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ORIGINAL ARTICLE

Gene spectrum and clinical traits of 10 patients with primary carnitine deficiency

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Abstract

Background: Rare studies focused on the tandem mass spectrometry (MS/MS) findings for the primary carnitine deficiency (PCD) in the neonates in China mainland. In this study, we aim to analyze the gene mutation spectrum of PCD in Fujian Province in China mainland.

Methods: Primary carnitine deficiency (PCD) samples used in this study were selected from 95,453 cases underwent neonatal screening between May 2015 and February 2020. *SLC22A5* gene sequencing was performed on the neonates and their parents with C0 level of less than 8.8 μmol/L.

Results: Ten patients (male: 7; female: 3) were finally included in this study. Among these patients, nine were neonates, and one was maternal decline of C0 of less than 8.8 μmol/L. The maternal case showed two types of mutations of *SLC22A5* including c.760C>T(p.R254*) and c.1400C>G(p.S467C). The other nine neonates showed compound mutations involving nine types in 18 sites, among which two mutations [i.e., c.37G>T(p.E13*) and c.694A>G(p.T232A)] were novel that had never been reported before. Bioinformatic analysis indicated that c.37G>T(p.E13*) was a pathogenic mutation, while the c.694A>G (p.T232A) was considered to be likely pathogenic.

Conclusion: MS/MS screening on PCD contributed to the early diagnosis and screening. In addition, *SLC22A5* gene mutation analysis contributed to the PCD screening.

KEYWORDS
MS/MS analysis, neonatal screening, primary carnitine deficiency, *SLC22A5* gene

1 | INTRODUCTION

Primary carnitine deficiency (PCD, OMIM212140), also designated as carnitine transport deficiency, is caused by mutations of *SLC22A5* (OMIM603377, NM_001308122) encoding organic cation transporter 2 (OCTN2). *SLC22A5* mutation would lead to OCTN2 functional deficiency, and then result in decline of plasma carnitine and lack in the tissue cells. This triggered biochemical metabolism disorder, as well as injuries to the cardiac muscles, skeletal muscles, and hepatic tissues.

PCD gene was originally identified in 1975, and its gene mapping was performed in 1998. The estimated incidence of

PCD was in a range of 1:40,000–1:12,000 based on the newborn screening (Filippo et al., 2008). In the Faroe Islands, PCD is a common disease with a high incidence of 1:300 (Rasmussen et al., 2014). The incidence of PCD in China is approximately 1:8938–1:33,000 among diverse regions (Han et al., 2014; Lee et al., 2010; Lin et al., 2017). With the utilization of MS/MS in the neonatal screening, more and more neonates could be diagnosed at the early stage (Han et al., 2014; Lin et al., 2017; Lindner et al., 2011). In addition, the alternative therapy using L-carnitine contributes to the prognosis of PCD patients (Gu et al., 2004; Magoulas & El-Hattab, 2012). In this study, MS/MS was utilized to determine the dried blood spots. In addition, gene mutation analysis was carried out to the neonates suspected with PCD and their parents.

2 | MATERIALS AND METHODS

2.1 | Subjects

Ten samples including 9 infants and 1 mother were selected from neonatal screening samples between May 2015 and February 2020. Generally, 72 hours after birth, the neonates were normally fed, and the blood sample was collected as early as possible. In our center, the neonatal screening report was generated within 7 working days. The neonates' parents were informed by telephone upon a screening of C0 concentration of less than 8.8 μmol/L, followed by examination on the neonates and mothers upon achieving the patients' informed consent. Their C0 level was less than 8.8 μmol/L. At first, a concentration of 9.0 μmol/L was adopted in our center, but it was changed to 8.8 μmol/L in cases of a sample size of 10,000 in 2016. As the neonatal screening indices were not normally distributed, the method of percentiles was utilized to confirm the distribution of C0 in PCD population, in order to calculate the value suitable for the population in this region. Among these cases, one was diagnosed with maternal carnitine deficiency presenting c.760C>T(p.R254*).

Written informed consent was obtained from each child's parents. The study protocols were approved by the Ethical Committee of Fujian Maternity and Child Health Hospital, Affiliated Hospital of Fujian Medical University.

Inclusion criteria were: neonates with a birth duration of more than 72 hours, those with complete lactation, and received no administration of agents before neonatal screening, with a C0 level of less than 8.8 μmol/L after MS/MS. The exclusion criteria were as follows: (a) those with secondary carnitine deficiency induced by genetic diseases, such as isovaleric acidemia and methylmalonic academia; (b) those with inadequate intake or severe loss of carnitine such as severe malnutrition, congenital malformation of the alimentary tract, or peritoneal dialysis; or those with inadequate recovery of carnitine after nutrition uptake; (c) those with severe renal or hepatic diseases; and (d) those with administration of cyclosporin A or valproic acid previously.

2.2 | Blood acylcarnitine spectral analysis

The peripheral blood (three droplets) collected from the neonates was dropped onto the filter paper (Whatman S&S903), followed by drying under natural conditions. The amino acid and acylcarnitine analyses were carried out using the PE commercial kit and the TQD MS/MS screening system (Waters), respectively.

2.3 | DNA extraction

Peripheral whole blood (5 ml) was collected from each subject. Genomic DNA was extracted from peripheral whole blood (2 ml) using a blood DNA mini kit (Qiagen®), followed by preservation at −20°C. The rest peripheral whole blood was preserved at −80°C for further analysis.

2.4 | Genetic analysis

Targeted sequencing was conducted in the patients utilizing the basic edition panel of inherited metabolic diseases (Genuine Diagnostic, Hangzhou, China) to detect the 94 genes (e.g., *PAH*, *PTS*, *MUT*, and *SLC22A5*). The target sequences were enriched by multiple probe hybridization using commercial human exon sequence capture kit (SureSelect, Agilent). The captured products were purified using AMPure XP beads (Agencourt, Beckman), and the purified products were treated by DNA Library Prep Kit V2 for Illumina (TruePrep, Vazyme). Afterwards, special index was added using Index Kit V2 for Illumina (TruePrep, Vazyme). The quality of the DNA library was tested by Qubit and 2100 Bioanalyzer (Agilent). The sequencing libraries were quantified by Illumina DNA Standards and Primer Premix Kit (Kapa). Then the libraries were massively parallel-sequenced by Illumina MiSeq platform. The paired-end reads were trimmed using the Trimmomatic program (http://www.usadellab.org/cms/index.php), followed by aligning with the human genome reference sequence (UCSC Genome build hg19). Single-nucleotide polymorphisms (SNPs) and insertions or deletions were identified using GATK 4.0.0.0 (https://gatk.broadinstitute.org/hc/en-us/sections/360007407851-4-0-0-0) and the Samtools 1.8 software (https://sourceforge.net/projects/samtools/files/samtools/1.8/).

The identified variants were annotated to public databases, including ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/

ac/index.php), ClinGen (https://www.clinicalgenome.org/), the ExAC consortium (http://exac.broadinstitue.org/), dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/), as well as the 1000 Genome Project (http://www.1000genomes.org/). The missense variants were assessed for possible pathogenicity using bioinformatic programs including PolyPhen-2, SIFT, MutationTaster, and PROVEAN. For the pathogenic analysis, the variants were conducted in line with the guidelines proposed by American College of Medical Genetics and Genomics (ACMG).

2.5 | Sanger sequencing

Sanger sequencing was utilized to confirm the mutations using the specific primers listed in Table 1. PCR amplification was conducted using the TaKaRa LA PCR™ Kit (Ver.2.1, TaKaRa). PCR products were purified using NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL). Then the products were diluted into a concentration of 10 ng/μl for sequencing with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Then, Hi-Di (10 μl, Applied Biosystems) was added to each well. The DNA was denaturalized at 95°C for 5 min, and then was transferred to 96-well plates after cooling, and sequencing analysis was conducted using ABI 3500XL (Applied Biosystems).

2.6 | In silico analytical tools

The identified variants were checked for its presence in disease databases such as the HGMD (Stenson et al., 2014), ClinVar (Landrum et al., 2016), and the Leiden Open Variation Database (LOVD) (Fokkema et al., 2011). Then several bioinformatic programs, including SIFT, PolyPhen-2, MutationTaster, and PROVEAN, were utilized to predict the effects of a missense change on the protein structure and function (Adzhubei et al., 2010; Kumar et al., 2009; Schwarz et al., 2014; Yongwook et al., 2012) (Table 2). Meanwhile, multiple amino acid sequences were extracted from NCBI and aligned to verify the evolutionary conservation using ClustalX software (Larkin et al., 2007; Ramu et al., 2003).

3 | RESULTS

3.1 | Patient characteristics

Ten patients (male: 7; female: 3) aged from 3 days to 29 years were finally included in this study. The C0 screening scale was in a range of 1.07–8.29 μmol/L, which was significantly lower than that of the normal range (8.8–50 μmol/L, Table 2). Meanwhile, gene mutation analysis to the *SLC22A5* was performed to the nine neonates and their parents with lower C0.

TABLE 1 Primers used for *SLC22A5* verification of variations

Exon	Nucleotide change	Primer	Sequence (5′–3′)	Size (bp)
1	c.37G>T	Forward	TGTAAAACGACGGCCAGTGGAGGGGTGCGTTTTCAA	1022 bp
		Reverse	CAGGAAACAGCTATGACCGAGTGACAGCGTCCAGTGC	
3–4	c.694A>G	Forward	TGTAAAACGACGGCCAGTACTGTTCACACCCACTTACTG	1799 bp
		Reverse	CAGGAAACAGCTATGACCCAGCACACAGCCAGAACTA	

TABLE 2 Biochemical and gene sequencing results for the patients

					Mutations	
Case	Gender	Age	C0 (μmol/L)	Exon location	Allele 1	Allele 2
Case 1	Male	3d	5.17	4, 8	c.760C>T(p.R254*)	c.1400C>G(p.S467C)
Case 2	Male	3d	3.82	2, 4	c.428C>T(p.P143L)	c.695C>T(p.T232M)
Case 3	Female	29 years	1.07	4, 8	c.760C>T(p.R254*)	c.1400C>G(p.S467C)
Case 4	Male	6d	4.71	1, 8	c.51C>G(p.F17L)	c.1400C>G(p.S467C)
Case 5	Female	3d	4.95	8	c.1400C>G(p.S467C)	c.1411C>T(p.R471C)
Case 6	Female	7d	4.56	1, 4	c.51C>G(p.F17L)	c.760C>T(p.R254*)
Case 7	Male	5d	8.29	1, 4	c.37G>T(p.E13*)	c.694A>G(p.T232A)
Case 8	Male	3d	3.49	1, 4	c.51C>G(p.F17L)	c.760C>T(p.R254*)
Case 9	Female	4d	5.78	2, 4	c.428C>T(p.P143L)	c.695C>T(p.T232M)
Case 10	Male	3d	6.75	1, 3	c.51C>G(p.F17L)	c.506G>A(p.R169Q)

3.2 | Gene mutation analysis results

Gene sequencing was performed to the *SLC22A5* gene in the 10 patients with lower carnitine (1.07–8.29 μmol/L). *SLC22A5* mutation was identified in all the nine neonates and one mother. In total, nine gene mutation types were identified. In one case, only a single pathogenic mutation, c.760C>T (p. R254*), was identified. Her mother (Case 3) showed c.760C>T (p.R254*) and c.1400C>G(p.S467C). The patient's father showed no mutation in *SLC22A5*. In case 7, two novel gene mutations, including c.37G>T(p.E13*) and c.694A>G(p.T232A), were identified. The most frequent mutations that were identified were c.760C>T(p.R254*) and c.1400C>G(p.S467C), which represented 20% (4/20) of the alleles. Most of the mutant alleles were detected in exon 4 (35%) and were located mainly in the extracellular domains of the OCTN2 protein (Table 2). As the screening and diagnosis were given on time, there were no clinical symptoms in these neonates.

3.3 | ACMG score

According to the previous description on ACMG, there was a novel mutation c.37G>T. The pathogenic analysis revealed that the mutation was pathogenic (P, PVS1+PM2+PP4). Meanwhile, the c.694A>G pathogenic analysis was likely pathogenic (LP, PM2+PM3+PM5+PP4, Table 3).

3.4 | Preservative analysis on the new mutation sites

In this section, we conducted the preservative analysis on the c.37G>T (p.E13*) and c.694A>G (p.T232A) sites in different species. As shown in Figure 1, the base on the 37th position of exon 1 of *SLC22A5* was highly preservative. Meanwhile, the base on the 694th position of exon 4 was highly preservative.

4 | DISCUSSION

PCD is usually induced by OCTN2 functional defect triggered by *SLC22A5* gene mutation. *SLC22A5* is localized on 5q31 containing 10 exons with a full length of 25,906 bp. It encodes a polypeptide consisting of 557 amino acids (Li et al., 2010). The neonatal MS/MS screening could provide reference to the PCD patients and the guardians. According to a recent survey by HGMD, there were 150 mutations for

TABLE 3 Pathogenicity prediction analysis of *SLC22A5* c.37G>T and c.694A>G alteration

Variants	Bioinformatic program	URL	Score	Prediction effect
SLC22A5 c.37G>T/p.E13*	SIFT	http://sift.jcvi.org/	NA	NA
	PolyPhen-2	http://genetics.bwh.harvard.edu/pph2/	NA	NA
	PROVEAN	http://provean.jcvi.org/seq_submit.php	NA	NA
	MutationTaster	http://www.mutationtaster.org/	1.000	Disease causing
SLC22A5 c.694A>G/p.T232A	SIFT	http://sift.jcvi.org/	0.007	Damaging
	PolyPhen-2	http://genetics.bwh.harvard.edu/pph2/	0.516	Possibly damaging
	PROVEAN	http://provean.jcvi.org/seq_submit.php	−3.037	Deleterious
	MutationTaster	http://www.mutationtaster.org/	0.999	Disease causing

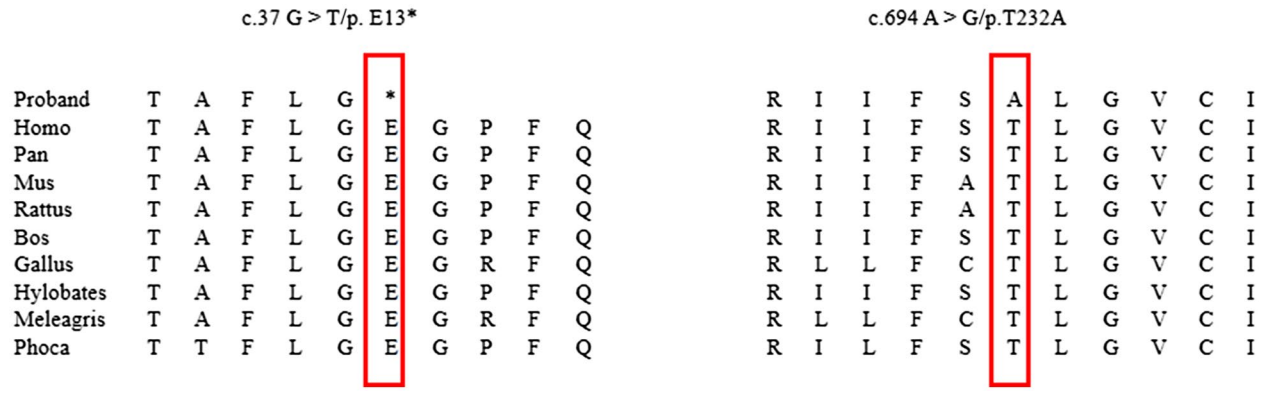

FIGURE 1 Preservative mutation analysis for the novel sites in different species

TABLE 4 Genotype and clinical symptoms of the confirmed cases in the previous literatures

Case	Genotype	Symptoms	Literature
Case 1	R169W/R289*	Muscle weakness, recurrent pneumonia	Han et al. (2014)
Case 2	R254*/R254*	Cardiomyopathy, muscle weakness	Han et al. (2014)
Case 3	R254*/F17L	Cardiomyopathy, fatigability on walking	Han et al. (2014)
Case 4	F17L/F17L	Cardiomyopathy, muscle weakness	Han et al. (2014)
Case 5	F17L/F17L	No	Lee et al. (2010)
Case 6	F17L/R471C	Hyperammonemia, hypoglycemia, Reye-like syndrome	Lee et al. (2010)
Case 7	R254*/P143L	No	Lee et al. (2010)
Case 8	F17L/S467C	No	Lee et al. (2010)
Case 9	R254*/S467C	No	Lee et al. (2010)

SLC22A5, most of which were missense mutations and the rest were nonsense mutation, frameshift mutation, and mutation of the splicing sites (Stenson et al., 2014). There were variations in the mutational spectrum in different regions and races. For instance, the most common spectra in the Asian population were c.396G>A(p.W132*) and c.849G>T(p.W283C) (Akio et al., 1999), while in the Caucasian population was c.844C>T (p.R282*) (Burwinkel et al., 1999). The c.760C>T (p.R254*) was the most common in Southern part of China including Taiwan (Lee et al., 2010; Tang et al., 2002). Interestingly, Lamhonwah et al. (2004) reported this mutation in a Saudi Arabian family and two American neonates, which implied that it was a recurrent mutation in diverse genetic backgrounds or an ancient founder mutation (Lamhonwah et al., 2004).

In this study, nine mutation types of *SLC22A5* were identified in the 10 cases, including c.760C>T(p.R254*), c.1400C>G(p.S467C), c.51C>G(p.F17L), c.428C>T(p.P143L), c.695C>T(p.T232 M), c. 694A>G(p.T232A), c.506G>A(p.R169Q), c.37G>T(p.E13*), and c.694A>G(p.T232M). To our best knowledge, seven mutations [i.e., c.760C>T(p.R254*), c.1400C>G(p.S467C), c.51C>G(p.F17L), c.428C>T(p.P143L), c.695C>T(p.T232M), c.694A>G(p.T232A), and c.506G>A(p.R169Q)] had been acknowledged to be responsible for the pathogenesis of PCD (Akio et al., 1999; Burwinkel et al., 1999; Lee et al., 2010; WANG et al., 1999). Nevertheless, the other two mutations [i.e., c.37G>T(pE13*) and c.694A>G(p.T232A)] in our study were not reported in the previous studies, which were considered as new variations for *SLC22A5*. The most abundant mutations were c.760C>T(p.R254*) and c.1400C>G(p.S467C) representing 20% (4/20) in the whole mutations. The carrier rate for this mutation in Hong Kong was estimated to be as high as 1 in 125, and the estimated incidence of PCD due to c.760C>T(p.R254*) mutation was 1 in 62,500. In addition, Lee et al. (2010) estimated a high incidence of the c.760C>T(p.R254*) mutation of approximately 1 in 67,000 (95% CI: 1 in 31,600–512,000) among neonates and the prevalence in mothers of 1 in 33,000 (95% CI: 1 in 18,700–169,000) at the National Taiwan University Hospital screening center (Lee et al., 2010). The c.760C>T(p.R254*) mutation led to generation of a premature termination codon at amino acid residue 254 in the extracellular domain of OCTN2. The clinical symptoms of PCD patients showed large variations. For instance, patients with slight conditions were symptom free in their life span, while those with severe conditions may present cardiomyopathy and/or sudden cardiac death. In this study, some of the PCD neonates were confirmed through neonatal screening before the presence of symptoms. Thus, the patients did not present the clinical symptoms lethal to the neonatal growth and development in the previous literatures (Table 4). According to the previous description (Gu et al., 2004), there was no significant correlation between PCD genotype and phenotype, and its clinical symptoms were affected by the genetic background, environment, eating habits, and other acquired factors. We could not evaluate the disease situation of the neonates as the symptoms were not available during the neonatal screening, but we think it is quite necessary to pay attention to the prevention of PCD.

Homology analysis to the novel variations indicated that there was a high conservation in the Tyr 84 of *SLC22A5*. Such variation was not identified in the 100 normal samples and the 1000 genome database (www.1000genomes.org/), which excluded the possibility of polymorphism. Then functional prediction was given to the c.694A>G(p.T232A) using SIFT and PolyPhen-2, which indicated that such variation may affect the function of organic cation transporter (SIFT Score = 0.007, damaging; PolyPhen-2 Score = 0.516, possibly damaging). According to the score standard for the ACMG, the mutation was considered to be likely pathogenic. The mutation c.37G>T (p.E13*) led to termination of amino acid synthesis in advance, which was considered to be pathogenic mutation according to the guidelines proposed

by ACMG. Moreover, no relationship was identified between blood C0 level and OCTN2 genotype in the present work on the analysis of case studies.

All the 10 cases with relatively low C0 were confirmed in the diagnosis within 10 days, among which nine were neonates, and one was maternal decline of C0. As these patients were screened on time, no clinical symptoms were shown in these children. For the treatment, levocarnitine (50–100 mg/kg per day) was given in the non-acute stage, followed by regular MS/MS once per month. Upon the stability of the C0 level after treatment, follow-up was performed once every 3 months. Remind guardians to keep the children away from fasting, frequent high-carbohydrate, low-fat meals, and keep on carnitine administration when the children grow up.

In summary, we identified nine *SLC22A5* gene mutations and characterized the spectrum of *SLC22A5* mutations in Chinese patients with PCD. Two mutations, c.760C>T(p.R254*) and c.1400C>G(p.S467C), were the most frequent in the cases. In this study, we identified one mother carrying two *OCTN2* mutations that were clinically asymptomatic through newborn screening. Further studies with a large sample size are required to validate the functional changes induced by the two novel missense mutations.

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CONFLICT OF INTEREST

The authors report no conflict of interest or any financial interest. This study did not receive any commercial funding.

AUTHOR CONTRIBUTIONS

Chen Y: Study design and data interpretation. Lin QY: Data collection. Zeng YL and Qiu XL: did the experiments. Liu GH: clinical supervisor. Zhu WB: Technical supervisor and crucial revision of the manuscript.

DATA AVAILABILITY STATEMENT

All the data were available upon appropriate request.

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