A newly identified heterozygous lipoprotein lipase gene mutation ($Cys^{239} \rightarrow stop/TGC^{972} \rightarrow TGA$; LPL_{obama}) in a patient with primary type IV hyperlipoproteinemia

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Abstract We investigated measures for identification of heterozygous lipoprotein lipase (LPL) deficiency in unrelated subjects with primary type IV hyperlipoproteinemia in order to acquire a helpful clue for understanding the correlation between hypertriglyceridemia and the status of being a heterozygous carrier of an LPL gene variant. Identification of heterozygous LPL deficiency was performed by monitoring the immunoreactive LPL mass in postheparin plasma (PHP) using our developed sandwich-enzyme immunoassay technique for first screening. Then, in subjects found to have half or less than half of the control LPL mass value in PHP, the polymerase chain reaction-single strand conformation polymorphism method was used to detect LPL gene aberrations as a second screening. This approach was evaluated as being useful as it succeeded in identifying a subject (proband KD) with heterozygous LPL deficiency. The mutation in the LPL gene of proband KD was newly characterized as a nucleotide C^{972} to A transversion in exon 6, resulting in substitution of a premature termination codon (TGA) for Cys^{239} (TGC). This nonsense mutation, designated as LPL_{obama}, creates an *Mbo*I restriction site and eliminates an *Hgi*AI restriction site, and this allows rapid screening of subjects with type IV as well as type I hyperlipoproteinemia for the mutation. The homozygous state for the LPL_{obama} allele resulted in neither detectable LPL activity nor immunoreactive LPL mass in PHP, and this was seen in two of proband KD's siblings.—**Takagi, A., Y. Ikeda, A. Mori, Z. Tsutsumi, K. Oida, T. Nakai, and A. Yamamoto.** A newly identified heterozygous lipoprotein lipase gene mutation ($Cys^{239} \rightarrow stop/TGC^{972} \rightarrow TGA$; LPL_{obama}) in a patient with primary type IV hyperlipoproteinemia. *J. Lipid Res.* 1994. **35:** 2008–2018.

Supplementary key words type I hyperlipoproteinemia • hypertriglyceridemia • enzyme immunoassay • nonsense mutation • polymerase chain reaction • single strand conformation polymorphism • lipoprotein metabolism

Lipoprotein lipase (LPL, EC 3.1.1.34) is a glycoprotein enzyme that plays a key role in hydrolyzing triglycerides in chylomicrons and very low density lipoproteins (VLDL) as the first step in their metabolism (1, 2). In this reaction, LPL requires apolipoprotein (apo) C-II as an essential cofactor and produces chylomicron and VLDL remnants, thereby releasing free fatty acids that are used as either energy or reesterified for storage. The resulting lipoprotein remnants are thought to be further catabolized by hepatic triglyceride lipase (HTGL) (3, 4). Both LPL and HTGL are believed to be anchored to the surface of the capillary endothelium, and they are released into the circulation after intravenous injection of heparin (1, 4). Recently, both of these lipases were purified from human postheparin plasma (PHP) and characterized (5, 6). Human PHP-LPL is catalytically active in a monomeric form, and its apparent molecular weight is 61,000 (6), whereas there is a report that human LPL is catalytically active in a dimeric form (7). Human LPL cDNA has been cloned, and its sequence predicts a translated molecular weight of 50,394 with 448 amino acid residues for the mature form of the protein in the absence of any sugar moiety (8). The human LPL gene has been identified to be approximately 30 kb in length and consists of 10 exons (9, 10): exons 1 through 9 contain coding regions, whereas exon 10 contains a 3'-noncoding region.

Abbreviations: LPL, lipoprotein lipase; VLDL, very low density lipoprotein; apo, apolipoprotein; HTGL, hepatic triglyceride lipase; PHP, postheparin plasma; EIA, enzyme immunoassay; SIIA, selective immunoinactivation assay; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; MAb, monoclonal antibody.

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Homozygous or compound heterozygous LPL deficiency has been demonstrated in subjects with type I hyperlipoproteinemia, which is a rare autosomal recessive disorder characterized by severe fasting chylomicronemia and recurrent pancreatitis (11–13). To date, analysis of the LPL gene in such subjects has revealed several types of mutations occurring predominantly in exons 4, 5, and 6: missense, nonsense, deletion, addition, and splicing mutations (14–19). Within one family in which homozygous LPL deficiency was identified in a proband and carrier status of the LPL gene variation was genetically assured, some heterozygous LPL-deficient subjects were identified as hypertriglyceridemic (type IV hyperlipoproteinemia) that was characterized by mild accumulation of serum triglycerides as a consequence of an elevated VLDL concentration and a normal level of LDL cholesterol (18, 20–22). Also, type IV hyperlipoproteinemia has been reported in both carriers and noncarriers when such secondary factors as age, obesity, and hyperinsulinemia are also involved (23). Although these family studies suggest that this type of hyperlipoproteinemia is closely related to the heterozygous state for LPL deficiency, the underlying etiology of this disease remains unclear. Thus, the reverse approach of identifying heterozygous LPL deficiency in unrelated subjects with primary type IV hyperlipoproteinemia is important in order to understand the correlation between hypertriglyceridemia and heterozygous defective LPL gene carrier status.

Recently, we developed a sandwich-enzyme immunoassay (EIA) technique for quantification of the immunoreactive mass of LPL (24) and a selective immunoinactivation assay (SIIA) for measuring the LPL activity in human PHP (6, 24). The EIA uses a combination of two distinct types of anti-human LPL monoclonal antibodies that recognize different epitopes on the LPL molecule (24). These assay systems enable us to discriminate heterozygotes of LPL deficiency from normal and homozygous subjects (18).

In the present study, we have systematically searched for heterozygous LPL deficiency in unrelated subjects with primary type IV hyperlipoproteinemia by monitoring the LPL activity and mass in PHP using the SIIA and sandwich-EIA techniques for the first screening, followed by a second screening for LPL gene aberrations using the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method (25, 26). By this approach, we were able to detect a proband KD with heterozygous LPL deficiency due to a newly identified nonsense mutation (LPL_{obama}) which was a C→A transversion at nucleotide position 972 of exon 6 that resulted in a premature termination codon (Cys→stop) at amino acid residue 239.

MATERIALS AND METHODS

Materials

Human LPL and human HTGL were purified from PHP according to methods described previously (6). Pure human PHP-LPL (7.08 μmol/h per ml; specific activity, 45 mmol/h per mg (24)) was used as an enzyme source to check whether or not the patient's plasma contained functionally active apoC-II or inhibitors of LPL. A monospecific, polyclonal antiserum and monoclonal antibodies (MAb) were raised in rabbits and mice against human PHP-LPL and human PHP-HTGL, as previously reported (6, 24). An HLC601 LPL cDNA probe (from nucleotide numbers 32 to 1643) containing the complete coding region for human LPL cDNA was prepared from the human THP-I (monocytic leukemia cell line) macrophage cDNA library (27). HLG37-D, containing the 3'-noncoding region of the LPL gene (from nucleotide numbers 1629 to 3154 of LPL cDNA), was isolated from the human gene library and also used as a probe (18).

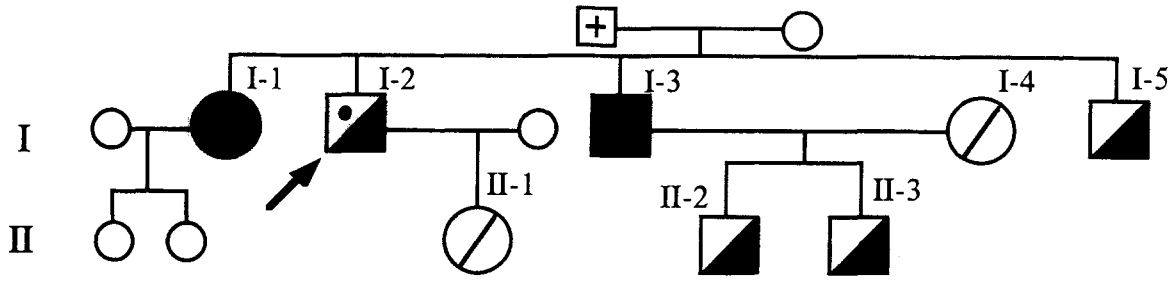
Subjects

Thirty-two unrelated Japanese patients, including proband KD, were diagnosed as primary type IV hyperlipoproteinemia with moderate accumulation of serum triglycerides (more than 150 mg/dl) and a normal level of LDL cholesterol (less than 150 mg/dl) according to the WHO classification of phenotype expression (11, 12). These patients were determined to be normal for glucose metabolism as evidenced by a 75 g of oral glucose tolerance test. They did not show clear evidence of thyroid, liver, and renal diseases. Their mean ages, serum triglyceride and cholesterol concentrations were 51.2 ± 11.8 years, 311.6 ± 144.9 mg/dl, and 204.5 ± 27.9 mg/dl (mean ± SD), respectively. No patients received lipid-lowering drugs at the time of this study. Ninety-three healthy volunteers (49 males and 44 females) between 23 and 69 years of age with a mean ± SD of 43.9 ± 13.1 years were studied as a control group. Their mean serum triglyceride and cholesterol concentrations were 86.8 ± 31.0 mg/dl and 195.5 ± 26.2 mg/dl (mean ± SD), respectively.

The proband KD and his family (D-pedigree) members were referred from Fukui Medical School. The D-pedigree and its members' clinical data are shown in **Fig. 1.** None of the tested members showed clear evidence of diabetes mellitus. The status of consanguinity was unclear.

Analysis of serum lipids, lipoproteins, and apolipoproteins

Blood samples were obtained after the subjects had fasted overnight, and they were allowed to stand for at



Subject's ID		I-1	I-2	II-1	I-3	II-2	II-3	I-4	I-5
Subject's name		Sister	Proband	Daughter	Brother	Son	Son	Spouse	Brother
		KM	KD	HM	KO	YO	JO	TO	KiD
Age	(year)	57	54	27	49	17	15	47	41
Sex		Female	Male	Female	Male	Male	Male	Female	Male
BMI	(Kg/m^2)	17.33	23.59	25.71	21.77	17.99	20.7	24.56	20.57
Serum-TG	(mg/dl)	1187	394	108	381	76	104	98	121
(CM + VLDL)-TG	(mg/dl)	1097	275	58	241	34	75	50	69
Serum-Chol	(mg/dl)	155	130	180	83	109	152	213	176
(CM + VLDL)-Chol	(mg/dl)	114	53	20	34	10	13	19	13
LDL-Chol	(mg/dl)	21	26	113	22	60	93	153	111
HDL-Chol	(mg/dl)	14	25	45	17	34	38	40	41
ApoC-II	(mg/dl)	5.1	3.2	4.3	4.4	1.4	2.1	2.9	3.7
Alcohol intake	(g/day)	0	33	0	0	0	0	0	0
WHO type		I	IV	Normal	V	Normal	Normal	Normal	Normal

Fig. 1. Proband KD and his family members' clinical data. Proband KD is indicated by the arrow. ●, type I and ■, type V hyperlipidemic subjects who are homozygous for the LPL_{obama} allele, resulting in no detectable LPL activity or immunoreactive LPL mass in PHP; ◪, normolipidemic subjects who are heterozygous for the LPL_{obama} allele; ◪, a type IV hyperlipidemic subject, who is heterozygous for the LPL_{obama} allele; ∅, normal; □, not studied; ⊞, deceased subject. ID, identification number; BMI, body mass index; TG, triglyceride; CM, chylomicron; Chol, cholesterol. Depictions of the genotypes are derived from the results presented in Fig. 4 as well as Table 1.

least 2 h at room temperature. Serum was prepared by centrifugation at 1500 *g* for 15 min at 4°C. Serum lipoproteins were fractionated into four classes according to density by sequential flotation ultracentrifugation as described by Schumaker and Puppione (28): chylomicron and VLDL (d < 1.006 g/ml), intermediate density lipoprotein (IDL, d 1.006 to 1.019 g/ml), LDL (d 1.019 to 1.063 g/ml), and HDL (d 1.063 to 1.21 g/ml). The triglyceride and cholesterol concentrations of the serum and the fractionated lipoproteins were measured by enzymatic methods according to the suppliers' directions (Triglyceride G-test Wako, Wako Pure Chemical, Osaka, Japan; Determiner TC5, Kowa Medex Co., Tokyo, Japan). ApoA-I, A-II, B, C-II, C-III, and E were measured by a single radial immunodiffusion assay (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) according to the manufacturer's directions.

Measurement of LPL and HTGL activities and immunoreactive masses in plasma

The subjects were fasted overnight, and blood samples were collected in tubes containing Na_2 EDTA (1 mg/ml) before and 10 min after the injection of heparin (30 units/kg of body weight) for determination of the LPL and HTGL activities as well as masses. The total PHP lipolytic activity, and the LPL and HTGL activities in PHP were measured with gum arabic-emulsified tri[9,10-^{3}H]oleoylglycerol (sp act 330.4 GBq/mol) as a substrate by SIIA using rabbit anti-human PHP-LPL antiserum and anti-human PHP-HTGL antiserum, as previously reported (6, 24). The enzyme activity was expressed as μmoles of free fatty acid released per hour per ml of enzyme solution, unless otherwise mentioned.

Sandwich-EIA for LPL mass quantification was performed using two distinct anti-human PHP-LPL monoclonal antibodies (MAbs) that recognize different epitopes on the LPL molecule. The immunoreactive mass of LPL in PHP was specifically measured using a β-galactosidase-labeled anti-human PHP-LPL MAb (1(1)D2B2) as an enzyme-linked MAb, an anti-human PHP-LPL MAb (2(10)F8F9) linked with bacterial cell walls as an insolubilized MAb, and pure human PHP-LPL (0, 100, 200, 300, and 400 ng/ml) as a standard, as previously reported (24). The immunoreactive mass of HTGL in PHP was also measured by sandwich-EIA using a combination of two distinct MAbs: a β-galactosidase-labeled anti-human

PHP-HTGL MAb (2(4)F12C12) and an anti-HTGL MAb linked with bacterial cell walls (1(11)A3H3) (24). The mass values were expressed as ng/ml of plasma.

DNA analysis

Southern blot analysis. Genomic DNA was isolated from the whole blood of subjects, and was subjected to Southern blot analysis after digestion with the appropriate restriction enzymes. Hybridization was performed with probes of ^{32}P-labeled HLC601 or ^{32}P-labeled HLG37-D according to the standard procedure (29). The blotted sheets were exposed to Kodak XOMAT™ AR film for several days at −80°C with intensifying screens.

Detection of LPL gene variation by PCR-SSCP. DNA fragments containing each exon and the exon-intron boundaries, and the 5'-upstream region of the LPL gene were enzymatically amplified from the genomic DNA of subjects by the PCR method using each pair of primers listed below (30, 31). Each primer was labeled with [γ-^{32}P]ATP (370 MBq/ml, 111 TBq/mmol; New England Nuclear; Boston, MA) using T4 polynucleotide kinase (Takara; Kyoto, Japan). The PCR mixture contained 2.5 μl of labeled forward-primer (1 pmol), 2.5 μl of labeled reverse-primer (1 pmol), 1.6 μl of a 1.25 mM solution of each of the four deoxynucleotides, 1 μl of 10-fold Taq polymerase buffer, 1 μl of 50 ng/μl genomic DNA, and 0.05 μl of 5 U/μl Taq polymerase (AmpliTaq®; Perkin Elmer Cetus; Norwalk, CT) in a total volume of 10 μl. The reaction was performed with 0.5 min of denaturation at 94°C, 1 min of primer annealing at 60°C for exon 1, 70°C for exons 2, 8, and 9 or 65°C for other exons, 2 min of extension at 72°C, and 30 cycles using a DNA Thermal Cycler (Perkin Elmer-Cetus). In the PCR of exon 1, primer Exon-1F and -1R, designed by Monsalve et al. (32), were used, because longer PCR products of more than 350 bp were not suitable for the detection of mutations by the PCR-SSCP method. The labeled PCR products were mixed with a dye solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). Immediately after heating at 80°C for 5 min, 2 μl of each sample was loaded onto a 5 or 6% polyacrylamide gel equilibrated with Tris-borate-EDTA buffer (0.045 M Tris-borate, pH 8.3, 0.001 M EDTA) with or without 10% glycerol. Electrophoresis was performed at 40 watts for 2–6 h at 4°C or 25°C. After electrophoresis, the gel was dried on Whatman 3 MM paper, and the bands were visualized by autoradiography at −80°C with intensifying screens after 6–12 h of exposure.

The primers used were synthesized using an Applied Biosystems 380A DNA synthesizer (Applied Biosystems; Foster City, CA) and are listed below:

Exon-1F (5'-CCTTGCAGCTCCTCCAGAGGG-3')
Exon-1R (5'-AGGGGAGTTTGCGCGCAAAA-3')
Exon-1UF (5'-GCCGGATCCAAATGGAATCATACAATATGTGTCTTTTG-3'),
Exon-1UR (5'-GGCCTGCAGTAAATTCACTTCTAAACGTTTGAGC-3'),
Exon-1DF (5'-TTCGGATCCAGCGAACAGGAGCCTAACAAAGCAA-3'),
Exon-1DR (5'-CGGGTCTGCAGGTGGAGGGTAGTTT-3'),
Exon-2F (5'-GGTGGATCCAACCCTCCAGTTAACCTCATATCCAA-3'),
Exon-2R (5'-GTGCTGCAGCACCACCCCAATCCACTCTTCCCCA-3'),
Exon-3F (5'-ACCGGATCCTAGGTGGGTATTTTAAGAAAGCTTGTG-3'),
Exon-3R (5'-GAGCTGCAGCACTGCTTTGGACACATAAGTCTCC-3'),
Exon-4F (5'-GAGGGATCCGCAGAACTGTAAGCACCTTCATTTTC-3'),
Exon-4R (5'-GGTCTGCAGTTCACCTCTTATGATAAGACCAACGAA-3'),
Exon-5F (5'-GGAGGATCCAAATTTACAAATCTGTGTTCCTGCTTTTT-3'),
Exon-5R (5'-GCCCTGCAGGATAAGAGTCACATTTAATTCGCTTCTA-3'),
Exon-6F (5'-AGAGGATCCTTCTGCCGAGATACAATCTTGGTGTC-3'),
Exon-6R (5'-AGGCTGCAGGACTCCTTGGTTTCCTTATTTACAACA-3'),
Exon-7F (5'-GGCGGATCCATAAAGATTGATCAACATGTTCGAATTTC-3'),
Exon-7R (5'-TATCTGCAGGGGACTGGTGCCATGATGACCGCCC-3'),
Exon-8F (5'-GCCGGATCCGATCTCTATAACTAACCAAATTTATTGCT-3'),
Exon-8R (5'-TCCCTGCAGTGGGGGTCTAAAGTGAAGGAAGAAAA-3'),
Exon-9F (5'-GAAGGATCCTTGTTCTACATGGCATATTCACATCCA-3'),
Exon-9R (5'-TAACTGCAGAGCTCAGGATGCCCAGTCAGCTTTA-3').

These primers were designed on the basis of the 5'-upstream DNA sequence (10) and the intron-exon boundaries (33).

Cloning and sequencing of the genomic DNA. DNA fragments containing each of exons 2 through 9 were enzymatically amplified as described above in the section of "Detection of LPL gene variation by PCR-SSCP." Exon 1, containing the 5'-upstream region, was amplified with the Exon-1UF and -1UR primer pair, and the Exon-1DF and -1DR primer pair for cloning and sequencing. The amplified DNA was purified using a GENECLEAN Kit (BIO 101 Inc.; La Jolla, CA) according to the manufacturer's directions. The purified DNA was sequenced

either directly or after cloning into a Bluescript SK II M13(−) vector (Stratagene; La Jolla, CA). In the latter case, the purified DNA was digested with *Pst*I and *Bam*HI, and then cloned into the vector. DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger, Nicklen, and Coulson (34) using Sequenase® T7 DNA polymerase (United States Biochemical Corporation; Cleveland, OH) or VentR™ exo- DNA Polymerase (New England BioLabs; Beverly, MA).

Restriction enzyme digestion of PCR-amplified DNA. LPL gene-exon 6 was amplified by PCR using the Exon-6F and -6R primer pair. The amplified DNA was digested with 10 units of *Mbo*I (Bethesda Research Laboratories; Gaithersburg, MD) and 5 units of *Hgi*AI (New England Biolabs) overnight at 37°C. The resulting DNA fragments were analyzed on 4% NuSieve agarose gel (FMC BioProducts; Rockland, ME). DNA bands were visualized by staining with ethidium bromide.

Haplotype analysis. The haplotype of the LPL gene was constructed on the basis of the *Hin*dIII (35, 36), *Pvu*II (37), and *Bam*HI (37) restriction fragment length polymorphism (RFLP) at the LPL gene locus. The genome was digested with *Bam*HI or *Pvu*II, and Southern hybridization was carried out with LPL cDNA probe HLC601 and HLG37-D for RFLP analysis. The RFLP pattern with *Hin*dIII was determined by digesting the PCR products amplified with the Exon-8F and Exon-9R primers by *Hin*dIII, because the polymorphic *Hin*dIII site is reported to be in intron 8 of the LPL gene (38). A common mutation (Ser^{447}→stop) in exon 9 of the human LPL gene (39) was also examined for haplotype determination. This mutation was detected by PCR amplification of exon 9 using a modified 3'-primer and digestion with *Hin*fI, as described by Stocks, Thorn, and Galton (40).

RESULTS

Detection of heterozygous LPL deficiency in proband KD by monitoring LPL mass and gene of subjects with type IV hyperlipoproteinemia using the sandwich-EIA and PCR-SSCP methods

Thirty-two unrelated subjects, including proband KD, who had been diagnosed as having primary type IV hyperlipoproteinemia were investigated in an attempt to identify heterozygous LPL deficiency. Their immunoreactive LPL mass and LPL activity in PHP were measured using the sandwich-EIA and SIIA methods, and 24 subjects were found to have LPL mass and activity values approximately half or less than half of the mean values of normal subjects: these subjects were selected for a 5th percentile cut-off (130 ng of LPL mass/ml of plasma) of normal LPL mass increment values (22). **Table 1** presents the data for the LPL mass and activity of one subject, proband KD. He showed about half the normal values for both LPL mass and activity, suggesting that proband KD is heterozygous for an LPL gene mutation.

DNA was available from 18 subjects among the 24 subjects having about half of the control LPL mass. Their DNA samples were searched for LPL gene abnormalities

TABLE 1. LPL and HTGL activities and immunoreactive masses in preheparin plasma and postheparin plasma (PHP) of proband KD and his family members

		LPL					HTGL				
		Activity	Immunoreactive Mass				Activity	Immunoreactive Mass			
Number[a]	Subject	PHP	Preheparin Plasma	PHP	Increment[b]	S.A.[c]	PHP	Preheparin Plasma	PHP	Increment[b]	S.A.[c]
		U	*ng/ml*	*ng/ml*	*ng/ml*	*U/mg*	*U*	*ng/ml*	*ng/ml*	*ng/ml*	*U/mg*
I-2	Proband KD	5.4	23	133	110	49	24.0	16	1181	1165	21
I-1	Sister KM	ND[d]	4	12	8		10.1	28	741	713	14
I-3	Brother KO	ND[d]	0	15	15		15.0	18	1022	1004	15
I-4	KO's spouse TO	8.2	19	161	142	58	24.0	29	1228	1199	20
I-5	Brother KiD	4.9	14	108	94	52	13.4	49	834	785	17
II-1	Daughter HM	8.0	30	200	170	47	27.0	40	1467	1427	19
II-2	KO's son YO	6.5	35	151	116	56	39.4	61	2188	2127	19
II-3	KO's son JO	6.4	21	145	124	52	40.4	17	2439	2422	17
Normal (mean ± (n = 93)		9.8 ± 2.5	30 ± 9	216 ± 50	186 ± 46	53 ± 8	22.5 ± 9	31 ± 11	1273 ± 517	1242 ± 517	18 ± 3

The enzyme activities and immunoreactive masses were quantified by SIIA and sandwich-EIA, respectively. The unit (U) of activity is defined as μmoles/h/ml, as described in Materials and Methods.

[a]Numbers are the same as in Fig. 1.

[b]The increment represents the difference between the preheparin plasma and PHP immunoreactive masses.

[c]S.A., specific activity; calculated by dividing the PHP activity by the increment.

[d]ND, not detectable.

by screening for LPL gene variants in exons 1 through 9 by the PCR-SSCP method. LPL gene variants were identified in several subjects (21, 22), and one of them, proband KD, was studied in detail. Abnormalities were found in exons 6 and 9 of proband KD's LPL gene under the conditions used. The PCR-SSCP pattern of proband KD's LPL gene-exon 6 was different from the patterns of normal subjects and the other 17 subjects with type IV hyperlipoproteinemia. A part of the autoradiograph of PCR-SSCP of exon 6 is shown in **Fig. 2.** Proband KD exhibited three distinct bands: two SSCP-bands from the normal allele and an additional SSCP-band from the variant allele (lane 4) which was easily distinguishable from the others, i.e., two control subjects (lanes 1 and 6) and three hyperlipoproteinemic subjects (lanes 2, 3, and 5), suggesting that proband KD is heterozygous in exon 6 of the LPL gene. It should be noted that one strand of the variant allele showed an abnormal SSCP-band, while the other strand could not be distinguishable from the normal SSCP-band. The variation in proband KD's LPL gene-exon 9 was a common premature termination codon ($Ser^{447} \rightarrow$ stop) which has been reported to be identified in both normolipidemic and hyperlipoproteinemic subjects (41), and proband KD was also heterozygous for this variation (data not shown). Furthermore, the gross structure of proband KD's LPL gene was examined by Southern blot analysis, and no appreciable deletion or insertion in the LPL gene was detected (data not shown). These findings indicate that a heterozygous LPL deficiency in LPL gene-exon 6 of proband KD resulted in an LPL mass value in PHP which was about half of the control value.

Fig. 2. Identification of proband KD as a case of heterozygous LPL deficiency by the PCR-SSCP method. A portion of autoradiograph of LPL gene-exon 6 analyzed by the PCR-SSCP method is shown. Amplified, radiolabeled PCR products of exon 6 were loaded onto a 6% polyacrylamide gel containing 10% glycerol and Tris-borate-EDTA buffer (pH 8.3) and electrophoresed at 25°C, as described in Materials and Methods. Proband KD was detected by the presence of an aberrant band in lane 4. Lanes 1 and 6 are normolipidemic subjects, and lanes 2 through 5 are type IV hyperlipidemic subjects.

Identification of the mutation in LPL gene of proband KD

The mutation sites in proband KD's LPL gene were determined by directly sequencing each DNA segment encompassing an exon and the exon-intron boundaries and the 5'-upstream region of the LPL gene amplified with primers, as described in Materials and Methods. In all of the coding exons (1 through 9) examined, only one apparent nucleotide substitution other than the common variant of exon 9 was identified at position 972 of exon 6 (**Fig. 3A**): two distinct bands were detected at that position, one derived from the normal allele and the other from the variant allele. The nucleotide C^{972} at the third position of the TGC codon coding Cys^{239} in the normal allele was substituted to A in the variant allele, thereby generating a stop codon (TGA). This mutation was designated as LPL_{obama} on the basis of the birthplace of proband KD. This result was further confirmed by sequencing exon 6 after subcloning into Bluescript SK II M13 (−) vector, as shown in Fig. 3B. As expected, the subcloned normal allele of exon 6 showed a band for nucleotide C (TGC) at position 972 (left panel in Fig. 3B), whereas the LPL_{obama} allele exhibited a band for nucleotide A (TGA) at the same position (right panel in Fig. 3B). This one base substitution resulted in the loss of an *Hgi*AI restriction enzyme recognition site present in the normal LPL gene and the generation of an *Mbo*I site (**Fig. 4A**). Detection of the LPL_{obama} allele by *Mbo*I digestion was applied to screen for this mutation in the other 17 subjects with primary type IV hyperlipoproteinemia. Proband KD was ascertained to be only a heterozygous carrier of the LPL_{obama} allele, which is in agreement with the observation that the PCR-SSCP pattern of proband KD was distinguishable from the others.

Family studies of proband KD

Analysis of carrier status of LPL_{obama} allele and phenotypic expression of carriers. As we were able to obtain DNA samples from proband KD's sister KM (I-1 in Fig. 1), brothers KO (I-3) and KiD (I-5), daughter HM (II-1) and KO's spouse TO (I-4), the carrier status of each for the LPL_{obama} allele was determined by *Mbo*I digestion of PCR-amplified

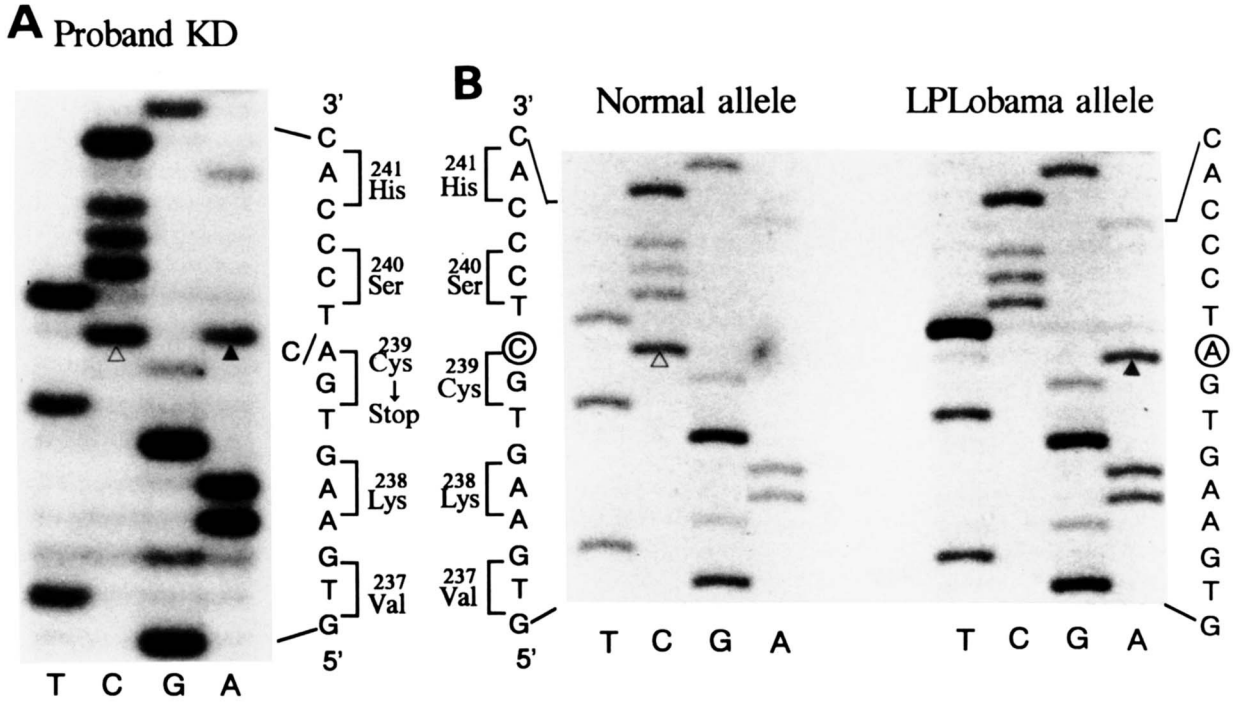


Fig. 3. Identification of the mutation site in LPL gene-exon 6 of proband KD using both direct sequencing (A) and sequencing methods after subcloning (B). Nucleotide sequences of a part of the sense strand from exon 6 of proband KD are shown. (A) Two distinct bands were detected by the direct sequencing method: nucleotide C^{972}, indicated by an open triangle, is from the normal allele, while nucleotide A^{972}, indicated by a solid triangle, is from the LPL_{obama} allele. (B) PCR-amplified exon 6 was subcloned into the Bluescript SK II M13 (−) vector, and five independent clones were isolated and sequenced. Nucleotide C^{972} was detected in the subcloned normal allele (left panel), whereas nucleotide A^{972} was identified in the subcloned LPL_{obama} allele (right panel). Both the nucleotide sequence and the amino acid numbers were taken from the human LPL cDNA sequence reported by Wion et al. (8).

DNA from the LPL gene-exon 6. As shown in Fig. 4B, sister KM (lane 3) and brother KO (lane 5) exhibited three distinct bands of 151-, 66-, and 120-bp fragments after *Mbo*I digestion, which indicated that they were homozygous for LPL_{obama} allele. The homozygous state for this mutation resulted in no detectable LPL activity or mass in PHP (Table 1) as a consequence of a nonsense mutation in LPL gene-exon 6. Sister KM manifested type I hyperlipoproteinemia, as evidenced by fasting hyperchylomicronemia with triglycerides of more than 1,000 mg/dl and a low cholesterol concentration for both LDL and HDL, whereas brother KO showed type V hyperlipoproteinemia (Fig. 1).

Proband KD (lane 7) and his brother KiD (lane 6) exhibited four distinct bands of 151- and 66-bp fragments from LPL_{obama} allele, and a 217-bp band from the normal allele in addition to a constant band of 120 bp from both alleles, indicating that both proband KD and brother KiD were heterozygous for the LPL_{obama} allele (Fig. 4B). They showed about half of the control LPL activity and mass levels (Table 1). Brother KO's sons YO (II-2) and JO (II-3) were genetically estimated to have obligate heterozygous LPL deficiency because brother KO was homozygous for LPL deficiency. As expected, their LPL activities and masses in PHP were in almost the same range as those of proband KD (Table 1). The phenotypic expressions of the four heterozygous LPL_{obama} deficient subjects (KD, YO, JO, and KiD) were different; proband KD was the only subject with type IV hyperlipoproteinemia, while the others showed normolipidemia (Fig. 1).

Proband's daughter HM (lane 4) and brother KO's spouse TO (lane 2) showed 217- and 120-bp bands after the digestion with *Mbo*I, which was the same as in the normal subject (lane 1) (Fig. 4B), and they were normolipidemic (Fig. 1). The *Hgi*AI restriction enzyme digestion gave the same results in the genotype determination as the *Mbo*I digestion (data not shown).

For all members of the D-pedigree tested here, the possibility of existence of inhibitors of LPL in their plasma was ruled out because their plasma did not inactivate pure human LPL enzyme activity (data not shown). The apoC-II mass values of their plasma were within the normal range (0.9–5.9 mg/dl) (Fig. 1). The biological function of their apoC-II was also found to be normal as their plasma properly activated pure human PHP-LPL in vitro (data not shown). The HTGL activities and mass values of all of the D-pedigree members were within or above the normal ranges (Table 1).

Analysis of haplotype of the LPL_{obama} allele. As sister KM

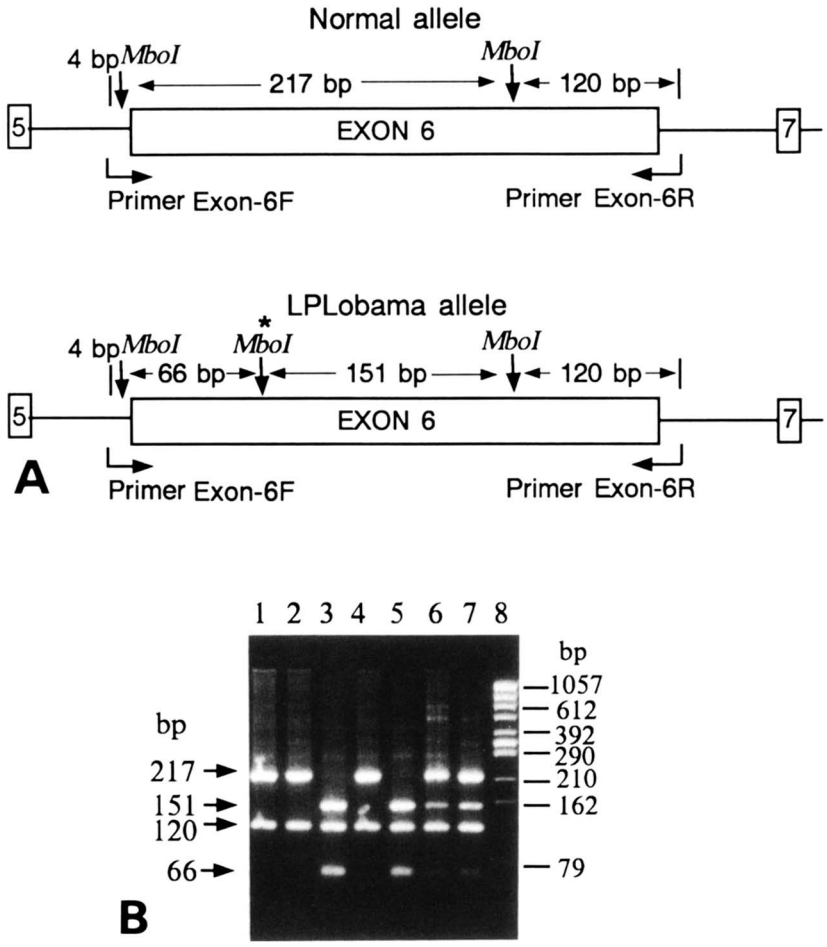


Fig. 4. Schematic illustration of a newly generated *Mbo*I restriction site in LPL_{obama} allele of proband KD (A) and the D-pedigree's carrier status for this mutation (B). LPL gene-exon 6 was amplified using an Exon-6F and -6R primer pair by the PCR method. (A) The DNA fragments of the normal allele produce 217-bp and 120-bp bands by digestion with the *Mbo*I restriction enzyme, whereas those of the LPL_{obama} allele with a newly generated *Mbo*I site, indicated by the asterisk, produce 66-bp, 151-bp, and 120-bp bands. (B) The carrier status of the D-pedigree for the LPL_{obama} allele was analyzed by 4% Nusieve agarose gel electrophoresis after *Mbo*I digestion of the PCR-amplified DNA of each subject. DNA bands were visualized by staining with ethidium bromide. Lane 1, normal subject; lane 2, brother KO's spouse TO; lane 3, sister KM; lane 4, daughter HM; lane 5, brother KO; lane 6, brother KiD; lane 7, proband KD; lane 8, ΦX174 DNA digested with *Hinc*II as a size marker.

and brother KO were homozygous for LPL_{obama} deficiency, the haplotype of the LPL_{obama} allele was easily determined by monitoring three RFLPs with *Pvu*II, *Hin*dIII, and *Bam*HI digestion and the common variant of LPL gene-exon 9 with *Hin*fI digestion, as described in Materials and Methods. The haplotype of their LPL_{obama} allele was *Pvu*II (−), *Hin*dIII (−), *Bam*HI (+), and *Hin*fI (+), where (−) and (+) mean that the DNA is "not digested" and "digested" by the enzymes, respectively (data not shown). The LPL_{obama} mutation was linked to the common variation in LPL gene-exon 9. Knowledge of the haplotype of LPL_{obama} allele is helpful for analyzing the origin of this gene mutation when subjects with the same mutation are detected in unrelated families.

DISCUSSION

We systematically investigated whether heterozygous LPL deficiency can be identified in unrelated subjects with primary type IV hyperlipoproteinemia by combined application of the sandwich-EIA method for quantifying the LPL mass in PHP and the PCR-SSCP method for detecting LPL gene-exons having aberrations. With this approach, a number of heterozygous LPL deficiencies were indeed detected in subjects with type IV hyperlipoproteinemia (21, 22). One of the subjects with heterozygous LPL deficiency, proband KD, was extensively studied to determine the site of mutation in his LPL gene. The LPL gene mutation, leading to half of the control LPL mass

value in its heterozygous state, was found in LPL gene-exon 6. By the direct sequencing method, the mutation site in LPL gene-exon 6 was identified to be a C to A transversion at nucleotide position 972, resulting in a premature termination codon in place of cysteine at amino acid residue 239. There was loss of an *Hgi*AI restriction enzyme site, and generation of a new *Mbo*I site. This mutation, designated as LPL_{obama}, was newly identified as a case of nonsense mutation. The LPL_{obama} allele can be easily identified by the digestion of PCR-amplified LPL gene-exon 6 with an *Mbo*I or *Hgi*AI restriction enzyme. Such LPL_{obama} allele-specific restriction enzyme assays provide one of the probes for detecting LPL gene variations in subjects with type IV as well as type I hyperlipoproteinemia.

The correlation between heterozygous LPL deficiency and type IV hyperlipoproteinemia is important for understanding the underlying etiology of this disease. In the members of the D-pedigree tested here, four of them, i.e., proband KD, brother KiD, and brother KO's sons YO and JO, were assured to be heterozygous carriers of the LPL_{obama} allele based on genetic and/or biochemical observations. Proband KD was the only subject with type IV hyperlipoproteinemia, whereas the others were normolipidemic. Significant differences between proband KD and the other three heterozygous subjects were age and alcohol consumption under the available clinical data. Proband KD was older than the others and had a history of mild alcohol consumption. Thus, the manifestation of type IV hyperlipoproteinemia in proband KD may have been due to the superimposition of secondary factors such as aging and/or alcohol intake on the background of heterozygous LPL deficiency, as reported with respect to age by Wilson et al. (23). This is also compatible with our previous observation that three of eight heterozygous carriers of the LPL_{arita} allele exhibited type IV hyperlipoproteinemia and had a history of alcohol consumption (18, 21).

The homozygous state of the LPL_{obama} allele results in neither LPL activity nor immunoreactive LPL mass in PHP, as observed for two of proband KD's siblings (KM and KO). We were unable to determine whether the LPL_{obama} allele homozygosity results in no detectable LPL protein due to the absence of LPL mRNA or due to production of a truncated LPL protein which escapes detection with the monoclonal antibodies used here because of the limited amount of sample. However, it is most likely that LPL transcripts become unstable due to the nonsense mutation in exon 6 of the LPL_{obama} allele, as was seen with the LPL_{arita} mutation (18). The LPL_{arita} allele generates a stop codon at amino acid position 224 within exon 5 due to a frameshift caused by a one-base deletion at nucleotide 916. This mutation results in no LPL protein synthesis due to the absence of LPL mRNA, instead of truncated LPL protein synthesis. Therefore, the occurrence of a premature termination in centrally located exons of the LPL gene might be closely related to reduced stability of LPL mRNA, although the mechanism remains to be elucidated.

With respect to understanding the underlying genetic disorder of type IV hyperlipoproteinemia, the detection of heterozygous LPL deficiency in unrelated subjects with this disease provides a helpful clue. LPL gene aberrations could be directly searched for by various either mutant allele-specific oligonucleotide or restriction enzyme assays reported to date, but these measures might be laborious. Our sandwich-EIA method, applied for the first screening, was effective in identifying subjects who might have a heterozygous LPL deficiency based on the results of the LPL mass level in PHP, which is half or less than half of the control value. After identification of such subjects, the PCR-SSCP method was effective as a second screening for detecting LPL gene variations. Our system was surmised to be useful for identifying heterozygous LPL deficiency in unrelated subjects with primary type IV hyperlipoproteinemia.

We would like to express our gratitude to Drs. Masanori Bunya and Tomikazu Fukuoka for their technical assistance. This research was supported in part by a Grant-in-Aid for Encouragement of Young Scientists (No. 05770112) from the Ministry of Education, Science and Culture of Japan, and research grants for Aging and Health (12023A) and Cardiovascular Diseases (5C-1) from the Ministry of Health and Welfare of Japan, Takeda Medical Research Foundation, and ONO Medical Research Foundation for 1991.

Manuscript received 18 February 1994 and in revised form 12 May 1994.

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