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Biochemical and Biophysical Research Communications 341 (2006) 82–87
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A novel substitution at the translation initiator codon (ATG → ATC) of the lipoprotein lipase gene is mainly responsible for lipoprotein lipase deficiency in a patient with severe hypertriglyceridemia and recurrent pancreatitis

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Received 16 December 2005
Available online 9 January 2006

Abstract

A patient with severe hypertriglyceridemia and recurrent pancreatitis was found to have significantly decreased lipoprotein lipase (LPL) activity and normal apolipoprotein C-II concentration in post-heparin plasma. DNA analysis of the *LPL* gene revealed two mutations, one of which was a novel homozygous G → C substitution, resulting in the conversion of a translation initiation codon methionine to isoleucine (LPL-1). The second was the previously reported heterozygous substitution of glutamic acid at residue 242 with lysine (LPL-242). In vitro expression of both mutations separately or in combination demonstrated that LPL-1 had approximately 3% protein mass and 2% activity, whereas LPL-242 had undetectable activity but normal mass. The combined mutation LPL-1-242 exhibited similar changes as for LPL-1, with markedly reduced mass, and for LPL-242, with undetectable activity. These results suggest that the homozygous initiator codon mutation rather than the heterozygous LPL-242 alteration was mainly responsible for the patient phenotypes.

Keywords: Lipoprotein lipase deficiency; Gene mutation; Initiation codon; Polymerase chain reaction-single-strand conformation polymorphism; DNA sequencing; Expression analysis

Lipoprotein lipase (LPL) functions as a monodimer bound to heparan sulfate proteoglycans at the surface of capillary endothelium to hydrolyze triglycerides (TGs), using apolipoprotein C-II (ApoC-II) as a cofactor [1]. When lipoprotein lipase is defective, this results in massive accumulation of chylomicrons and profound fasting hypertriglyceridemia (HTG) due to delayed plasma clearance, as well as markedly reduced cholesterol concentrations in low- and high-density lipoproteins. Patients typically exhibit severe chylomicronemia, hepatosplenomegaly, and episodes of abdominal pain and eruptive xanthomas, sometimes complicated by acute pancreatitis [2].

At present, more than 120 types of mutations that lead to LPL deficiency have been investigated. The majority of the mutations have been located in exons 5 and 6 [3], the most conserved regions of the *LPL* gene, with a minority located in exon 3, and one mutation reported in exon 1 [4]. To the best of our knowledge, there have been no reports of LPL deficiency in relation to the translation initiation codon of the *LPL* gene.

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doi:10.1016/j.bbrc.2005.12.165

Here we describe a patient who presented with HTG, recurrent pancreatitis, and significantly decreased post-heparin plasma (PHP) LPL activity, and was shown to have a previously reported heterozygous mutation $Glu^{242} \rightarrow Lys$ in exon 6 [5]. However, in general, single heterozygous mutation only leads to slight or partial reduction in LPL activity or mass [6,7]. The severe symptoms of this patient led us to suspect another unidentified homozygous or heterozygous mutation. Further DNA analysis of the *LPL* gene revealed the novel homozygous mutation $Met^{1} \rightarrow Ile$ in the translation initiation codon. In vitro expression of this mutation produced a wild-type enzyme with normal LPL mRNA but severely decreased enzyme activity and mass, which is in accordance with the in vivo data for the patient. Therefore, the homozygous initiation codon mutation rather than the heterozygous $Glu^{242} \rightarrow Lys$ alteration is mainly responsible for the phenotypic expression of the chylomicronemia syndrome in this patient.

Materials and methods

Subject. The patient was hospitalized at the age of 18 with acute pancreatitis, and severe HTG was discovered. During subsequent years, the patient suffered recurrent episodes of acute pancreatitis and responded poorly to lipid-lowering medicines (niacin, clofibrate, and Chinese herbal medicine) and a fat-restricted diet (TGs were still 11.29–36.94 mmol/L). Diagnosis of LPL deficiency was established by demonstrating a serious reduction in PHP LPL activity: 1.4 μmol FFA mL^{-1} h^{-1} (control value: 21.7 ± 1.7 μmol FFA mL^{-1} h^{-1}). Hepatic lipase (HL) activity was normal (22.3 μmol FFA mL^{-1} h^{-1}). Plasma lipid-related parameters were: total cholesterol (TC) 3.73 mmol/L; apo C-II 0.08 g/L; apo C-III 0.32 g/L [8]. In addition, a heterozygous mutation $Glu^{242} \rightarrow Lys$ in exon 6 was detected [5].

DNA analysis. Genomic DNA was extracted from peripheral venous blood samples from the patient and a control using a Genome DNA Extraction Kit (TaKaRa BIOTECH). The upstream non-coding sequence of the *LPL* gene (917 bp in length from −743 to +174) and exons 1–9 were amplified by polymerase chain reaction (PCR). The cycle profile included denaturation at 94 °C for 45 s, annealing at 54–57 °C for 45 s, and extension at 72 °C for 1 min for a total of 30 cycles. Aliquots of 2 μL of each PCR product were assayed by low ionic strength-single strand conformation polymorphism (LIS-SSCP) [9]. In the case of an abnormal electrophoresis profile on an exon of the *LPL* gene on 8% polyacrylamide-silver stained gel in LIS-SSCP, DNA sequencing of this exon was performed using a TaKaRa BIOTECH kit after being subcloned into pMD18-T vector according to the supplier's instructions.

In vitro expression of site-directed mutations of LPL cDNA. A 1433-bp fragment of wild-type LPL cDNA that spanned the signal peptide through the termination codon was amplified from a human LPL-cDNA clone by PCR. The fragment was subcloned into the *Hin*dIII and *Xba*I sites of pcDNA3 to construct the wild-type expression vector, pcDNA3-wt (LPL-wt). Mutants of LPL cDNA, pcDNA3-1 (LPL-1) containing G177C ($Met^{1} \rightarrow Ile$) at the translation initiation codon, pcDNA3-242 (LPL-242) containing G979A ($Glu^{242} \rightarrow Lys$) in exon 6, and pcDNA3-1-242 (LPL-1-242) containing both mutations were generated from pcDNA3-wt by site-directed mutagenesis [10]. All the primers used for constructing expression vectors are shown in Table 1. A total of 30 cycles of 30 s at 94 °C for denaturation, 30 s at 54 °C for annealing, and 2 min at 72 °C for extension were used.

The wild-type and mutant LPL cDNA constructs were sequenced before transfection into COS-7 cells. Preparation of the wild-type and mutant LPL constructs was performed using a Midi Plasmid Kit (Qiagen). Transfection was carried out by mixing 4 μg of the expression vectors for LPL cDNA or pEGFP (used as mock transfection) with 8 μL of Lipofectamine 2000 (GIBCO BRL Invitrogen, Carlsbad, CA), which was then added to 1×10^{6} COS-7 cells/well (in 6-well plates) maintained in DMEM containing 10% fetal bovine serum. For LPL activity and protein mass determinations, culture medium supplemented with heparin 200 mU/mL was collected at 36 h after transfection. Cells were collected, washed in PBS, and solubilized in lysis buffer containing heparin as previously described [11]. Media and cell extracts were stored at −70 °C until analysis.

Table 1
Synthetic oligonucleotides for constructing expression vectors

LPL-wt
5′-C[a]AAGCTTCCGAG[c]ATGGAGAGCAAAGCC-3′
5′-C[b]TCTAGA[d]TCAGCCTGACTTCTTATTCAG-3′

LPL-1
5′-C[a]AAGCTTCCGAGAT[e]C[e]GAGAGCAAAGCC-3′

LPL-242
5′-CTAGTGAAGTGCTCCCAC[f]A[f]AGCGCTC-3′

LPL-1-242
5′-C[a]AAGCTTCCGAGAT[e]C[e]GAGAGCAAAGCC-3′
5′-CTAGTGAAGTGCTCCCAC[f]A[f]AGCGCTC-3′

[a] The artificial *Hin*dIII site.
[b] The artificial *Xba*I site.
[c] The translation initiation codon.
[d] The stop codons are underlined.
[e] The single base substitution at the translation initiation codon is highlighted by a box.
[f] The single base substitution at the residue 242 is highlighted by a box, respectively.

Real-time quantitative PCR. Fluorescent real-time quantitative PCR for human LPL cDNA was performed to determine the level of LPL mRNA. The first-strand cDNA was generated by reverse transcription of 1 μg RNA with random hexamers using an Invitrogen kit. The following primer sets were used: LPL, 5′-GCGTGATTGCAGAGAGAGGAC-3′ and 5′-TCAGGCAGAGTGAATGGGATG-3′; 18S rRNA, 5′-GGAA GGGCACCACCAGGAGT-3′ and 5′-TGCAGCCCCGGACATCTAA G-3′. A 1-μL aliquot of the RT product and 1× SYBR green (Molecular Probes, Eugene, USA) was included in the reaction mixture. A total of 35 cycles of amplification was performed using an Opticon continuous fluorescence detection system (MJ Research, MA, USA). Each cycle consisted of a 40-s denaturation at 94 °C, 40-s annealing at 58 °C, and a 60-s extension at 72 °C. The mRNA levels were normalized to 18S RNA using the comparative cycle threshold (CT) method [12].

LPL mass analysis. LPL mass was measured by ELISA as described elsewhere [13]. Briefly, 96-well plates were coated with a chicken anti-human antibody. After application of samples, the plates were incubated overnight at 4 °C. Following washes, bound LPL was detected with monoclonal antibody 5D2, kindly provided by Dr. Brunzell of Washington University, conjugated to horseradish peroxidase. The plates were developed by addition of σ-phenylene diaminedihydrochloride substrate and read in a microtiter plate reader at dual wavelengths (490–405 nm). Purified bovine milk LPL was diluted as standard.

LPL activity analysis. A 10-μL aliquot of supernatant of cell homogenate, or 100 μL of culture medium, was added to a total 200-μL mixture containing [^{3}H]triolein emulsion substrate prepared as previously described [14] and incubated at 37 °C for 60 min. Enzyme activity was expressed as milliunit/milliliter (1 milliunit corresponds to 1 nanomolar of free fatty acid generated per minute).

Western blot. To enrich LPL, samples of culture media from transfected cells were precipitated using heparin–Sepharose beads. After washing and release of LPL from the beads by addition of SDS–PAGE sample buffer, the media and cell extracts were electrophoresed, blotted onto nitrocellulose membranes, and detected with 5D2 as previously described [15].

Statistical analysis. Statistical analysis was performed using Version 10 of the Statistical Package for the Social Sciences (SPSS) for Windows. The statistical significance for the mean values between mutant and normal types was determined by Student's t test. The threshold for statistical significance was set at the 0.05 level.

Results

LIS-SSCP analysis and DNA sequencing of the LPL gene

LIS-SSCP analysis of the *LPL* genes from the patient and a control subject showed an aberrant migration of exon 1 and exon 6 on electrophoresis (Fig. 1). DNA sequencing revealed that a G at nucleotide 177 was substituted to a C, which was a novel mutation found in the *LPL* gene. This missense mutation resulted in a substitution of Met for Ile in the initiation codon. The patient was found to be homozygous for this mutation. Another G → A heterozygous mutation at nucleotide 979 was found, which resulted in a substitution of Glu^{242} for Lys in exon 6 (Fig. 2).

Assessment of in vitro-expressed LPL mutants

mRNA abundance

Quantitation of mRNA extracted from transfected COS-7 cells by fluorescent real-time PCR showed a steady state of normal and mutant LPL mRNA expression (Fig. 3). These data indicate that the amount of LPL-1, LPL-242, and LPL-1-242 mRNA expressed in the cells was almost equal to that of the LPL-wt mRNA ($P > 0.05$).

Protein mass and catalytic activity

Media and cell extracts harvested from cells transfected with the pEGFP vector alone showed a mild background level of both LPL mass and activity, whereas medium and cell extracts obtained from cells transfected with LPL-wt demonstrated normal LPL expression in these cells (Table 2). There was very low LPL mass and catalytic activity in the media and cell extracts in the cells transfected with LPL-1, approximately 3% and 2% of the wild-type values, respectively, which indicates that the initiation codon mutation ATG → ATC generated normal LPL but at a low level. The LPL-242 mutation in exon 6 resulted in the generation of mutant LPL at protein levels similar to those of wild-type LPL but without enzyme activity. The mutant protein could also be secreted into medium normally. The combination of both mutations LPL-1-242 produced markedly decreased LPL mass and undetectable LPL activity. Western blot of media and cell extracts (Fig. 4) further confirmed that mutant LPL-242 produced a band of 55 kDa of similar size and level as observed for LPL-wt, whereas mutant LPL-1 produced only a faint band of 55 kDa. There was no band detected in the cell extracts and media from cells transfected with LPL-1-242.

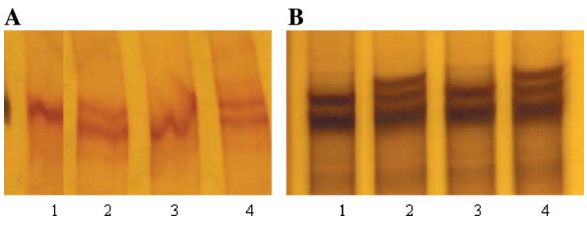

Fig. 1. LIS-SSCP analysis of the *LPL* gene showed aberrant migration of exon 1 and exon 6 on 8% polyacrylamide-silver stained gel. (A) LIS-SSCP electrophoresis of the exon 1 fragment. Lanes 1 and 3: normal subject; lanes 2 and 4: patient. (B) LIS-SSCP electrophoresis of the exon 6 fragment. Lanes 1 and 3: normal subject; lanes 2 and 4: patient.

Discussion

We report the finding of a novel *LPL* gene mutation in a patient who manifested massive chylomicronemia and recurrent pancreatitis with serious reduction in PHP LPL activity. Although initial analysis of the *LPL* gene failed to reveal a key mutation responsible for the significantly reduced LPL activity of PHP, the early onset of the disease (diagnosed at 18 years of age), and the severe symptoms prompted us to perform further analysis. We therefore carried out PCR-LIS-SSCP and direct DNA sequencing of the *LPL* gene, which revealed a novel homozygous mutation that substituted methionine for isoleucine G177C (Met^1 → Ile) in the translation initiation codon.

Initiation codon mutations are relatively uncommon compared to other types of mutations. Waye et al. [16] and Beris et al. [17] reported initiation codon mutations of the β-globin gene that resulted in β-thalassemia in a northern European boy and a Swiss family separately. Fukao et al. [18] reported an initiator codon mutation in mitochondrial acetoacetyl-CoA thiolase (*mACT*) gene deficiency and further examined translation efficiencies of nine one-base substitutions in the initiator methionine codon using in vitro transient expression analysis. They demonstrated that all the mutants produced normal polypeptide, with various degrees of translation efficiency (wild-type ATG as 100%): CTG, 66%; ACG, ATC, and ATT, 22%; ATA and GTG, 11%; and AAG, AGG, and TTG, 7.4%. In this study, we showed that transient expression of the *LPL* gene with the initiation codon mutation ATG → ATC generated LPL protein of a size similar to those of wild-type LPL but at much lower levels in terms of mass quantity compared to cells expressing wild-type LPL that were identically transfected. The Kozak sequence [19] of the *LPL* gene in this patient is $GCC^{-3}GCC^{1}AUC^{4}G$. According to the translation rules for non-AUG initiation codons in mammalian cells, the mutant AUC codon occurs in a favorable context with the most highly conserved purine in position −3 and the guanine in position +4 [20], ribosomes initiate translation and the translation efficiency may be reduced [21]. However, our result is much lower than that for the ATG → ATC mutation in the *mACT* gene reported by Fukao (3% vs. 22%).

Transient expression of mutant LPL-242 using COS-7 cells established that the Glu^{242} → Lys substitution resulted in the synthesis of a non-functional LPL enzyme

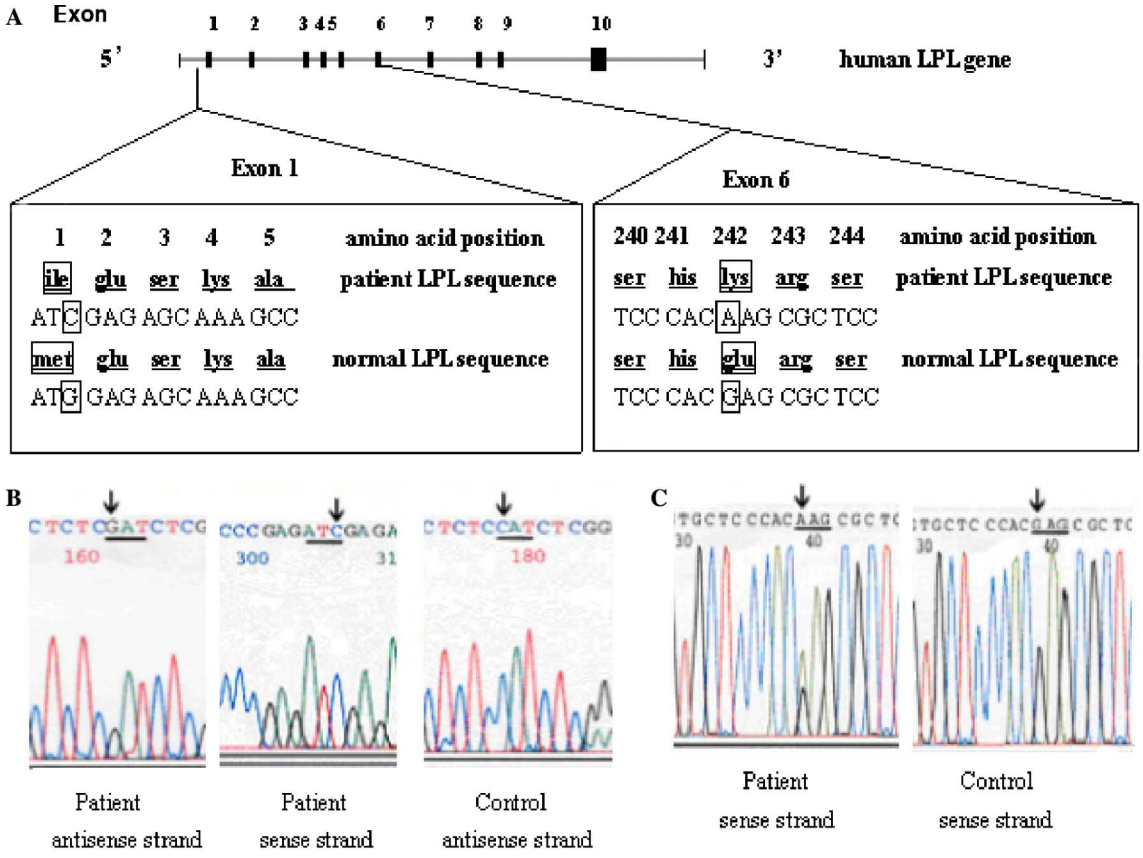

Fig. 2. (A) Schematic representation of the human *LPL* gene. Exons are illustrated by solid bars interrupted by lines that represent introns. The G → C and G → A substitutions, which lie on exons 1 and 6, respectively, in the patient's *LPL* sequence, are highlighted by a box. (B) Direct DNA sequencing of exon 1 from a normal subject and the patient. The G → C substitution is indicated by an arrow. (C) Direct DNA sequencing of exon 6 from a normal subject and the patient. The G → A substitution is indicated by an arrow.

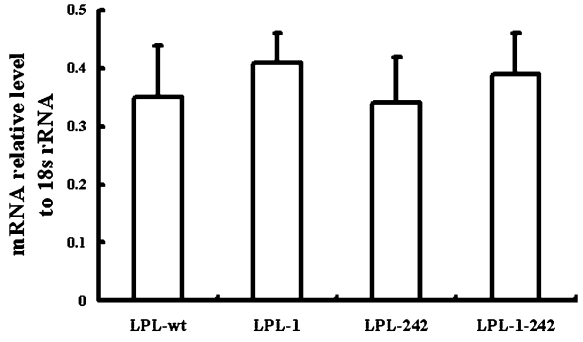

Fig. 3. Quantitation of mRNA extracted from transfected COS-7 cells and normalized to 18S RNA. The mutant mRNA levels of LPL-1, LPL-242, and LPL-1-242 were almost equal to that of the normal LPL-wt, and there was no significant difference between the wild-type and each of the mutants ($P > 0.05$).

of normal mass. Several mutations adjacent to residue 242 in the *LPL* gene have been reported. In vitro expression of both the $Arg^{243} \rightarrow His$ [22] and the $Ser^{244} \rightarrow Thr$ [23] substitutions produced inactive enzymes. The mutant Lys^{242}, as well as His^{243} and Thr^{244}, was directly adjacent to the catalytic triad Asp^{156}-Ser^{132}-His^{241} of the LPL enzyme, and replacement of the negatively charged glutamic acid (GAG) with a positively charged lysine (AAG) would be predicted to significantly disrupt formation of the LPL catalytic triad. A conformational change appears to be the most plausible explanation for the complete loss of catalytic activity in media and cell extracts. However, since it was heterozygous in this patient, the LPL-242 mutation can hardly play a major role in leading to remarkably diminished LPL activity.

In vitro expression of the combination of both mutations (LPL-1-242) produced markedly decreased LPL mass and undetectable LPL activity. Therefore, the compound homozygote for $Met^1 \rightarrow Ile$ and $Glu^{242} \rightarrow Lys$ would be a serious mutation resulting in LPL enzyme of significantly reduced mass and absolute loss of activity.

In conclusion, here we analyzed the molecular basis of a Chinese female patient with LPL deficiency and identified a novel homozygous mutation of $Met^1 \rightarrow Ile$ in the initiation codon in exon 1 and confirmed again the heterozygous $Glu^{242} \rightarrow Lys$ substitution in exon 6 of the *LPL* gene. In vitro expression studies showed that $Met^1 \rightarrow Ile$ led to an enzyme with significantly decreased mass and correspondingly reduced activity, and $Glu^{242} \rightarrow Lys$ resulted in the synthesis of a catalytically inactive enzyme of normal mass. Since the patient was compound homozygous LPL-1 and heterozygous LPL-242, and single heterozygous mutation only leads to a slight or partial reduction in LPL activity

Table 2
LPL enzyme activity and immunoreactive mass in COS-7 cells transfected with wild-type and mutant expression vectors

Plasmid	N	Media		Cell extracts	
		Activity (mU/ml)	Mass (ng/ml)	Activity (mU/ml)	Mass (ng/ml)
Mock	4	$0.16 \pm 0.068^{***}$	$2.3 \pm 1.06^{***}$	$0.18 \pm 0.07^{***}$	$2.5 \pm 0.68^{***}$
LPL-wt	4	36.6 ± 3.71	571.7 ± 32.82	26.7 ± 1.65	395.5 ± 28.62
LPL-1	4	$0.63 \pm 0.20^{***}$	$20.0 \pm 6.99^{***}$	$0.19 \pm 0.12^{***}$	$50.2 \pm 2.46^{***}$
LPL-242	4	$0.12 \pm 0.12^{***}$	$391.0 \pm 13.76^{*}$	$0.12 \pm 0.04^{***}$	$584.0 \pm 23.42^{*}$
LPL-1-242	4	$0.16 \pm 0.079^{***}$	$18.7 \pm 2.69^{***}$	$0.13 \pm 0.12^{***}$	$49.7 \pm 2.58^{***}$

Values are reported as means ± SD. N is the number of samples of COS-7 transfected cultures. Significance level of difference with wild-type: $^{*}P < 0.05$, $^{***}P < 0.001$.

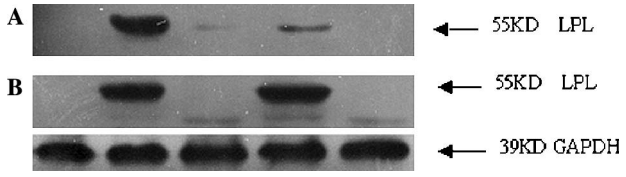

Fig. 4. Western blot of wild-type and mutant LPL from media and cell extracts of transfected COS-7 cells. (A) LPL in 1 mL of culture media was precipitated using heparin–Sepharose beads. Lane 1: mock; lane 2: LPL-wt; lane 3: LPL-1; lane 4: LPL-242; lane 5: LPL-1-242. (B) Aliquots of 10 μL of cell extract mixed with SDS loading buffer were used for direct loading. Lane 1: mock; lane 2: LPL-wt; lane 3: LPL-1; lane 4: LPL-242; lane 5: LPL-1-242. Western blots of media and cell extracts showed that mutant LPL-242 produced a band of 55 kDa of similar size and level as observed for LPL-wt, whereas mutant LPL-1 produced only a faint band of 55 kDa. There was no band detected in the cell extracts and media from cells transfected with LPL-1-242.

or mass [6,7], it was predicted that the homozygous initiation codon mutation rather than the heterozygous LPL-242 alteration in exon 6 was mainly responsible for the phenotypic expression of very low LPL activity and the chylomicronemia syndrome in this patient.

Acknowledgments

We thank Dr. Hiroaki Hattori, BML Inc., Japan for PCR primers for the *LPL* gene, and Y. Zhang, B. Zhang, J.Y. Wang, X.H. Zhang, and W. Huang for expert technical assistance. W.C. Hu is supported by the National Natural Science Foundation of China.

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