A compound heterozygote for a novel missense mutation (G105R) in exon 3 and a missense mutation (D204E) in exon 5 of the lipoprotein lipase gene in a Japanese infant with hyperchylomicroanaemia

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ABSTRACT

We systematically investigated the molecular defects resulting in primary lipoprotein lipase (LPL) deficiency in a Japanese male infant (hereafter called ‘the patient’) with severe fasting hypertriglyceridaemia (type I hyperlipoproteinaemia). The primary LPL deficiency was diagnosed on the basis of the findings that no LPL activity was detected in post-heparin plasma (PHP) and that the immunoreactive LPL mass in PHP was less than 2% of the control level. The patient was a compound heterozygote for a novel missense mutation (G568GA→AGA/Gly105→Arg; G105R) in exon 3 and a missense mutation (GAC867→GAG/Asp204→Glu; D204E) in exon 5 of the LPL gene. The biological significance of both missense mutations was examined by an in vitro study of the expression of the mutant G105R LPL cDNA and D204E LPL cDNA in COS-1 cells. Both mutant LPLs were catalytically inactive and were barely released by heparin from the expressing COS-1 cells. These findings explain the failure to detect LPL activity and immunoreactive LPL mass in the patient’s PHP. The G105R allele could be detected by digestion with the BsmAI restriction enzyme, and the D204E allele by digestion with Hincll. The patient inherited the G105R allele from his mother and the D204E allele from his father. His parents were heterozygotes for the corresponding mutant allele, but normolipidaemic. The novel G105R missense mutation could not be detected by conventional analysis of single-strand conformation polymorphism, but it was identified by extensive sequencing of the entire exons and their flanking regions in the LPL gene.

INTRODUCTION

Type I hyperlipoproteinaemia is a rare autosomal recessive disorder characterized by severe fasting hypertriglyceridaemia due to massive plasma accumulation of chylomicrons, which are triacylglycerol-rich lipoprotein (TGRL) particles derived from the absorption and transport of dietary fat [1]. Conversely, plasma chol-
estrol levels are either normal or moderately elevated. Other characteristics of this disorder are a normal or marginally increased very-low-density lipoprotein (VLDL) level, and decreased low-density lipoprotein (LDL) and high-density lipoprotein cholesterol levels. This disease often presents in infancy or early childhood with episodes of abdominal pain, pancreatitis, xanthomatosis, lipaemia retinalis and hepatosplenomegaly. Acute pancreatitis is sometimes a fatal complication of the disease.

A primary deficiency of lipoprotein lipase (LPL; EC 3.1.1.34), the enzyme responsible for hydrolysing the triacylglycerols in chylomicrons and VLDL, has been reported to be a major cause of type I hyperlipoproteinaemia [1]. Human LPL is a glycoprotein enzyme with a molecular mass of 61 kDa [2]. The LPL cDNA predicts a translated molecular mass of 50394 Da, comprising 448 amino acid residues, for the mature form of the protein in the absence of any sugar moiety [3]. The human LPL gene, 30 kb in length, is located on chromosome 8p22 [4] and consists of 10 exons [5–7]: exons 1–9 contain coding regions, whereas exon 10 contains a 3'-non-coding region. LPL requires apolipoprotein (apo) C-II as an essential cofactor for the hydrolysis of triacylglycerols in TGRL particles, and produces chylomicron and VLDL remnants, thereby releasing non-esterified fatty acids, which are either used for energy or re-esterified for endogenous triacylglycerol storage in adipocytes [8]. The resulting lipoprotein remnants are thought to be catabolized further by hepatic triacylglycerol lipase (HTGL) [9]. In the plasma, small amounts of LPL and HTGL are detected as catalytically inactive forms [10], whereas intravenous injection of heparin releases significant amounts of both catalytically active lipases from the surface of cells into the circulation [10,11].

Clinically, a first screening for primary LPL deficiency is performed by analysing the LPL activity [1], as well as the immunoreactive LPL mass [10,12], in plasma obtained before (pre-heparin) and after (post-heparin plasma; PHP) injection of heparin. Abnormalities in the LPL gene of subjects with LPL deficiency have been detected by analysis [13] of single-strand conformation polymorphism (SSCP), and mutation sites in the LPL gene have subsequently been identified by DNA sequencing. To date, homozygous or compound heterozygous mutations in the LPL gene have been identified, and over 60 distinct mutations, including gene rearrangements and missense, nonsense and splice mutations, have been reported in various ethnic groups, including Japanese [1,14]. Most missense mutations that result in a non-functional LPL have been identified in exons 4, 5 and 6 of the LPL gene. Most of the mutations are specific to a particular family [14], and this makes it difficult to easily scan for LPL gene aberrations in patients with type I hyperlipoproteinaemia. Further identification and characterization of novel mutations in the LPL gene is important for developing a simple diagnostic method for identifying the mutations.

In the present paper we describe in detail a compound heterozygote for a novel missense mutation (G105R) in exon 3 and a missense mutation (D204E) in exon 5 of the LPL gene detected in a Japanese male infant (hereafter called ‘the patient’) with type I hyperlipoproteinaemia due to primary LPL deficiency.

**MATERIALS AND METHODS**

**Patient and family members**

The patient was a 21-month-old Japanese male with no apparent family history of consanguinity who was referred from Kobe Children’s Hospital because of fasting hyperchylomicronaemia. His height and weight were 84.0 cm and 12.0 kg respectively. Physical examination revealed no significant signs, such as a pale face, hepatosplenomegaly, eruptive xanthomata, lipaemia retinalis or abdominal pain. The plasma concentrations of triglycericcerols and total cholesterol were 28.8 mmol/l and 7.1 mmol/l respectively (normal upper limits of triglycericcerols and total cholesterol are 1.7 and 5.9 mmol/l respectively). All examinations of the patient were performed with permission from his parents. The patient’s father, mother and brother were also investigated. As a control group for the patient, four healthy male subjects (age 2.1±0.9 years; mean±S.D.) were recruited with their parents’ agreement. Plasma, obtained from two of the volunteers after a meal, was used for assay of functional significance of apo C-II and possible inhibitors of LPL.

**Laboratory and biochemical tests**

Blood samples were obtained after the subjects had fasted overnight, and plasma and serum were prepared. These samples were used for routine laboratory tests for endocrinological disorders, liver dysfunction, renal disease, autoimmune disease, serum lipids and apolipoproteins. Serum lipoproteins were fractionated into five classes according to their density by sequential flotation ultracentrifugation [15]: chylomicrons (d < 1.006), VLDL (d = 1.006–1.019), LDL (d = 1.019–1.063) and high-density lipoprotein (d = 1.063–1.21). The triacylglycerol and cholesterol concentrations in the serum and the levels of fractionated lipoproteins were measured by enzymic methods (Triglyceride G-test Wako and Determiner TC5 kits) according to the suppliers’ directions. Apo A-I, A-II, B, C-II, C-III and E were measured by a single radial immunodiffusion assay (Daiichi Pure Chemicals Co.) according to the manufacturer’s directions. The apo E genotype was determined by the method reported by Hixson and Vernier [16].
Assay of the functional significance of apo C-II and possible inhibitors of LPL in plasma

Plasma samples obtained from the patient after fasting, and from two healthy male control subjects after a meal, were centrifuged at 356,000 g for 2 h using a Beckman TL-100 ultracentrifuge to float TGRL containing most of the apo C-II, such as chylomicrons and VLDL. The apo C-II was prepared from the TGRL by delipidation with acetone/ethanol (1:1, v/v), and the prepared apo C-II (44 µg) was dissolved in 0.3 ml of 0.4 M guanidine/HCl.

The apo C-II (4.4 µg/30 µl) was mixed well with 30 µl of gum arabic-emulsified tri[9,10−3H]olein (specific radioactivity 330.4 GBq/mol) to prepare a substrate for the LPL assays. Using this substrate, assay of purified human PHP LPL (0.16 µg of protein) was performed in a final volume of 0.3 ml of 0.2 M Tris/HCl buffer (pH 8.5) containing 5% (w/v) BSA and 0.15 M NaCl, as described previously [2]. In the assay for possible inhibitors of LPL in the patient’s and controls’ plasma, TGRL-depleted plasma (30 µl) and 30 µl of purified human PHP LPL (5.4 µg of protein/ml) were mixed in a test tube and allowed to stand for 1 h in an ice bath. Then the LPL activity was measured using 30 µl of gum arabic-emulsified tri[9,10−3H]olein in the presence of pure apo C-II (4.4 µg) [2].

Measurement of LPL and HTGL activities and immunoreactive masses in plasma

Blood samples were collected into tubes containing Na2EDTA (1 mg/ml) before and 10 min after the injection of heparin (30 units/kg body weight). LPL and HTGL activities in PHP were measured using gum arabic-emulsified tri[9,10−3H]olein as the substrate by selective immunoinactivation assay, as reported previously [2,10]. Both enzyme activities were expressed in units of µmol of non-esterified fatty acid released·h−1·ml−1 enzyme solution, unless stated otherwise.

A one-step sandwich enzyme immunoassay for LPL mass quantification was performed using a Markit-F LPL kit (Dainippon Pharmaceutical Co.), and a sandwich enzyme immunoassay for HTGL mass quantification was also carried out as described previously [10]. The masses of immunoreactive LPL and HTGL in pre-heparin plasma and in PHP were measured, and the increments in the LPL and HTGL masses were calculated as the difference between the masses in pre-heparin plasma and PHP. The enzyme mass values were expressed as ng of LPL or HTGL/ml of plasma.

Southern blot analysis and haplotype analysis

Genomic DNA was isolated from the peripheral whole blood of the patient’s family members and from normal controls. Southern blot analysis was performed after digestion of the DNA with the appropriate restriction enzymes (HindIII and PvuII), using 32P-labelled HLC601 [17] as a probe according to the standard procedure [18]. The haplotype of the LPL gene was determined by analysis of the restriction fragment length polymorphism using HindIII and PvuII, and tetrarepeat (TTTA) polymorphism at intron 6 [19] in the LPL gene locus, as described previously [20,21].

Fluorescence-based SSCP analysis

The individual exons and their flanking regions (1DU and 1DD–9; Table 1) in the LPL gene were each amplified enzymically from the genomic DNA by the PCR method using a pair (F and R) of fluorescent substance (Cy5)-labelled primers, in which Cy5 was attached to the 5’ end of individual 5’ flanking and intron sequences (without the tag sequences) of the LPL gene, as listed in Table 1. Fluorescence-based SSCP analysis was performed by electrophoresis using a 6% (w/v) polyacrylamide gel containing 10% (w/v) glycerol and Tris/boric acid/EDTA buffer, pH 7.7, at various temperatures (15, 20, 25 and 30 °C). The SSCP pattern was analysed with an ALFExpress DNA sequencer equipped with AlleleLinks® software (Pharmacia Biotech), according to the manufacturer’s directions.

DNA sequencing

Templates (1DU and 1DD–9; see Table 1) for DNA sequencing were amplified from the genomic DNA by PCR using the appropriate pair (F and R) of the 5’ flanking and intron sequences of the LPL gene with the tag sequences listed in Table 1. The sequencing reaction was carried out using the template DNA, 5’ Cy5-labelled primer (Seq-F and -R in Table 1) and a Thermo Sequence fluorescent-label labelled primer cycle Sequencing Kit (Amersham Life Science). The reaction mixture was mixed with the stop/loading dye and electrophoresed on a 6% (w/v) polyacrylamide/7 M urea gel. The DNA sequence pattern was determined with an ALFExpress DNA sequencer equipped with ALFwin® software, according to the manufacturer’s directions. Both the nucleotide sequence and amino acid positions of the LPL gene mutations detected here correspond to the human LPL cDNA sequence [3].

Construction of LPL cDNAs subjected to site-directed mutagenesis

Normal human LPL cDNA (HLC601), subcloned into the phagemid Bluescript SK II M13 (−) vector, was used as a template for site-specific mutagenesis. Mutant G105R LPL cDNA was generated with the mutagenic oligonucleotide primer (no. 359) 5’-ACCAAACTGGTGACAGGATGTGG-3’, and mutant D204E LPL cDNA was generated with the primer (no. 360) 5’-TGGGCATGTTGAGATTACCCGAAT-3’, using a...
Mutant-b-Kit (Takara). The underlined bases of the primers are the specifically mutated bases. The normal, G105R or D204E LPL cDNA fragment was subcloned into an expression vector (pRC/CMV; Invitrogen) with the cytomegalovirus early promoter.

### Expression of normal LPL and mutant LPL cDNAs in COS-1 cells, LPL enzyme assay, and Northern blot analysis

Normal LPL cDNA-, G105R-LPL cDNA- or D204E-LPL cDNA-pRC/CMV, or a pRC/CMV expression vector, was transfected into COS-1 cells by an electroporation method using a Bio-Rad Gene Pulser II according to the supplier’s directions. A β-galactosidase expression plasmid (pCH110; Pharmacia) was co-transfected into the above expression system as an internal standard in order to assess the efficiency of expression. The transfected COS-1 cells were plated into 10-cm dishes containing 10 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, and cultured. After 2.5 days of culture, the medium was removed, and the cells were washed with PBS. The washed cells in one dish were treated for 3 × 5 min at room temperature with 0.5 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 10 units of heparin in order to release LPL bound to the cell surface. The pooled medium (a total volume of 1.5 ml) containing heparin-released LPL was immediately frozen in liquid nitrogen and stored at −80°C until used for the assay. The remaining cells in the dish were washed with PBS, harvested in 2.0 ml of cell-solubilizer solution (20 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 10% glycerol, 3 mM benzamidine, 3.1 μM aprotinin, 10 units/ml heparin and 0.15% Brij-35), and solubilized by 1 min of sonication using a Branson sonicator. The cell lysate (2.0 ml) was centrifuged to remove cell debris, flash-frozen, and stored at −80°C. The LPL activity and immunoreactive LPL mass were determined as described above, and these values were corrected based on the β-galactosidase activity co-expressed in the COS-1 cells.

Poly(A)+ RNA was prepared from COS-1 cells transfected with normal LPL cDNA-, G105R-LPL cDNA- or D204E-LPL cDNA-pRC/CMV, or pRC/CMV ex-
expression vector, using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) according to the supplier’s directions. Northern blot analysis of the poly(A)$^+$ RNA was performed by using $^{32}$P-labelled HLC601 [17] for LPL mRNA and $^{32}$P-labelled human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (Cayman Chemical) as probe, according to the standard procedure [18].

RESULTS

Laboratory data, LPL activity and immunoreactive LPL mass for the patient and his family

Figure 1 shows the laboratory data, LPL and HTGL activities and immunoreactive masses for the patient and members of his family. The patient exhibited an extremely high serum triacylglycerol concentration (28.8 mmol/l), which was 17 times higher than the highest value (1.7 mmol/l) of the control subjects. His serum cholesterol (7.1 mmol/l) was slightly higher than the highest value (5.95 mmol/l) of the controls, while LDL and high-density-lipoprotein cholesterol were both approx. one-quarter of those of the controls. The patient was diagnosed as having primary type I hyperlipoproteinaemia according to the WHO classification of phenotype expression [1]. Apo C-II and apo E, known to be possible factors affecting the plasma triacylglycerol level, were examined. The apo C-II concentration was above the normal range (Figure 1), and its biological function was found to be normal, since the patient’s plasma activated pure human LPL in vitro (Table 2). The genotype of apo E was E3/3, which is known to be the normal type.

Analysis of the LPL activity and immunoreactive LPL

<table>
<thead>
<tr>
<th>Subject’s ID</th>
<th>I-1</th>
<th>I-1</th>
<th>I-2</th>
<th>I-2</th>
<th>Control (n=4)</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (yr)</td>
<td>35</td>
<td>4</td>
<td>1.8</td>
<td>33</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>BMI (Kg/m$^2$)</td>
<td>21.02</td>
<td>1.64</td>
<td>28.78</td>
<td>1.62</td>
<td>1.14 ± 0.09</td>
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<tr>
<td>Serum-TG (mmol/L)</td>
<td>1.58</td>
<td>0.99</td>
<td>27.61</td>
<td>4.40</td>
<td>0.43 ± 0.10</td>
</tr>
<tr>
<td>Serum-Chol (mmol/L)</td>
<td>4.73</td>
<td>5.51</td>
<td>7.14</td>
<td>3.38</td>
<td>4.33 ± 0.35</td>
</tr>
<tr>
<td>HDL-Chol (mmol/L)</td>
<td>3.38</td>
<td>0.70</td>
<td>0.26</td>
<td>0.70</td>
<td>2.63 ± 0.42</td>
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<tr>
<td>Serum-apo A-I (g/L)</td>
<td>1.46</td>
<td>0.26</td>
<td>0.08</td>
<td>0.38</td>
<td>1.20 ± 0.19</td>
</tr>
<tr>
<td>Serum-apo A-II (g/L)</td>
<td>0.08</td>
<td>0.38</td>
<td>0.46</td>
<td>0.38</td>
<td>0.97 ± 0.08</td>
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<tr>
<td>Serum-apo A-III (g/L)</td>
<td>0.04</td>
<td>0.46</td>
<td>0.04</td>
<td>0.46</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Serum-apo E (g/L)</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Alcohol intake (g/day)</td>
<td>6.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>WHO-type</td>
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<td>Normal</td>
<td>Type 1</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>LPL activity (µmol/h/ml)</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td>NT</td>
<td>9.8 ± 2.5</td>
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<tr>
<td>LPL mass (ng/ml)</td>
<td>NT</td>
<td>NT</td>
<td>3.0</td>
<td>NT</td>
<td>186 ± 46</td>
</tr>
<tr>
<td>HTGL activity (µmol/h/ml)</td>
<td>NT</td>
<td>NT</td>
<td>23.7</td>
<td>NT</td>
<td>22.5 ± 9.0</td>
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<tr>
<td>HTGL mass (ml)</td>
<td>NT</td>
<td>NT</td>
<td>1628</td>
<td>NT</td>
<td>1242 ± 517</td>
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</tbody>
</table>

Figure 1 Clinical data, LPL activity and immunoreactive LPL mass for the patient and members of his family

The patient, a type I hyperlipoproteinaemic subject who is a compound heterozygote for the G105R and D204E mutations, is indicated by the arrow (II-2). The patient's normolipidaemic mother (I-2) is heterozygous for the G105R mutation. The patient's normolipidaemic father (I-1) is heterozygous for the D204E mutation. The patient's brother (II-1) is a non-carrier of these mutations. Abbreviations: ID, identification number; BMI, body mass index; TG, triacylglycerol; CM, chylomicron; Chol, cholesterol; HDL, high-density lipoprotein; NT, not tested; ND, not detectable. The control subjects’ LPL and HTGL activities and masses were from data reported previously [21]. Depictions of the LPL genotypes are derived from results presented in Figures 2–5.
Table 2  Assay of biological function of apo C-II and possible inhibitors of LPL in the patient’s plasma

These assays were carried out as described in the Materials and methods section. The unit of LPL activity is defined as \( \mu \text{mol} \cdot \text{h}^{-1} \cdot \text{ml}^{-1} \). Control values represent the average of individual measurements, which differed by less than 5%.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Biological function of apo C-II in plasma (units) (% of control)</th>
<th>Possible inhibitors in plasma (units) (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>7.04 (99.4)</td>
<td>7.02 (99.6)</td>
</tr>
<tr>
<td>Controls (n = 2)</td>
<td>7.08 (100)</td>
<td>7.05 (100)</td>
</tr>
</tbody>
</table>

mass in plasma from the patient revealed values for both parameters to be less than 2% of the control values (Figure 1); these data are compatible with LPL deficiency. The existence of inhibitors of LPL in the patient’s plasma was excluded, as his plasma did not inactivate pure human LPL activity (Table 2). HTGL activity and immunoreactive mass were within the normal range for each parameter.

The patient was placed on alimentary treatment with a diet containing 5020 kJ (1200 kcal) of energy (the fat content represented 15% of total energy) consisting of milk containing medium-chain triacylglycerols, in order to avoid development of pancreatitis. This treatment reduced the serum triacylglycerol concentration to 5.44 mmol/l after 9 days. Thereafter he was maintained on a low-fat diet (less than 15 g/day). His serum triacylglycerol concentration was kept at \(<4.5\text{ mmol/l}\), although this was still 2.6 times higher than the control value. Growth and development have been normal, without any significant episodes of abdominal pain or pancreatitis, until his present age of 3 years.

Identification of the sites of mutation in the LPL gene of the patient, and carrier status and haplotype of the mutant allele in members of his family

The gross structure of the patient’s LPL gene was examined by Southern blot analysis, and no appreciable deletions or insertions were detected (results not shown). Each DNA segment, encompassing an exon and its flanking regions, and the 5′-upstream region of the LPL gene were amplified and analysed by the SSCP method. An anomalous PCR-SSCP pattern was found for exon 5, indicating that the patient had a heteroallelic mutation in exon 5 (results not shown). This heteroallelic mutation was thought to be one of the causes underlying his LPL deficiency, but the near absence of LPL activity and immunoreactive LPL mass in the PHP could not be explained on the basis of this mutation alone. Therefore another latent mutation was searched for by direct sequencing of all of the LPL gene exons and their flanking regions. As expected, heteroallelic mutations were identified, in exon 3 and exon 5, indicating that the patient was a compound heterozygote for a mutation in exon 3 and a mutation in exon 5. Apparent nucleotide substitutions were identified at position 568: nucleotide \( C \) indicated by the open triangle, is from the normal allele, while nucleotide \( T \) indicated by the solid triangle, is from the G105R allele. This mutation is a \( G \rightarrow A \) transition in the sense strand of exon 3. (B) One single peak was detected at position 568, representing nucleotide \( C \) (indicated by the open triangle) of the normal allele.

![Identification of the mutation site in exon 3 of the LPL gene](image-url)

The nucleotide sequence patterns of a part of the antisense strand from exon 3 of the patient (A) and a control (B) are shown. (A) Two significant peaks were detected at position 568: nucleotide \( C \) indicated by the open triangle, is from the normal allele, while nucleotide \( T \) indicated by the solid triangle, is from the G105R allele. This mutation is a \( G \rightarrow A \) transition in the sense strand of exon 3. (B) One single peak was detected at position 568, representing nucleotide \( C \) (indicated by the open triangle) of the normal allele.

![Identification of the mutation site in exon 3 of the LPL gene](image-url)
Novel mutation (G105R) in the lipoprotein lipase gene

Figure 3  Schematic illustration of detection of the G105R mutation with BsmAI enzyme (A), and carrier status of the patient’s family for this mutation (B)
Exon 3 of the LPL gene was amplified using an Exon-3F and -3R primer pair (see Table 1) by the PCR method. (A) The DNA fragments of the PCR-amplified LPL gene exon 3 with the normal allele produce bands of 47 bp and 249 bp on digestion with the BsmAI restriction enzyme. The DNA fragments of the G105R allele with a newly generated BsmAI site, indicated by the asterisk, produce bands of 47 bp, 53 bp and 196 bp. (B) The carrier status of the patient’s family for the G105R allele was analysed by 4% NuSieve agarose gel electrophoresis after BsmAI digestion of PCR-amplified DNA from each subject. DNA bands were visualized by staining with ethidium bromide. Lane 1, X174 DNA, digested with HincII, as a size marker; lane 2, normal subject (N); lane 3, father (F); lane 4, brother (B); lane 5, patient (P); lane 6, mother (M).

Figure 4  Schematic illustration of detection of the D204E mutation with HincII enzyme (A), and carrier status of the patient’s family for this mutation (B)
Exon 5 of the LPL gene was amplified using an Exon-5F and -5R primer pair (see Table 1) by the PCR method. (A) The DNA fragments of the PCR-amplified LPL gene exon 5 with the normal allele produce bands of 143 bp and 207 bp on digestion with the HincII restriction enzyme. The DNA fragments of the D204E allele with the loss of the HincII site, indicated by the asterisk, produce a band of 350 bp. (B) The carrier status of the patient’s family for the D204E allele was analysed by 4% NuSieve agarose gel electrophoresis after HincII digestion of PCR-amplified DNA from each subject. DNA bands were visualized by staining with ethidium bromide. Lane 1, X174 DNA, digested with HincII, as a size marker; lane 2, normal subject (N); lane 3, father (F); lane 4, brother (B); lane 5, patient (P); lane 6, mother (M).

The G105R mutation resulted in the generation of a BsmAI restriction enzyme recognition site (marked by the asterisk in Figure 3A). The existence of this mutation was determined by detecting a 350 bp band after digestion of the PCR-amplified exon 5 with HincII (Figure 4B). The patient and his father were heterozygous for the D204E mutation, while his mother and brother were not carriers.

The haplotypes of the patient’s G105R and D204E alleles were determined to be PvuII (+), HindIII (+) and (10) repeats of TTTA; and PvuII (+), HindIII (+) and (11) repeats of TTTA respectively.

Functional assay of mutant G105R and D204E LPLs expressed in COS-1 cells
The functional significance of the mutant G105R LPL was examined by analysing LPL activity and immuno-
The enzyme activity and immunoreactive mass in the medium (1.5 ml) and the cell lysate (2.0 ml), recovered from one dish, were quantified by selective immunoinactivation assay and sandwich enzyme immunoassay methods respectively. The unit of activity is defined as μmol·h⁻¹·1.5 ml⁻¹ for the medium and μmol·h⁻¹·2.0 ml⁻¹ for the cell lysate, and the mass value is defined as ng/1.5 ml for the medium and as ng/2.0 ml for the cell lysate. The total LPL activity is the sum of the activities in the medium and the cell lysate, and the total LPL mass is the sum of the masses in the medium and the cell lysate. Each experiment was performed using four distinct dishes, and both the activity and mass values are shown as the means ± S.D. S.A., specific activity; calculated by dividing LPL activity by LPL mass; ND, not detectable.

<table>
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<tr>
<th>cDNA</th>
<th>Medium containing LPL released by heparin</th>
<th>LPL in cell lysate after heparin treatment</th>
<th>Total LPL activity (units)</th>
<th>Total LPL mass (ng)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Activity (units)</td>
<td>Mass (ng)</td>
<td>S.A. (units/µg)</td>
<td>Activity (units)</td>
</tr>
<tr>
<td>Normal LPL cDNA</td>
<td>42.7 ± 1.4</td>
<td>783 ± 7</td>
<td>55 ± 2</td>
<td>17.3 ± 0.2</td>
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<tr>
<td>Mutant G105R LPL cDNA</td>
<td>ND</td>
<td>12 ± 2</td>
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<td>ND</td>
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<td>Mutant D204E LPL cDNA</td>
<td>ND</td>
<td>34 ± 9</td>
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<tr>
<th>pRL (pmol)</th>
<th>LPL mRNA activity</th>
<th>LPL mRNA mass</th>
<th>S.A. (pmol/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42.7</td>
<td>688 ± 34</td>
<td>25 ± 2</td>
<td>60.0 ± 1.3</td>
</tr>
<tr>
<td>17.3</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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</tbody>
</table>

Figure 5  Detection of LPL mRNA by Northern blot analysis
Poly(A)+ RNA was isolated from COS-1 cells transfected with normal LPL, G105R LPL or D204E LPL cDNA-pRc/CMV, or vector pRc/CMV, and subjected to Northern blot analysis as described in the Materials and methods section. LPL and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNAs were visualized by 32P-labelled HPLC601 and G3PDH probes. Lane 1, cells transfected with G105R LPL cDNA; lane 2, cells transfected with D204E LPL cDNA; lane 3, cells transfected with a vector of pRc/CMV; lane 4, cells transfected with normal LPL cDNA.

Phenotypic expression of heterozygous carriers of the G105R and D204E alleles
The clinical phenotypes of a heterozygous carrier (the proband’s mother; 33 years old) of the G105R allele and a heterozygous carrier (the proband’s father; 35 years old) of the D204E allele were examined. Both carriers’ serum triacylglycerol and cholesterol concentrations were lower than the upper limit of the normal range for both parameters, i.e. a normolipidaemic state (Figure 1). Their lifestyle was healthy, and they did not show any signs of disease affecting the plasma triacylglycerol concentration. This was in good agreement with the previously reported phenotypic expression of some heterozygous carriers of the LPLarita allele [20] or the LPLobama allele [21] in other families, in terms of the gene-environment interaction. That is, subjects with heterozygous LPL deficiency can usually maintain a normal triacylglycerol level, provided that they are not exposed to factors, such as high alcohol intake and/or a
Novel mutation (G105R) in the lipoprotein lipase gene

In the present study, we describe in detail the biochemical and genetic characterization of two missense mutations in the LPL gene of a Japanese male infant with fasting hyperchylomicronaemia due to primary LPL deficiency. The patient was a compound heterozygote for a novel missense mutation (G568GA→AGA/Gly105→Arg; G105R) in exon 3 and a previously described missense mutation (GAC867→GAG/Asp281→Glu; D204E) [22] in exon 5 of the LPL gene. Both missense mutations were confirmed to be responsible for the LPL deficiency by examining the in vitro expression of the mutant LPL cDNAs in COS-1 cells. Both of the expressed mutant LPLs were catalytically inactive, as well as being almost non-releasable from the expressing cells by heparin treatment. These data readily explain why neither LPL activity nor immunoreactive LPL mass was detected in the PHP of the patient, and also explain his clinical expression of severe fasting hypertriglyceridaemia due to massive accumulation of chylomicrons in the plasma.

The structure–function relationship of human LPL has been deduced from a number of missense mutations that result in non-functional LPLs due to the substitution of single amino acids [23,24], based on the three-dimensional crystallographic structure of human pancreatic lipase [25], which is catalytically active as a monomeric form [26]. Most missense mutations have been found in exons 4, 5 and 6 (residues 117–312), which constitute a large N-terminal domain (residues 1–312), and this region’s importance for catalytic function has been established. In contrast, little information is available regarding the missense mutations in exon 3 (residues 57–116) of the LPL gene, which result in a catalytically abnormal LPL, as shown by in vitro expression studies in COS-1 cells. These mutations are V69L [27], R75S [28], W86R [29] and T101A [30]. The G105R (Gly→Arg substitution) mutation, newly identified here by us, results in an enzymically completely inactive LPL and provides clues to an important role for this region in the catalytic function of LPL. The residue Gly105 (indicated by underlining) is located in the centre of an α-helix (α3; Tyr94-Phe118; Y94PVSAGYTKLVGDVARFI-NWMEEFF119) [23]. The α3 helix plays a role in packing against the strands of the central β-sheet in the N-terminal domain of LPL [23]. The Val-Gly105-Xaa-Xaa-Val-Ala motif situated in the centre of the α3 helix is highly conserved among the three lipases LPL, HTGL and pancreatic lipase in all species that have been studied [31]. The replacement of Gly105 by Arg has a significant effect on the molecular nature, i.e. conversion of a neutral hydrophobic small amino acid into a positively charged hydrophilic large amino acid. This substitution, therefore, may change the conformation of the α3 helix and subsequently disrupt the spatial configuration of this critical region for catalysis in the N-terminal domain. As a result, the mutant G105R LPL results in a completely inactive enzyme. Similarly, the known mutant W86R and R75S LPLs, which involve substitution of a charged amino acid with a neutral one and the reverse change respectively in exon 3, have almost no LPL activity [28,29]. On the other hand, the mutant V69L and T101A LPLs, which involve substitution with an amino acid having a similar nature in exon 3, showed 20–30% of normal LPL activity [27,30].

The D204E mutation in exon 5 of the LPL gene was identified previously in a homozygous state in a Japanese patient with hyperchylomicronaemia [22]. In that study, LPL activity was not detectable in the patient’s PHP, but the LPL mass was 30% of that in the normal control. This was confirmed by an expression study in COS cells [22]. In our case, the D204E LPL was synthesized as a catalytically inactive form which was barely released from the expressing COS cells by heparin. This was in good agreement with the findings that LPL activity and immunoreactive LPL mass were almost absent from the proband’s PHP. The discrepant immunoreactive PHP LPL mass values might be due to a difference in specificity for LPL in human PHP between our monoclonal antibodies and those used in the previous study [22]. Notably, the amount of mutant D204E LPL synthesized in COS-1 cells was approx. 50% of that of the normal LPL, but the amount of D204E LPL mRNA was almost equal to that of the normal LPL mRNA. This indicates that a C→G transversion at position 867 of exon 5 in the LPL gene does not affect the efficiency of transcription and/or the stability of the transcripts. Thus we speculate that the reduced amount of the D204E LPL in the COS cells stems primarily from a post-translational unstable structure in the mutant LPL due to structural alterations.

A remarkable finding is that the novel missense mutation (G105R) was not detected as an aberrant pattern by SSCP analysis of the PCR-amplified product (260 bp) of exon 3 and its flanking regions in the LPL gene. This missense mutation was identified as being heterozygous in exon 3 by extensive sequencing of all coding exons (1–9) and their flanking regions of the proband’s LPL gene. In general, as a first screening for identification of mutations in a gene, SSCP analysis is commonly utilized to detect alterations in DNA fragments ranging from 100 bp to 300 bp after amplification by PCR [13]. However, the efficiency of detection of alterations in DNA fragments is sometimes poor, although the causes of this are unclear. Thus simple detection of the G105R allele by BsmAI digestion might be useful for elucidation of the aetiology underlying severe hypertriglyceridaemia in unrelated Japanese patients and those in other ethnic
groups in whom this mutation has escaped detection by SSCP analysis.

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REFERENCES

10 Ikeda, Y., Takagi, A., Ohkura, Y. et al. (1990) A sandwich-enzyme immunoassay for the quantification of lipoprotein lipase and hepatic triglyceride lipase in human postheparin plasma using monoclonal antibodies to the corresponding enzymes. J. Lipid Res. 31, 1911–1924

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