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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The mutation spectrum of the SLC25A13 gene in Chinese infants with intrahepatic cholestasis and aminoacidemia

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Abstract
Background SLC25A13 gene mutations cause citrin deficiency, which leads to neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). Information on the mutation spectrum of SLC25A13 in the Chinese population is limited. The aim of this study was to explore the mutation spectrum of the SLC25A13 gene in Chinese infants with intrahepatic cholestasis and various forms of aminoacidemia.
Methods Sequence analyses were performed on 39 infants with intrahepatic cholestasis and various forms of aminoacidemia. Novel mutations were subjected to homology and structural analyses. Western blots were performed when liver specimens available.
Results Genetic testing revealed the presence of SLC25A13 gene mutations (9 heterozygotes, 6 homozygotes and 13 compound heterozygotes) in 28 infants. Subsequent Western blot analysis revealed 22 cases of citrin deficiency, accounting for 56.4% of the 39 patients. Twelve types of mutations, including nine known mutations and three novel mutations, were found. Of the 49 mutated alleles, known ones include 851del4 (26 alleles, 53.1%), 1638ins23 (6 alleles, 12.2%), IVSl6ins3kb (3 alleles, 6.1%), IVS6+5G>A (2 alleles, 4.1%), E601K (2 alleles, 4.1%) and IVS11+1G>A, R184X, R360X and R585H (1 allele each, 2.0%). The three novel mutations were a splice site change (IVS6+1G>A), a deletion mutation (1092_1095delT) and a missense mutation (L85P), each in one allele.
Conclusions The mutation spectrum of the SLC25A13 gene in a Chinese population of infants with intrahepatic cholestasis with various forms of aminoacidemia was found to be different from that of other population groups in East Asia. The SLC25A13 gene mutation is the most important cause of infantile intrahepatic cholestasis with various forms of aminoacidemia.

Keywords Aminoacidemia · Infants · Intrahepatic cholestasis · Mutation · NICCD

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Introduction

Citrin protein, consisting of 675 amino acid residues with a molecular weight of 74 kDa and harboring four EF-hands and six mitochondrial transmembranous (TM) spanners, has been identified as a mitochondrial aspartate–glutamate carrier protein [1, 2]. Citrin deficiency causes not only adult-onset type II citrullinemia (CTLN2, MIM #603471)

[1] but also neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, MIM #605814) [3, 4]. The symptoms of NICCD include intrahepatic cholestasis, mild liver dysfunction, an elevated aspartate aminotransferase/alanine aminotransferase ratio, failure to thrive, fatty liver, multiple forms of aminoacidemia, including citrullinemia, hypoproteinemia, hypoglycemia, coagulation disorders, and/or high levels of plasma α-fetoprotein [5–13]. Although the symptoms of most NICCD patients may spontaneously disappear by 12 months of age or after dietary adjustment, liver failure may occur, necessitating liver transplantation in a small proportion of such patients in early life [6, 14]. In less fortunate cases, CTLN2 may develop one or more decades later and may lead to death if treated inappropriately [15]. Early diagnosis of NICCD may prevent progression to CTLN2 by dietary adjustment or prevent serious consequences by close follow-up and timely treatment before the onset of symptoms; hence, early detection is extremely important in such patients [16]. Because the symptoms of NICCD are transitory and complex, it is not so easy to establish definite clinical diagnostic criteria, and the best diagnostic test for NICCD is a genetics test.

Citrin is encoded by the SLC25A13 gene located on chromosome 7q21.3 [1, 17]. This gene, 160 kb in length, consists of 18 exons and encodes a 3.4-kb transcript. It is expressed ubiquitously, but most abundantly in the liver. To date, more than 50 mutations have been identified [18], and all, with the exception of P632L, are pathogenic.

Citrin deficiency was thought to be restricted to the Japanese population when it was first reported in Japan [1, 19]. However, recent studies have indicated that the disease may be distributed worldwide [12, 20–24], especially in the East Asian region [25]. More than 100,000 individuals may be homozygous for SLC25A13 mutations in the total population of East Asia [23]. Only a few cases of NICCD have been reported in the Chinese population to date [9, 13, 18, 23, 25–27]. Details on the spectrum of the SLC25A13 gene mutation in Chinese infants with intrahepatic cholestasis is still under investigation. In this study, the SLC25A13 gene mutation spectrum was studied in Chinese infants with neonatal intrahepatic cholestasis and various forms of aminoacidemia.

Materials and methods

Definition of intrahepatic cholestasis

In this study, conjugated hyperbilirubinemia was defined as serum total bilirubin (TBil) >5 mg/dL, with a conjugated fraction that accounted for more than 20% of the total or conjugated bilirubin >1 mg/dL where total serum bilirubin <5 mg/dL. Intrahepatic cholestasis was defined as conjugated hyperbilirubinemia following the exclusion of diseases affecting the extrahepatic biliary system, such as biliary atresia, choledochal cyst, tumor, inspissated bile, or hemangioma, among others, by imaging of the hepatobiliary system. The imaging procedures included ultrasound scan and hepatobiliary iminodiacetic acid (HIDA) scintigraphy in each case and laparotomic cholangiography in selected cases.

Definition of aminoacidemia

The plasma amino acid spectrum was analyzed by tandem mass spectrometry (MS/MS). The concentrations of 19 amino acids, including alanine, valine, leucine, methionine, phenylalanine, tyrosine, aspartic acid, glutamic acid, glycine, ornithine, citrulline, arginine, serine, proline, threonine, tryptophan, cysteine, asparagine, and histidine, were determined. Aminoacidemia was defined as either of the following two conditions: (1) an elevation in the concentration of any one of the screened amino acids to twofold higher than the upper normal reference point; (2) elevation of multiple amino acids, with the concentration of at least one of the amino acids being 1.5-fold higher than the upper limit of normal.

Subjects

Patients who were referred to the Children's Hospital of Fudan University, a tertiary referral pediatric hospital in eastern China, for investigation of conjugated hyperbilirubinemia before 1 year of age between June 2003 and September 2009 were eligible for enrollment if both of the definitions of intrahepatic cholestasis and aminoacidemia (see above) were satisfied. The exclusion criteria were:

1. Patients with persistent cholestasis and low γ-glutamyl transpeptidase (GGT; no more than 50 U/L), which may be indicative of progressive familiar intrahepatic cholestasis or bile salt synthesis defects [28, 29].
2. Patients with low free T4 and elevated thyroid stimulating hormone.
3. Patients with obvious extrahepatic abnormalities, such as abnormal facies, heart disease, butterfly vertebrae, etc.
4. Patients with positive serology that may indicate infection of hepatitis B, hepatitis C, hepatitis A and E, toxoplasmosis, rubella, herpes simplex, human immunodeficiency virus-1 or syphilis. Patients with cytomegalovirus (CMV) infection were not excluded because it is highly prevalent in Chinese infants, and patients infected with CMV have the same outcome as those without the infection [30, 31]. The presence of

CMV infection has been found not to rule out other causes of intrahepatic cholestasis [26].

5. Patients whose parents were unwilling to take part in the study.

In total, 39 patients (22 male and 17 female infants) fulfilled the above inclusion and exclusion criteria (Table 1) and were enrolled in the study. With the except of one patient, who was born of consanguineous parents (P2394, Table 1), no consanguinity was found among the parents of the enrolled infants.

An additional 50 infants with intrahepatic cholestasis but a normal plasma amino acid profile served as controls for the screening of the novel mutations using direct sequencing or real time fluorescent (RTD)-PCR with dual-labeled probes.

Mutation detection

The study protocol conforms to the ethical guidelines of the Declaration of Helsinki of 1975 and was approved by the Ethics Committee on human research of the Children's Hospital of Fudan University. Informed consent was obtained from the parents or guardian of every participant. About 1 ml whole blood from each participant was obtained. Genomic DNA of peripheral blood leucocytes was extracted using routine methodology. The entire 18 coding exons together with its flanking sequence of the SLC25A13 gene of all 39 patients were amplified by PCR and directly sequenced. A list of primers is available upon request. Purified PCR products were detected by laser-induced fluorescence on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using BIOEDIT software (North Carolina State University, Raleigh, NC) and double-checked by two of the investigators. All sequences were blasted to the gene bank. Genomic sequences were obtained at the National Center for Biotechnology Information (NCBI), and sequence RefSeq NG_012247.1 was used as the SLC25A13 gene reference. Possible mutations were confirmed by direct sequencing from both ends of a second independent PCR fragment. The known large fragment mutations Ex15dup (IVS14_15), IVS16ins3kb, and Ex16+74_IVS17-32del516 were tested as reported previously [20, 23, 32].

Homology and structural predictions

MaxEntScan was used to evaluate the role of splice site mutations (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html). The homology between human citrin protein and that of other species was surveyed using software Clustal X (European Bioinformatics Institute, Hinxton, Saffron Walde, UK). Secondary structures were predicted with YASPIN secondary structure prediction (http://www.ibi.vu.nl/programs/yaspinwww/). The program Polyphen (Polymorphism Phenotyping), available at: http://genetics.bwh.harvard.edu/pph/, was used to predict the possible impact of an amino acid substitution on the structure and function of citrin proteins. Polyphen calculates PSIC (position-specific independent counts) scores for two amino acid variants in the polymorphic position. A PSIC score difference of less than 0.5 denote benign variants, PSIC scores that differ by between 1.5 and 2 indicate the possibility of damaging variants, and PSIC scores that differ by >2 indicate the probability of damaging variants [33].

Western blot analysis

Western blot analysis was performed on the biopsied liver specimens of nine patients. Liver tissues were homogenized in radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and the proteins extracted routinely. Western blotting was performed using anti-citrin immunoglobulin G as the first antibody [34] and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies as the secondary antibody. Fluorescence was effected with ECL+Plus kit (Thermo Fisher Scientific, Waltham, MA). HRP-conjugated monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; KangChen Bio-tech Inc., China) was used served as loading control (detecting band at approx. 36 kDa).

Statistical analysis

The frequency of citrullinemia among the three groups, including patients with definite diagnosis of citrin deficiency, with probable citrin deficiency and patients without mutation were assessed using Fisher's exact test. A two-tailed P value of <0.05 was considered to be significant.

Results

The incidence of citrin deficiency

Among the 39 cases of intrahepatic cholestasis and various forms of aminoacidemia, SLC25A13 gene mutations were found in 28 patients cases, including six patients with a homozygous mutation 13 patients with a compound heterozygous mutation, and nine patients with a heterozygous mutation (Table 1).

Table 1 Relationship between the aminoacidemia and mutation types of SLC25A13 gene

Mutations	Case/ Patient	Gender	Province/ City	Age[a] (months)	>2× UNL[b]	1.5–2× UNL[b]	Mutation types	Citrin protein
Homozygotes ($n = 6$)	P2509	F	Shanghai	3	Cit Met	Tyr Thr Asn	851del4/851del4	Absent
	P4412	M	Hubei	1	Cit Thr	Met His Tyr	851del4/851del4	N/A
	P2394	M	Anhui	3	Cit	Met	IVS6+1G>A/ IVS6+1G>A	63, 68 kDa
	P4295	F	Sichuan	4	Cit	Met	851del4/851del4	N/A
	P3383	F	Jiangxi	7	Cit	His	851del4/851del4	N/A
	P4163	F	Shanghai	4	Met	None	851del4/851del4	N/A
Compound heterozygotes ($n = 13$)	P4554	F	Sichuan	24	Cit Met	Tyr Arg Orn Thr	851del4/IVS6+5G>A	N/A
	P1541	F	Zhejiang	7	Cit Met	Arg	851del4/1638ins23	Absent
	P2078	F	Jiangsu	4	Cit Thr	Met Tyr	851del4/R184X	Absent
	P3163	F	Zhejiang	3	Cit Tyr	Phe	851del4/1638ins23	Absent
	P4740	M	Zhejiang	3	Cit Met	None	851del4/E601K	N/A
	P2525	F	Shanghai	4	Cit	Met Tyr	851del4/R585H	N/A
	P3174	F	Jiangsu	3	Cit	Gly	E601K/L85P	Absent
	P4405	M	Henan	3	Cit	Arg	IVS11+1G>A/R360X	N/A
	P2383	M	Jiangsu	6	Met Tyr	Cit Gly	851del4/1638ins23	N/A
	P3013	M	Zhejiang	4	None	Tyr Gly	851del4/1092_5delT	N/A
	P4463	M	Zhejiang	3	Cit	Glu Met His	851del4/IVSl6ins3kb	N/A
	P2586	M	Zhejiang	8	Met	Cit Orn Cys	851del4/IVSl6ins3kb	N/A
	P4068	M	Jiangxi	5	Met	Tyr	IVS6+5G>A/ IVSl6ins3kb	N/A
Heterozygotes ($n = 9$)	P3156	M	Jiangxi	3	Cit Met	Tyr Ser Gly	851del4/?	Absent
	P2625	M	Jiangsu	6	Cit Met	Orn Asn	851del4/?	Absent
	P2434	M	Shanghai	6	Cit Met	Phe	1638ins23/?	Absent
	P2439	M	Jiangsu	2	Cit Met Tyr	Thr	1638ins23	N/A
	P2556	F	Zhejiang	1	Cit Met	Tyr Asn Arg Thr	851del4	N/A
	P519	F	Sichuan	5	Cit Met	Tyr Arg Thr	851del4	N/A
	P4749	F	Zhejiang	3	Cit Met	Arg	851del4	N/A
	P4461	M	Anhui	5	None	Cit Met	1638ins23	N/A
	P2516	F	Jiangxi	2	None	Tyr Gly	851del4	N/A
No mutation ($n = 11$)	P420	M	Jiangsu	3	Cit Thr	Arg	ND	N/A
	P1628	M	Jiangsu	4	Cit Met	None	ND	N/A
	P1684	F	Anhui	5	Cit	Met Ser	ND	N/A
	P4509	M	Hubei	1	Cit	None	ND	N/A
	P4684	M	Henan	2	Met	Tyr Trp Orn Ser Thr	ND	N/A
	P2769	M	Anhui	5	Tyr	Ser	ND	N/A
	P4542	M	Hubei	3	Thr	Glu His Met	ND	N/A
	P2338	F	Jiangsu	4	Arg	Met Tyr	ND	N/A
	P4487	M	Shanghai	5	Trp	Ser Orn Glu	ND	N/A
	P4115	M	Jiangsu	4	Ala Gly Tyr	Glu Met Pro Ser	ND	N/A
	P4129	F	Hubei	6	Pro	None	ND	N/A

F Female, *M* male, *ND* not detected, *N/A* liver specimen not available, *Cit* citrulline, *Met* methionine, *Arg* arginine, *Tyr* tyrosine, *Thr* threonine, *Ser* serine, *Pro* proline, *Asn* asparagine, *Gly* glycine, *Orn* ornithine, *His* histidine, *Lys* lysine, *Phe* phenylalanine, *Ala* alanine, *Trp* tryptophane, *Glu* glutamic acid, *Cys* cysteine

[a] Patient's age at tandem mass spectrometry

[b] Upper normal limit

Western blotting were performed on the biopsied liver specimens from nine patients, of whom six had homozygous or compound heterozygous mutations (P2509, P2394, P1541, P2078, P3163, and P3174) and three had heterozygous mutations (P3156, P2625, and P2434). Citrin protein was absent in all specimens except that from the patient with homozygous mutation IVS6+1G>A (P2394), in which approximately 63- and 68-kDa immunoreactive bands were detected (Fig. 1). Western blot analysis was not performed for the other patients due to the lack of a liver specimen.

When the results of the Western blot of citrin protein and the genetic tests were analyzed together, at least 22 cases of citrin deficiency could be diagnosed, accounting for 56.4% of all the subjects. The other six patients in whom only a mutation was detected in an allele were diagnosed as probable citrin deficiency, although there is a possibility that some of these are really carriers. The diagnosis of citrin deficiency is unlikely in the 11 patients for whom no mutation was found because all 18 exons were tested.

SLC25A13 gene mutation spectrum

Twelve mutations (49 mutated alleles) were detected, of which three mutations were novel. These three novel mutations were splice site change IVS6+1G>A in one allele and missense mutation L85P and frameshift mutation 1092_1095delT in one allele each. Two other mutations, R585H and IVS11+1G>A, each in one allele, were detected in Chinese patients for the first time. The remaining seven mutations identified were: 851del4 (26 alleles, 53.1%), 1638ins23 (6 alleles, 12.2%), IVSl6ins3kb (3 alleles, 6.1%), IVS6+5G>A (2 alleles, 4.1%), E601K (2 alleles, 4.1%), and R184X and R360X (each in 1 allele, account for 2.0%, respectively).

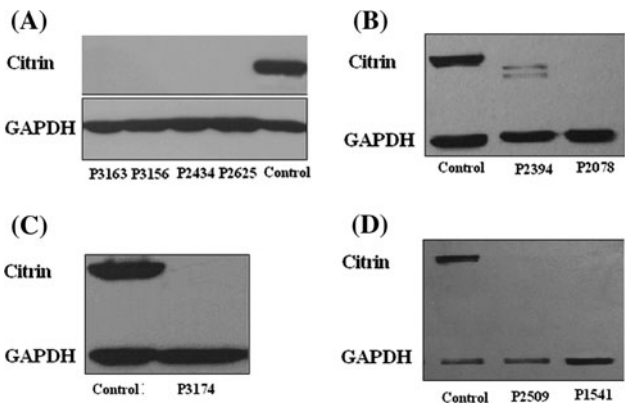

Fig. 1 Western blot analyses of biopsied liver specimens of six patients with homozygous or compound heterozygous mutations (*P2509*, *P2394*, *P1541*, *P2078*, *P3163*, and *P3174*) and three patients with heterozygous mutations (*P3156*, *P2625*, and *P2434*). *GAPDH* Glyceraldehyde-3-phosphate dehydrogenase

The effect of novel mutations

The splice-site mutation IVS6+1G>A identified in P2394 represents a base substitution from G to A at the first position of the 5′-end in intron 6. The patient with homozygous mutation IVS6+1G>A/IVS6+1G>A (P2394) was born of parents who were second-generation cousins. Genetic tests on the parents indicated that both were heterozygotes of IVS6+1G>A. The scores of predicted splicing sites decreased significantly compared with the wild sequence (Table 2). Western blot analysis of the liver protein of this patient revealed the disappearance of the normal band but the appearance of two size-decreased bands (about 63 and 68 kDa in Fig. 1b).

The mutation L85P, which represents a T>C substitution at position 254 in exon 4 and an amino acid change from leucine to proline at position 85, was found in P3174. Analysis on the alignment of amino acids residual reservation in different species showed that the locative amino acid is highly conserved (Fig. 2). L85P was found in a compound heterozygote with E601K (Table 1). Western blot analysis with anti-human citrin antibody revealed the absence of citrin protein in the liver specimen (Fig. 1c), indicating that this mutation leads to citrin deficiency.

R585H, first reported in two Japanese patients with CTLN2 or NICCD [18, 35] with no detailed description, represents a G>A substitution at position 1754 in exon 17 and leads to an arginine to histidine substitution at position 585. Conservation analysis in different species indicated that the amino acid in this position is highly conserved [23], except in *Danio rerio* (Fig. 2). This is the first report of a Chinese patient with the R585H mutation.

Secondary structural prediction of the two missense mutations, L85P and R585H, using YASPIN showed that these variations in amino acids did not affect the secondary structure of citrin protein. The PSIC for the normal amino acid L85 and R585 is 1.54 and 2.133, respectively, and for the variant amino acid 85P and 585H, −1.007 and −0.335, respectively; thus, the absolute difference between the two

Table 2 The score change of splice sites of splicing mutations deduced using MaxEntScan

Exon 6	Scoring models			
	MAXENT	MDD	MM	WMM
Wild sequence	8.56	12.68	6.46	5.41
IVS6+1G>A	0.38	4.50	−1.73	−2.78
IVS6+1G>C	0.29	4.40	−1.82	−2.87

IVS6+1G>C is a mutation type reported by Lu et al. [25]

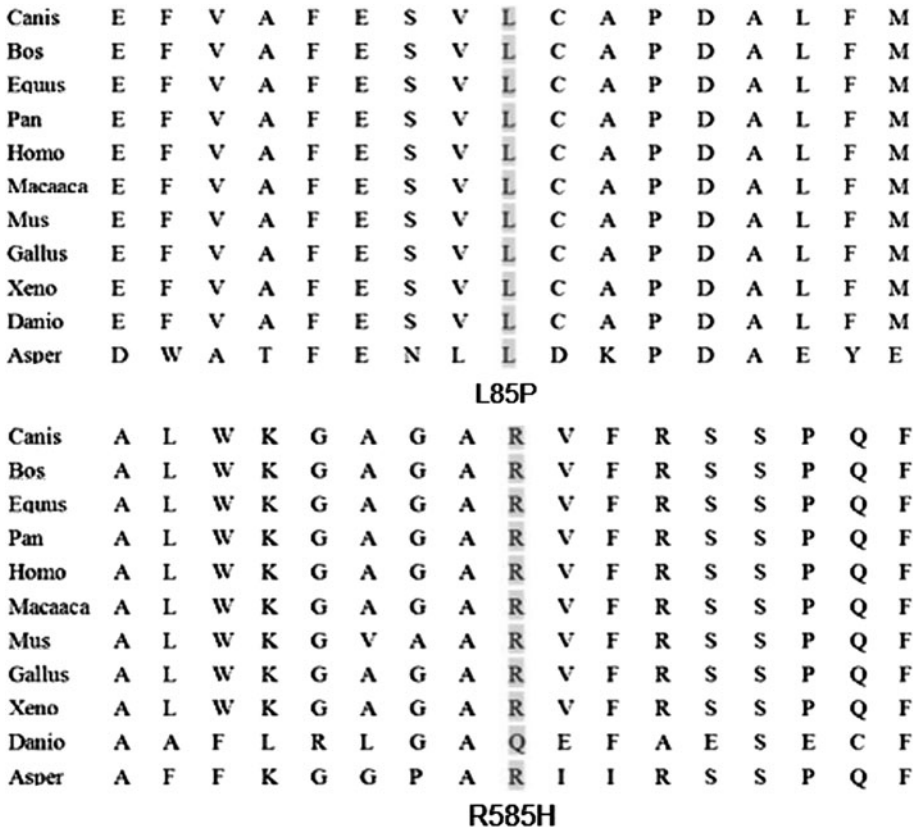

Fig. 2 Conservation analysis of SLC25A13 gene mutations L85P and R585H among different species

profile scores is 2.547 and 2.468, respectively, indicating that both missense mutations have a high chance of affecting protein function.

The mutation 1092_1095delT (one of 4 conjoint bases of T deletion within nt 1092–1095 in exon 11) found in P3013, predicting a frame shift and the introduction of a stop codon at position 407, may lead to premature truncation of the citrin protein.

Mutation 1092_5delT and IVS6+1G>A were detected by PCR and the direct sequencing method. Mutation L85P was screened by RTD-PCR with dual-labeled probes L85P-FP 5′-AATATCTTTTCAAGAATTTGTTGCC-3′, L85P-RP 5′-GGTAGCCTTTCAGCTGTTTGAC-3′; fluorescence-labeled probes are MAR-TCTGTCCCGTGTGC CC-MAR and JUP-TCTGTCCTGTGTGCCC-JUP. Each of the above mutations was screened in 50 control infants, and none was detected in the 100 control alleles.

Aminoacidemia and the incidence of SLC25A13 gene mutations

To explore the relationship between SLC25A13 mutations and citrullinemia, we compared the data among three patient subgroups. Citrulline elevated more than 1.5-fold the upper normal limit (UNL) was found in 28 patients, including 19 of the 22 cases that could be definitely diagnosed as citrin deficiency, five of the six patients with probable citrin deficiency, and four of the 11 infants with unlikely citrin deficiency (no mutation was detected) (Table 1). Citrullinemia was more frequently found in patients with a definite diagnosis of citrin deficiency than in those in whom citrin deficiency was unlikely (definite diagnosis vs. unlikely, $P = 0.006$; probable vs. unlikely, $P = 0.131$).

Among the 11 patients with normal levels of plasma citrulline (P4129, P4115, P4487, P2338, P4542, P2769, P4684, P2516, P4068, P3013, P4163), the homozygous mutation was found in one patient (P4163) and the compound heterozygous mutation was found in two patients (P3013 and P4068).

Discussion

To the best of our knowledge, this is the first study on the SLC25A13 gene mutation spectrum in infants with intrahepatic cholestasis and various forms of aminoacidemia in mainland China. The design is different from those of the previous studies in which mutation analysis was generally only carried out on individuals for whom a diagnosis of citrin deficiency is highly suspected based on citrullinemia detected by MS analysis [6, 20, 32–39]. In contrast, we tested all cases of various forms of aminoacidemia, including those patients with citrulline in normal range and

those who were given a possible diagnosis of tyrosinemia or aminoacidemia secondary to liver diseases on the basis of MS amino acid analysis. More than one half of the patients were given a definitive prognosis of citrin deficiency, including not only those with citrullinemia but also those with forms of aminoacidemia other than citrullinemia. This means that the SLC25A13 gene mutation is the most important cause of infantile intrahepatic cholestasis and various forms of aminoacidemia in this region.

Mutations 851del4 and IVS11+1G>A are two of the most prevalent mutations in Japan and Korea [25]. However, mutation IVS11+1G>A has never been reported in the Chinese population [23, 25], and we found only one mutant allele with IVS11+1G>A (P4405) in our study. Among our patients, mutations 851del4 and 1638ins23 were the two most common mutations. Mutation IVS6+5G>A, which had been identified to be the second most common mutation in the south area of China [23, 25], was found in only two patients (P4554 and P4068) of our study from the south area of China. This result suggests that mutation types have regional specificity within China.

Mutation IVS6+1G>A has not been reported in other countries to date. However, mutation IVS6+1G>C at the same site (named mutation XIV), but not G>A, has been reported [25]. Theoretically, the deduced product of mutation IVS6+1G>A should be much smaller than approximately the 63- and 68-kDa products revealed by Western blot analysis. The size difference may be caused by abnormal splicing, leading to the deletion of exons 7, 8, and 9 (total 318 nt, about 11 kDa) or the deletion of exon 6 (147 nt, about 6 kDa).

The presence of mutation L85P indicates a base substitution in exon 4, located in the middle part between the second and the third EF-hand domain (http://srs.ebi.ac.uk/cgi-bin/wgetz?-id+newId+-e+[SWALL-ACC:Q9UJS0]#Features), which is conserved in calcium-binding proteins [1]. To date, no mutation has been reported in this exon [18]. Western blot analysis on this patient (P3174; see Fig. 1c), with compound heterozygote E601K/L85P showing no detectable peptide, indicated that the mutation led to citrin deficiency. R585H is located in the sixth TM spanning one of the most functional domains of citrin protein. Mutations in this area may cause the abnormal function of citrin protein and result in clinical manifestations. 1092_1095delT leads to a premature truncated protein.

NICCD is a complicated metabolic disorder that is difficult to distinguish from other causes of hepatic disease. Aminoacidemia is one of the more important features of NICCD, but the diagnosis is difficult without early monitoring of amino acid levels [24]. MS to detect citrin deficiency is useful in identifying the clinical course, treatment, and prevention of this disease [38]. In this study, citrin deficiency was not only found in patients with citrullinemia, but also in patients with aminoacidemia other than citrullinemia, suggesting that although citrullinemia is a very useful parameter for the diagnosis of citrin deficiency, the diagnosis cannot be ruled out even if the level of citrulline is within normal range.

Based on amino acid profile, there were three suspected cases of possible tyrosinemia (P3013, P4068 and P2769). However, fumarylaceteoacetic acid hydrolase (FAH) gene sequencing did not reveal any mutation (data not shown). The SLC25A13 compound heterozygote was found in two patients (P3013 and P4068), and patient P2769 had no mutation (Table 1). Therefore, citrin deficiency should be considered in patients with any form of aminoacidemia, including tyrosinemia.

No mutation was detected in 11 patients with aminoacidemia. The amino acid profile of these patients is significantly different from that of patients with a definite diagnosis of citrin deficiency (Table 1). These 11 patients may have other metabolic disturbances, such as tyrosinemia, galactosemia [40] or just secondary to liver diseases other than citrin deficiency. Hence, the diagnosis of NICCD cannot be made based solely on the various forms of aminoacidemia, similar to the diagnosis of NICCD not being established based only on clinical manifestations and biochemical changes [13].

One limitation of this study was that we did not perform Western blotting on lymphocytes. We did attempt this in a previous study [41], but unfortunately failed. Consequently, we were unable to determine whether those patients that carried only one mutation allele were carriers or citrin-deficiency patients. Another limitation to our study was that direct sequencing may miss the mutation occurring in the primers and that the deletion/insertions of a large fragment also could be determined. This may explain why the second mutation was not find in patients cases with citrin deficiency (P3156, P2625 and P2434).

In conclusion, the results of this study indicate that SLC25A13 gene mutations play an important role in Chinese infants with intrahepatic cholestasis and various forms of aminoacidemia. 851del4 and 1638ins23 are the most common mutation types. Three novel mutations were found in our cohort of patients, which has expanded the SLC25A13 gene mutation spectrum.

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