表 1　対象者の QOL および倦怠感得点

	子ども（自己評価）			親（代理評価）		
	n	平均点±SD	α	n	平均点±SD	α
倦怠感						
合計	39	68.8±14.9	0.81	48	77.7±18.2	0.94
一般的疲労	39	69.8±21.8	0.82	49	78.0±20.9	0.92
睡眠／休息	39	70.0±22.8	0.81	48	76.4±21.6	0.90
認知的疲労	39	66.8±21.9	0.80	48	77.7±18.2	0.96
QOL 得点						
合計	37	78.1±12.4	0.70	51	82.2±14.6	0.91
身体的機能	37	80.9±14.6	0.68	51	85.9±17.9	0.86
感情の機能	37	71.1±17.0	0.49	51	78.3±18.2	0.77
社会的機能	37	82.8±14.6	0.62	51	83.9±16.7	0.81
学校	37	75.9±17.2	0.63	51	78.7±16.9	0.65

α；クロンバックのα係数

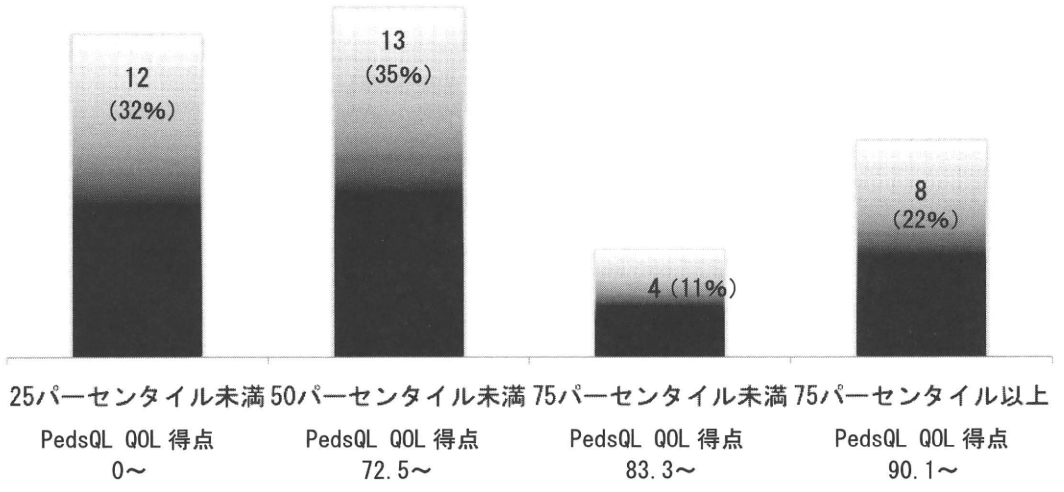

図 1　健康児の PedsQL QOL 尺度得点より算出のパーセンタイルに対するシトリン欠損症患者の分布

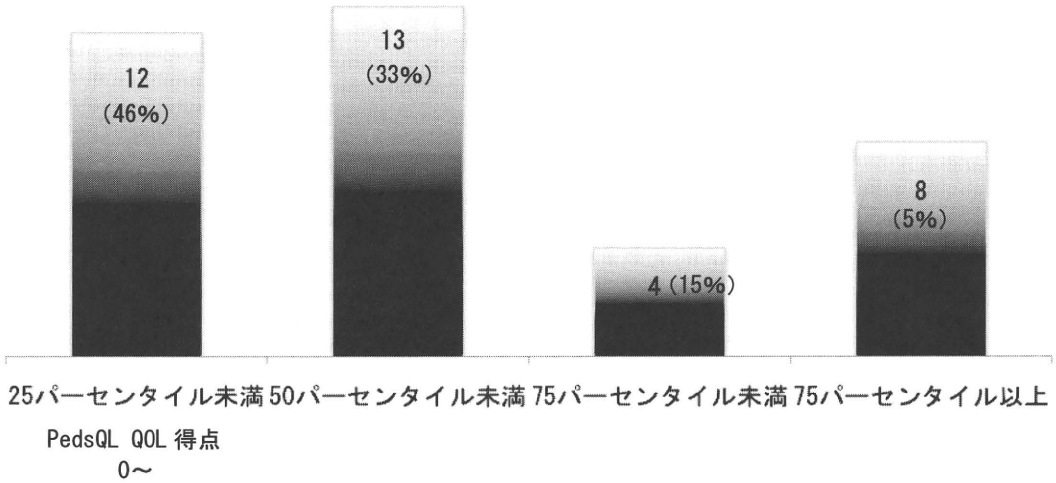

図2　健康児のPedsQL 倦怠感尺度得点より算出のパーセンタイルに対するシトリン欠損症患者の分布

2．倦怠感とQOLとの関連

表2にPedsQL 倦怠感尺度得点とPedsQL QOL尺度得点間の相関係数を示した．倦怠感尺度とQOL尺度の下位尺度ごとの相関係数は，シトリン欠損症患者自己評価では，全般的疲労0.26–0.45，睡眠／休息0.34–0.57，認知的疲労0.09–0.41で，QOLの合計得点および，すべての下位尺度について，睡眠／休息の疲労が最も強い関連を示した．また，倦怠感の合計点は，学校に最も強い相関を示した（$r = 0.52$）一方，親の代理評価では，全般的疲労0.59–0.70，睡眠／休息0.38–0.52，認知的疲労0.52–0.66であった．親の代理評価では，QOL合計得点は，全般的疲労との関連が最も強く，身体的機能，感情の機能，社会的機能についても同様であった．学校のみ，認知的疲労との関連が最も強かった．

3．シトリン欠損症患者と親の評価間差

患者–親間の倦怠感およびQOL認識についての評価間差を示すSpearmanの順位相関係数（ρ），級内相関係数（ICC），絶対差，差の方向性を表3に示した．相関係数および，級内相関係数では，患者–親間の一致は，中～大の程度であった．得点の差を示す絶対差と差の方向性に関しては，PedsQL疲労尺度で，認知的疲労において患者–親間の得点差が大きく，PedsQL QOL尺度で，感情の機能で最も得点差が大きかった．また，PedsQL疲労尺度，QOL尺度とも差の方向性では，親は患者の状態を患者よりも良好であると捉えていることを示す，正の得点差を示した．

表 2　PedsQL 倦怠感尺度と PedsQL QOL 尺度の関連

	n	倦怠感合計	全般的疲労	睡眠／休息	認知的疲労
シトリン欠損症患者（自己評価）					
QOL 合計	37	0.58	0.45	0.57	0.28
身体的機能	37	0.24	0.26	0.57	0.24
感情の機能	37	0.28	0.32	0.34	0.09
社会的機能	37	0.31	0.28	0.42	0.41
学校	37	0.52	0.31	0.49	0.17
親（代理評価）					
QOL 合計	37	0.71	0.70	0.47	0.63
身体的機能	37	0.60	0.62	0.38	0.52
感情の機能	37	0.59	0.59	0.39	0.54
社会的機能	37	0.68	0.65	0.41	0.64
学校	37	0.69	0.57	0.52	0.66

Spearman の順位相関係数 r

表 3　評価間差

	n	相関係数 ρ	ICC	絶対差 平均点±SD	差の方向性 平均点±SD	d
倦怠感						
合計	34	0.52	0.60	14.3±9.9	9.7±14.5	0.67
全般的疲労	35	0.37	0.51	14.0±12.6	5.8±17.9	0.32
睡眠／休息	34	0.58	0.73	15.3±11.7	10.0±16.5	0.61
認知的疲労	34	0.57	0.56	21.2±15.7	13.4±22.8	0.59
QOL 得点						
合計	36	0.55	0.53	10.5±8.9	5.5±12.7	0.43
身体的機能	36	0.47	0.44	12.9±13.6	2.3±18.7	0.12
感情の機能	36	0.50	0.42	16.7±14.5	9.6±20.0	0.48
社会的機能	36	0.35	0.38	14.2±13.9	5.7±19.1	0.30
学校	36	0.52	0.52	10.3±11.5	3.6±15.3	0.24

D. 考察

シトリン欠損症患者の倦怠感尺度およびQOL得点は，健康児と比較して低く，より低い QOL とより強い倦怠感をもっていることが明らかになった．特に，倦怠感においては，対象者の約 7 割が健康児の得点の 50 パーセンタイル値以下であり，この対象の倦怠感に対する介入の必要性が示されたと考える．先行研究で報告されているPedsQL 倦怠感尺度合計得点と比較すると，糖尿病患者（73.5 点）（Varni, et al., 2009），リウマチ患者（73.8 点）（Varni, Burwinkle, & Szer, 2004），がん患者（71.0 点）（Varni, et al., 2002）より低く，肥満患者（67.7 点）（Varni, et al., 2010），脳腫瘍患者（69.7 点）（Palmer, et al., 2007）とほぼ同等の得点であった．

倦怠感と QOL に関連において，患者自己評価では，小から中程度の相関がみられたのに対して，親の代理評価では，中から大の相関がみられシトリン欠損症の親が患者自身よりも倦怠感が QOL と強く関連していると考えていることが明らかになった．

患者と親の評価間差に関して，相関係数および ICC が中程度（倦怠感 0.51～0.73，QOL0.42～0.53）の効果量を示したことは，先行研究と比して（Varni, et al., 2009,Varni, et al., 2010）高い一致を示す結果であった．しかし，評価者間の得点の差をみると，差の方向では合計得点およびすべての下位尺度で正の差がみられ，親が患者よりも倦怠感を過小評価していること，QOL を過大評価していることが明らかになった．

E. 結論

シトリン欠損症患者の倦怠感は，健康な子どもに比して強く，QOLは低いことが明らかになった．また，倦怠感合計点とQOL合計点間に，強い相関が示され，両者の関連が明らかになった．さらに，シトリン欠損症患者と親の倦怠感およびQOL評定の評価者間差は，中程度であり，親は患者自身よりも，倦怠感を低く，QOLを高く評定しており，患者自身よりも患者の健康状態を良好であると捉えていることが明らかになった．

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F. 健康危険情報

なし.

G. 研究発表

なし.

H. 私的財産権の出願・登録状況

なし.

〔III〕　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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〔IV〕　研究成果の刊行物・別刷

J Inherit Metab Dis
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CASE REPORT

Neonatal intrahepatic cholestasis associated with citrin deficiency (NICCD): a case series of 11 Malaysian patients

Hui Bein Chew · Lock Hock Ngu · Md Yunus Zabedah · Wee Teik Keng · Shanti Balasubramaniam · Mohd Jamil M. Hanifah · Keiko Kobayashi

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Abstract Citrin deficiency, aetiologically linked to mutations of SLC25A13 gene, has two clinical phenotypes, namely adult-onset type II citrullinaemia (CTLN2) and neonatal/infantile intrahepatic cholestasis, caused by citrin deficiency (NICCD). Malaysian patients with NICCD, especially of Malay and East Malaysian indigenous descent, have never been reported in the literature. We present the clinical features, biochemical findings and results of molecular analysis in 11 Malaysian children with NICCD. In this case series, all patients manifested prolonged cholestatic jaundice and elevated citrulline levels. The other more variable features included failure to thrive, bleeding diathesis, hypoproteinaemia, abnormal liver enzymes, prolonged coagulation profile, hyperammonaemia, hypergalactosaemia, multiple aminoaciduria, elevated α-feto protein and urinary orotic acid as well as liver biopsies showing hepatitis and steatosis. DNA analysis of SLC25A13 revealed combinations of 851del4 (Ex9), IVS16ins3kb and 1638ins23. Most of our patients recovered completely by the age of 22 months. However, one patient had ongoing symptoms at the time of reporting and one had died of liver failure. Since a small percentage of children with NICCD will develop CTLN2 and the mechanisms leading to this is yet to be defined, ongoing health surveillance into adulthood is essential.

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Competing interests: None declared.

References to electronic databases: NICCD: OMIM #605814
Citrin: Solute Carrier Family 25, Member 13; SLC25A13 OMIM *603859

H. B. Chew (✉)
Genetic and Metabolic Unit, Department of Paediatrics,
University Malaya Medical Centre,
50603 Kuala Lumpur, Malaysia
e-mail: hbchew155@hotmail.com

H. B. Chew · L. H. Ngu · W. T. Keng · S. Balasubramaniam
Department of Medical Genetics, Institute of Paediatrics,
Kuala Lumpur Hospital,
Jalan Pahang,
50586 Kuala Lumpur, Malaysia

M. Y. Zabedah
Biochemistry Unit, Specialised Diagnostic Centre,
Institute for Medical Research,
50586 Kuala Lumpur, Malaysia

M. J. M. Hanifah
Department of Paediatrics, Hospital Raja Perempuan Zainab II,
15590 Kota Bahru, Malaysia

K. Kobayashi
Department of Molecular Metabolism and Biochemical Genetics,
Kagoshima University Graduate School of Medicine and Dental Sciences,
Kagoshima 890-8544, Japan

Introduction

Citrin, a bipartite protein located in the mitochondrial inner membrane, functions as a calcium-binding/stimulated aspartate-glutamate carrier that is a component of the malate-aspartate NADH (nicotinamide adenine dinucleotide) shuttle. It is mainly expressed in the liver and plays an important role in the metabolic pathways of aerobic glycolysis, gluconeogenesis, urea cycle, and synthesis of proteins and nucleotides (Kobayashi et al. 1999; Saheki and Kobayashi 2002; Saheki et al. 2002, 2004; Tamamori et al. 2004). The deficiency of citrin results in two main clinical phenotypes: the adult-onset type II citrullinaemia (CTLN2) that mainly presents with neuropsychiatric symptoms of confusion, seizure, coma and death associated with hyperammonaemia, and the neonatal/infantile form of transient intrahepatic cholestatic hepatitis (NICCD: OMIM #605814) Table 1 and Table 2.

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Table 1 Summary of clinical characteristics of 11 infants with NICCD

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Sex	Male	Female	Male	Female	Female	Female	Male	Female	Male	Male	Female
Ethnic group	Chinese	Malay	Chinese	Chinese	Malay	Malay	Iban	Kadazandusun	Malay	Chinese	Malay
Birth weight (kg)	2.5	2.3	2.46	2.95	2.44	2.1	2.8	2.5	2.5	2.6	2.5
Consanguinity	No	No	?	No	No	Yes	No	No	No	No	No
Type of feeding (duration)	BF (3 weeks)	Mix	IF	Mix	BF (5 weeks)	BF (6 months)	BF (3 months)	BF	Mix	Mix	BF
Age at Px[a] (weeks)	3	8	8	8	5	20	4	10	8	8	4
Px[a] complaint	J	J	J	J	J	J	J	J	J	J	J
FTT[b]	+	+	+	-	-	+	-	+	-	-	-
Hepatomegaly	+	+	-	-	-	+	+	+	-	+	+
Splenomegaly	+	+	-	-	-	+	-	+	-	-	-
Development	N	N	Mild delay	N	N	N	N	Mild delay	N	N	N
Resolution of symptoms and biochemistry (age in months)	6½	14	16	5	Ongoing	9	6	Died at 9 months	Ongoing	5	Ongoing

BF Breast fed, *IF* infant formula, *J* jaundice, *N* normal

[a] *Px* Presentation

[b] *FTT* Failure to thrive

Children with NICCD, a recessively inherited condition, are typically small for gestational age and present in infancy with prolonged cholestatic jaundice and failure to thrive. Biochemical findings include hypoproteinaemia, decreased coagulation factors, haemolytic anaemia, mild liver dysfunction, ketotic hypoglycaemia, hypergalactosaemia, multiple aminoacidaemia including high citrulline, arginine, threonine, methionine and tyrosine, and high levels of α-feto protein. Liver biopsy may show diffuse fatty infiltration and hepatic fibrosis. These symptoms generally disappear by 1 year of age and are followed by a peculiar aversion to carbohydrate-rich food and a preference for protein and lipid-rich food (Tazawa et al. 2004; Ohura et al. 2007). Although benign in most, some children with NICCD may develop liver failure or CTLN2 decades later requiring liver transplant (Tamamori et al. 2002; Tomomasa et al. 2001).

Citrin deficiency was initially reported solely in Japanese subjects. However, recent literature reports of non-Japanese patients have emerged, including those of Chinese origin (residing in China and Taiwan), Korean, Vietnamese (residing in Taiwan, China, Korea, Australia, France and Czech Republic), Palestinian, Israeli and Caucasian (residing in the United Kingdom and United States of America) (Ben-Shalom et al. 2002; Luder et al. 2006; Yeh et al. 2006; Dimmock et al. 2007; Ko et al. 2007). The SLC25A13 gene encodes citrin and, to date, 32 different mutations have been identified including the very specific IVS16ins3kb found relatively frequently in Japanese patients but not outside the East Asian population (Tabata et al. 2008).

Malaysia has a heterogenous population, with Malay being the predominant ethnic group followed by Chinese, Indian and indigenous subpopulations such as Iban and Kadazandusun. NICCD patients of Malay and East Malaysian indigenous descent have never been reported in the literature. We present 11 patients, including 5 Malay, 4 Chinese and 2 East Malaysian indigenous children, with clinical and biochemical features consistent with NICCD. DNA diagnosis for SLC25A13 mutations revealed combinations of the mutations 851del4(Ex9), IVS16ins3kb and 1638ins23 in our patients. The recently described mutation IVS16ins3kb was found solely in Japanese, Chinese and Korean patients. In this study, we report for the first time the IVS16ins3kb mutation in 4 patients of Malay descent.

Patients

Patient 1 had jaundice on day 3 of life which resolved with 4 days of phototherapy. He was the youngest of 4 children, all of whom had physiological jaundice in infancy. At 3 weeks old, he was re-admitted with increasing cholestatic jaundice, hypoproteinaemia and mildly elevated liver trans-

Table 2 Summary of laboratory data of 11 infants with NICCD at presentation

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Citrulline (6 – 22 μmol/l)[a]	30	59	332	965	355	NA	128.63	61	176.12	265	440
threonine (89 – 232 μmol/l)	304	472	N	1054	352.1	NA	NA	126	NA	595	465
methionine (17 – 43 μmol/l)	74	149	311	360.1	123.4	NA	81.98	325	113.04	146	456
tyrosine (30 – 193 μmol/l)	145	130	N	365	N	NA	217.89	320	219.87	N	N
arginine (46 – 172 μmol/l)	237	164	220	364.3	124	NA	2.66	125	16.85	199	146
Urine reducing sugar	+	-	+	+	+	-	-	+	-	-	+
Total Galactose (DBS[b]) (NR <552 μmol/l)	99	NA	NA	NA	4,245	NA	NA	157	4,287	NA	7,060
T. bili[c] (3 – 17 μmol/l) [direct]	130.3 [68]	164 [53.3]	277 [173]	136 [75]	277 [111]	75 [31]	153 [76]	809 [368]	181 [127]	202 [158]	218 [168]
Albumin (35 – 50 g/l)	27	25	22	27	27	34	4.3	29	31	25	23
ALP[d] (117 – 390U/l)	1,058	611	1,379	1,144	958	780	1,015	661	1,374	1,475	1,815
AST[e] (0 – 50U/l)	104	153	95	112	NA	127	67	202	97	187	30
ALT[f] (6 – 39U/l)	53	49	29	48	20	41	27	67	35	48	31
Ammonia (14 – 55 μmol/l)	22.9	41	151	76.4	NA	124.5	46.9	110	NA	NA	NA
Hypoglycaemia	No	No	No	No	No	No	No	No	No	No	No
α-fetoprotein (ng/ml)[g]	30716.3	27480.5	NA	>35,000	NA	1307.6	NA	NA	NA	NA	NA
Hepatobiliary Ultrasound	Hepato-splenomegaly	Hepato-splenomegaly	N	N	N	Enlarge, fatty liver+ splenomegaly	NA	Hepatosplenomegaly +R hydronephrosis	N	NA	NA
Liver biopsy	NA	NA	NA	NA	NA	Hepatitis with steatosis	NA	Neonatal hepatitis	NA	NA	NA
SLC25A13 Mutation	Htz[i] 851del4 (Ex9)	Hmz[h] IVS16ins3kb	Hmz[h] 851del4 (Ex9)	Htz[i] 851del4 (Ex9)	None found	Hmz[h] IVS16ins3kb	None found	None found	Htz[i] 851del4 (Ex9) and Htz[h] IVS16in3kb	Htz[i] 851del4 (Ex9) and Htz[h] 1638ins23	Htz[i] IVS 16ins3kb

N Normal, *NA* Not applicable

[a] Normal range in parentheses

[b] Dried blood spot

[c] Total bilirubin

[d] Alkaline phosphatase

[e] Aspartate transaminase

[f] Alanine transaminase

[g] Normal range of α-fetoprotein in adult is <50 ng/ml. At birth, infants have 4 or more orders of magnitudes above this normal range, decreasing to it within the first 2 years of life

[h] Homozygote

[i] Heterozygote

aminases. A diagnosis of breast milk jaundice was made in light of normal G6PD, thyroid function, full blood count, negative Coomb's test and normal infective hepatitis screen. However, an increasing trend of conjugated hyperbilirubinaemia, unsatisfactory weight gain and hepatosplenomegaly prompted further investigations at 10 weeks old. Radio-imaging excluded biliary atresia. α-feto protein was markedly elevated and plasma amino acid profile was consistent with citrin deficiency. He was commenced on lactose free milk, vitamin K and multivitamins. Over the course of the next 4 months, he sustained good weight gain with normal developmental milestones and resolution of jaundice, hepatosplenomegaly and α-feto protein. Plasma amino acid normalised at 15 months old. At 17 months of age, he suffered 4 episodes of generalised tonic clonic convulsions, 2 of them associated with fever and 2 episodes unexplained. Electroencephalogram was normal. Growth, physical examination and biochemical data remained normal at 7 years old.

Patient 2 was first noticed to be jaundiced at 2 months old. Her older sister reportedly suffered physiological jaundice in infancy. Jaundice persisted at 4½ months old associated with 3-cm hepatomegaly and 2-cm splenomegaly below the costal margin. Abdominal ultrasonography excluded biliary atresia. Abnormal investigations were liver function tests, coagulation profile, lactate and α-feto protein. Urine orotic acid was mildly elevated at 5.2 Mmol/mol creat (NR 1.0–3.2). Extensive investigations ruled out infective hepatitis, storage diseases and neonatal haemachromatosis. She was empirically treated as having a mitochondrial respiratory chain disorder, and received multivitamins, folic acid, vitamin B complex and advice on avoidance of fasting. Serum amino acid subsequently revealed a profile consistent with NICCD and the diagnosis was supported by DNA analysis of SLC25A13. Mitochondrial cocktail was discontinued. Growth was slow in the first year of life with an average increase of 400 g per month; however, this eventually normalised. Citrullinaemia resolved at 8 months of age with normalisation of liver function and urine orotic acid at 14 months of age. In her second year of life, she was noticed to have a peculiar fondness for protein-rich food like peanuts and seafood and an aversion to carbohydrate-rich food. She is currently 6 years old and enjoys excellent health.

Patient 3 is a 4-year-old Chinese boy from the east of Malaysia. Antenatal and family history was unobtainable as he was an adopted child. He was admitted at 1 month old with cholestatic jaundice and elevated liver transaminases that were attributed to an overdose of Dothiepin and Diazepam, antidepressants taken by his biological mother. His urine reducing sugars were recorded as 4+. He was discharged well after this event but re-presented at 2 months old with increasing cholestatic liver dysfunction. NICCD was diagnosed based on serum amino acid and DNA analysis of SLC25A13. He was commenced on lactose free milk with supplementation of fat-soluble vitamins and MCT oil. Cholestasis resolved at 16 months old and Citrulline level normalised at 22 months of age. This patient also demonstrated a preference for protein-rich foods with an aversion to sugary foods. Currently, his growth is normal, but development is delayed especially in the area of speech.

Patient 4 is a 3½-year-old Chinese girl who had transient neonatal physiological jaundice on day 3 of life. This resolved with natural sunlight but subsequent intermittent jaundice led to further investigations at 2 months of age. NICCD was diagnosed based on clinical phenotype, conjugated hyperbilirubinaemia, plasma amino acid profile and markedly elevated α-feto protein. She received lactose free formula milk, supplementation of fat soluble vitamins and MCT oil. All investigations normalised at 5 months old. Her physical examination, growth and development are normal at the time of reporting.

Patient 5 had jaundice on day 4 of life which persisted at 5 weeks old. She was otherwise clinically well and thriving. Investigations including plasma amino acid profile and elevated total galactose level were consistent with NICCD. Breast feeding was continued supplemented by lactose free milk. At her current age of 11 months old, development and physical examination are normal. Plasma citrulline level remained high at 328.2 μmol/l.

Patient 6 is a 6-year-old Malay girl born via emergency caesarean section to parents who are second cousins. Of her 4 siblings, one older brother (current age 19 years) had a history of prolonged neonatal jaundice that resolved without sequelae. She had jaundice on day 3 of life which resolved with 2 days of phototherapy. She re-presented at 5 months old with cholestatic jaundice, poor weight gain, 3-cm hepatomegaly and 1-cm splenomegaly below the costal margins. Ultrasonography of the hepatobiliary system showed hepatosplenomegaly with features of fatty change. A liver biopsy showed generalised macrovesicular steatosis with bile stasis, portal tract expansion by lymphocytic infiltrate with associated interface hepatitis. Unfortunately, plasma amino acid was not performed at the acute stage, but elevated ammonia and α-feto protein accompanied by homozygocity for IVS16ins3kb mutation in SLC25A13 gene led to the diagnosis of NICCD. Urine orotic acid was elevated at 8 Mmol/mol creat. In addition, she had hypokalaemia and hypophosphataemia with normal anion gap metabolic acidosis that was attributed to renal tubulopathy. This resolved spontaneously without intervention. Jaundice resolved at the age of 9 months with normalisation of liver transaminases at the age of 1 year and 3 months. Plasma amino acid is now normal.

Patient 7, the 3rd child of Ibanese (Malaysian aborigines) parents, presented at 1 month old with jaundice which persisted to 6 months of age. Examination revealed 5-cm hepatomegaly below the costal margin. NICCD was diagnosed based on clinical phenotype and amino acid profile. He was commenced on lactose-free diet with supplementation of multivitamins. At his current age of 3 years, he remains clinically well.

Patient 8, the 4th child of Kadazandusun (Malaysian aborigines) parents, presented at 10 weeks old with prolonged conjugated hyperbilirubinaemia. Investigations ruled out biliary atresia and liver biopsy showed neonatal hepatitis. She was followed up in the district hospital as liver disease progressively worsened. At 7 months old, she was admitted in a decompensated state with liver failure, coagulopathy and gastrointestinal bleed precipitated by sepsis. Physical examination revealed growth parameters below the 3rd percentile, jaundice, ascites and hepatosplenomegaly recorded at 4.5 cm and 5 cm below the costal margins, respectively. Doppler ultrasound of the abdomen suggested cirrhotic changes in the liver, gross splenomegaly and mild right hydronephrosis. Liver enzymes and coagulation profile were abnormal with ongoing bleeding requiring rescue with fresh frozen plasma. Full blood count showed normocytic normochromic anaemia. Investigations for infective hepatitis, autoimmune hepatitis, hypothyroidism, Wilson's disease, α-1-Anti-Trypsin deficiency yielded negative results. Urine reducing sugars were positive but total galactose level was normal. Serum amino acid profile was consistent with citrin deficiency. She was treated with lactose free diet, multivitamins, folate and vitamin D. Unfortunately, she passed away from liver failure shortly after.

Patient 9 is a 6-month-old Malay boy, who had transient physiological jaundice at 2 days old, re-presented at 2 months old with conjugated hyperbilirubinaemia. Physical examination was unremarkable. Investigations ruled out biliary atresia, hypothyroidism and congenital infections. Plasma amino acid profile, elevated galactose level and DNA analysis of SLC25A13 led to the diagnosis of NICCD. He was commenced on lactose-free milk as a supplement with continuation of breast feeding.

Patient 10 is a 5-month-old Chinese boy who presented with prolonged cholestatic jaundice at 2 months old. Physical examination revealed a well and thriving child with jaundice and 3-cm hepatomegaly below the costal margin. Plasma amino acid profile was consistent with NICCD and this was supported by DNA analysis of SLC25A13.

Patient 11 is a 3-month-old Malay girl who presented with prolonged cholestatic jaundice at 1 month old. Clinically, she was jaundiced, has bilateral cataract and 3-cm hepatomegaly. Citrin deficiency was diagnosed based on clinical and biochemical data. She was commenced on lactose-free diet and examination at 3 months of age showed resolving jaundice and cataracts.

Molecular analysis

Fifteen known common mutations among Asians were screened according to the methods described previously by using GeneScan/SNaPshot and/or PCR/gel running (Tabata et al. 2008). These mutations are: (1) [851del4 (Ex9)]; (2) (g.IVS11+1 G>A); (3) [1638ins23 (Ex16)]; (4) [S225X (Ex7)], (5) (g.IVS13+1 G>A); (6) [1800ins1 (Ex17)]; (7) [R605X (Ex17)]; (8) [E601X (Ex17)]; (9) [E601K (Ex17)]; (10) (g.IVS6+5 G>A); (11) [R184X (Ex6)]; (14) (g.IVS6+1 G>C); (16) [G531D (Ex16)]; (19) (g.IVS16ins3kb); (20) (g.Ex16+74_IVS17-32del516).

Discussion

The diagnosis of NICCD can be reliably achieved by molecular studies as mutations of the SLC25A13 gene had been established as the cause of citrin deficiency. However, studies have estimated that mutations are not detected in about 15% of cases (Tokuhara et al. 2007). So far, 32 mutations have been described and novel mutations are still being discovered. The phenotype of NICCD patients have been delineated in studies over the past decade (Ohura et al. 2007; Saheki et al. 2002, 2004; Tazawa et al. 2004). The diagnosis of NICCD in our series is confirmed by molecular studies in 5 patients (patient 2, 3, 6, 9, 10); in the other 6 patients, published clinical phenotype and biochemical data form the basis of our diagnosis. Interestingly, all our patients who carry the mutation IVS16ins3kb are from the Malay ethnic group. This mutation was thought to have occurred historically very early on in East Asia and has never been reported outside the Japanese, Chinese and Korean populations. This may lend support to the theories regarding the origin of Malaysians of Malay descent. One theory postulates migration from Yunnan, China; another states that they are sea migrants originally from Taiwan. Genetic heterogeneity exists among different races in the mutations of SLC25A13. This may be the other reason that abnormal mutations were not found in patients 7 and 8, children of Iban and Kadazandusun descent. The Ibans are the original inhabitants of Borneo Island and Kadazandusun is an ethnic group indigenous to the state of Sabah in East Malaysia (North Borneo). With advancement in this field, these samples should be re-tested for novel mutations of SLC25A13.

The characteristic clinical feature of NICCD is neonatal hepatitis associated with cholestasis. Some patients are

picked up on newborn screening with hypercitrullinaemia, hypermethioninaemia, and hypergalactosaemia prior to emergence of symptoms. All our patients presented with cholestatic jaundice. Three of these patients had citrullinaemia detected on high risk dried blood spot screening using tandem mass spectrophotometry (TMS) on presentation (patients 5, 7, 9). Asymptomatic infants with NICCD may be picked up when nationwide newborn screening is established in Malaysia.

The age of presentation ranged from 3 weeks to 5 months, occurring most commonly around 2 months old. Previous reports have suggested that symptoms and signs in NICCD resolve completely by 1 year old if uncomplicated by liver failure. In 2 of our patients, cholestasis resolved after the age of 1 year at 14 and 16 months respectively (patients 2 and 3) and in patient 3, citrulline level normalised at the age of 22 months. 3 other patients had ongoing symptoms at the time of reporting (patients 5, 9, 11) and patient 8 died from liver failure at the age of 9 months.

Children with NICCD are generally small for their gestational age; this is thought to be due to intrauterine citrin deficiency (Tamamori et al. 2004). Our patients, all born at term, had birth weights ranging from 2.1 to 2.95 kg. Mean birth weight was 2.5 kg (± 0.32 SD), at the lower limit of normal. However, since Asian children may be smaller than Caucasians, the birth weights in our case series may be considered normal rather than small at birth. Five out of 11 of our patients failed to thrive. Four of these 5 patients demonstrated catch up growth upon resolution of liver dysfunction and citrullinaemia, while one had passed away.

Patient 1 had 2 episodes of febrile convulsions and 2 episodes of unexplained afebrile convulsions. Afebrile convulsions have previously been reported in a patient with NICCD by Tazawa et. al. (2004). It is uncertain whether this is related to the disease or a separate entity. In Tazawa's case, the patient also suffered developmental delay which resolved with time. Development was normal in all our patients except for patient 3 who had concurrent psychosocial factors which may have contributed to his delay and patient 8 who was very ill and spent much time in the hospital.

In our series, the amino acid profile showed an elevated citrulline level 1.3–43.8 times the upper limit of normal at presentation. The concentration of threonine, methionine, tyrosine and arginine ranged from normal to 8.3 times the upper limit of normal. It has previously been suggested that a higher protein, proline and asparagine content in formula milk may replenish the depleted intracellular stores of amino acid and stimulate urea synthesis under citrin deficiency, thus conferring a beneficial effect compared to breast milk (Saheki et al. 2002; Ben-Shalom et al. 2002). The level of hyperamino acidaemia was not related to the mode of feeding in our patients, suggesting that other factors may be responsible, for example endogenous production. Previous studies have recommended a high protein/low carbohydrate diet because a high carbohydrate diet may increase cytosolic NADH, overloading the defective malate aspartate shuttle resulting in difficulty in conversion to NAD^+ and the subsequent fatty liver (Saheki et al. 2002). Patients with NICCD therefore naturally exhibit a fondness for protein-rich food and a dislike for carbohydrate-rich food. Dietary modifications have not been studied with structured protocols, and therefore have not been applied in our patients. However, high levels of galactose may have adverse effects as shown in patient 11 who had cataract at presentation. The galactosaemia present is thought to be due to the high $NADH/NAD^+$ ratio since there is no abnormality in the enzymes of galactose metabolism (Saheki et al. 2004). Tazawa et. al. (2004) have proposed that lactose is a toxic substance in NICCD and may worsen cholestasis. As such, when infant formula was introduced in our patients, we have used lactose-free formula. In addition, dietary management is directed at treating the consequences of cholestasis with supplementation of fat soluble vitamins and MCT oil.

Elevated levels of urinary orotic acid and involvement of the renal system have not previously been reported in NICCD. Orotic acid was elevated in patients 2 and 6. Elevated orotic acids would be expected since the deficiency of citrin directly affects the detoxification of ammonia to urea at the start of urea cycle. Two patients had renal diseases; patient 6 had biochemical evidence of renal tubulopathy and patient 8 had an incidental radiological finding of mild right hydronephrosis of which the aetiology is unknown. Since there was no single unifying factor, it is possible that these renal pathologies are unrelated to their metabolic problem.

In several of our patients, many other diagnoses were considered before the final diagnosis of NICCD. These included biliary atresia, breast milk jaundice, drug toxicity, infective hepatitis, galactosaemia, mitochondrial cytopathies, glycogen storage diseases, urea cycle defects, tyrosinaemia, organic acidaemias and neonatal haemachromatosis. This led to many expensive, unnecessary and on occasion invasive investigations. NICCD should be considered in all children who present with prolonged conjugated hyperbilirubinaemia, and all investigations should include a serum/plasma amino acid profile with galactose level and urine organic acid. If citrulline level is normal but suspicion of NICCD still high, repeat of amino acid profile should be considered followed by molecular studies. Achieving an accurate diagnosis rapidly translates to prompt treatment, counselling and expectation of prognosis. Although most patients with NICCD experience

a benign course and recover completely, a small percentage may develop the more severe CTLN2 in adulthood or liver failure (Tamamori et al. 2002, 2004). One of our patients (patient 8) suffered liver failure and subsequently succumbed at 9 months old. These patients should have long-term follow-up as the mechanisms that lead to the development of CTLN2 are yet to be elucidated. They should also avoid alcohol, acetaminophen and certain anti-inflammatory drugs that are implicated as triggers of CTLN2.

Conclusion

Our study has identified 11 cases of NICCD presenting to a tertiary unit in Malaysia. These patients are made up of 4 different ethnic groups: Malay, Iban, Kadazandusun and Malaysian Chinese. This has implications for the prevalence of the condition, suggesting that it may be commoner in South East Asians than previously thought.

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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

Hai-Yan Fu · Shao-Ren Zhang · Xiao-Hong Wang · Takeyori Saheki · Keiko Kobayashi · Jian-She Wang

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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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H.-Y. Fu · S.-R. Zhang · X.-H. Wang · J.-S. Wang (✉)
The Center for Pediatric Liver Diseases,
Children's Hospital of Fudan University,
399 Wanyuan Road, Minhang District, Shanghai 201102,
People's Republic of China
e-mail: jshwang@shmu.edu.cn; jianshewang@sina.com

H.-Y. Fu · S.-R. Zhang · X.-H. Wang · J.-S. Wang
The Department of Pediatrics, Shanghai Medical College
of Fudan University, Shanghai 201102,
People's Republic of China

T. Saheki
Institute for Health Sciences, Tokushima Bunri University,
Tokushima 770-8514, Japan

K. Kobayashi
Department of Molecular Metabolism and Biochemical
Genetics, Kagoshima University Graduate School of Medical
and Dental Sciences, Kagoshima 890-8544, Japan

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)

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[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).