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RESEARCH ARTICLE

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Two novel *CPS1* mutations in a case of carbamoyl phosphate synthetase 1 deficiency causing hyperammonemia and leukodystrophy

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Background: Carbamoyl phosphate synthetase 1 deficiency (CPS1D) is a rare autosomal recessive disorder of the urea cycle, mostly characterized by hyperammonemia and the concomitant leukodystrophy. The onset of CPS1D can be at any age, and the clinical manifestations are variable and atypical. Genetic tests are indispensable for accurate diagnosis of CPS1D on the basis of biochemical tests.

Methods: Blood tandem mass spectrometric analysis and urea organic acidemia screening were performed on a Chinese neonatal patient with low activity, recurrent seizures, and hyperammonemia. Next-generation sequencing and Sanger sequencing were followed up for making a definite diagnosis. Bioinformatics tools were used for the conservation analysis and pathogenicity predictions of the identified mutations.

Results: Increased lactate in urea and decreased citrulline in blood were detected in the patient. Two novel mutations (c.173G>T, p.G58V in exon 2 and c.796G>A, p.G266R in exon 8) in *CPS1* identified in the neonatal patient were found through coseparation verification. Both of the two mutations were predicted to be deleterious, and the two relevant amino acids exerted highly evolutionarily conserved. The final diagnosis of the patient was compound heterozygous CPS1D.

Conclusion: This study described the specific clinical characteristics and the variations of physiological and biochemical indices in a Chinese neonatal patient with CPS1D, which facilitated the diagnosis and mechanism research of the disease. Two novel causative missense mutations were identified, which enriched the mutation spectrum of CPS1D in China and worldwide. Advice of prenatal diagnosis was given to the family for a new pregnancy.

KEYWORDS
carbamoyl phosphate synthetase 1 deficiency, hyperammonemia, leukodystrophy, molecular diagnosis, next-generation sequencing

1 | INTRODUCTION

Carbamoyl phosphate synthetase 1 deficiency (CPS1D; MIM237300) is an autosomal recessive genetic disorder, characterized by devastating metabolic disease dominated by severe hyperammonemia, and some patients also suffer from brain white matter changes.[1,2] The time of onset presents as either in neonates or in a more insidious late-onset. The morbidity of CPS1D is estimated 1:50 000-1:300 000 in worldwide, of which 1:800 000 in Japan and 1:50 000-1:100 000 in European population.[1,3-6] To our knowledge, the frequency of CPS1D has not been observed in Chinese cohorts and only three Chinese patients with CPS1D were reported.[1,3]

The pathogenic gene of CPS1D is *CPS1* (*CPS1*; NC_000002.12), which encodes carbamoyl phosphate synthetase 1 (CPS1). As a

rate-limiting enzyme in the urea cycle, CPS1 catalyzes the synthesis of carbamoyl phosphate from bicarbonate, ammonia, and adenosine triphosphate (ATP).[7] *CPS1* maps on chromosome 2q35 and comprises 38 exons that span more than 120 kb region, which encodes 1500 amino acids.[8] More than 240 changes distributed along the whole *CPS1* sequence, including missense, nonsense, deletion or insertion, and other types of mutations resulting in enzyme destruction, have been confirmed in unrelated individuals with CPS1D in the world population, while only 6 mutations were reported in Chinese patients.[1,3,4]

Based on the next-generation sequencing (NGS), we report two novel mutations causing compound heterozygous *CPS1* in a fourteen-month-old girl with hyperammonemia crisis and leukodystrophy of neonatal onset from a Chinese family. CPS1D was diagnosed according to the clinical characteristics and genetic analysis. On the basis of the diagnosis, prenatal diagnosis of CPS1D by amniotic fluid sampling should be performed on the patients' mother during next pregnancy, to deliver a healthy baby.

2 | MATERIALS AND METHODS

2.1 | Clinical characterization of patients (Table 1)

The proband of this study is a fourteen-month-old girl, who was admitted to hospital at three-month age due to cerebral injury and recurrent seizures for two months. The girl was the second child born to the parents who did not have a consanguineous marriage, and no congenital diseases were documented in the family. The patient has a healthy 3-year-old sister, and a younger brother was born recently. The boy presented low activity, somnolence, and tachypnoea after birth, and further examination indicated severe hyperammonemia (serum ammonia level > 1000 μmol/L). He died at 35 days.

Similar symptoms had been observed on the proband. Poor feeding, low activity, and tachypnoea with groaning were observed within 4 days after birth. At three months of age, she was admitted to hospital because of recurrent hypertonia, with convulsion and head lag. Her serum ammonia level was above 500 μmol/L.

Brain magnetic resonance imaging (MRI) and cranial computed tomography (CT) (Figure S1) revealed a formation of softening lesion in bilateral deep frontal lobe and parietal lobe. Enlarged ventricular, decreased density of bilateral cerebral white matter and widened extracerebral space were detected, which suggested leukodystrophy. Electroencephalogram (EEG) returned abnormal electroencephalogram: multifocal low-middle amplitude spike wave and distributed apex wave can be observed dramatically in the left frontal, central, and posterior temporal regions during sleeping stages.

The primary diagnosis of this case was metabolic acidosis and brain damage. Blood and urine samples of the patient were then collected for metabolic screening and gene testing. Meanwhile, protein-free milk was given to the patient every 3 hours via a nasal tube, 60 mL per time. Acid correction treatment was administered with alkali solution. L-arginine and levocarnitine were used to improve metabolism. Ganglioside and vitamin-B6 were used for neurotrophic therapy. After 4 days' treatment, the patient's serum ammonia level decreased dramatically with activity improved and recurrent hypertonia reduced.

2.2 | Blood tandem mass spectrometric analysis

Sample preparation for MS-MS (API4000, AB Sciex, Massachusetts, USA) was carried out as described previously with some modifications.[9,10] A single 3-mm dried blood spot (equivalent to 3.2 μL of blood) was placed in a polypropylene filter plate. Then, methanolic internal standard solution (100 μL) was added. After standing for 20 minutes, the extract liquor was filtered into the polypropylene microtiter plate and dried by a hot air blower at 55°C. Finally, add 100 μL acetonitrile (80%) and cover the plate with aluminum foil. Through these processes, the sample was ready for MS/MS analysis.

2.3 | Urea organic acidemia screening

Sample preparation for GC-MS (7890A-5975C, Agilent, California, USA) was based on the previous described by Xiaowei Fu.[10,11] Urine creatinine determination was performed by ultraviolet spectrophotometer (UV-1750, Shimadzu, Bergen, Japan). After determination, appropriate amounts of urine (equal to 25 μmol creatinine) were loaded in a 10-mL centrifuge tube (tube 1). 50 μL of tropic acid and heptadecanoic acid were each added as internal standards, 100 μL 5% hydroxylamine hydrochloride, 200 μL 25% NH_2SO_4, and 500 μL saturated ammonium chloride were then added, respectively. Oscillated the mixture for 2 minutes and then made it stood for 1 hour at room temperature. Continuously added 3 mL ethyl acetate in tube 1 and oscillated for 2 minutes. After centrifuge at 4500 rpm for 5 minutes, the supernatant was collected to another centrifuge tube (tube 2). Continuously added 3 mL diethyl ether in tube 1 and collected the supernatant into tube 2 as above. Oscillated the mixture for 5 minutes to combine the organic phases. Next, appropriate amount of anhydrous sodium sulfate was added into tube 2 to wipe off redundant

TABLE 1 Clinical features of the present patient with CPS1D

Common features of patients with CPS1D	Features of the present patient
Poor feeding, low activity, and somnolence	Yes
Tachypnoea	Yes
Vomiting	No
Recurrent hypertonia	Yes
Hyporeflexia	Yes
Hyperammonemia	Yes
Hyperbilirubinemia	No
Hypernatremia and hyperchloremia	No
Leukodystrophy	Yes

water, 5 minutes oscillated and 4500 rpm centrifugation was followed, removed the supernatant into a third centrifuge tube (tube 3). After drying the aliquot with a pressure blowing concentrator, 100 μL of a BSTFA and TMCS (99:1, v:v) mixture were added for derivation at 60°C for 60 minutes. 400 μL n-hexane was then added to tube 3 and oscillated for 3 minutes after restoring to room temperature. Through the above processes, the sample was ready for GC/MS analysis.

2.4 | Genotyping by next-generation sequencing (NGS)

Genomic DNA was isolated from peripheral blood samples using standard methods. High-throughput sequencing (Illumina Hiseq 2000) of candidate genes from the patient's genomic DNA was performed after library preparation, solution hybridization, and beads capture (Figure S2) to identify potential disease-causing gene mutations. Neonatal metabolic disease screening V3-panel (MyGenostics, M006) was used for the enrichment of exonic and adjacent intronic sequences. Mutations founded by NGS, especially those in the urea cycle disorder and hyperammonemia-related genes (including *CPS1*, *OTC*, *ASS1*, *ASL*, *ARG1*, *OAT*, *NAGS* and *SLC25A13*, *SLC25A15*, *GLUD1*, and *GLUL*), were then verified by Sanger sequencing and cosegregation in the family. The base pair numbers of mutation sites were determined according to the GenBank mRNA reference sequences.

3 | RESULTS

3.1 | Blood tandem mass spectrometric analysis and urea organic acidemia screening

Urine organic acidemia screening showed elevated urinary lactate, acetoglycocoll, malic acid, alkapton, 4-hydroxyphenyl-lactic acid, and 4-hydroxyphenylpyruvic acid. Blood tandem mass spectrometry revealed dramatically decreased citrulline and threonine. These results suggested liver dysfunction or urea cycle disorder.

3.2 | Sequence analysis and cosegregation in the family

Sequence analysis revealed that the patient has two heterozygous missense mutations in the *CPS1* (II-2; Figure 1B): c.173G>T (p.G58V) in exon 2 and c.796G>A (p.G266R) in exon 8. Both of them are novel mutations which have neither been described in other patients nor in controls.

The two mutations were subsequently confirmed by Sanger sequencing of the patient. The following cosegregation of the two *CPS1* alleles in the family pedigree demonstrated that the c.173G>T allele was paternal, and the c.796G>A allele was maternal (I-1, I-2; Figure 1A,B). Furthermore, c.173G>T allele was found in the patient's sister, while c.796G>A allele was not (II-1, Figure 1A,B). Her brother's genotype cannot be confirmed because the samples were not available.

3.3 | Structure-function correlations of the *CPS1* mutations

Mature CPS1 enzyme composes a 40 kDa N-terminal and a 120 kDa C-terminal moiety. The 40 kDa N-terminal moiety is homologous to the small subunit of bacterial CPS, which composes intersubunit interaction domain and glutaminase subdomain (ISD and GSD) according to the bacteria enzyme.[4] In bacteria, this small enzyme subunit catalyzes the transfer of the amide nitrogen from glutamine to the catalytic center. But human CPS1 cannot use glutamine because of the lack of cysteine residue for aminotransferase activity.[12] The

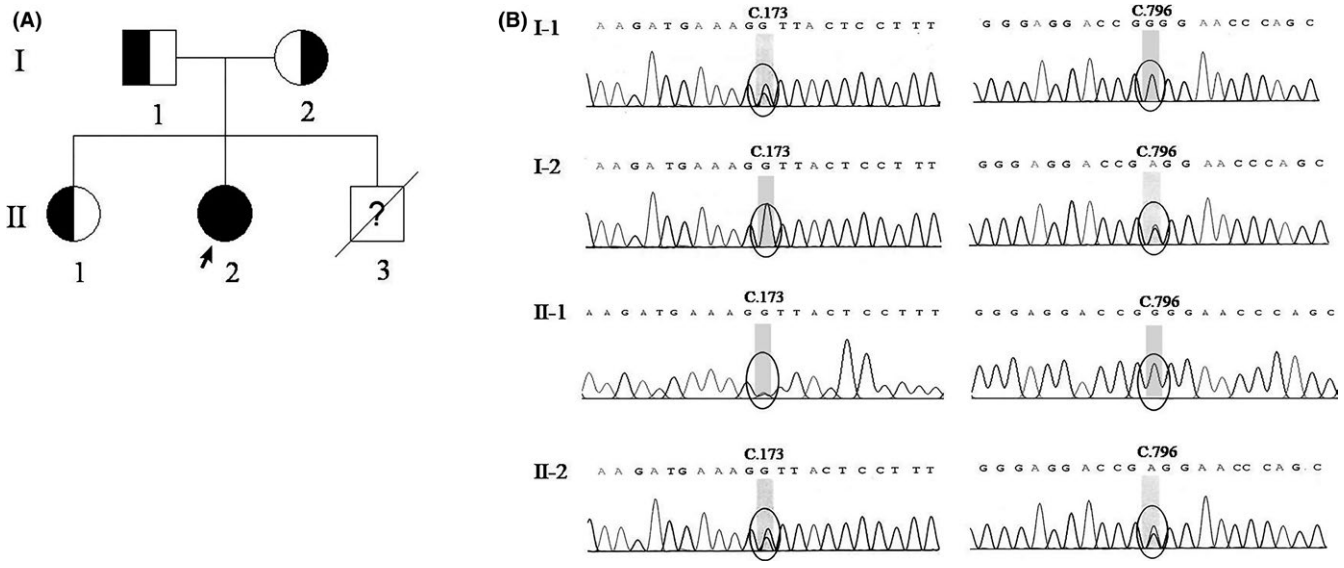

FIGURE 1 *CPS1* mutations inheritance in the family. (A) The pedigree of the family. Symbols are as follows: Square, male; circle, female; filled, affected; dotted, heterozygous carrier; empty, unaffected; arrow, proband; question mark, genotype unknown. (B) Mutations in the family members

120 kDa is homologous to the large subunit of Escherichia coli CPS and can be halved into two repeats. The N-terminal repeat region contains a bicarbonate phosphorylation domain (BPSD) and a 20 kDa function unknown domain, and the C-terminal repeat region contains a carbamate phosphorylation domain (CPSD) and an allosteric domain (ASD) which binds N-acetylglutamate (NAG) (Figure 2A).[13] The two mutations in this study are located in ISD and GSD, respectively, and the substituted amino acids are highly conserved throughout evolution (Figure 2A,B). Both mutations were predicted to be deleterious by SIFT (http://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and PROVEAN (http://provean.jcvi.org) prediction tools. Significant amino acid and conformation changes of the affected polypeptide were simulated with SWISS-MODEL (https://swissmodel.expasy.org) (Figure 2C).

4 | DISCUSSION

Cases of CP1D are usually sporadic. Diagnosis of CP1D is difficult due to its comprehensive symptoms spanning multiple organs. *CPS1* has high expressions in liver and intestine which catalyzes the entry of ammonia into the urea cycle in the first and limiting step of the cycle.[14] A deficiency in *CPS1* will lead to urea cycle disorder and accumulation of ammonia.[15] Therefore, marked hyperammonemia and decreased downstream production of the urea cycle can be observed in patients with CPS1D. As the superabundant ammonia can enter the central nervous system and exerts its toxic effects on the brain, hepatic encephalopathy can also be detected as a complication of hyperammonemia.[16,17] MRI data of the patient are reported in our study which is always absent in other reports because of untimely diagnosis and limited lifetime of patients with CPS1D. Moreover, as a crucial activator of CPS1 by producing N-acetylglutamate, defects in N-acetylglutamate synthase (NAGS) gene can also lead to similar metabolize dysfunction.[18,19] Therefore, genetic examination is the key element in making a definite diagnosis of CPS1D.

The severity of the manifestations in patients with CPS1D depends on the degree of enzymatic activity deficiency. The uneven distribution of mutations in *CPS1* causing CPS1D implied that some regions in this enzyme take main charge of enzyme folding and functionality.[4] Mutations in the large-subunit-like moiety involving the two phosphorylation domains and the NAG binding domain are highly important to the activation of the enzyme and more likely to be pathogenic. CPS1 defects located in the NAG binding domain (c.4088_4099del), bicarbonate phosphorylation domain (c.1799G>A), carbamate phosphorylation domain (c.3443T>A), ISD subdomain (c.323G>A), and even the function unknown domain (c.2537C>T, c.2407C>G) have already been reported in the Chinese CPS1D cohorts.[1,3] The two novel mutations in this study, c.173G>T (p.G58V) and c.796G>A (p.G266R), are, respectively, fall into the ISD and GSD subdomains in the 40-kDa small-subunit-like domain of CPS1, which has been proved to lost its role in binding and hydrolyzing glutamine in human body.[4,20,21] The enzyme deficiency caused by these two mutations strongly suggests that small-subunit-like region of CPS1 also has its indispensable role to contribute the catalysis reaction. Moreover, a pathogenic mutation located in GSD domain is first reported in Chinese. This may support the hypothesis raised by Saeed-Kothe and Powers-Lee that the GSD domain in human CPS1 remains the entry site for ammonia substrate and may lower the ammonia Km in CPS1.[22]

Identification of pathogenic mutations in *CPS1* would give guidance for the aristogenesis of affected families. The patients in this study appeared a typical high serum ammonia level and white matter change. Further test results suggested urea cycle disorder. The family

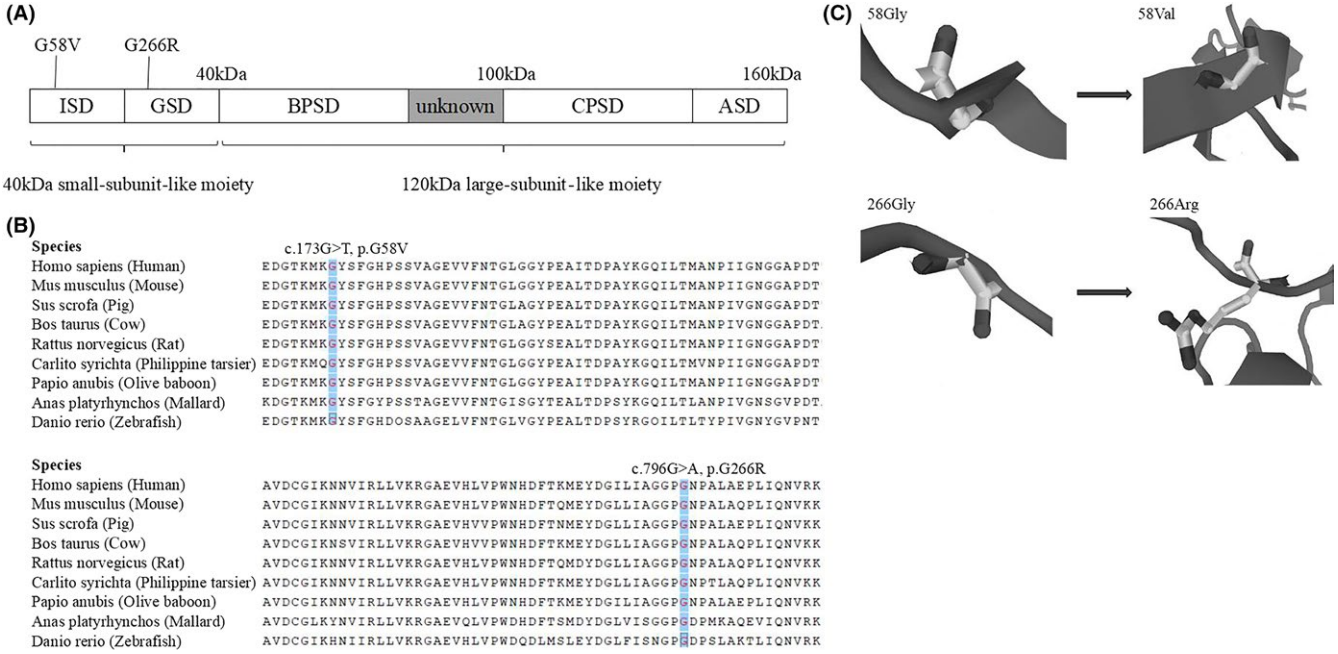

FIGURE 2 (A) Linear map of the mutations in *CPS1*. (B) Conservation of the two missense changes found in this study. (C) Amino acid and conformation changes of the polypeptide

was advised to perform genetic examination due to a second patient with similar symptoms appeared, and they were preparing the next pregnancy. The result of NGS and cosegregation assay in the family confirmed two causative mutations in the girl, respectively, derived from her parents. Depending on this situation, amniocentesis should be performed between 15 and 23 weeks of the next pregnancy in this family for prenatal diagnosis.[23] Analysis of *CPS1* exons 2 and 8 is necessary to find out whether the fetus has inherited any of the mutations present in the proband. Furthermore, the two girls should also accept genetic counseling at their childbearing age in light of eugenics.

The clinical symptoms of the patient had been relieved through some basic treatment. However, the only cure for CPS1D is liver transplantation, while other treatments aim at managing hyperammonemia. Dietary protein restriction is required to patients with CPS1D. Drugs such as L-arginine, sodium benzoate, sodium phenylbutyrate, and sodium phenylacetate can be used for detoxification of long-term hyperammonemia, while hemodialysis should be considered for severe acute hyperammonemia.[15,24] Besides, N-carbamoyl-L-glutamate (NCG), an allosteric activator of CPS1, has been tested to decrease ureagenesis in minority patients with CPS1D.[25] A recent study also indicates flavonoids can rescue CPS1 levels and reduce serum ammonia in *CPS1*-repressed mouse models.[26] Moreover, with the genome-editing strategies become gradually mature, for instance, CRISPR/Cas9 system, gene therapy will be a potential approach to correct *CPS1* mutations in patients with CPS1D.[24]

In conclusion, CPS1D is a rare monogenic inheritance disorder that affected multiple organs originally owing to different extent of destruction in urea cycle. Two novel potentially pathogenic mutations in *CPS1* were first identified in a Chinese patient with compound heterozygous mutations, and her clinical characteristics are comprehensively described. Genetic counseling implications for the family are also stated in this study. These results will facilitate diagnoses of patients with CPS1D at an early stage, further characterize the genotype-phenotype correlations of the disease, and, moreover, contribute to the prevention of birth defects.

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SUPPORTING INFORMATION

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