Identification and functional characterization of a novel heterozygous missense variant in the \textit{LPL} associated with recurrent hypertriglyceridemia-induced acute pancreatitis in pregnancy

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\textbf{Abstract}

\textbf{Background:} Acute pancreatitis in pregnancy (APIP) is a life-threatening disease for both mother and fetus. To date, only three patients with recurrent hypertriglyceridemia-induced APIP (HTG-APIP) have been reported to carry rare variants in the lipoprotein lipase (\textit{LPL}) gene, which encodes the key enzyme responsible for triglyceride (TG) metabolism. Coincidentally, all three patients harbored \textit{LPL} variants on both alleles and presented with complete or severe LPL deficiency.

\textbf{Methods:} The entire coding regions and splice junctions of \textit{LPL} and four other TG metabolism genes (\textit{APOC2}, \textit{APOA5}, \textit{GPIHBP1}, and \textit{LMF1}) were analyzed by Sanger sequencing in a Han Chinese patient who had experienced two episodes of HTG-APIP. The impact of a novel \textit{LPL} missense variant on LPL protein expression and activity was analyzed by transient expression in HEK293T cells.

\textbf{Results:} A novel heterozygous \textit{LPL} missense variant, p.His210Leu (c.629A > T), was identified in our patient. This variant did not affect protein synthesis but significantly impaired LPL secretion and completely abolished the enzymatic activity of the mutant protein.

\textbf{Conclusion:} This report describes the first identification and functional characterization of a heterozygous variant in the \textit{LPL} that predisposed to recurrent HTG-APIP. Our findings confirm a major genetic contribution to the etiology of individual predisposition to HTG-APIP.

\textbf{KEYWORDS}

HTG-APIP, lipoprotein lipase (\textit{LPL}) gene, missense variant, recurrent acute pancreatitis in pregnancy

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Acute pancreatitis in pregnancy (APIP) is a life-threatening disease for both mother and fetus, with an incidence of 1 in 1,000–10,000 pregnancies (Igbinosa, Poddar, & Pitchumoni, 2013; Zhang et al., 2013). Over the past decades, significant improvements in diagnosis and management have reduced the mortality rate from 37% to 3.3% for mothers and from 60% to 11.6% for the fetus, respectively (Ducarme, Maire, Chatel, Luton, & Hammel, 2014; Fan et al., 2018; Ramin, Ramin, Richey, & Cunningham, 1995; Tang, Xu, Song, Mei, & Zhang, 2018; Vilallonga, Calero-Lillo, Charco, & Balsells, 2014; Xu, Wang, & Zhang, 2015). Gallstones are the leading cause of APIP worldwide while hypertriglyceridemia (HTG) ranks second in China and third in Western countries, respectively (Luo et al., 2018; Papadakis, Sarigianni, Mikhailidis, Mamopoulos, & Karagiannis, 2011). Compared to biliary APIP, HTG-induced APIP (HTG-APIP) often has a more severe disease course, is associated with a poorer outcome, and causes more maternal and fetal deaths (Deng, Wang, Wu, Tang, & Chen, 2014; Liu, Lun, Lv, Hou, & Wang, 2016; Luo et al., 2018; Sun, Fan, & Wang, 2013; Tang et al., 2018).

A normal triglyceride (TG) level is less than 1.7 mmol/L (150 mg/dl). During the late stages of gestation, the maternal TG concentration normally increases two- to fourfold due to a high-fat diet, lack of exercise, increase in estrogen, and other factors (Alvarez, Montelongo, Iglesias, Lasuncion, & Herrera, 1996; Basaran, 2009). However, the TG level in HTG-APIP patients can increase to more than 11.3 mmol/L (1,000 mg/dl), the threshold for defining severe HTG (Garg, Garg, Hegele, & Lewis, 2019), which may be determined by genetic factors associated with TG metabolism. In this regard, variants in the lipoprotein lipase (LPL) gene (OMIM# 609708), which encode the key enzyme responsible for TG metabolism, have been reported in a dozen of HTG-APIP patients (Bartha et al., 2009; Gilbert, Rouis, Griglio, de Lumley, & Laplaud, 2001; Goldberg & Hegele, 2012; Hieronimus, Benlian, Bayer, Bongain, & Frederich, 2005; Keilson, Vary, Sprecher, & Renfrew, 1996; Liu et al., 2016; Ma et al., 1993, 1994; Murugasu et al., 1998; Suga et al., 1998; Xie et al., 2015). Of these patients, only three had experienced a recurrence of HTG-APIP. Interestingly, all three of these recurrent HTG-APIP patients harbored LPL variants on both alleles and exhibited either no or very low-plasma post-heparin LPL activity (Liu et al., 2016; Murugasu et al., 1998; Suga et al., 1998). Herein, we report the first identification and functional characterization of a heterozygous variant in the LPL predisposing to recurrent HTG-APIP.
taking medications between the two episodes of acute pancreatitis and from the end of 2017 to the end of 2018 (Figure 1).

2.3 | Plasma lipid profile analysis

A blood sample was taken from the patient after fasting for 12 hr. Serum TG and other lipid levels were measured enzymatically on an automatic analyzer (Hitachi High-Tech, 7600–120).

2.4 | Variant screening

Genomic DNA was extracted from peripheral blood cells using the Gentra Puregene Blood kit (Qiagen) according to the manufacturer’s instructions. All exons and splicing junctions of the LPL as well as the four other canonical TG metabolism genes, namely APOC2 (OMIM# 608083), APOA5 (OMIM# 606368), LMF1 (OMIM# 611761), and GPIHBPI (OMIM# 612757), were amplified from genomic DNA, and analyzed by Sanger sequencing (Chen et al., 2019). The identified variants were confirmed by independent PCR amplification and sequencing.

2.5 | Reference sequence and variant nomenclature

NM_000237.3 and NC_000008.11 were employed as the LPL mRNA and genomic reference sequences, respectively. Nomenclature for the description of LDL variants was in accordance with the recommendations of the Human Genome Variation Society (HGVS) (den Dunnen et al., 2016).

2.6 | In silico analysis

The Combined Annotation-Dependent Depletion (CADD) tool (Kircher et al., 2014) and the PP3 rule established by VarSome (Kopanos et al., 2019) were employed to predict the pathogenicity of the LPL missense variant detected. CADD integrates more than 60 diverse annotations into a single measure (C-score) for each variant, with a C-score of ≥20 being regarded as evidence of pathogenicity. VarSome’s PP3 rule was based upon a combined consideration of predictions from 10 in silico programs, namely DANN, MutationTaster, dbNSFP.FATHMM, MetaSVM, MetaLR, GERP, LRT, MutationAssessor, PROVEAN, and SIFT.

2.7 | Analysis of LPL mass and activity in the patient’s plasma

A blood sample was collected from the patient after overnight fasting and 10 min after an intravenous injection of heparin (60 IU/kg body weight). Blood plasma was then prepared by centrifugation at 400 g for 30 min. Plasma LPL mass was determined with a human LPL Elisa kit (TSZ Biological Trade). Plasma total lipase activity and plasma hepatic lipase activity were both

FIGURE 1 Timeline of the patient’s two episodes of hypertriglyceridemia-induced acute pancreatitis during pregnancy and her triglyceride (TG) levels. Note that (i) only some of the treatment procedures adopted have been illustrated and (ii) the patient was transferred to the Surgical Intensive Care Unit (SICU) at Nanjing on the 5 July 2017. AP, acute pancreatitis; EN, enteral nutrition; PN, parenteral nutrition
determined using a Free Fatty Acid (FFA) assay kit (Wako kit, NEFA-HR(2)). In the case of the plasma hepatic lipase activity assay, the sample was pretreated with 1 M NaCl and incubated for 60 min at 4°C to inactivate the LPL. Plasma LPL activity was then calculated by subtracting total lipase activity from hepatic lipase activity. All assays were performed in triplicate.

2.8 | Cell culture and transfection

Human wild-type and mutant LPL cDNAs were synthesized and cloned into the pcDNA3.1 vector by GeneArt Gene Synthesis (Vigene Biosciences), and confirmed by DNA sequencing. HEK293T (ATCC, CRL-3216) cells, which have no endogenous LPL expression, were cultured in DMEM medium with 10% FBS and 1% PS at 37°C in a humidified chamber supplemented with 5% CO₂. Transfection was performed using Lipofectamine 3,000 (Thermo, L3000015) in 6-well plates (Costar, 3,516) according to the manufacturer’s instructions. Six hours after transfection, the medium was changed into fresh DMEM with 2% FBS and the cells were then cultured for 48 hr before LPL mass and activity analysis. In the case of heparin treatment, the medium was further changed to a 500-µl heparin-DMEM mixture (the ratio of DMEM and heparin is 500:8, heparin is 20 units/ml) per well, and the cells were cultured for an additional 30 min.

2.9 | Analysis of LPL mass and activity in transfected cell medium and lysate

The transfected cell medium was centrifuged to remove cells and cell debris, and stored at −20°C for later analysis. The transfected cells were treated with RIPA Lysis Buffer (Beyotime, China, P0013) and centrifuged at 4°C for 10 min at 12,000 g; the resulting supernatant was stored at −20°C for later analysis.

The expression of LPL protein in cell medium and lysate was analyzed by Western blot. The antibodies used were as listed below: 1:200 primary rabbit LPL antibodies (Cell Signaling Technology), 1:5000 primary mouse GAPDH antibody (Santa Cruz Biotechnology), 1:2000 secondary anti-mouse, and 1:5000 anti-rabbit HRP-conjugated IgGs (Santa Cruz Biotechnology). Band intensities were quantified using the ImageJ software.

LPL activity in cell medium and lysate was determined using the abovementioned FFA assay (Wako kit, NEFA-HR(2)).

3 | RESULTS

3.1 | Genetic findings

We analyzed the entire coding regions and proximal flanking intronic sequences of the LPL, APOC2, APOA5, GPIHBP1, and LMF1 in the patient by means of Sanger sequencing. We focused on the rare coding variants that resulted in missense, frameshift, or nonsense mutations and rare intronic variants that altered canonical splice sites. Rare variants are defined here as having an allele frequency of <1% in the East Asian population according to the gnomAD database (Lek et al., 2016). We identified only one such variant, c.629A > T, in exon 5 of the LPL, which was predicted to result in a missense variant, p.His210Leu (Figure 3a). This variant is absent from the gnomAD database, has not been previously reported in the literature, and was also not found in any of our other 28 Chinese HTG-APIP patients.

3.2 | Pathogenicity prediction

The histidine residue at LPL amino acid position 210 is highly conserved from zebrafish (Danio rerio) to humans (Figure 3b). Moreover, leucine, an aliphatic and uncharged residue, is significantly different from histidine, a polar and positively charged
residue, in terms of physicochemical properties. Taken together, these observations suggest that the p.His210Leu missense variant is highly likely to affect LPL protein structure and function. Indeed, it was predicted to be pathogenic by both CADD (C-score, 26.2) and VarSome (in accordance with its PP3 rule).

3.3 | Analysis of LPL mass and activity in the patient’s plasma

Plasma LPL mass and activity were measured when the patient was admitted to our center. Her LPL mass was determined to be 185.7 ± 12.49 U/L, approximately half of the mean value of 10 normal controls (382 ± 75.62 U/L). Her LPL activity was 0.071 ± 0.025 mEq L\(^{-1}\) hr\(^{-1}\), approximately 60% of the mean value of 10 normal controls (0.118 ± 0.055 mEq L\(^{-1}\) hr\(^{-1}\)).

3.4 | In vitro functional characterization of the LPL missense variant

The LPL wild-type and p.His210Leu mutant coding sequences were transiently expressed in HEK293T cells. We first compared LPL expression levels in cell lysates and media of wild-type and mutant transfected cells with heparin treatment by means of Western blotting. Exogenous heparin triggers the release of cell surface-bound LPL into the medium. Significantly higher LPL expression was found in the cell lysate but significantly lower LPL expression was noted in the medium of the mutant transfected cells as compared to those of the wild-type transfected cells (Figure 4). These results indicated that the p.His210Leu variant greatly reduced LPL secretion. We then compared LPL expression levels in the cell lysates of wild-type and mutant transfected cells without heparin treatment, finding no significant difference (Figure 5). This latter finding indicated that the p.His210Leu variant has no effect on LPL synthesis. By contrast, LPL activity was not detectable in either the cell lysate or the medium of mutant vector-transfected cells with heparin treatment. This indicated that the p.His210Leu variant completely abolished the enzymatic activity of the mutant LPL protein. For comparison, LPL activities in the cell lysate and medium of wild-type transfected cells with heparin treatment were 0.35 ± 0.24 mEq L\(^{-1}\) hr\(^{-1}\) and 0.33 ± 0.19 mEq L\(^{-1}\) hr\(^{-1}\).

4 | DISCUSSION

In the present study, we identified a novel heterozygous LPL missense variant, p.His210Leu (c.629A > T), in a Chinese patient with recurrent HTG-APIP. The
pathogenicity of this variant was supported by predictions made using CADD and VarSome, then demonstrated by virtue of the significantly reduced LPL mass and activity in the patient's plasma (compared to that of normal controls), and finally confirmed by in vitro functional analyses. The in vitro analysis demonstrated that the p.His210Leu missense variant did not affect protein synthesis but significantly impaired LPL secretion and completely abolished enzymatic activity of the mutant protein. It should be noted that two other missense variants, p.His210Asp (c.628C > G) and p.His210Arg (c.629A > G), have been reported in LPL amino acid residue 210. p.His210Asp also caused a complete loss of LPL activity when tested in vitro (Holzl et al., 2000). However, in vitro protein analysis was not performed on this variant and no data on plasma LPL mass in the carrier were available. Therefore, it remains unclear whether the complete loss of enzyme activity caused by the p.His210Asp missense variant is due to its impact on enzymatic activity or alternatively whether it exerts its effects on protein synthesis and/or secretion. p.His210Arg was not functionally characterized in vitro; however, the patient, who was homozygous for this variant, exhibited a LPL activity deficiency but a LPL mass value within the normal range (Ariza et al., 2018). In short, all three missense variants caused a complete or almost complete functional loss of the affected allele, highlighting the importance of the evolutionarily strictly conserved residue 210 for LPL structure and function. In this regard, it is pertinent to note that based upon the LPL crystal structure, “the active site cleft is lined by the hydrophobic side chains of W82, V84, W113, Y121, Y158, L160, A185, P187, F212, I221, F239, V260, V264, and K265, which would form van der Waals interactions with (and stabilize) the hydrophobic tails of lipid substrates in the active site” (Birrane et al., 2019). The spatial proximity of residue 210 to these active site-defining residues or structures speaks for itself.

As mentioned earlier, only three patients with recurrent HTG-APIP have so far been reported to carry rare LPL variants (Liu et al., 2016; Murugasu et al., 1998; Suga et al., 1998). Coincidentally, all three patients had severe LPL deficiency and were each found to have two affected LPL alleles. One patient, who had complete LPL deficiency, was homozygous for a nonsense variant, p.Trp409* (c.1227G > A) (Suga et al., 1998); one patient, with almost complete LPL deficiency, was homozygous for a missense variant, p.Cys291Tyr (c.872G > A) (Murugasu et al., 1998); and the third patient, whose post-heparin LPL activity was <17% that of normal controls, was compound heterozygous for p.Glu269Lys (c.805G > A) and p.Leu279Val (c.835C > G) (Liu et al., 2016). The p.Trp409* homozygous patient, when not pregnant, managed to maintain a mild HTG state with a plasma TG level ranging from 5.6 to 11.3 mmol/L by ingesting a low-fat diet (Suga
et al., 1998); after discharge, the compound heterozygous [p.Glu269Lys and p.Leu279Val] patient maintained a consistently elevated TG level (ranging from 7 to 11 mmol/L) by following a lipid-lowering drug regimen (Liu et al., 2016); finally, in the case of the p.Cys291Tyr homozygous patient, her TG level at 4 weeks postpartum (25.5 mmol/L) exhibited no significant decrease by comparison to that measured at 18 weeks of gestation (29.6 mmol/L) (Murugasu et al., 1998). These findings are consistent with recent observations suggesting that (a) an episode of acute pancreatitis may predispose to the risk of developing more severe metabolic derangements (Singh et al., 2018) and (b) more severe HTG may result in more severe disease (Jo et al., 2019).

There is one final point to make. Our patient bore two healthy children owing to timely cesarean section. It follows that early diagnosis and clinical intervention are vitally important to acute pancreatitis patients caused by gestational HTG and should help to improve the prognosis of the fetus.

5  |  CONCLUSIONS

The present study describes the first identification and functional characterization of a heterozygous variant in the LPL that predisposed to recurrent HTG-APIP. Our findings not only underscore the importance of genetic defects in predisposing to HTG and/or acute pancreatitis but also shed new light on the complexity underlying the etiology of these phenotypes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Qi Yang, Xiao-Lei Shi, and Wei-Qin Li conceived and designed the study. Xiao-Lei Shi performed the experiments. Qi Yang, Xiao-Lei, Na Pu, Xiao-Yao Li, Wei-Wei Chen,
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