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

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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)

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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).

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).

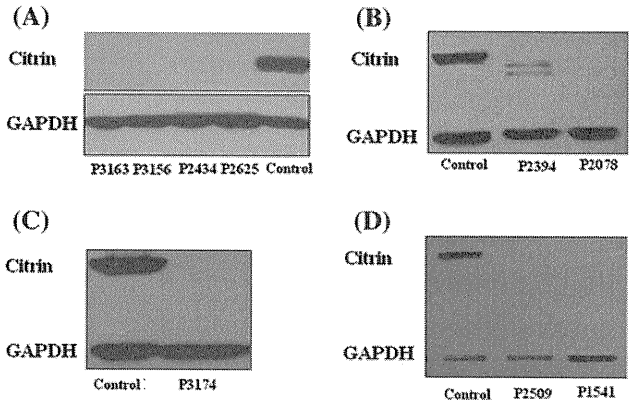

Fig. 1 Western blot analyses of biopsied liver specimens of six patients with homozygous or compound heterozygous mutations (*P2509*, *P2394*, *P1541*, *P2078*, *P3163*, and *P3174*) and three patients with heterozygous mutations (*P3156*, *P2625*, and *P2434*). *GAPDH* Glyceraldehyde-3-phosphate dehydrogenase

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]

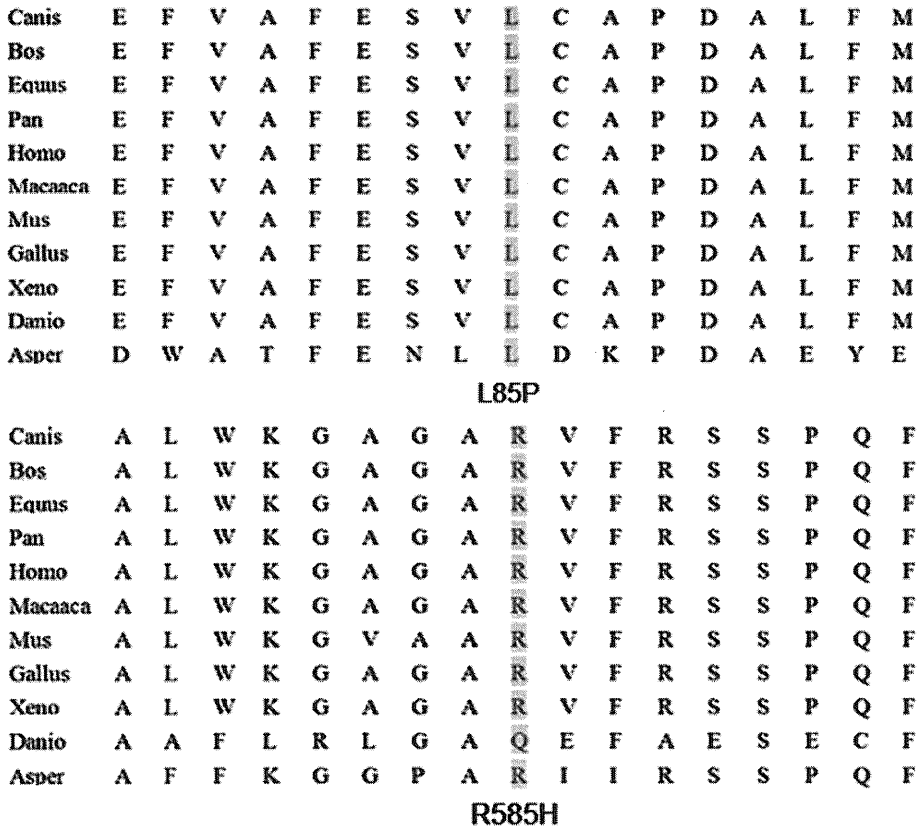

Fig. 2 Conservation analysis of SLC25A13 gene mutations L85P and R585H among different species

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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Research Article

Etiological Analysis of Neurodevelopmental Disabilities: Single-Center Eight-Year Clinical Experience in South China

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Etiology determination of neurodevelopmental disabilities (NDDs) currently remains a worldwide common challenge on child health. We herein reported the etiology distribution feature in a cohort of 285 Chinese patients with NDDs. Although concrete NDD etiologies in 48.4% of the total patients could not be identified, genetic diseases (with the proportion of 35.8% in the total cases) including inborn errors of metabolism (IEM) and congenital dysmorphic diseases, constituted the commonest etiology category for NDDs in this study. The two key experimental technologies in pediatric metabolomics, gas chromatography-mass spectrometry (GC-MS), and tandem mass spectrometry (MS-MS), proved to be substantially helpful for the exploration of the NDD etiologies in this clinical investigation. The findings in this paper provided latest epidemiologic information on the etiology distribution of NDDs in Chinese, and the syndromic NDDs caused by citrin deficiency and the novel chromosomal karyotype, respectively, further expanded the etiology spectrum of NDDs.

1. Introduction

With global developmental delay (GDD) and mental retardation (MR) as two main clinical subtypes, neurodevelopmental disabilities (NDDs), which are defined as a group of chronic clinically distinct disorders that all share a documented disturbance, quantitative, qualitative, or both, in developmental progress in one or more developmental domains compared with established norms [1], are conventionally categorized into syndromic type which is characterized by associated clinical, radiological, metabolic or biological features, and nonsyndromic type in which NDD represents the only manifestation. The precise prevalence of NDDs remains unclear, but this entity has been estimated to affect 5% to 10% of children [2]. In developed countries, MR has become the most frequent cause of severe handicap in children and one of the main reasons for referral in clinical genetic practice [3]. Actually, 1% to 3% of children younger than 5 years have been reasonably given the prevalence of MR in a specific population [4]. As the largest developing country in the world with a population over 1.3 billion, China also faces the difficult challenge of NDDs on its child health. An investigation in the year 2000 has revealed that the MR incidence in children below 6 years of age was 0.931%,

and 136,000 children with MR were increased annually in mainland of our country [5]. Etiology determination of NDDs was essential not only for the option of therapeutic imperatives and evaluation of clinical outcomes and recurrence risks but also for other benefits including avoidance of unnecessary tests and access to appropriate patients for accumulating management experiences, however, this issue also remains far from resolved at the current stage in pediatric practice. In this paper, we reported our eight-year findings on NDD etiologies in a medical center in south China.

2. Subjects and Methods

2.1. Patients. The research subjects recruited in this study were all patients referred to, from April 2002 to March 2010, Department of Pediatrics, First Affiliated Hospital, Jinan University, Guangdong, China. The GDD/MR diagnosis in most cases was made by at least 2 pediatric physicians in different hospitals in accordance with the updated concepts in the review [1]. For some cases who suffered from other clinical problems such as liver diseases and malformations, NDD was noticed and then confirmed in our department. The patients in this study came from 22 provinces, municipalities, and autonomous regions in China, respectively, with most of them from Guangdong Province.

2.2. Clinical Data. History inquiry and physical examination were performed on all the NDDs patients in our pediatric clinic or award and the positive findings were recorded and preserved by the authors. Most of the laboratory and imaging results were collected from the corresponding databases in our hospital, besides some provided by parents of the patients at their referral to the authors for clinical counseling. In this study we, by means of a cross-sectional study, retrospectively analyzed and summarised the clinical information collected in the past 8 years.

2.3. Gas Chromatography-Mass Spectrometry (GC-MS). Selective screening of inborn errors of metabolism (IEMs) in this study was conducted by analysis of the urinary components, using an urease pretreatment GC-MS procedure, mainly with a Finnigan GC-MS instrument (TRACE DSQ), with detailed information described previously by our group [6].

2.4. Tandem Mass Spectrometry (MS-MS). Amino and acyl carnitine in dried blood stains was analyzed by means of a MS-MS procedure, and sample preparation, apparatus settings, and data analysis were based on the detailed information described in [7]. The analysis was conducted with an API 3200 tandem mass spectrometer purchased from Applied Biosystems. Neutral loss scan and precursor scan were used for the analysis of most amino acids and acyl carnitines, respectively, while multiple reaction monitoring (MRM) was utilized for the detection of glycine, ornithine, arginine, and citrulline as well.

Table 1: Main clinical manifestations besides NDDs and the positive laboratory and imaging findings in syndromic NDDs.

No.	Positive findings	Cases
01	Failure to thrive	81
02	Seizure/convulsion	37
03	Hearing disability	28
04	Dysmorphic facial features	25
05	Abnormal urine odor	20
06	Eye movement obstacles	19
07	Vomitting	18
08	Hair depigmentation	15
09	Microcephaly	11
10	Skin abnormalities	10
11	Hepato/splenomegaly	10
12	Impaired swallowing and chewing	5
13	Fondus ocili abnormalities	4
14	Vision problem	4
15	Abnormal lens	3
16	Genitalia malformation	3
17	Metabolic acidosis	46
18	Hyperammonemia	12
19	Abnormal EEG	22
20	Skeleton abnormality on X ray	5
21	CT/MRI abnormal findings	85

2.5. Chromosome Karyotype Analysis. Traditional chromosomal banding was performed in NDD patients suspected to have chromosomal abberations. Fluorescence in situ hybridization (FISH) was further used, when necessary, to determine the complex karyotypes as previously described [8]. Briefly, the peripheral blood lymphocytes were cultured under phytohemagglutinin (PHA) stimulation and treated with colcemid and harvested by standard methods. Metaphases were spread on clean slides, and standard G-banding with trypsin-Giemsa was performed. The slides for FISH were stored at −20°C before use. The denatured FISH probe (Abbott-Vysis, Downers Grove, IL, USA) was added to the denatured slides with metaphase spreads in a moist chamber for hybridizing over night. After washing, the slide was counterstained with DAPI in an antifade solution. The hybridized metaphase chromosomes were captured and analyzed using a digital image analysis system containing an Olympus BX51 microscope equipped with LUCIA Cytogenetics system (Prague, Czech Repuplic).

2.6. SLC25A13 Gene Mutation Analysis. The diagnosis of citrin deficiency was confirmed by mutation analysis of the causative gene SLC25A13. Four hotspot mutations, 851-854del(851del4), IVS6 + 5G > A, IVS16ins3kb, and 1638-1660dup were screened by means of the routine approaches described in reference [9]. In this study, the sequences of the forward and backward primers for PCR amplification of the mutation 851del4 were 5′-ggtatatttgttgcttgtgtttg-3′ and 5′-tcttccagaggagcaatccg-3′, respectively.

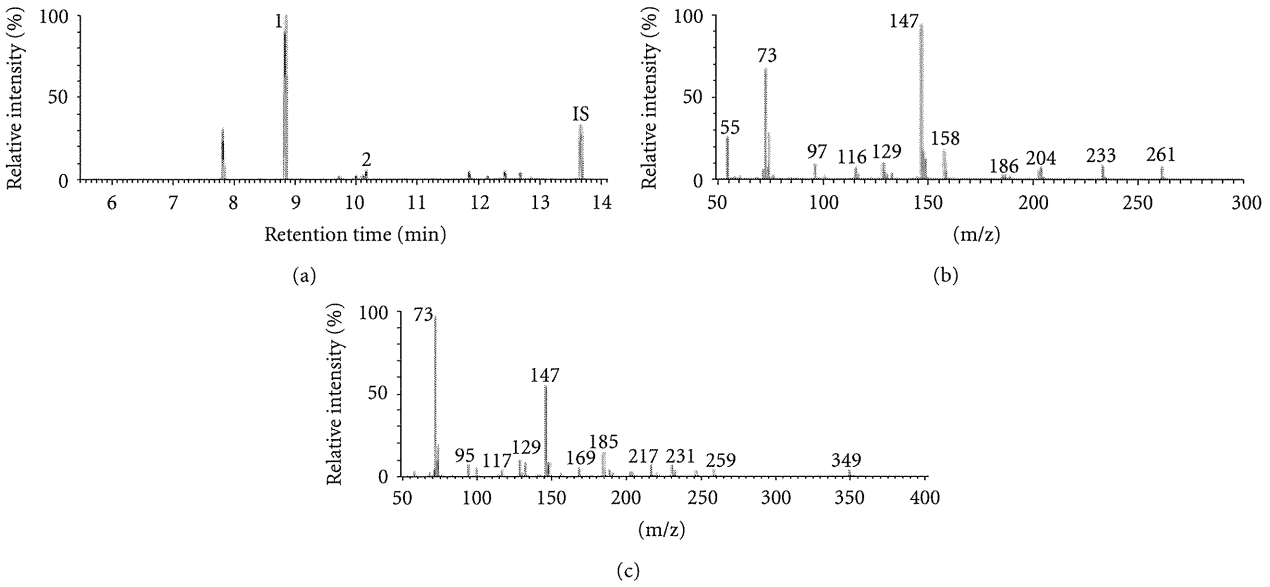

FIGURE 1: Chemical diagnosis of glutaric acidemia type I by GC-MS analysis of urinary metabolites for a 9-month-old female with motor and language retardation. Figure 1(a) is a representative GC-MS total ion current (TIC) profile, in which intensities of peak 1 and 2 were both dramatically increased. IS is the abbreviation of internal standard. Figures 1(b) and 1(c), the mass spectra for peak 1 and 2 in Figure 1(a), revealed their identifications as trimethylsilyl derivatives of glutarate and 3-hydroxyglutarate, respectively.

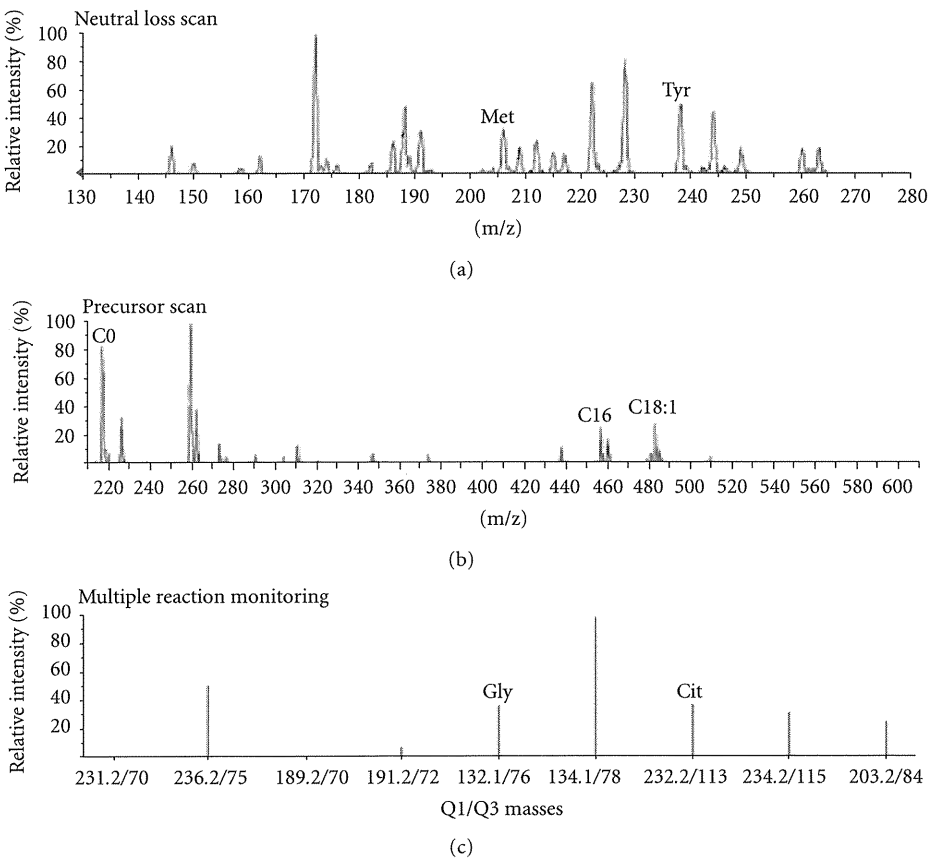

FIGURE 2: Chemical diagnosis of citrin deficiency by MS-MS analysis of amino and acyl carnitines in dried blood stain from a 16-month-old male toddler (C0013) with syndromic GDD. Figures 2(a), 2(b), and 2(c) are profiles of neutral loss scan, precursor scan, and multiple reaction monitoring, respectively. The amino and acyl carnitines with increased levels were labeled as abbreviations, with Met, Tyr, Gly, Cit, C0, C16, and C18 : 1 representing methionine, tyrosine, glycine, citrulline, free carnitine, palmitate, and hydroxypalmitate, respectively.

Table 2: Etiology distribution in the whole cohort of NDDs.

Etiology categories	Disease types	Case number	Proportion	Concrete disease (case number)
Genetic diseases	50	102	35.8%	Detailed information in Table 3
Psychobehaviour	3	23	8.1%	Autism (21, including 5 cases of Rett syndrome); ADHD (2)
Acquired brain injuries	2	7	2.4%	Kernicterus (4); HIE (3)
Other etiology	2	15	5.3%	Cerebral palsy (7), Epilepsy (8)
Unknown	1 (NDDs)	138	48.4%	No concrete etiologies were identified at the current stage
In total	58	285	100%	—

Table 3: Etiology distribution in the patients with genetic diseases in Table 2.

Etiology categories	Disease types	Case number	Proportion	Concrete diseases (case number)
IEMs	27	66	64.7%	Detailed information in Table 4
Congenital dysmorphic diseases	14	22	21.6%	Detailed information in Table 5
Chromosomal abberations	4	6	5.9%	Detailed information in Section 3.5
Endocrine disorders	3	4	3.9%	Hypoparathyroidism (1), Pseudohypoparathyroidism (1), Congenital hypothyroidism (2)
Others	2	4	3.9%	Congenital muscular dystrophy (3), Progressive muscular dystrophy (1)
In total	50	102	100%	—

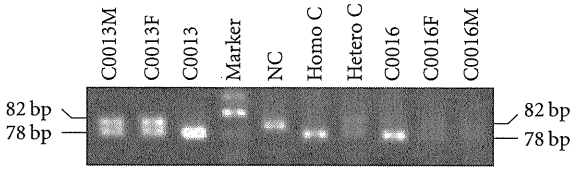

Figure 3: PCR-gel electrophoresis analysis of mutation 851del4 in the gene SLC25A13 of the two families with citrin-deficient patients C0013 and C0016. NC, Homo C and Hetero C in this figure are abbreviations of Normal Control, Homozygous Control and Heterozygous Control, respectively. F and M in the two families represent Father and Mother, respectively. The 78 bp PCR products in both patients are 4 bp shorter than the normal size 82 bp, suggesting that the 2 patients are both 851del4 homozygotes, and their parents all carriers of the same mutation.

2.7. Electronic Microscopy. The muscular ultrastructure changes were observed by transmission electronic microscopy in patients suspected to have Leigh syndrome and muscular dystrophy, with biopsy samples from musculi gastrocnemius. Muscular tissue was fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide solution, and then embedded in epoxy resin before semithin sectioning, as described in reference [10]. Two electronic microscope instruments (JOEL-CX100 and Philips-Tecai-10) were utilized for ultrastructure observation in our investigation.

This study was performed with the informed consents from the parents of the patients, adhering to the principles of the Declaration of Helsinki. In particular, SLC25A13 mutation analysis has been approved by the Committee for Medical Ethics in the First Affiliated Hospital, Jinan University, while chromosome karyotype analysis by that in Reproductive and Genetic Hospital of Citic-Xiangya, Central South University.

3. Results

3.1. General Information and Semiology. The NDDs cohort in this study was composed of 285 cases in total, including 191 males and 94 females. The median age at referral was 1 year and 7 months, with minimum 1 week, and maximum 16 years. The whole cohort included 240 syndromic and 45 nonsyndromic NDD cases, with the relative proportion of 84.2% and 15.8%, respectively. The main clinical manifestations besides NDD and the laboratory and imaging findings in the syndromic NDDs were summarized in Table 1.

3.2. Etiology Distribution. As shown in Table 2, no concrete etiologies, unfortunately, were found for 48.4% of the total patients, but NDDs in the remaining 51.6% could be attributed to complex and diverse etiologies, among which genetic diseases were on top of the list of the identified causes. Further analysis in Table 3 revealed that IEMs took the first place in genetic etiologies, and then came congenital dysmorphic disorders. These 2 entities constituted

Table 4: Feature of etiology distribution in the patients with IEMs in Table 3.

Categories	Diseases*	Cases	Major diagnostic evidences	Clinical outcomes
Disorders of Carbohydrate metabolism	Galactosemia	1	Clinical features including congenital cataract and leukodystrophy, and GC-MS analysis	Lost contact
	Fructosuria	2	GC-MS analysis	Both lost contact
Disorders of Amino acid metabolism	Phenylketonuria	7	GC-MS analysis in 6 cases, and PAH gene analysis in 1 case	Referred to local network of management and 2 died after treatment withdrawal
	Histidinemia	1	Repeated MS-MS analysis	Lost contact
	Hyperhomocysteinemia	2	Total plasma homocysteine levels and MS-MS analysis	1 died, and 1 stable without obvious clinical or biochemical improvement
	Pyroglutamic acidemia	1	GC-MS analysis	Lost contact
	Tyrosinemia type I	1	GC-MS and MS-MS findings	Died due to acute liver failure
	Hyperglycinemia	1	GC-MS and MS-MS analysis	Intractable seizures and behavioral problem
	Canavan's disease	1	GC-MS analysis	Lost contact
Organic acidemia	Methylmalonic acidemia	11	GC-MS, MS-MS and MMACHC gene analysis, with 5 combined with hyperhomocysteinemia	5 died after withdrawal of treatment, 3 improved and 3 lost contact
	Maple syrup urine disease	2	GC-MS and MS-MS analysis	Both died
	Ethylmalonic acidemia	1	GC-MS analysis	Lost contact
	Propionic acidemia	3	GC-MS analysis	2 stable with episodic hyperammonemia, and 1 lost contact
	Glutaric acidemia type I	2	GC-MS and MS-MS analysis	1 lost contact and 1 stable
	Glutaric acidemia type II	1	GC-MS analysis	Stable
	2-hydroxyglutaric acidemia	1	GC-MS analysis	Lost contact
	4-hydroxybutyric aciduria	1	GC-MS and ALDH5A1 gene analysis	Stable but with seizure episodes
	Multiple carboxylase deficiency	4	GC-MS, biotinidase activity, and HLCS gene analysis	1 died, 3 recovered/improved clinically
Urea cycle disorders	OTCD	2	GC-MS and MS-MS analysis	Recovered clinically
	Hyperammonemia	4	Markedly increased serum ammonia levels, but with etiologies undetermined yet	All lost contact
	Citrin deficiency	2	SLC25A13 mutation analysis	1 died due to liver cirrhosis, 1 improved
Mitochondrial disease	Leigh syndrome	5	Clinical and imaging features, serum/CSF lactate levels, and electronic microscopy findings on muscle biopsy samples	3 died already, and the remaining 2 stable at follow-up
Lysosome storage diseases	Mucopolysaccharidosis type I	1	Typical clinical manifestations	Improved after bone marrow transplantation
	Mucopolysaccharidosis type II	2	Activity analysis of iduronate-2-sulphatase	Lost contact
Peroxisomal disorders	X-linked adrenoleukodystrophy	2	Clinical manifestations, CT/MRI findings, and MS-MS analysis of VLCFA	Both Died
Others	Glyceroluria	4	GC-MS analysis	1 died after severe infection, 3 lost contact
	3-aminoisobutyric aciduria	1	GC-MS analysis	Lost contact

* Some diseases have been reported in [6] as GC-MS screening results, and this list herein is the latest update of our findings, just focusing on the IEMs associated with NDDs.

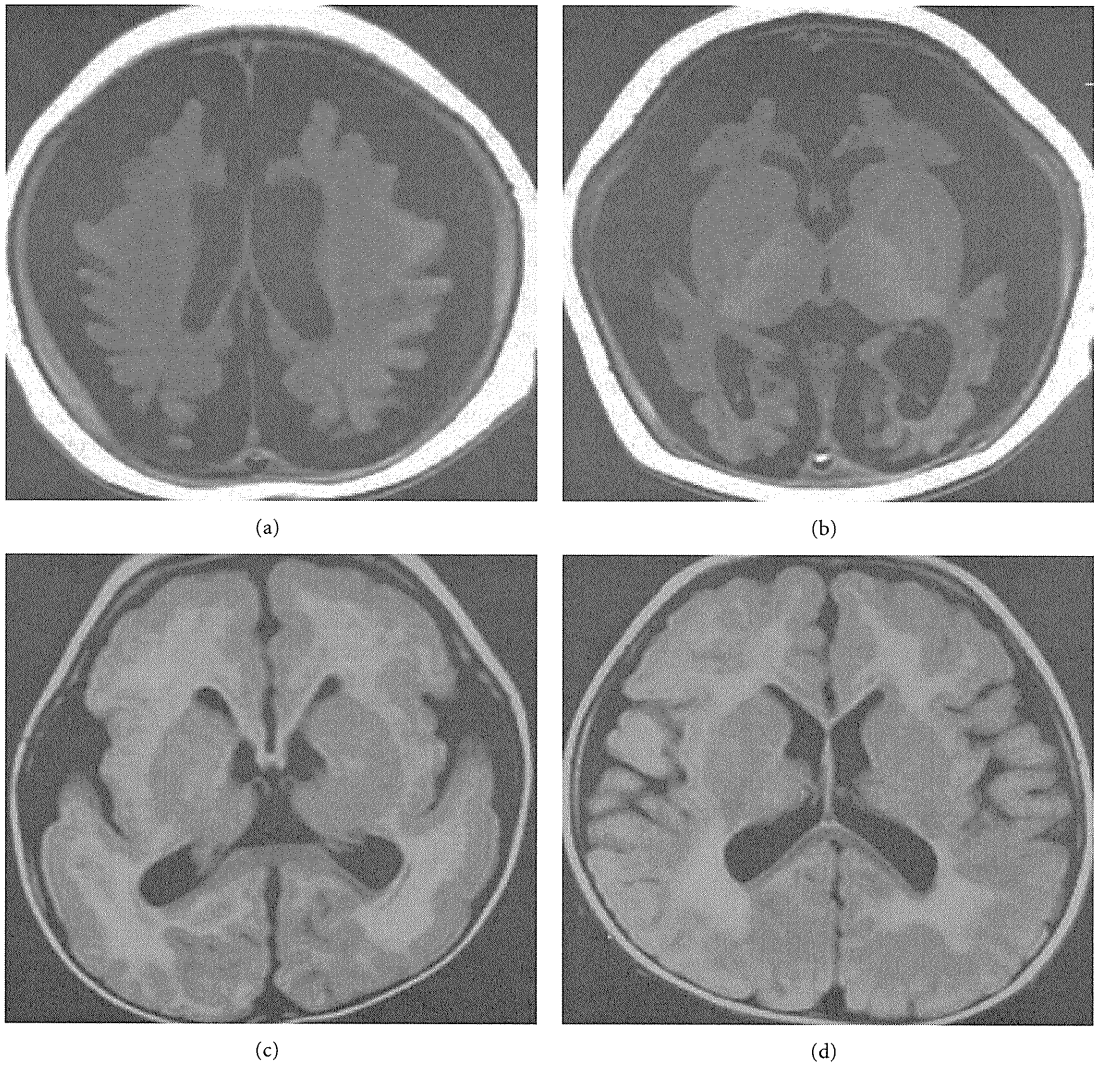

FIGURE 4: Representative MRI findings in different malformations of cortical development (MCD). Figures 4(a) and 4(b) showed severe cortex dysplasia in bilateral parietal lobes and frontal, temporal and occipital lobes, respectively, in the telencephalon of a 5-month-old male with NDD. Figure 4(c) demonstrated typical lissencephaly in a 9-month-old female with Miller-Dieker syndrome. The white matter volume was decreased while the cortex was thick and smooth due to lack of enough sulcation, forming the so-called pachygyria malformation, and the thickened and irregular cortex in Figure 4(d) revealed the cobblestone cortical malformation in a patient with muscle-eye-brain disease.

the overwhelming majority in the genetic diseases. Other causes such as chromosome and endocrine abnormalities were also identified, just accounting for a minority less than 14% in the total genetic etiologies.

3.3. IEMs. As listed in Table 4, 66 patients with IEMs of 27 types and 8 categories were diagnosed in this NDD cohort. The traditional clinical, biochemical, and imaging findings were indispensable during the diagnostic processes, however, the applications of two metabolome tools, GC-MS and MS-MS, were substantially helpful in the exploration of the NDD etiologies in this study. Figure 1 demonstrated the diagnostic evidences of glutaric acidemia type I by GC-MS analysis of urine sample. In particular, we diagnosed 2 GDD patients secondary to citrin deficiency. Figure 2 illustrated the MS-MS findings suggestive of citrin deficiency in a male toddler (C0013) at his age of 1 year and 4 months, who presented with persistent GDD due to prolonged hepatosplenomegaly and recurrent ascites that progressed into lethal hepatic encephalopathy at his age of 1 year and 10 months. GDD was transient in another 7-month-old infant (C0016) with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM #605814), who demonstrated catch-up development after recovery of dyslipidemia and abnormal liver function indices. As shown in Figure 3, mutation analysis of the causative gene SLC25A13 clearly confirmed their diagnosis of 851del4 homozygotes.

3.4. Congenital Dysmorphic Disorders. In this NDDs cohort, 14 kinds and 22 cases of congenital dysmorphic disorders

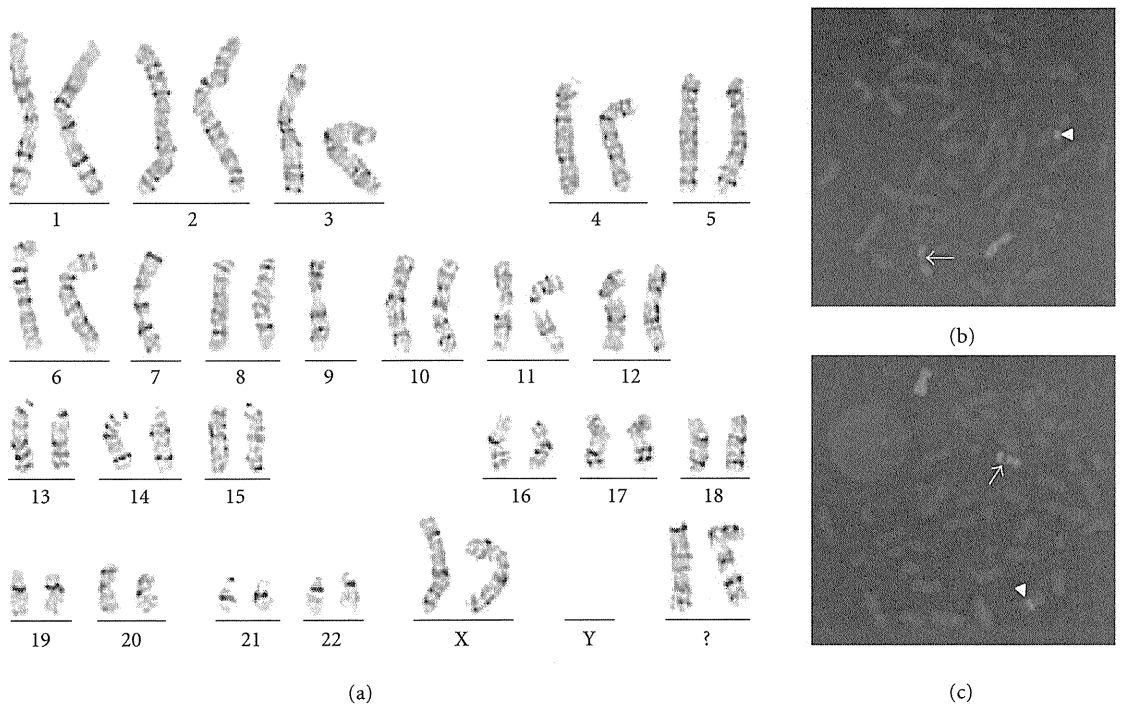

FIGURE 5: Chromosome abberation in a 6-year-old female with mental retardation (MR). High-resolution GTG-banding in Figure 5(a) revealed the derivative chromosomes 7 and 9 (question mark), and their detailed identities were further illustrated by the results of FISH analysis. Figure 5(b) showed four red signals in a metaphase, by means of utilization of whole chromosome 7 painting probe (WCP 7, red). The normal chromosome 7 had one intact red signal, but the derivative chromosome 7 (arrow) had two dispersed red signals, and a fragment of chromosome 7(arrowhead) was inserted into a chromosome 9, forming a derivative chromosome 9. Similarly, FISH analysis with WCP 9 (red) in Figure 5(c) showed the normal chromosome 9 with intact red signal, the derivative chromosome 9 (arrow) with two dispersed red signals, and the derivative chromosome 7 with a inserted fragment of chromosome 9 (arrowhead). Finally, the chromosome karyotype in this patient was identified as 46, XX, ins (7;9) (p13; q32q22) inv(7) (p11.2 q11.23), ins (9;7) (q22; q22q32), ish ins(7;9) (WCP7+, WCP9+), ins(9;7) (WCP7+,WCP9+).

were diagnosed. Most of them were, as shown in Table 5, malformations of cortical development (MCD) such as Miller-Dieker syndrome, Muscle-Eye-Brain disease, and isolated lissencephaly sequence. Figure 4 demonstrated representative MRI findings in the different MCD types. Other CNS malformations like Dandy-Walker syndrome and spinocerebellar ataxia, and some rare syndromes including Silver-Russell syndrome, Noonan syndrome, Poland–Moebius syndrome, and Crisponi syndrome, were also found in our clinical practice. To our knowledge, the patient with Crisponi syndrome reported here is the first case in China.

3.5. Chromosome Karyotypes. By traditional chromosome analysis, karyotype abnormalities were found in 6 patients. Among them, 3 patients were diagnosed as having Down's syndrome (trisomy 21) and 1 Patau's syndrome (trisomy 13), respectively. The remaining 2 were both complex karyotypes, with mos. 47, XX, +der (15) (pter → ql4::q14 → pter) [11]/48, XX, +der (15) (pter → q14::ql4 → pter)/×2 [12]. ish der(15) (WCP15+, UBE3A++, PML-) in 1 case, and 46, XX, inv ins (7;9) (p13; q32q22) inv(7) (p11.2 q11.23), ins (9;7) (q22; q22q32), ish(7;9) (WCP7+,WCP9+), ins(9;7) (WCP7+,WCP9+) in another, as illustrated in Figure 5. So far as we know, the last karyotype is a novel one that has never been reported in any other references. The patient with this novel abnormal karyotype was a 6-year-old female with mental retardation. History inquiry revealed motor retardation, and examination uncovered dysmorphic facial features including hypertelorism, downward eyeslant, and low-set ears, constituting a clinical phenotype of syndromic NDD.

3.6. Endocrine and Other Genetic Disorders. As shown in Table 3, endocrine and other genetic disorders also played important roles in NDD development in this cohort. Figure 6 clearly demonstrated not only the muscular lesions on electronic microscopy observation, but also the existence of leukodystrophy in MRI which indicated the involvement of central nervous system and thus explained in part the neurodevelopmental retardation in the patient with congenital muscular dystrophy. It is also noteworthy that a hypothyroidism patient in this study was combined with hypophosphatasia with remarkable delay in bone ossification as the radiological feature due to the reduced activity of alkaline phosphatase. To our knowledge, the combined clinical spectra of hypothyroidism and hypophosphatasia in the same patient, once again, have never been reported in other references.

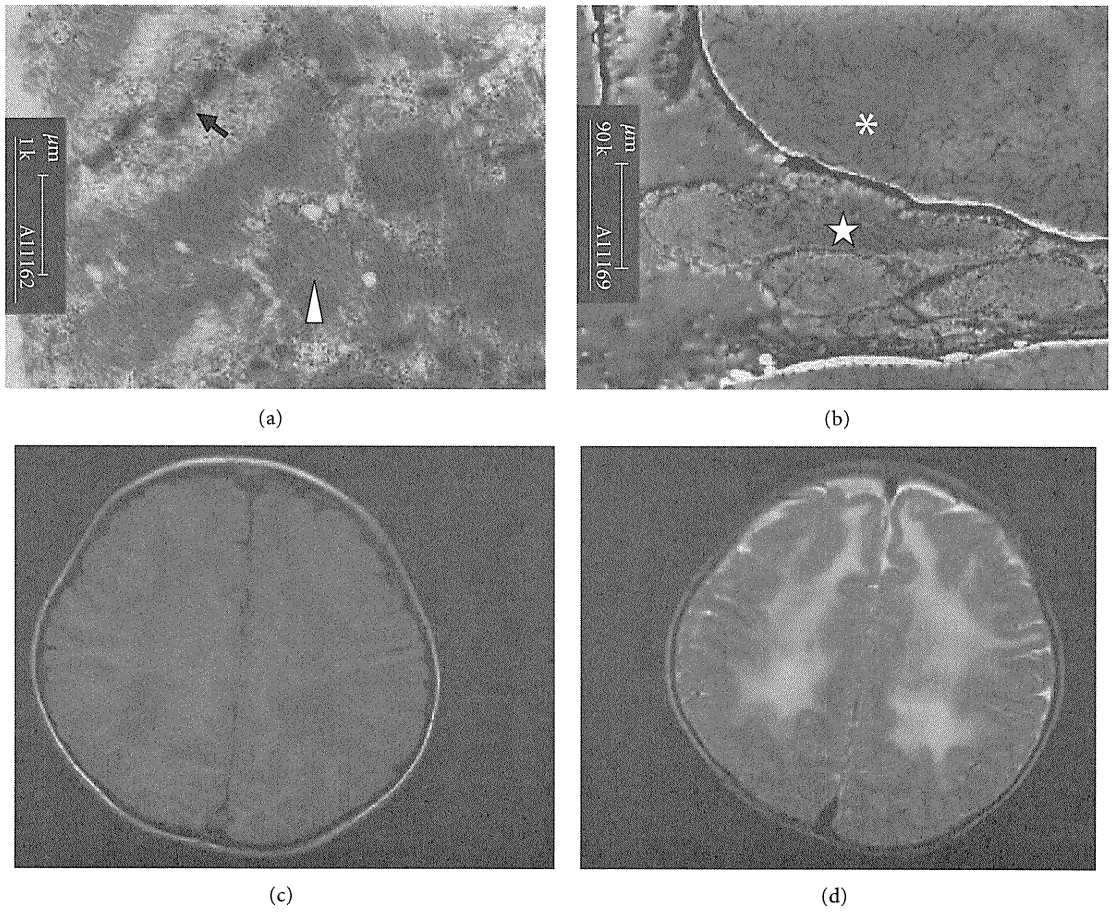

FIGURE 6: Muscular ultrastructure and brain MRI findings in a patient with congenital muscular dystrophy. In Figure 6(a), the myofibril Z lines in a muscle cell were ruptured (↑), and the filaments between Z lines were arranged disorderly (△). Figure 6(b) showed the fatty degeneration of myofibrils (∗) in a muscle cell with completely vanished sarcolemma, and large quantity of collagenous fibers (☆) were observed in the endomysium (Bar = 1 μm). Figures 6(c) and 6(d) were cranial MRI findings revealing the reduced signal intensity in T1WI while increased one in T2WI, respectively, in bilateral frontal and parietal lobes, both indicating leukodystrophy.

4. Discussion

The findings in this study provided the latest clinical epidemiology information on the etiology distribution of NDDs in Chinese children. As shown in Table 2, nearly half of the NDD cases could not be attributed to concrete etiologies. This finding indicated the current issue or challenge that we are facing and was consistent with the well-known fact that the etiologies in a substantial percentage of NDDs patients are undiagnosed even after a comprehensive evaluation [13]. On the other hand, concrete etiologies were identified for 51.6% of the total NDD cases, and the etiology distribution in this cohort demonstrated a rather heterogeneous feature. Autism spectrum disorders (ASDs) have been well recognized as pervasive NDDs entities, and there have been evidences suggesting that some genes or chromosomal abberations are associated with ASDs [14–16]. However, genetic studies have not provided substantial insight into the 90% of cases of autism whose cause is idiopathic, and the relative genetic contribution to a susceptibility to autism from de novo mutations, rare mutations, and common polymorphisms has been debated extensively [17]. Therefore, autism was categorized into psychobehavioral disorders other than genetic diseases in this paper. This investigation also found 7 NDD cases caused by kernicterus and hypoxemic and ischemic encephalopathy (HIE) which were secondary to 2 curable and even preventable diseases, hyperbilirubinemia and birth asphyxia, respectively. The existence of these 7 cases further suggested the brain vulnerability to exogenous injuries in children, especially in fetuses and neonates. We also found cerebral palsy and epilepsy as NDD etiologies in this NDD cohort, with several cases of refractory epilepsy. However, it is noteworthy that, considering the brain vulnerability once again and the possible effects of antiepileptic drugs (AEDs) on neurodevelopment [18, 19], every child with epilepsy must be evaluated on an individual basis as to the risk and benefit of any particular AED used and the role of ongoing treatment [20].

In this eight-year clinical study, various laboratory technologies were conducted in the fields of clinical biochemistry, neuroimaging, biochemical genetics, cytogenetics, molecular

Table 5: Congenital dysmorphic disorders in the patients with genetic diseases in Table 3.

Disorders	Cases	Main clinical/imaging features
Cortical dysplasia	1	Motor developmental retardation and microcephaly. Severe cortex dysplasia in parietal lobe and frontal, temporal and occipital lobes, respectively, on cranial MRI scanning.
Tuberous sclerosis	1	Intelligence and motor retardation, seizures, cutaneous hypomelanotic macules, fondus ocili depigmentation, and subependymal nodules and calcified lesions in the cortex of parietal and temporal lobes on cranial CT scanning
Miller-Dieker syndrome	2	Intelligence and motor retardation, microcephaly, prominent occiput, narrow forehead, small nose and chin. Seizure in 1 case and hypertonia in another one. Agyria/pachygyria cortical malformations on MRI.
Muscle-Eye-Brain disease	2	Sibling sisters with global developmental delay. Abnormal pupils and vitreous bodies in both cases on ophthalmologic examination. Convulsions in 1 case and small right eyeball in another. Both have increased creatine kinase levels and cobblestone cortical malformations on MRI.
Isolated lissencephaly sequence	1	Intelligence and motor retardation, and bilateral thickened and irregular cortex on MRI
Lissencephaly with cerebellar hypoplasia	1	Mental/language retardation, drooling, and bilateral pachygyria malformation and hypoplasia of cerebellum revealed by MRI.
Other malformations of cortical development	6	All have intelligence and motor retardation. Including 2 cases of cobblestone cortical malformations and 1 classic lissencephaly revealed by CT/MRI.
Dandy-Walker syndrome	1	Intelligence and motor retardation with low-set and everted ears, and hypoplasia and upward rotation of the cerebellar vermis and cystic dilation of the fourth ventricle on MRI.
Spinocerebellar ataxia	1	Intelligence and motor retrogression, and severe cerebellar and pons atrophy together with tiger-eye-like sign at the basal ganglia level and cross-sign at pons level, respectively, on MRI.
Neurofibromatosis type I	1	Intelligence and motor retardation, and 7 cutaneous cafe-au-lait patches with diameter over 10 mm
Silver-Russell syndrome	2	Both have dysmorphic facial features including triangular face, low-set ears, flat nasal bridge with extroversion of nostrils and down-curving mouth corners. Normal head circumference. Asymmetry of the lower extremities. Postnatal failure to thrive. Intrauterine retardation in 1 case and linea alba hernia in another.
Noonan syndrome	1	Short stature, short neck with redundancy of skin, hypertelorism, downward eyeslant, low-set ears, cryptorchidism, and poor sucking. Atrial septal defect and right pulmonic stenosis on ultrasonography.
Poland–Moebius syndrome	1	Signs of facial palsy, disappeared corneal reflex, and poor sucking and swallowing. Micrognathia and high-arched palate. Small left hand, ipsilateral brachydactyly and hypoplasia of the nails and pectoralis major muscle.
Crisponi syndrome	1	Convulsions in response to stimuli like crying and bathing. Camptodactyly in hands. Round face, broad nose with anteverted nostrils, and micrognathia. Major sucking difficulty and frequent apnea. Hyperthermia that led to death.

genetics, and electronic microscopy as well. Our experiences strongly supported the viewpoint that detailed history inquiry and physical examination are paramount in the evaluation of NDDs, while judicious investigations can be useful adjunct in determining etiology [21]. As the commonest etiology category in this NDD cohort, genetic diseases, as shown in Table 3, also covered a wide profile of different entities, with IEMs on top of the list. The application of two metabolomic technologies, GC-MS and MS-MS, played irreplaceable roles in the identification of various IEM entities in this study (Table 4). Although other skills such as enzymatic activity and gene mutation analysis help us to confirm the diagnosis of IEM in some cases, selective screening for IEMs by means of GC-MS and MS-MS provides the basis or prerequisite for further intensive investigation. The clinical application of these metabolomic tools in mainland of China was initiated from the beginning of this century, however, their indispensable function in chemical diagnosis of IEMs has been affirmed by more and more clinical evidences in our pediatric practice [6, 22, 23]. Actually, mass spectrometry has occupied an increasingly prominent place in clinical chemistry and laboratory diagnostics during the past few decades [24], and nowadays has been recognized as one of the most frequently