

ORIGINAL ARTICLE

Genotypic and clinical analysis of 49 Chinese children with hepatic glycogen storage diseases

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Abstract

Background: Glycogen storage disease (GSD) is a relatively rare inborn metabolic disorder, our study aims to investigate the genotypic and clinical feature of hepatic GSDs in China.

Methods: The clinical and genotypic data of 49 patients with hepatic GSDs were collected retrospectively and analyzed.

Results: After gene sequencing, 49 patients were diagnosed as GSDs, including GSD Ia (24 cases), GSD IIIa (11 cases), GSD IXa (8 cases), GSD VI (3 cases) and GSD Ib (3 cases). About 45 gene variants of *G6PC*, *AGL*, *PHKA2*, *PYGL*, and *SLC37A4* were detected; among which, 22 variants were unreported previously. c.648G>T (p. Leu216Leu) of *G6PC* exon 5 is the most common variant for GSD Ia patients (20/24, 83.33%), splice variant c.1735+1G>T of *AGL* exon 13 is relatively common among GSD IIIa, while novel variant accounts for the majority of GSD IXa and GSD VI patients. As for clinical features, there was no significant difference in the onset age among group GSD Ia, GSD IIIa, and GSD IXa, but the age at diagnosis and average disease duration from diagnosis of GSD Ia were significantly higher than GSD IIIa and GSD IXa. Body weight of GSD patients was basically normal, but growth retardation was relatively common among them, especially for GSD Ia patients; and renomegaly was only found in GSD Ia. Besides, serum cholesterol, triglyceride, lactic acid, and uric acid in GSD Ia were significantly higher than those with GSD IIIa and IXa ($p < 0.05$); but ALT, AST, CK, and LDH of GSD III and GSD IXa were significantly higher when compared to GSD Ia ($p < 0.05$).

Conclusions: All hepatic GSDs patients share similarity in clinical and biochemical spectrum, but delayed diagnosis and biochemical metabolic abnormalities were common in GSD Ia. For family with GSD proband, pedigree analysis and genetic testing is strongly recommended.

KEYWORDS

gene, hepatic glycogen storage diseases, variant

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1 | INTRODUCTION

Glycogen storage diseases (GSDs) are a group of inherited metabolic disorders resulting from enzymes deficiency in the pathway of glycogen synthesis or glycolysis. The estimated incidence is 1 in 20,000–43,000 live births (Colonetti et al., 2019). Sixteen different subtypes of GSDs are classified, based on the deficient enzymes, affected tissues, and clinical manifestations. Majority of them are autosomal recessive disorders (Burda & Hochuli, 2015; Kishnani et al., 2010, 2014, 2019).

Hepatic GSDs is characterized by hepatomegaly and metabolic abnormalities such as hypoglycemia, hyperlipemia, lactic acidosis, and hyperuricemia. The most common types of hepatic GSDs are GSD Ia, GSD IIIa, and GSD IXa, which were caused by deficiency of glucose 6-phosphatase enzyme (*G6PC*; OMIM *613742), glycogen debranching enzyme (*AGL*; OMIM*610860), and phosphorylase kinase (*PHKA2*; OMIM *300798), respectively (Özen, 2007). Diagnosis of hepatic GSDs is complicated because of heterogeneous clinical presentations and distinguishing from each other is more difficult, which often leads to misdiagnosis or missed diagnosis (Burda & Hochuli, 2015; Kishnani et al., 2010, 2014, 2019; Roscher et al., 2014). Raw cornstarch is the common treatment for all subtypes of hepatic GSDs. However, for different subtypes of hepatic GSDs, specific treatment plans and symptomatic treatment are different, and the key issues concerned during the follow-up are also different. Therefore, diagnosis and classification early are critical to treatment and prognosis.

In order to further understand the genotypic and clinical characteristics of hepatic GSDs, so as to facilitate early diagnosis and treatment, 49 Chinese cases with hepatic GSDs were recruited retrospectively and their clinical features were analyzed.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

This study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Approval number: TJ-IRB20180703) and adhered to the tenets of the Declaration of Helsinki. Informed written consent was obtained from the parents of the patient.

2.2 | Subjects

About 49 patients with hepatic GSDs, admitted to Tongji Hospital, Tongji Medical College, Huazhong University of

Science and Technology from September 2002 to February 2019, were included. These patients came from seven provinces in China, most of them from Hubei Province (7 from Wuhan City, 29 from 7 other cities in Hubei Province), seven from Henan Province, three from Jiangxi Province, and the remaining three were from Anhui Province, Shanxi Province, and Guangdong Province.

The 46 patients were diagnosed clinically and biochemically with the following features: (a) Hepatomegaly with or without splenomegaly; (b) Fasting hypoglycemia; (c) Growth and development delay; (d) Metabolic abnormalities: metabolic acidosis, lactic acidosis, hyperlipidemia, and hyperuricemia; and/or (e) Liver biopsy suggesting GSDs. In addition, three patients were diagnosed by genetic analysis because of their families had a proband.

Clinical data of patients were collected, including history, age of disease onset, initial symptoms, age of diagnosis, height, weight, and serum biochemical parameters (such as fasting glucose, liver and kidney functions, creatine kinase, serum lipids, lactic acid, pyruvic acid, uric acid, serum gas analysis, coagulation function, etc.). Adrenaline test and pathological examination of liver biopsy were also collected if available.

The 49 patients were from 46 unrelated families. All patients and their parents, as well as siblings were analyzed for GSDs gene panel. About 17 GSD-related genes reported by OMIM were included: *G6PC*, *LDHA* (OMIM*150000), *GYS2* (OMIM*138571), *GYG1* (OMIM*603942), *PHKG2* (OMIM*172471), *AGL*, *ENO3* (OMIM*131370), *GYS1* (OMIM*138570), *GBE1* (OMIM*607839), *SLC37A4* (OMIM*602671), *PRKAG2* (OMIM*602743), *PHKA2*, *PFKM* (OMIM*610681), *ALDOA* (OMIM*103850), *PYGL* (OMIM*613741), *PGAM2* (OMIM*612930), and *GAA* (OMIM*606800). The GenBank accession numbers in this study were: *G6PC* (NG_011808.1), *LDHA* (NG_008185.1), *GYS2* (NG_016167.1), *GYG1* (NG_027677.1.1), *PHKG2* (NG_016616.2), *AGL* (NG_012865.1), *ENO3* (NG_012063.2), *GYS1* (NG_012923.1), *GBE1* (NG_011810.1), *SLC37A4* (NG_013331.1), *PRKAG2* (NG_007486.1), *PHKA2* (NG_016622.1), *PFKM* (NG_016199.2), *ALDOA* (NG_008010.1), *PYGL* (NG_012796.1), *PGAM2* (NG_013016.1), and *GAA* (NG_009822.1).

2.3 | Genetics analysis

2.3.1 | Next generation sequencing

DNA of all patients, their parents and siblings was extracted from peripheral leukocytes using QIAamp Whole Blood DNA Extraction Kit (Qiagen, Germany). About 3 µg of DNA was diluted and fragmented into approximately 150 bp in size using a Covaris S220 ultrasonicator (Covaris, USA). Whole-genome

libraries were prepared using standard library construction kits. The exon regions of the target genes were captured using the GenCap liquid phase target gene capture technology. Double-ended sequencing was performed with an Illumina NextSeq 500 high-throughput sequencer with a read length of 150 bp.

2.3.2 | Data analysis

Filtered sequences were aligned to the human genome reference sequence (hg19) in the NCBI database using the BWA software (<http://bio-bwa.sourceforge.net/>). GATK software was used to analyze and obtain information on single nucleotide variant (SNV) and insertions and deletions (INDEL) (<https://software.broadinstitute.org/gatk/>). All SNPs and INDELS were then annotated by the ANNOVAR software (<http://annovar.openbioinformatics.org/en/latest/>). Mutant sites with a frequency of less than 0.05 in the normal human database (including the Thousand Genome Project and EXAC, etc.) were screened. Pathogenicity and conservation prediction of the missense variants were made using software such as REVEL, SIFT, PolyPhen-2, MutationTaster, and GERP++. Those of the splice site variants were analyzed by software such as SPIDEX. A comprehensive analysis was performed based on the hereditary pattern of the disease and clinical characters of the patients to screen for suspected candidate variants.

2.3.3 | Sanger sequencing verification

Candidate variant sites as the result of screening and analysis were verified by PCR and Sanger sequencing. PCR primers were designed using Primer 3.0 online software (<http://primer3.ut.ee/>). The PCR amplification products were subjected to capillary electrophoresis sequencing using a 3130 XL sequencer and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems). Verification of co-segregation was performed among members of the family.

2.4 | Statistical analysis

Statistical analysis was performed on SPSS 24.0 software (SPSS Inc Chicago IL, USA). The mean and standard deviation (SD) of different variables in each group was calculated from the collected data. The Shapiro–Wilk test was used to test for normality of the data. For data following a normal distribution, one-way analysis of variance (ANOVA) with Bonferroni's post hoc test was performed for multiple comparisons. For data with non-normal distribution, Kruskal–Wallis test was used for multiple group comparisons. $p < 0.05$ (two-tailed) were considered statistically significant.

3 | RESULTS

3.1 | General clinical characteristics

Among 49 patients, 46 patients came from 46 unrelated families, the other 3 cases were diagnosed by genetic analysis due to affected siblings. A total of 49 patients, including 29 males and 20 females, aged between 3 months and 11.17 years old. The average age of onset was 1.51 ± 1.06 years, and the age of diagnosis was 3.49 ± 2.53 years.

The chief complaints included abdominal distension or hepatomegaly (20 cases), abdominal distension and increased liver transaminase level (16 cases), hepatosplenomegaly (3 cases), abnormal liver function detected during upper respiratory tract infection visit (4 cases), abnormal liver function detected by kindergarten enrollment health screening (2 cases), and growth retardation (1 case).

3.2 | Genetic analysis results

Gene sequencing showed that 24 patients were diagnosed with GSD Ia, 11 patients GSD IIIa, 8 patients GSD IXa, 3 patients GSD VI, and 3 patients GSD Ib.

3.2.1 | Genotype of GSD Ia

About 11 different *G6PC* gene variants were identified in 24 patients (Figure 1a and Table S1). c.648G>T, p. Leu216Leu within exon 5 of *G6PC* gene was detected in 20 (83.33%) patients (15 cases were homozygous and 5 cases were heterozygous), which suggests that it may be a hotspot variant in Chinese patients with GSD Ia. We also found three unreported variants of *G6PC* gene, including c.230+1G>T (exon 1), c.262delG (p. Val88PhefsTer14, exon 2) and c.550G>A (p. Gly184Arg, exon 4), which were all classified into likely disease-associated or disease-associated variants according to ACMG standards and guidelines.

Of the 24 patients, 22 patients inherited *G6PC* gene variants from their parents; 2 patients inherited one variant from one of their parents, and the remaining was a de novo variant. Genetic analysis was performed on four siblings from four families with proband, and two were diagnosed as patients and two as carriers.

3.2.2 | Genotype of GSD IIIa

About 15 different variants, involving 13 exons of the *AGL* gene, were detected in 11 patients with GSD IIIa. Three patients had a c.1735+1G>T splice site variant in exon 13 and 3 patients had a c.2001+5G>A splice site variant in exon 15. 7 variants were unreported, including variants c.1484A>G, p. Tyr495Cys in

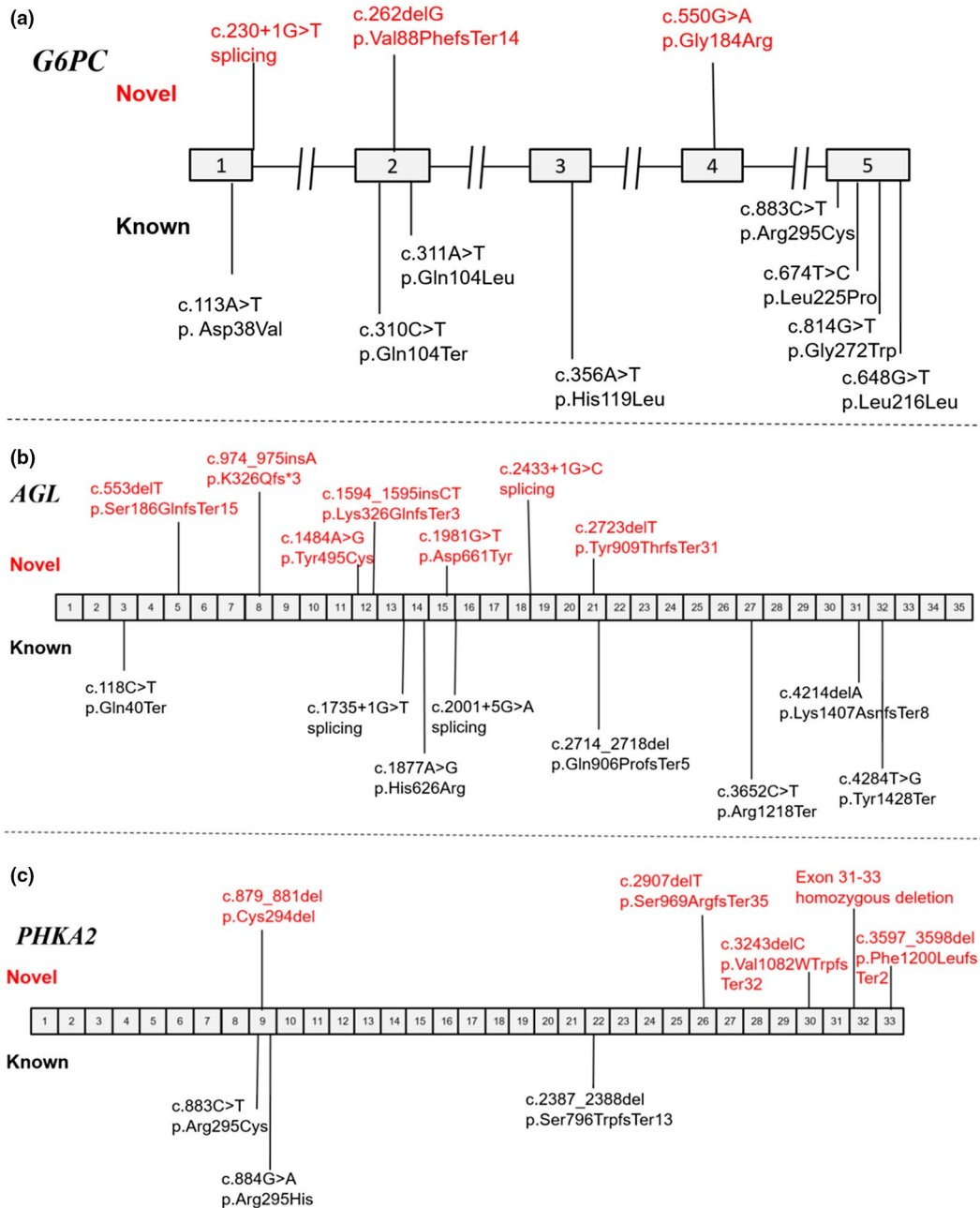


FIGURE 1 (a) Spectrum of *G6PC* variants in 24 GSD Ia patients. Schematic representation of the *G6PC* gene consist of 5 exons. (b) Spectrum of *AGL* variants in 11 GSD IIIa patients. Schematic representation of the *AGL* gene consist of 35 exons. (c) Spectrum of *PHKA2* variants in eight GSD IXa patients. Schematic representation of the *PHKA2* gene consist of 33 exons. Exons are not drawn in scale. The variants identified in this study are depicted in red, and known variants previously reported are in black

exon 12, c.1981G>T, p. Asp661Tyr in exon 15, c.553delT, p. Ser186GlnfsTer15 in exon 5, c.2723delT, p. Tyr909ThrfsTer31 in exon 21, c.1594_1595insCT, p. His534PhefsTer3 in exon 12, C.2433+1G>C, splicing in exon 18 and c.974_975insA, p. Lys326GlnfsTer3 in exon 8 (Figure 1b and Table S2).

The variants of the 11 patients were all inherited from their parents. Two siblings from families with proband were tested, and one patient and one carrier were found.

3.2.3 | Genotype of GSD IXa

Eight variants of *PHKA2* gene were detected in eight patients with GSD IXa. Except for two variants on exon 9 (c.883C>T, p. Arg295Cys and c.884G>A, p. Arg295His) and a variant on exon 22 (c.2387_2388del, p. Ser796TrpfsTer13) have been previously reported, the others were unreported variants. (Figure 1c and Table S3).

TABLE 1 General clinical conditions of the GSD patients at the time of diagnosis

Characteristic	Ia	IIIa	IXa	P-value
Cases (n)	24	11	8	
Male/Female	11/13	6/5	8/0	
Age of onset (Years) ^a	1.38 ± 1.27	1.75 ± 0.82	1.70 ± 0.93	0.109
Age at diagnosis (Years) ^a	4.84 ± 3.16	2.22 ± 0.86	2.61 ± 1.19	<0.05
Disease course at the time of diagnosis (Years) ^a	3.46 ± 3.11	0.47 ± 0.67	0.91 ± 1.08	<0.001
Height at diagnostic (SDS)	-3.20 ± 0.91	-2.29 ± 0.64	-1.69 ± 1.80	<0.05
Blood chemistry				
ALT ^a	88.04 ± 45.913	502.73 ± 245.78	383.63 ± 418.79	<0.001
AST ^a	109.10 ± 47.90	926.60 ± 563.06	528.63 ± 732.09	<0.001
TC ^a	5.58 ± 2.01	4.36 ± 1.39	3.31 ± 1.13	<0.01
TG ^a	7.36 ± 5.22	3.67 ± 1.72	1.71 ± 0.74	<0.001
HDL	0.96 ± 0.34	0.52 ± 0.22	0.88 ± 0.40	<0.05
LDL ^a	2.44 ± 1.00	2.89 ± 1.26	1.87 ± 1.06	0.134
CK	66.43 ± 17.52	213.5 ± 152.44	81.67 ± 20.60	<0.05
LDH ^a	280.50 ± 69.18	710.00 ± 432.89	419.60 ± 110.46	<0.001
Creatinine ^a	19.82 ± 19.04	22.59 ± 10.96	22.39 ± 2.20	<0.01
BUN ^a	4.94 ± 3.49	3.45 ± 0.90	5.39 ± 1.26	<0.05
Fasting blood glucose	3.05 ± 1.16	3.54 ± 2.01	3.57 ± 1.17	0.56
Lactic acid	11.17 ± 5.01	5.07 ± 3.36	3.57 ± 1.34	<0.001
Pyruvate ^a	172.16 ± 168.29	100.95 ± 51.42	43.38 ± 50.92	0.185
Blood ammonia ^a	73.83 ± 31.68	72.48 ± 30.26	72.48 ± 25.28	0.938
Uric acid	528.37 ± 134.30	335.02 ± 112.21	348.70 ± 113.97	<0.001
PH value	7.34 ± 0.07	7.38 ± 0.06	7.42 ± 0.08	<0.05
BE	-9.40 ± 4.86	-6.48 ± 3.37	-6.66 ± 2.09	0.15
Liver B-scan ultrasound				
Hepatomegaly (cm)	7.17 ± 2.40	8.25 ± 2.16	5.80 ± 1.92	0.09
Splenomegaly (cm)	2.42 ± 0.35	2.20 ± 0.46	2.40 ± 0.28	0.47
Adrenaline test (+)	14/14	8/8	4/6	
Liver biopsy				
Ratio	37.5% (9/24)	72.72% (8/11)	75% (6/8)	
PAS staining (+)	5	7	4	
Accompanying splenomegaly	50% (12/24)	63.63% (7/11)	50% (4/8)	
Kidney involvement rate	12.5% (3/24)	0	0	

Note: All values are given as mean ± SD.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BE, base excess; BUN, blood urea nitrogen; CK, creatine kinase; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low density lipoprotein; PAS, periodic acid Schiff; TC, total cholesterol; TG, triglyceride.

^aData not normally distributed.

Four patients inherited the variants from their mother and the other four have de novo variants. In the family pedigree analysis, one of the patient's sisters was a carrier.

3.2.4 | Genotypes of GSD VI and GSD Ib

There are six variants of *PYGL* gene detected in three patients with GSD VI and five variants of *SLC37A4* in three patients with GSD Ib. All of the six *PYGL* gene variants

were unreported. The variant, c.680G>A p. Trp227Ter of *SLC37A4*, was novel, and the variant c.572C>T p. Pro191Leu only reported in Chinese (Table S4).

3.3 | Results of clinical data analysis after genotyping

Forty-three patients were divided into three groups by genotypes after gene analysis, group GSD Ia (24 patients),

group GSD IIIa (11 patients), and GSD IXa (8 patients) (Table 1). Data of patients with GSD VI and GSD Ib were not included in the table because each subtype only has three cases.

The main complaints of hepatic GSDs were related to subtypes. For GSD Ia, 91.67% patients (22/24) had a clinical evidence of abdominal bulging with hepatomegaly or hepatosplenomegaly and the other two cases confirmed with genetic screening due to affected siblings. For GSD IIIa and GSD IXa, only two patients had abdominal bulging with hepatomegaly or hepatosplenomegaly, the main reason of other 17 patients (89.47%) to see doctor was found liver function abnormal, among them, 6 patients (31.58%) were found high level liver enzyme accidentally due to respiratory infections or routine physical examination at kindergarten enrollment.

There was no significant difference in the onset age between three groups. The age at diagnosis, and average disease duration from diagnosis of patients in group GSD Ia was 4.84 ± 3.16 years and 3.46 ± 3.11 years, respectively, which was significantly higher than that in group GSD IIIa and GSD IXa ($p < 0.05$). It indicates that delayed diagnosis was more common among patients with GSD Ia.

As we know, growth retardation was a common presentation in GSD. In our data, the average height of the patients in three groups was -3.20 ± 0.91 SDS, -2.29 ± 0.64 SDS, and -1.69 ± 1.80 SDS, respectively. It showed significant growth retardation in group GSD Ia than in the other groups. In group IXa, only one patient had a height shorter than -2 SDS, the rest of the patients were normal. It seems that patients with GSD IXa were unlikely to have growth retardation.

Except the characteristic of growth retardation in GSDs, weight of patients with hepatic GSDs was basically normal. In group GSD Ia, weights of 10 patients were less than the 10th percentile, the others were all within the normal range (10–75th percentile). In group GSD IIIa, weights of nine patients were heavier than 10th percentile (six of them were heavier than the 50th percentile), while only two weighted less than the 10th percentile. In group GSD IXa, two patients weighted less than the 3rd percentile, and the remaining six patients were at the upper normal range (four patients had a weight within the 75–90th percentile). Compared with height, there was no obviously development retardation in weight.

By abdominal ultrasound examination, all 49 patients had hepatomegaly, 23 patients accompanied with splenomegaly. Three patients with GSD Ia developed kidney enlargement. The age of onset of the three patients with kidney involvement was 3, 12, and 24 months, respectively. The duration of symptoms before diagnosis was 10, 10.17, and 6.92 years, respectively. It suggests that younger patients with a longer course of disease are more likely to

have kidney involvement. Echocardiography and electrocardiogram of all patients showed no obviously abnormalities at the time of diagnosis.

In biochemical examination, there were significant statistical difference among the three group in alanine transaminase (ALT), aspartate transaminase (AST), total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), creatine kinase (CK), low density lipoprotein (LDH), lactic acid, uric acid, and PH ($p < 0.05$). The levels of serum cholesterol, triglyceride, lactic acid, and uric acid in patients with GSD Ia were significantly higher than those in type IIIa and IXa ($p < 0.05$). The levels of ALT, AST, CK, and LDH in patients with GSD IIIa and GSD IXa were significantly higher than those of GSD Ia. These results suggest that biochemical abnormalities (hypercholesterolemia, hypertriglyceridemia, lactic acidosis, and hyperuricemia) in patients with GSD Ia was more serious, while abnormal of liver enzymes (ALT, AST) and muscle enzymes (CK, LDH) were more pronounced anomalies in patients with GSD IIIa and GSD IXa (Table 1 and Figure 2). Fasting hypoglycemia was observed in all three groups, but there was no significant difference. There was no statistical difference in the levels of pyruvic acid, serum ammonia, and base excess (BE) among three groups either ($p > 0.05$).

Adrenaline test was performed in 29 patients (14 GSD Ia, 8 GSD IIIa, 6 IXa, and 1 GSD Ib). All results were positive except two patients with IXa and one patient with GSD Ib.

Liver biopsy was performed in nine patients with GSD Ia, eight patients with GSD IIIa, six patients with GSD IXa. In GSD Ia, the features found under electron microscopy were: hepatic steatosis (8/9), increased glycogen content (8/9), cholestatic vacuoles in hepatocytes (6/9), and regional collagen deposition (6/9), reduced or dissolved organelle structures except the mitochondria (5/9). Five patients had positive periodic acid–schiff staining (PAS), two with negative PAS staining and two patients without described PAS staining results. In GSD IIIa, it was found dissolution of hepatocyte cytoplasmic structure (8/8), increased glycogen particles (8/8), intracellular lipid droplet deposition (8/8), and regional fibrotic proliferation (3/8). In GSD IXa, electron microscopy showed disappearance of cytoplasmic structure of hepatocytes (3/6) and a large amount of glycogen deposition in the small number of remaining hepatocytes (3/6). Four patients had positive PAS.

4 | DISCUSSION

GSD is a relatively rare inborn metabolic disorder determined by the accumulation of glycogen in different tissues. In 1929, von Gierke first described the clinical

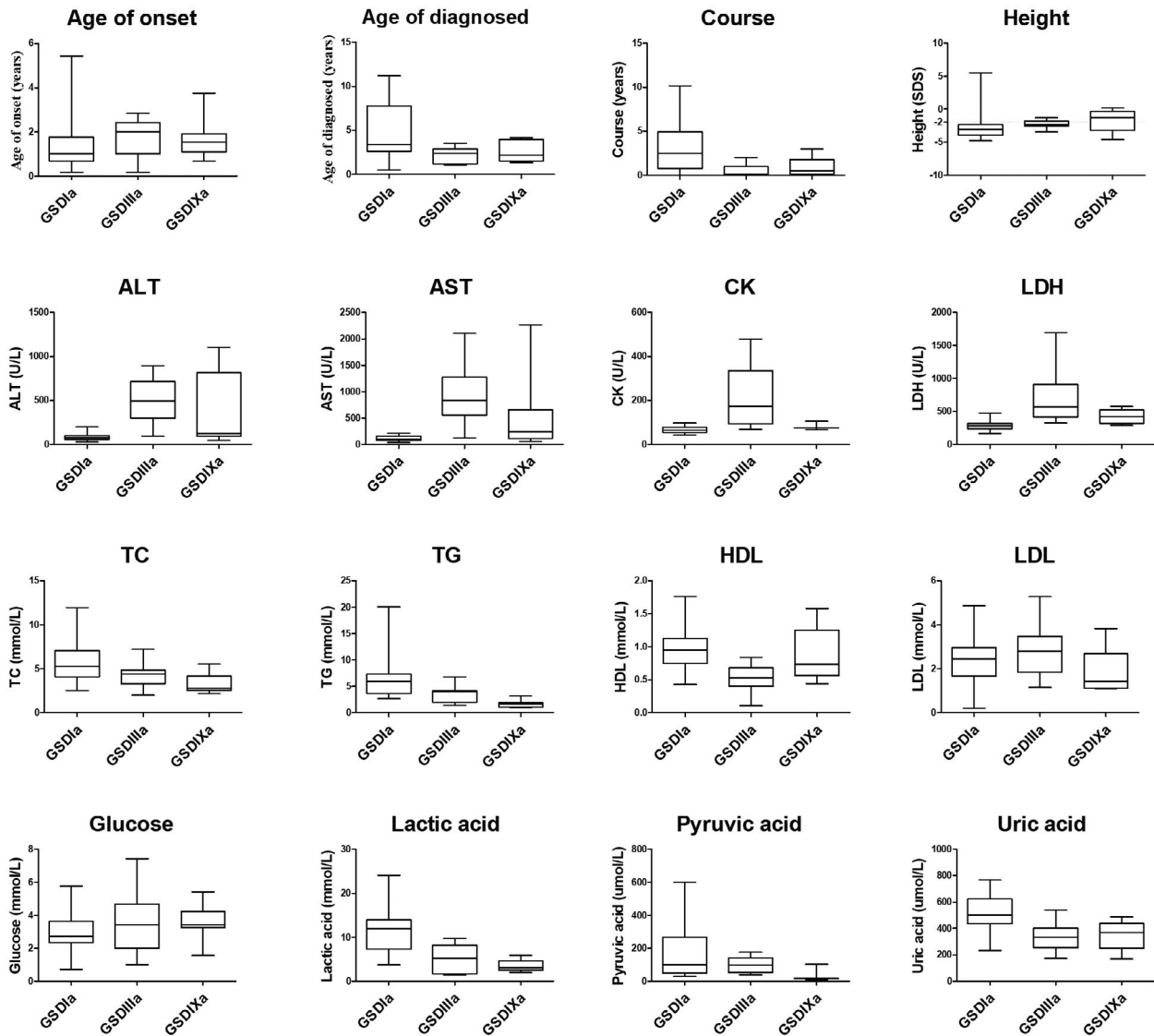


FIGURE 2 Comparison of general conditions and biochemical parameters of the three groups of GSD patients

manifestations of GSD and the pathological changes of glycogen accumulation in liver and kidney. In 1952, Cori et al found that the disease was caused by a deficiency of glucose-6-phosphatase in the liver. In 1953, Lei et al. located the pathogenic gene *G6PC* in 17q21. There are at least 8 enzymes necessary for glycogen synthesis and catabolism, and 16 subtypes caused by these enzyme defects. Hepatic GSDs (mainly affect the liver), included GSD Ia, IIIa, and IXa, are caused by *G6PC*, *AGL*, and *PHKA2* variants, respectively.

Until now, about 500 gene variants of hepatic GSDs have been reported, including 119 variants of the *G6PC* gene, 162 variants of the *AGL* gene, 108 variants of the *PHKA2* gene, 85 variants of the *SLC37A4* gene, and 31 variants of the *PYGL* gene. These variants were included missense, nonsense, frame shift, splice-site variants, small deletions, and rare gene rearrangements. However, there is no report regarding on ethnic differences in variants types and proportions, the

correlation between genotype and clinical phenotype is not clear (Albash et al., 2014; Bali et al., 2014; Ban, Sugiyama, Goto, Mizutani, & Togari, 2003; Beyzaei & Geramizadeh, 2019; Choi et al., 2016; Davit-Spraul et al., 2011; Kishnani et al., 2014; Liang et al., 2013; Lu et al., 2016; Sentner et al., 2016; Sperb-Ludwig et al., 2019; Szymańska et al., 2018; Zhang et al., 2017, 2018).

In this study, a total 45 different gene variants, including 22 unreported gene variants in 49 patients with hepatic GSDs from different cities in many provinces of China, mainly from the central and southern regions of China, were detected. The variant, c.648G>T (p. Leu216Leu) in *G6PC* was found in 83.33% patients with GSD Ia. The proportion was higher than that reported in Chinese patients (36%~40%). However, it's similar to the variant rate reported in Japan (85%~88%) (Kishnani et al., 2014). This suggests maybe this variant was a hotspot variant in Asian GSD Ia patients.

We also found that 15 variants of *AGL*, which involving 13 exons, in 11 cases with GSD IIIa. Three patients (27%) had c.1735+1G>T splice variant in exon 13, which was first reported in Japan and relatively common in Chinese and Korea population. (Ko et al., 2014; Zhang et al., 2018). Therefore, it might be the most prevalent variant in Asians.

Till now, there are no reported common variants in *PHKA2* gene. The majority variants of patients with GSD IXa were novel variants. In our study, five variants of *PHKA2* gene were detected in eight patients with GSD IXa. Two variants were inherited from patient's mother, and the other variants were de novo variants. This was consistent with other studies. So, it is not easy to diagnosed GSD IXa by sequencing targeted hotspot variant.

Among three patients with GSD VI and three patients with GSD Ib, eleven variants were found. Seven of the variants were unreported. The variant of c.572C>T in exon 6 of the *SLC37A* gene was only reported in Chinese GSD Ib patients in published studies (Beyzaei & Geramizadeh, 2019).

In our study, 22 novel variants were found, which greatly expanded the spectrum of the gene variants in hepatic GSDs. However, due to the limited cases in each subtype of hepatic GSDs, the relationship of genotypes and phenotypes was not analyzed. In the future, it is expected to accumulate more patients or to do multicenter studies to solve the problem.

Genetic analysis should emphasize on families with GSDs proband. As we know, most types of GSDs are autosomal recessive disease except Type IXa which was X-linked recessive inherited disease. In our study, gene sequencing was performed on seven siblings of seven families with hepatic GSDs, three patients and four carriers were detected. Pedigree analysis and genetic testing was strongly recommended for families with GSD proband.

The typical clinical manifestations of hepatic GSDs commonly present with hepatomegaly, metabolic abnormalities, and growth development delay. However, there was no specific clinical manifestation or biochemical marker to differentiate them (Beyzaei & Geramizadeh, 2019; Burda & Hochuli, 2015; Kishnani et al., 2014; Matern, Seydewitz, Bali, Lang, & Chen, 2002). Patients with different types of hepatic GSDs have different chief complaints. In our study, 91.67% (22/24) patients with GSD Ia have main complaint about abdominal distension with hepatomegaly. The other two diagnosed by gene sequencing analysis also have abdominal distension and hepatomegaly that their parents did not find. On the contrary, only two patients with GSD IIIa and GSD IXa had abdominal bulging with hepatomegaly or hepatosplenomegaly, the main complaint of the patients was abnormal liver function. Liver dysfunction was found occasionally in six patients during their respiratory infections or routine physical examination that required by kindergarten enrollment. That was different from the previous reports. It suggests us that the possibility of GSD, especially GSD IIIa and GSD IXa, should be

considered in patients of liver dysfunction with hepatomegaly with unknown reasons, and GSD gene analysis should be performed. Renal involvement was only found in GSD Ia patients. Renomegaly is a characteristic diagnostic clue for GSD I (Kishnani et al., 2014; Matern et al., 2002). Growth retardation was more seriously in patients with GSD Ia, but weight was usually normal. It shows deviation between the curves of height and weight.

Early diagnosis of GSD remains a huge challenge. We found that the age of onset of GSD Ia patients was relatively young, but the age at diagnosis was significantly older than that of GSD IIIa and IXa patients. This indicates that delayed diagnosis was common in patients with GSD Ia. The probable reasons for that may be: (a) Severe hypoglycemia episodes were not found in our patients, although hypoglycemia has been appeared in all three subtypes of GSDs. It is less likely to find abnormal for caregivers because of asymptomatic hypoglycemia. (b) There was no obvious liver dysfunction of patients with GSD Ia, although abdominal bulging and hepatomegaly was found, so it couldn't attract the attention of parents. (c) Liver enzymes increased significantly in patients with GSD IIIa and IXa. It's easy to cause the doctor and caregiver's alarm to do further examination, which facilitate early diagnosis of these two subtype of hepatic GSDs.

Biochemical results suggest that there were significant metabolic abnormalities in all three types of GSD. GSD Ia patients existed obvious metabolic abnormalities, such as lactic acidemia, hyperlipidemia, and hyperuricemia, while GSD IIIa and GSD IXa patients were mainly characterized by elevated enzyme indicators, especially ALT, AST, LDH, and CK. Patients with GSD IIIa had a more severe increase in CK. The abnormalities of serum lipids in GSD patients were mainly hypertriglyceridemia and hypercholesterolemia. In some patients, high-density lipoprotein levels were reduced. Hypertriglyceridemia was more pronounced in GSD Ia patients.

There were three GSD Ib patients in our study, whose metabolic and clinical manifestations were quite similar to GSD Ia. But, all patients had neutropenia and repeated respiratory and digestive tract infections (Chou, Cho, Kim, & Mansfield, 2018; Kishnani et al., 2014). Patients with GSD VI had mild hypoglycemia, ketosis, hyperlipidemia, elevated transaminases, and often normal levels of lactic acid and uric acid (Kishnani et al., 2019; Roscher et al., 2014). But, we still have one case with normal serum glucose and 1 case with normal liver enzymes. These findings were similar to previous literature reports.

For adrenaline tests, all patients with GSD Ia and IIIa showed positive results, while the result was negative for two patients with IXa and one patient with GSD Ib. We think the adrenaline test is helpful for the diagnosis of type Ia and type IIIa GSD (Dai et al., 2009; Rosenfeld, Chibisov, Chistova, Leontjev, & Karmansky, 1978).

Although the diagnosis of GSD can be confirmed by pathological examination of liver biopsy, the current diagnosis and treatment guidelines clearly state that liver biopsy is only referred to for the diagnosis and differential diagnosis of hepatomegaly. PAS staining of liver biopsy tissue was once associated with glycogen storage. In our data, there were some patients with negative PAS staining in all three types of GSD biopsies, indicating a negative PAS staining does not rule out the possibility of GSD. When GSD suspected clinically, liver biopsy was not required because genetic analysis can confirm the diagnosis (Kishnani et al., 2014; Matern et al., 2002). However, diagnosis of GSD and its variants is challenging because it is a genetically heterogeneous disorder. For example, there was no common variant of *PHKA2* gene in patients with GSD IXa, major individuals have a de novo variant, so it is not easy to diagnose GSD IXa by sequencing targeted hotspot variant.

In conclusion, although different subtypes of hepatic GSDs have some distinct clinical and biochemical characteristics, it is still difficult to differentiate the subtypes of hepatic GSDs according to clinical manifestations. Attention should be paid to medical history, symptoms, and biochemical indicators during clinical diagnosis. Confirmed diagnosis and classification are depended on genetic testing, which is a key method for providing a definitive diagnosis of GSDs. Today, we detected a total 45 different GSD gene variants including 22 unreported gene variants in 49 Chinese patients with hepatic GSDs. GSDs gene panel analysis used in this study is a noninvasive, reliable, efficient diagnosis method to determine the subtype of GSDs. For family with GSD proband, pedigree analysis, and genetic testing is strongly recommended to detect patients and carriers for interventions and consultant as early as possible.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Yan Liang and Xiaoping Luo designed and organized the study. Hong Wei, Min Zhang, Minghui Hu, Cai Zhang, and Feng Fang cared for the patients, acquired the clinical data, and prepared the samples from the family members. Yan Liang and Caiqi Du wrote the manuscript that was edited by all other authors. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

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

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