PCR products were amplified with the primer pair (APOE-F3 and APOE-R3). *Dots* indicate the bands of the novel apo E5ss

Discussion

We present a novel apo E5 variant, E5ss, that was detected by PCR-SSCP analysis and sequencing using newly designed primer pairs (Table 1). The reaction conditions for PCR and SSCP, in particular the temperature for electrophoresis during SSCP analysis, were strictly regulated. The primers included exons 3 and 4 and the neighboring intron, which contain the entire protein-coding region of the *apo E* gene. Thus, this method was useful for rapid detection of almost all the variants of the apo E allele, as shown in Fig. 2. In lane 5, apo E3/5ss from this proband was clearly distinguished from the common apo E3/3 in lane 2, as the two dots indicate new bands of apo E5ss. In lane 4, apo E4/5s from our previous case (Yamanouchi et al. 1994) was also distinguished from the common apo E3/3 in lane 2. Moreover, apo E2/3 (lane 1) and apo E3/4 (lane 4), which had the classical apo E2 and E4 missense mutations at nucleotide positions 158 and 112, were distinguished from apo E3/3.

The denaturing gradient gel electrophoresis method was also used to detect apo E variants (van den Maagdenberg et al. 1993). Methods using the allele-specific oligonucle-

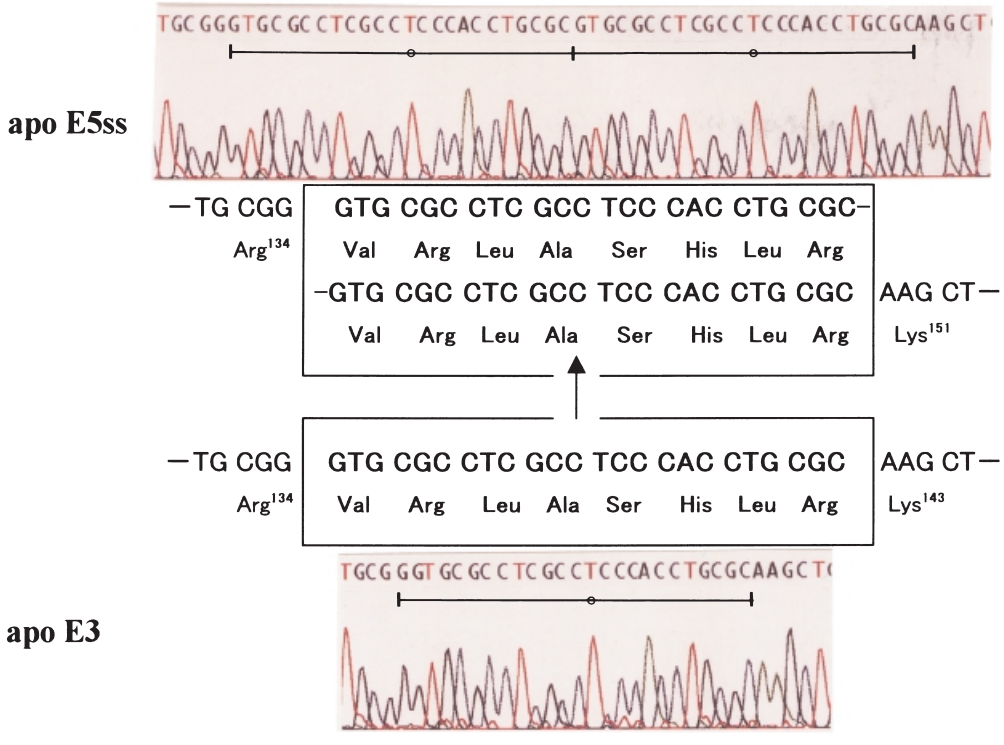

Fig. 3. Partial sequence chromatography of exon 4 in apo E5ss compared with that in apo E3. There is a 24-bp insertion at position 3813 or 3847. This tandem repeat led to insertions of eight amino acids at position 135 or 142

otide probe and PCR (Emi et al. 1988), or restriction fragment length polymorphism (Klasen et al. 1987; Tsai et al. 1993) enabled detection of the known variants only. There is a possibility that some unknown apo E variants are misclassified as apo E3.

DNA sequence analysis clarified that the novel apo E variant, apo E5ss, has a 24-bp insertion (Fig. 3). The 24-bp insertion in apo E5ss may be caused by slipped mispairing, resulting in a tandem duplication (Fig. 5). Amino acid residues 135–142 [24-BP INS, DUP CODONS 135–142] were duplicated. Another mechanism of duplication is homologous unequal recombination (Antonarakis et al. 2001). Variations of partial duplication of 45 bp (COL2A1) have been reported. The duplication of 24 bp of apo E5ss may be too small for homologous unequal recombination. In the case of slipped mispairing, a similar sequence exists in the original DNA sequence and mispairing causes a tandem repeat in that sequence (Efstratiadis et al. 1980; Cooper and Krawczak 1991; Yamakawa-Kobayashi et al. 1993). This is consistent with the large size of the PCR product of apo E5ss using the primer pair APOE-F3 and APOE-R3 (data not shown). Moreover, based on the 2-DE pattern (Fig. 1), apo E5ss migrated more slowly than apo E3 in IEF, and had a larger molecular weight than apo E3. Furthermore, these inserted amino acids contain two arginine residues, which are charged amino acids, this causes the PI of apo E5ss shift by two units toward the anode compared with apo E3.

The proband with apo E3/5ss had a typical type IV hyperlipidemia. The TC/TG ratio was 0.5, which was consistent with the definition of type IV hyperlipidemia ($0.15 < TC/TG < 0.6$). In the proband's plasma, apo CII and apo CIII levels were elevated, but the TC level was normal. Agarose gel electrophoresis of plasma lipoproteins using the Pol-E film system by cholesterol staining showed high levels of the pre-β lipoprotein (apo E3/E5ss) (Fig. 4). The chylomicron peak was not elevated. Because of the increased pre-β lipoprotein level and normal TC level, the pre-β lipoprotein (VLDL) of the proband became rich in cholesterol. There seem to be two possible reasons for these findings. One might be a deficiency in conversion of VLDL to LDL, resulting in an increase in the level of cholesterol-rich VLDL. The other might be an abnormality in binding of VLDL with apo E5ss to the VLDL receptor because apo E is a ligand of the VLDL receptor. The VLDL receptor has five functional domains common to the LDL receptor. In the case of apo E5ss, the insertion might be located within the first half of the receptor-binding region of helix 4 (residues 136–150) (Wilson et al. 1991; Dong et al. 1998). Although the insertion may result in a helix 4 extension in the four-helix bundle of the amino-terminal domain, the

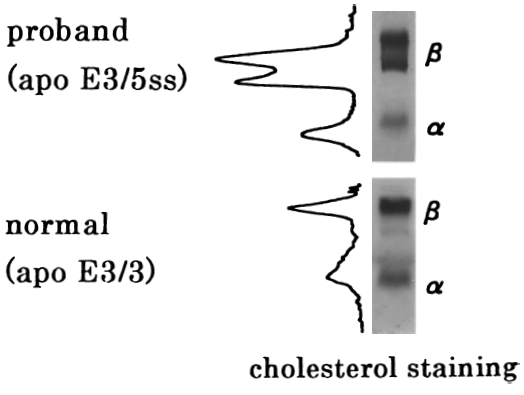

Fig. 4. Agarose gel electrophoresis of plasma lipoproteins visualized by cholesterol staining. This shows abundant pre-β lipoproteins in the proband (*apo E3/5ss*)

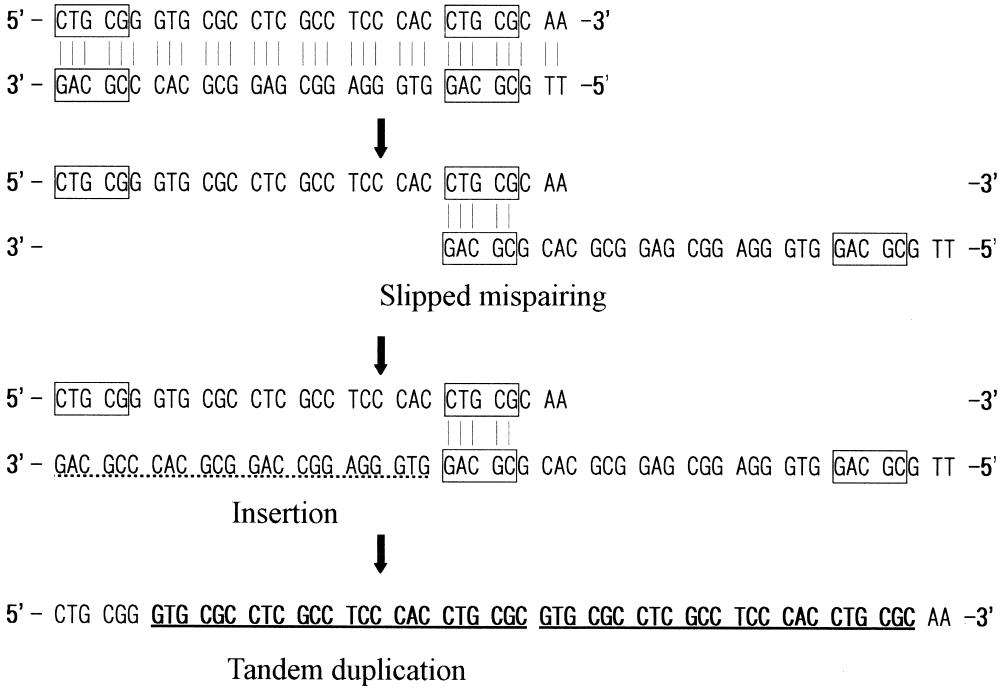

Fig. 5. Mechanisms for 24-bp insertion [APOE, 24-BP INS, DUP CODONS 135–142]. Slipped mispairing may be generated between one of the direct repeats (*CTGCG*) and the other complementary direct repeat (*GACGC*). Apo E5ss may have a 24-bp insertion caused by this slipped mispairing, resulting in a tandem duplication

Table 2. Summary of seven *apo E5* variants

Apo E genotype[a]	Lipid levels[b]		Binding affinity[c]	Electric mobility on SDS-PAGE[d]	Previous name	Reference
	TC (mg/dl)	TG (mg/dl)				
E3K	156–281	65–226	200%	fast	*E5*	Tajima et al. (1988) Matsunaga et al. (1995)
E13K	217–331	56–196		slightly fast	*E5*	Mailly et al. (1991)
Q81K AND C112R	226	191		same	*E5 Frankfurt*	Ruzicka et al. (1993)
P84R AND C112R	226	147	100%	same	*E5*	Ordovas et al. (1987) Wardell et al. (1991)
24-BP INS, DUP CODONS 135–142	181	394		slow	*E5ss*	Present report
E171K	240	98		slightly slow	*E5s*	Yamanouchi et al. (1994)
E212K	115–213	52–170	100%		*E5*	Feussner et al. (1996)

TC, total cholesterol; TG, triglyceride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
[a] Genotype was coded according to OMIM
[b] Lipid levels range of probands. Blood samples were taken after an overnight fast
[c] Binding affinity for low-density lipoprotein receptor compaired with apo E3/3
[d] Compaired to electric mobility of apo E3 on SDS-PAGE

original sequence of the receptor-binding region may be retained (Fig. 3). This may be one of the reasons that the LDL levels of the patient with apo E5ss were within the normal range.

The secretion level of apo E5ss seems to be lower than that of apo E3, although the plasma apo E level of the proband was within the normal range. This is because the apo E5ss spot on the 2-DE gel is smaller than the apo E3 spot (about one-third according to image analysis using NIH imaging software). This may be explained by the maturation process of the apo E protein rather than by the decrease in its expression level or by the degradation of the protein. Because of the extra eight amino acids on the site of the coding region in apo E5ss, the blood apo E level in the homozygote of apo E5ss is expected to be low, which is likely to result in severe symptoms. On the other hand, the proband's plasma levels of apo E, which is the heterozygote of apo E3, were normal (4.5 mg/dl; normal range, 2.2–6.4 mg/dl). It may be presumed that sufficient secretion of normal apo E3 may protect an individual against other lipid abnormalities.

As for the duplicate insertion of apo E, apo E3-Leiden has been reported (Wardell et al. 1989; Knijff et al. 1991). This is a tandem duplication of seven amino acids [APOE, 21-BP INS, DUP CODONS 121–127]. The heterozygote of E3-Leiden was characterized with a high cholesterol ratio, high VLDL fraction, high LDL fraction, and high apo E levels. All carriers of apo E3-Leiden present type III hyperlipidemia, and have different symptoms from those of apo E5ss carriers.

The allele frequency of apo E5 in Japan is estimated at about 0.005 (Yamanouchi et al. 1994; Matsunaga et al. 1995). This is high next to the three common types of apo E (apo E3, E4, E2) and apo E7. The incidence of apo E5ss may be rare. Among 806 persons having a physical examination in Tokyo, only 1 had apo E5ss. Seven apo E5 variants with different DNA sequences, including that in the present report, and their characteristics were compared (Table 2) (Tajima et al. 1988; Matsunaga et al. 1995; Mailly et al. 1991; Ruzicka et al. 1993; Ordovas et al. 1987; Wardell et al. 1991; Yamanouchi et al. 1994; Feussner et al. 1996). The apo E5s, which we described previously (Yamanouchi et al. 1994), was determined to be caused by a substitution, resulting in [APOE, E171K] using PCR-SSCP and DNA sequence analysis (data not shown). In apo E5 variants, most of the mutations were due to an E→K substitution. Although the electric mobility during SDS-PAGE of apo E5 [APOE, E3K] is fast compared with apo E3, that of apo E5s [APOE, E171K] is slow. The mobility of apo E5ss [APOE, 24-BP INS, DUP CODONS 135–142] is slower than that of apo E5s. There were no marked tendencies between the *apo E5* genotype and lipid abnormality. Thus, investigations of many more apo E5 variants are needed to elucidate the function of apo E.

Acknowledgments The authors would like to thank Takeo Juji, Yoshihide Ishikawa, Ling Lin (Japanese Red Cross Central Blood Center), Karo Tanaka (Teikyo University School of Medicine), Ryunosuke Miyazaki (Kudanzaka Hospital, Tokyo), Shigeru Tsuchiya, Tadao Arinami, Kimiko Kobayashi-Yamakawa, and Kaoru J. Takano (University of Tsukuba). This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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