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ORIGINAL ARTICLE

A novel *SLC37A4* missense mutation in GSD-Ib without hepatomegaly causes enhanced leukocytes endoplasmic reticulum stress and apoptosis

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Abstract

Background: Glycogen storage disease (GSD) type Ib is an autosomal recessive disease caused by defects of glucose-6-phosphate transporter (G6PT), encoded by the *SLC37A4* gene. To date, over 100 mutations have been revealed in the *SLC37A4* gene. GSD-Ib patients manifest a metabolic phenotype of impaired blood glucose homeostasis and also carry the additional complications of neutropenia and myeloid dysfunction.

Methods: Here, we present two daughters with an initial diagnosis of gout in a Chinese consanguineous family. Whole-exome sequencing was performed to identify the mutations. The mechanism of leukocytopenia was investigated.

Results: Whole-exome sequencing analysis of the proband identified a novel homozygous p.P119L mutation in *SLC37A4*, leading to a diagnosis of GSD-Ib. We found that the potential pathogenic p.P119L mutation leads to an unusual phenotype characterized by gout at onset, and GSD-Ib arising from this variant also manifests multiple metabolic abnormalities, leukocytopenia, and anemia, but no hepatomegaly. The leukocytes from the proband showed increased mRNA levels of sXBP-1, BIP, and CHOP genes in the unfolded protein response pathway, and enhanced Bax mRNA and caspase-3 activity, which might contribute to leukocytopenia.

Conclusion: Our findings broaden the variation spectrum of *SLC37A4* and suggest no strict genotype–phenotype correlations in GSD-Ib patients.

KEYWORDS

G6PT, glycogen storage disease type Ib, gout, leukocytopenia, *SLC37A4*

1 | INTRODUCTION

Glycogen storage disease (GSD) type I is an autosomal recessive disease caused by defects of glucose-6-phosphatase (G6Pase) and glucose-6-phosphate transporter (G6PT) (Chou et al., 2010a, 2015). The function of G6PT is to translocate glucose-6-phosphate (G6P) from the cytoplasm to the lumen of the endoplasmic reticulum (ER) and G6Pase catalyzes the hydrolysis of G6P to produce glucose and phosphate (Gerin et al., 1997; Lei et al., 1993). Therefore, G6PT and G6Pase

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work in concert to maintain glucose homeostasis (Chou et al., 2010b). G6Pase includes two isoforms, G6Pase-α and G6Pase-β (Guionie et al., 2003). The former one expresses restrictedly in liver, kidney, and intestine, while the latter one has ubiquitous activity (Shieh et al., 2003). Since G6PT is also expressed ubiquitously (Lin et al., 1998), different phenotypes of GSD-I lie on the tissue expression profiles of these three enzymes. Deficiencies in G6Pase-α, G6Pase-β, and G6PT cause GSD-Ia, GSD-I-related syndrome (GSD-Irs), and GSD-Ib, respectively (Boztug et al., 2009; Gerin et al., 1997; Lei et al., 1993). GSD-Ia patients manifest a metabolic phenotype of impaired blood glucose homeostasis, characterized by hypoglycemia, hepatomegaly, nephromegaly, hypertriglyceridemia, hypercholesterolemia, hyperuricemia, lactic acidosis, and growth retardation (Lei et al., 1993). GSD-Irs patients experience severe congenital neutropenia syndrome type 4, characterized by neutropenia and neutrophil/macrophage dysfunction (Boztug et al., 2009; Chou et al., 2010a). GSD-Ib shares the same metabolic phenotype of impaired glucose homeostasis with GSD-Ia but also carries the additional complications of neutropenia and myeloid dysfunction typical of G6Pase-β deficiency (Chou et al., 2018).

GSD-Ib arising from G6PT deficiency represents ~20% of GSD-I cases (Chou et al., 2010a, 2018). The human G6PT protein is encoded by the *SLC37A4* gene. To date, over 100 *SLC37A4* variants have been identified in GSD-Ib patients (Chou et al., 2015, 2018; Skakic et al., 2018). In the present study, two daughters with initial diagnosis of gout in a Chinese consanguineous family were enrolled. Whole-exome sequencing analysis of the proband identified a homozygous mutation in exon 4 of *SLC37A4* (c.356C>T, p.P119L), which has never been reported previously. We found that the novel *SLC37A4* mutation leads to an unusual phenotype characterized by gout at onset, and GSD-Ib arising from this variant also manifests a metabolic phenotype of disturbed glucose homeostasis and leukocytopenia with enhanced endoplasmic reticulum stress and increased rate of apoptosis, but without hepatomegaly.

2 | MATERIALS AND METHODS

2.1 | Patient information

We enrolled a Chinese consanguineous family from the Department of Rheumatology in Xiangya Hospital. The study is in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board at the Xiangya Hospital of Central South University. All study participants provided written informed consent prior to participation in the study. Peripheral blood samples were collected from the affected proband, her mother, and son.

2.2 | Whole-exome sequencing analysis

Genomic DNA was extracted from whole blood using a QIAamp Blood DNA Mini Kit (Qiagen GmbH) from the proband, her mother, and her son after signing an informed consent form. Her father refused genetic testing. The exome capture, high-throughput sequencing, and common filtering were performed on DNA samples from the proband in the Sinopath Institute. All the exomes were sequenced by the Illumina HiSeq X Ten platform. About 98.742% of target bases were covered to a total depth of >20×. The reads were aligned with the human genome reference sequence [University of California Santa Cruz, humangenome assembly 19 (UCSChg19)] by NextGENe V2.3.4. Target amplification PCR was performed on DNA from the proband, her mother, and her son using genetic-specific primers[1]. The whole-DNA sequence of *SLC37A4* was obtained from the available online database (UCSC: NM_00860.5). Forward primer 5′-AAGTAACCTGTCTCACCCCACTG-3′ and reverse primer 5′-CTCCCACATGCTCTTTAGGCATCC-3′) from *SLC37A4* gene were used to amplify the PCR product under the following conditions: 95°C for 3 min; 30× cycles of 30 s at 95°C, 30 s at 58°C, and 60 s at 72°C; followed by 5 min at 72°C. PCR products were purified and sequenced.

2.3 | Bioinformatics analysis

The PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) programs were used to predict the effects of mutations on the function of the protein. The conservation analysis was performed by comparing amino acid sequences of multiple species using CLUSTALW. The pathogenicity of variant was interpreted according to the American College of Medical Genetics and Genomics guideline (Richards et al., 2015).

2.4 | Isolation of leukocytes

The heparinized peripheral blood was collected and erythrocyte-depleted with BD Pharm Lyse™ lysing solution (Cat NO. 555899, BD Biosciences). Gently vortex each tube immediately and incubate at room temperature, protected from light, for 15 minutes. After centrifugation at 200× *g* for 5 min, leukocytes were washed twice with PBS.

2.5 | Real-time PCR

Total RNA was extracted from isolated leukocytes according to TRIzol (15596-026; Invitrogen) RNA isolation protocol. One μg of RNA was used to reverse-transcribe

into cDNA using PrimeScript RT reagent Kit (RR047A; Takara, Japan) and subjected to qRT-PCR using SYBR Premix Ex Taq kit (RR820A) with specific primers, as follows: sXBP-1 (sense) 5′-CTT GTA GTT GAG AAC CAG GAG T, (antisense) 5′- CCC AAC AGG ATA TCA GAC TCT G; BIP (sense) 5′-CAG TTG TTA CTG TAC CAG CCT A, (antisense) 5′-CAT TTA GGC CAG CAA TAG TTC C; CHOP (sense) 5′-GAG AAT GAA AGG AAA GTG GCAC, (antisense) 5′-ATT CAC CAT TCG GTC AAT CAG A; Bax (sense) 5′-TGG CAG CTG ACA TGT TTT CTG AC, (antisense) TCA CCC AAC CAC CCT GGT CTT; Bcl-2 (sense) 5′-TCG CCC TGT GGA TGA CTG A, (antisense) CAG AGA CAG CCA GGA GAA ATC; GAPDH (sense) 5′- CGG AGT CAA CGG ATT TGG TCG, (antisense) 5′- ATG TAA ACC ATG TAG TTG AGG TC. The mRNA expressions of genes (sXBP-1, BIP, CHOP, Bax, Bcl-2) were normalized to the "housekeeping" gene GAPDH, and the data were analyzed using the comparative Ct method to obtain relative quantitation values.

2.6 | Caspase-3 activity assay

Caspase activity of leukocytes was determined fluorometrically using Caspase-3 Fluorescence Assay Kit (No. 10009135, Cayman Chemical). The assay is based on the detection of the cleavage of substrate N-Ac-DEVD-N′-MC-R110. The leucocytes (5×10^5) were resuspended in 50 µl of chilled cell-based assay lysis buffer and transferred to a 96 well plate. Fifty microliters of substrate solution were added to each well and the cleavage of the substrate was followed at 37°C for 60 minutes. The fluorescence intensity was read with Flx 800 plate reader (BioTek).

3 | RESULT

3.1 | Clinical findings

The proband, a 23-year-old female, was admitted to the Department of Rheumatology at Xiangya Hospital due to recurrent joint pain and swelling since 9 years ago and exacerbation for 3 months. She experienced recurrent arthralgia and swelling since she was at age 14. Four years ago, her serum urate level was detected up to 1000 µmol/L and a diagnosis of gout was made. The patient was prescribed NSAIDs for pain relief and allopurinol and benzbromarone to lower urate level, but she discontinued allopurinol and benzbromarone because of pregnancy. Joint pain and swelling relapsed. The patient was transferred to our hospital due to the unusual age of onset of gout, especially in a female. She did not complain about blood in stools, chronic diarrhea or other intestinal signs and symptoms. She was 147 cm in height and 43 kg in weight. Multiple indices of laboratory tests were abnormal (Table 1). Complete blood count revealed leukocytopenia and anemia. Serum uric acid, creatinine, lactate, fasting triglyceride, and low-density lipoprotein were elevated in the presence of hypoglycemia. The elimination of uric acid was decreased. Abdominal ultrasonography showed normal size and texture of liver. Multiple monosodium urate deposits were detected by dual-energy computed tomography (CT) imaging of her knees and feet. Bone marrow aspiration smear revealed active proliferation with a left-shift in granulocyte (Table 1).

Her parents were cousins and she had an elderly sister showing the same symptoms as her (Table 1). Her sister presented with recurrent joints pain and swelling since she was 15 years old. She also had an increase in serum uric acid and creatinine when she was 23 years old. Complete blood count revealed panhematopenia as shown in Table 1. The diagnosis of pancytopenia, gout, gouty nephropathy, and chronic kidney disease (CKD) stage 3 was made. Though treated with allopurinol, coated aldehyde oxystarch capsules, medicinal carbon, and hemodialysis, she finally died of kidney failure at age of 27 before the proband was admitted into the Department of Rheumatology at Xiangya Hospital.

3.2 | Homozygous *SLC37A4* variant identified by Whole-exome sequencing analysis

Whole-exome sequencing analysis was performed on DNA samples from the proband. In the proband, a homozygous missense mutation (hg19_chr: 118898929_ NM001164278_c.356C>T_p.P119L) in the fourth exon of the *SLC37A4* gene was found (Figure 1a). This variant has not been reported from the Exome Aggregation Consortium database (http://exac.broadinstitute.org/). The Sanger sequencing data confirmed that the homozygous *SLC37A4* variant is present in the proband, and her mother and son carry the heterozygous *SLC37A4* variant (Figure 1b,c). Though her father refused to receive genetic testing, it is reasonable to suppose that he is also a carrier according to the law of segregation, which was consistent with the autosomal recessive inheritance mode of GSD-Ib. Altogether, the variant p.P119L would be interpreted as a likely pathogenic variant by ACMG standards and guidelines.

3.3 | Bioinformatics analysis

The ClustalW analysis revealed that the affected proline residue at site 119 of human G6PT shows absolute evolutionary conservation among all G6PT orthologs (Figure S1). This

TABLE 1 The laboratory parameters of the proband and her elder sister.

Items	Proband	Patient's elder sister
White blood cell ($\times10^9$/L)	2.1 (3.5–9.5)	1.5 (3.5–10)
Neutrophil ($\times10^9$/L)	0.6 (1.8–6.3)	0.77 (1.9–8.0)
Neutrophil percentage (%)	28.2 (40.0–75.0)	52 (50.0–75.0)
Red blood cell count ($\times10^{12}$/L)	2.58 (3.8–5.1)	1.88 (3.5–5.5)
Hemoglobin (g/L)	76 (115–150)	54 (110–165)
Platelet ($\times10^9$/L)	236 (125–350)	74 (100–300)
Transaminase (U/L)	Normal	Normal
24-h urine protein (g)	1.31 (<0.15)	2.19 (<0.15)
Blood urea nitrogen (mmol/L)	31.36 (5.2–15.0)	39.2 (5.80–14.28)
Serum creatine (μmol/L)	132.0 (48.0–100.0)	241.4 (44.0–133.0)
Blood uric acid (μmol/L)	512.5 (155–357)	646.8 (142.0–416.0)
Triglyceride (mmol/L)	5.32 (<1.7)	4.94 (<1.71)
Cholesterol (mmol/L)	5.10 (<5.18)	4.90 (2.90–5.20)
Fasting glucose (mmol/L)	3.39 (3.9–6.1×)	3.60 (3.9–6.1)
Blood lactic acid (mmol/L)	1.6 (1.42–1.90)	Unknown
Kidney ultrasonography	Left: 106 × 49 mm Right: 109 × 51 mm	Left: 105 × 39×10 mm Right: 108 × 45×13 mm
Liver size by ultrasonography	Normal	Normal
Bone marrow aspiration smear	Active proliferation	Active proliferation

mutation is predicted to be probably damaging with a score of 0.999 (sensitivity: 0.14; specificity: 0.99) by PolyPhen2 (Figure S2) and to affect protein function with a score of 0.00 by SIFT (Figure S3).

3.4 | Leukocytes exhibit ER stress and enhanced apoptosis in the proband

Several studies demonstrated that GSD-Ib patients and G6PT-deficient mice manifest neutropenia caused by endoplasmic reticulum stress and apoptosis, which is attributed to the disruption of G6PT/G6Pase-β complex activity (Chou et al., 2010b; Jun et al., 2014; Kuijpers et al., 2003). The leukocytes were isolated from the proband and her mother, and the mRNA levels of sXBP-1, BIP, and CHOP genes in the unfolded protein response pathway were compared between the proband and her mother. Real time-PCR showed that the expression of sXBP-1, BIP, and CHOP was obviously increased in leukocytes of the proband compared to her mother (Figure 2a). Furthermore, real time-PCR indicated that pro-apoptotic protein Bax was significantly increased in leukocytes of the proband compared to her mother, whereas the mRNA levels of anti-apoptotic protein Bcl-2 were downregulated (Figure 2b), and activity assays demonstrated that levels of active caspase-3 were increased in leukocytes from the proband (Figure 2c). These data suggested that the novel p.P119L mutation leads to enhanced endoplasmic reticulum stress and increased rate of apoptosis in leukocytes.

4 | DISCUSSION

Here we report on the molecular genetic analysis of one daughter with initial diagnosis of gout in a Chinese consanguineous family. This case was unusual in several ways. First, the proband was a female and she developed symptoms of gout since her teenage years. Second, she was born to a consanguineous parent and she had an elderly sister experiencing the same symptoms as her. Third, the patient exhibited hypoglycemia, hypertriglyceridemia, anemia, and leukopenia, but no hepatomegaly, just like her sister, which could not be explained simply by gout. Given the above, it is reasonable to presume that the patient and her elderly sister both had the same genetic disorder. Then whole-exome sequencing analysis and Sanger sequencing identified a homozygous variant c.356C>T (p.P119L) of the *SLC37A4* gene in the proband, leading to a diagnosis of GSD-Ib combined with her metabolic phenotype.

Since pathogenic mutations in the *SLC37A4* gene were identified in clinical cases of GSD type Ic (GSD-Ic) proposed to be deficient in an inorganic phosphate (P(i)) transporter, G6PT has been confirmed to have a dual role as a G6P and a P(i) transporter (Chen, Pan, Nandigama, et al., 2008; Chou & Mansfield, 2014; Chou et al., 2013). To date, over 100 mutations have been revealed in the *SLC37A4* gene, including 45 missense, 22 insertion/deletion, 21 splicing variants, and 12 nonsense (Chou et al., 2018; Skakic et al., 2018). These variants entirely span the helical transmembrane domains, cytoplasmic loops, and luminal loops of G6PT and show some ethnic variability. The G149E variant and P191L variant are the most frequent among Chinese patients. The W118R mutation is mainly prevalent among Japanese patients (Kido et al., 2013; Kojima et al., 2004). However, the p.P119L mutation in the present study was newly identified and predicted to be probably damaging. The proline at site 119 is highly conserved among all of the species and locates at the

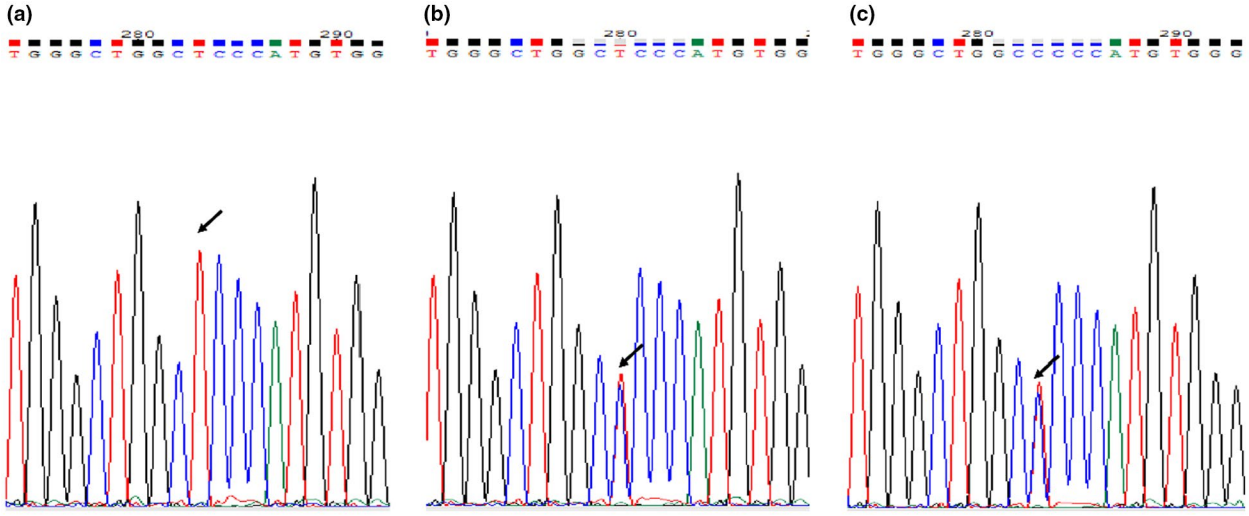

FIGURE 1 The molecular genetic analysis of the proband and her family members. The Sanger sequencing revealed that the homozygous *SLC37A4* variant is present in the proband (a); her mother (b) and son (c) carry the heterozygous *SLC37A4* variant. The arrow indicates the nucleotide change

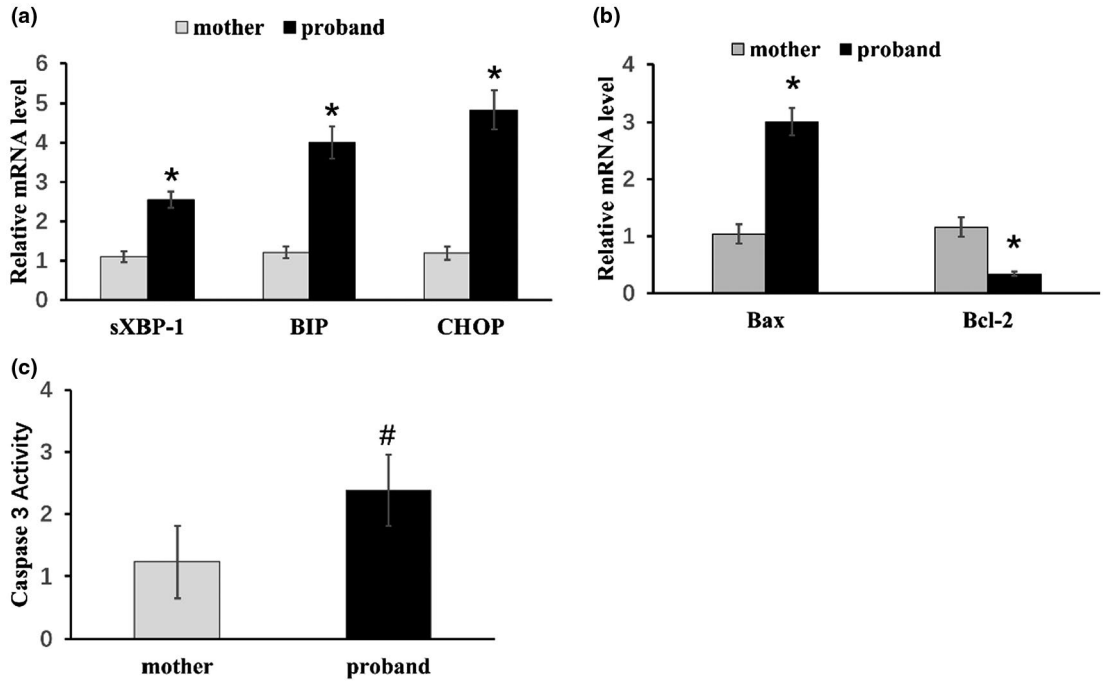

FIGURE 2 Increase in the expression of endoplasmic reticulum stress markers and apoptosis in leukocytes of the proband. (a) The relative mRNA levels of sXBP-1, BIP, and CHOP in peripheral leukocytes of the proband compared with the mother; (b) The relative mRNA levels of Bax and Bcl-2 in peripheral leukocytes of the proband compared with the mother; (c) The caspase-3 activity in protein extracts of peripheral leukocytes of the proband compared with the mother. Real-time PCR assay was performed in triplicates. Student's t test was used for statistical analysis. $^{*}p < 0.001$; $^{\#}p < .05$

cytoplasmic loops C1 of G6PT, in which there are four previously characterized variants G115R, W118R, Q133P, and G135D (Chou et al., 2018; Skakic et al., 2018). Among these mutations, 33 variants have been functionally characterized to have pathogenic effect using microsomal co-expression assay or reconstituted proteoliposomes assay (Chen et al., 2008; Chen, Pan, Nandigama, et al., 2008). The W118R variant was found devoid of both G6P and P(i) transport activity, while the Q133P mutation lacked G6P transport activity but retained 5% wild-type Pi transport activity (Chen, Pan, Lee, et al., 2008). It remains to be elucidated whether p.P119L variant has the same functional defect as W118R and Q133P.

Usually, there are no strict genotype–phenotype correlations in GSD-I patients (Chou et al., 2002). Not all GSD-Ib patients, diagnosed on their metabolic phenotype and genetic analysis, seem to present neutropenia (Chou et al., 2010a, 2010b). To our knowledge, we have reported for the first time that a GSD-Ib patient does not develop hepatomegaly. The suspected patient–patient's elder sister had no hepatomegaly as well, which further indicated that the mutation p.P119L of G6PT may be correlated with this uncommon phenotype. It is difficult to obtain liver biopsy from the proband to detect the total G6Pase activity and latency of G6Pase. In addition, tophaceous gout in these two patients at onset is not a typical manifestation in GSD- Ib although 17 cases of GSD- Ia with gout have been reported (Zhang & Zeng, 2016).

The mechanisms of neutropenia in GSD-Ib patients include maturation arrest and enhanced cell apoptosis caused by enhanced neutrophil endoplasmic reticulum stress and oxidative stress (Jun et al., 2014; Kuijpers et al., 2003). Apart from a decline in the number, impaired cell function characterized by impaired respiratory burst, chemotaxis, and calcium mobilization activities was also discovered in GSD-Ib (Kim et al., 2017). Deficiencies in the G6Pase-β/G6PT complex cause glucose deprivation in neutrophils, which affects protein folding leading to endoplasmic reticulum stress and triggers cell apoptosis (Boztug et al., 2009; Cheung et al., 2007; Jun et al., 2010). Consistently with other GSD-Ib patients and G6PT-deficient mice, our data showed that endoplasmic reticulum stress and cell apoptosis were enhanced in the leukocytes, which might contribute to leukocytopenia in the proband. Although the proband and her elder sister did not present hepatomegaly, both of them had elevated levels of blood urea nitrogen, creatinine, proteinuria, and multiple cysts in both kidneys, even the elder sister died of renal failure. Recent study demonstrated that the ER stress and increased apoptosis partly contributed to renal dysfunction in the GSD Ib in a kidney cell model system with a deficiency of *SLC37A4* by the CRISPR/Cas9-mediated precise gene editing method (Skakic et al., 2019). It is reasonable to deduce that the renal dysfunction in the proband and her sister is due to enhanced ER stress and apoptosis.

5 | CONCLUSION AND CLINICAL SIGNIFICANCE

Our study identified a novel pathogenic mutation p.P119L of G6PT in one female gout patient with multiple metabolic abnormalities and neutropenia, but without hepatomegaly, who was finally diagnosed with GSD-Ib based on her metabolic phenotype, genetic analysis, and functional abnormality of leukocytes. The molecular genetic detection of G6Pase-α and G6PT should be screened in young female patients with unusual gout. Our findings broaden the variation spectrum of *SLC37A4* and suggest no strict genotype–phenotype correlations in GSD-Ib patients.

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CONFLICTS OF INTEREST

None.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available.

ETHICS STATEMENT

This study was conducted in accordance with the Declaration of Helsinki, and we received approval from the Ethics Committee of Xiangya Hospital. A written informed consent was obtained from each participants.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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