Combining newborn metabolic and genetic screening for neonatal intrahepatic cholestasis caused by citrin deficiency

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Disclosure

Lin Zhu is currently an employee of Hangzhou Genuine Clinical Laboratory Co. Ltd. Chien-Hsing Lin is currently an employee of Feng Chi Biotech Corp. Chenggang Huang is currently an employee of Zhejiang Biosan Biochemical Technologies Corp. The other authors declare no conflicts of interest.

Abstract

Purpose: To evaluate the feasibility of incorporating genetic screening for neonatal intrahepatic cholestasis, caused by citrin deficiency (NICCD), into the current newborn screening (NBS) program.

Methods: We designed a high-throughput iPLEX genotyping assay to detect 28 *SLC25A13* mutations in the Chinese population. From March 2018 to June 2018, 237,630 newborns were screened by tandem mass spectrometry at six hospitals. Newborns with citrulline levels between $^{1/2}$cutoff and cutoff values of the upper limit were recruited for genetic screening using the newly developed assay.

Results: The sensitivity and specificity of the iPLEX genotyping assay both reached 100% in clinical practice. Overall, 29,364 (12.4%) newborns received further genetic screening. Five patients with conclusive genotypes were successfully identified. The most common *SLC25A13* mutation was c.851_854del, with an allele frequency of 60%. In total, 658 individuals with 1 mutant allele were identified as carriers. Eighteen different mutations were observed, yielding a carrier rate of 1/45. Notably, Quanzhou in southern China had a carrier rate of up to 1/28, whereas Jining in northern China had a carrier rate higher than that of other southern and border cities.

Conclusions: The high throughput iPLEX genotyping assay is an effective and reliable approach for NICCD genotyping. The combined genetic screening could identify an additional subgroup of patients with NICCD, undetectable by conventional NBS. Therefore, this study

demonstrates the viability of incorporating genetic screening for NICCD into the current NBS program.

Synopsis: Combining newborn metabolic and genetic screening for NICCD

Conflict of Interest

L Zhu is currently an employee of Hangzhou Genuine Clinical Laboratory Co. Ltd. CH Lin is currently an employee of Feng Chi Biotech Corp. CG Huang is currently an employee of Zhejiang Biosan Biochemical Technologies Corp. The other authors declare that they have no conflicts of interest.

Author contributions

YM Lin and YR Liu performed the data analysis, drafted and revised the manuscript; L Zhu and KX Le carried out the genetic tests, mutation analysis and paper editing; YY Shen, CJ Yang, XG Chen, HL Hu, QQ Ma, XQ Shi, ZZ Hu, JB Yang, and YP Shen followed the patients and collected the clinical data; CH Lin designed the study, analyzed and interpreted data, revised the manuscript; CG Huang participated in design and manuscript preparation; XW Huang designed and supervised the research study. All authors contributed to the data analysis, revising and approving the final manuscript to be published.

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Ethics statement

This study was approved by the Ethical Committee of Children's Hospital, Zhejiang University School of Medicine (reference number: 2018-IRB-077) and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents of all infants for collection of samples and publication of medical data.

Abbreviations

CD: citrin deficiency; NICCD: neonatal intrahepatic cholestasis caused by citrin deficiency; FTTDCD: failure to thrive and dyslipidemia caused by citrin deficiency; CTLN2: citrullinemia type II; NBS: newborn screening; MS/MS: tandem mass spectrometry; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; DHPLC: denaturing high performance liquid chromatography; HRM: high-resolution melting; MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; DBS: dried blood spot; SBE: single-base extension; SNP: single-nucleotide polymorphisms; SAP: shrimp alkaline phosphatase; MCT: medium chain triglyceride; CTLN1: citrullinemia type 1; ASLD: argininosuccinate lyase deficiency.

Key Words: neonatal intrahepatic cholestasis caused by citrin deficiency; *SLC25A13*; newborn screening; Agena iPLEX assay; MassARRAY genotyping

1. Introduction

Citrin deficiency (CD) is a pan-ethnic disorder caused by biallelic mutations of the *SLC25A13* gene on chromosome 7q21.3 and is relatively common in East Asian populations[1-5]. Three age-dependent phenotypes have been recognized, namely; neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM #605814) during infancy, failure to thrive and dyslipidemia caused by citrin deficiency (FTTDCD) in older children, and citrullinemia type II (CTLN2, OMIM #603471) in adults[6-10]. NICCD patients are characterized by intrahepatic cholestasis and diverse metabolic abnormalities, including hyperammonemia, citrullinemia, galactosemia, hypoglycemia, and coagulation disorders[11-14]. The onset of NICCD usually resolves spontaneously within the first year following appropriate treatment. Some affected individuals might conversely progress directly into FTTDCD, or step into severe-type CTLN2 decades later[11, 15-17].

Tandem mass spectrometry (MS/MS) is currently widely utilized in newborn screening (NBS) for NICCD. Elevated citrulline levels and several citrulline-based ratios such as citrulline/glutamine and citrulline/arginine are the primary screening markers. However, its performance was not satisfactory since citrulline levels may not be elevated immediately after birth, leading to several missed NICCD cases during NBS[18, 19]. Evidently, lowering the citrulline cutoff values could reduce the number of false negatives, but was expected to produce a significant number of false positives, which is also undesirable. Although plasma bile acid profiles are only used in the differential diagnosis of NICCD, they can serve as a biomarker for

NICCD screening[20]. Therefore, it is still challenging to perform MS/MS-based NBS to accurately detect NICCD, illustrating the urgent need to improve the effectiveness of NICCD NBS[11, 21].

Implementing genetic screening as part of NBS programs could drastically streamline the screening process, but only if appropriate methods are available. Various molecular diagnostic methods, such as direct sequencing, polymerase chain reaction-restriction fragment length polymorphism, multiple GeneScan/SNaPshot analysis, and denaturing high performance liquid chromatography, have been developed for clinical utility[1, 2, 5, 22, 23]. However, these methods are labor-intensive and time consuming, precluding their use in population screening. More recently, high resolution melting (HRM) curve analysis and HybProbe assays have been recommended for screening *SLC25A13* mutations[24-26]. Nonetheless, HRM analysis cannot directly detect specific variants, requiring post-HRM sequencing analysis to confirm the exact type. The high cost of probe synthesis is also a disadvantage of the HybProbe assay. Both methods also require experienced individuals to analyze the data since many parameters, especially the melting temperature (T_m), need to be adjusted periodically. It is therefore critical to develop a novel approach for the early identification of NICCD.

The Agena iPLEX assay is a MassARRAY genotyping platform, based on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry[27]. The MassARRAY assay consists of an initial locus-specific PCR reaction, followed by single base extension, using dideoxynucleotide terminators of a variant-specific oligonucleotide primer that anneals

immediately upstream of the target site[28]. Multiplexing application of the MassARRAY system, allowing for simultaneous assessment of multiple SNPs/variants, is a cost-efficient way to augment high-throughput genotyping output. High sensitivity and specificity have been demonstrated in NBS for Fabry disease, making this assay particularly attractive[29, 30]. Here, we initially developed an Agena iPLEX NICCD assay to identify 28 *SLC25A13* mutations in Chinese populations and evaluated the performance of this assay using 50 clinical positive samples. To explore the potential utility of iPLEX genotyping assay in NBS, we proposed a strategy of combining MS/MS with iPLEX assay for large-scale NICCD NBS. Newborns were first screened by MS/MS and those with citrulline levels between $^{1/2}$cutoff and cutoff values of the upper limit were scheduled for genetic screening.

2. Materials and Methods

2.1 Study subjects

To evaluate the sensitivity and specificity of the iPLEX genotyping assay, 50 clinical patients previously genotyped with NICCD were enrolled. The inclusion criteria for subjects participating in this study were newborns with citrulline levels between $^{1/2}$cutoff and cutoff values of the upper limit. The target sample size for newborn genetic screening was calculated based on the theoretical NICCD incidence of 1/16,639, which was derived according to the Hardy-Weinberg principle, with a carrier rate of 1/65 for China[2]. Using PASS software (package 11.0), with permissible error of 0.03% and a 2-tailed 95% confidence interval, we determined the necessary sample size needed to achieve statistical significance was 19,337. A total of

29,364 newborns from six hospitals were recruited, from a source of 237,630 screened newborns, between March 2018 and June 2018 to increase the screening reliability. The participating hospitals include the Children's Hospital from Zhejiang University School of Medicine (n=14,686, 14.6% of the total screening population), Quanzhou Maternity and Children's Hospital (n=2917, 9.4% of the total screening population), Huaihua Maternal and Child Health Care Hospital (n=2905, 11.7% the total screening population), Yancheng Maternity and Child Health Care Hospital (n=2965, 8.9% of the total screening population), Anhui Women and Child Health Care Hospital (n=2940, 12.5% of the total screening population) and Jining Maternal and Child Health Family Service Center (n=2951, 12.2% of the total screening population). As mentioned in previous articles, the Yangtze River is considered the historical border between northern and southern China[31, 32]. Accordingly, Zhejiang, Quanzhou, and Huaihua are classified as southern regions, Yancheng and Hefei as border regions and Jining as a northern region. This study was approved by the Ethical Committee of Children's Hospital, Zhejiang University School of Medicine (reference number: 2018-IRB-077) and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents of all infants for the collection of samples and publication of medical data.

2.2 Multiplex NICCD assay design

PCR and extension primers were designed for a total of 28 *SLC25A13* mutations, using MassArray Assay Design 3.1 software (Agena, San Diego, CA) with 80< amplicon length (bp)

<120 and 4,300< Mass Range (Da) < 9,400. These primers were run through BLAT and modified where necessary to avoid pseudogene amplification. Targeted primer design used in this study covers almost all the important *SLC25A13* gene mutation sites according to the mutation frequency identified by previous investigations[22, 32] and the mutations discovered in confirmed NICCD patients by our group (Supplementary file 1: Table S1). Two primer panels, namely core well (containing 18 mutations) and plus well (containing 10 mutations),were designed.

2.3 Genotyping using the MassARRAY platform

Reactions were carried out according to manufacturer instructions, using iPLEX reagents (Agena). Approximately 10 ng extracted DNA was then used as a template in each multiplex PCR reaction, which amplified an approximate 100 bp region, targeting the SNPs of interest. Unincorporated dNTPs were then inactivated by adding shrimp alkaline phosphatase (SAP) to the PCR reaction product. Single base extension onto the mutation site, using the extension primers and assay-specific iPLEX terminator nucleotide mixes, was performed after SAP treatment. The mass spectrum from time-resolved spectra was retrieved with a MassARRAY mass spectrometer (Agena). Each spectrum was then analyzed with SpectroTYPER software (Agena) to perform the genotype calling.

2.4 Sanger sequencing

The mutations were confirmed with Sanger sequencing, using the specific primers. PCR (polymerase chain reaction) conditions were carried out according to the TaKaRa LA PCR™

Kit Ver.2.1 (TaKaRa). The PCR products were recovered and purified form agarose gel using NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL). All PCR products were diluted to 10 ng/μL for sequencing with the BigDye® Terminator v3.1Cycle Sequencing Kit (Applied Biosystems, Foster City, California), for amplification and purification. A 10 μL Hi-Di (Applied Biosystems) solution was added to each well. The DNA was denatured at 95℃ for 5 min, transferred to 96-well plates after cooling and sequences analyzed with the ABI 3500XL (Applied Biosystems).

2.5 Newborn genetic screening

Newborn genetic screening was performed to further validate the applicability of our assay. The analytical workflow is summarized in Figure 1. Blood samples were collected by heel-stick and spotted on Whatman 903 filter paper. Dried blood spot (DBS) samples of 29,364 newborns were submitted to Hangzhou Genuine Clinical Laboratory (Hangzhou, Zhejiang, China) for *SLC25A13* mutation screening, after the center had completed NBS. Genomic DNA was extracted from the DBS samples using a Qiagen Blood DNA mini kit (Qiagen, Hilden, Germany) and then preserved and refrigerated at -20°C, after measuring the concentration. DNA quality and quantity was confirmed with a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The concentration of DNA extracted from DBS was 12.513+5.838 ng/μL.

3. Results

3.1 Clinical validation

To validate the performance of iPLEX genotyping assay, we performed a double-blind analysis of 50 positive clinical samples that were genotyped through Sanger sequencing. The iPLEX assay accurately detected all the genotypes from 50 samples, indicating its high sensitivity and specificity (Table 1).

3.2 Newborn genetic screening

Overall, 29,364 (12.4%) newborns received further genetic screening. Five patients with conclusive genotypes were successfully identified and confirmed through Sanger sequencing (Figures 2, 3). Four different *SLC25A13* mutations were detected. The most common mutation was c.851_854del with an allele frequency of 60%. Representative biochemical data for the five patients were collected. Notably, all patients had normal citrulline levels at the time of conventional NBS, but concentrations increased dramatically during the recall. Elevated levels of total bilirubin, direct bilirubin, ammonia and alpha-fetoprotein (AFP) were discovered in every patient, indicating the existence of intrahepatic cholestasis (Table 2).

Breast feeding was stopped once diagnosis was made and galactose-free and/or medium chain triglyceride (MCT)-enriched formula was initiated. The typical symptoms of all patients disappeared after treatment and the biochemical indicators gradually returned to normal, indicating that these 5 patients were diagnosed and treated in time, improving the prognosis.

3.3 Carriers and frequency of *SLC25A13* mutations

As shown in Table 2, a total of 658 individuals with 1 mutant allele were identified as carriers. Eighteen different mutations were observed, yielding a 1/45 carrier rate. The carrier

rates in the southern, border, and northern regions of China was 1/42, 1/56 and 1/43, respectively. Notably, Quanzhou in southern China had a carrier rate of 1/28. The c.851_854del mutation had the highest carrier rate with a relative frequency of 39.82%. The four common mutations exhibited a combined maximal relative frequency of 81.31%.

4. Discussion

NBS for NICCD, using MS/MS, is loaded with false-negative results that need to be urgently resolved. The inherent limitations of current metabolic screening could effectively be overcome with genetic screening. In this study, we successfully established a high throughput iPLEX genotyping assay to detect 28 *SLC25A13* mutations in Chinese populations. The detection rate was 100% in clinical practice. Five NICCD patients, that would have been missed by conventional NBS, were successfully identified by combining MS/MS with the iPLEX assay for large-scale NBS. Therefore, our study demonstrated that incorporating genetic screening into NBS programs could aid in the identification of an additional subgroup of NICCD patients.

Advantages of the iPLEX assay include the automation of high-throughput variants discovery and its capacity for large-scale population screening. Moreover, the iPLEX assay has the advantage of a fast turn-around time (7 h from DNA preparation to data report) and low-cost (approximate running costs of $4.6/sample). The iPLEX assay also has the advantage of panel flexibility. Incorporating novel variants to the assay panel improves convenience. Conversely, a limitation of the iPLEX assay is that it cannot identify de novo variants. This means that novel variants not targeted by this assay panel will be missed[33]. Nevertheless, the

28 mutations in our panel have already accounted for >90% of the reported *SLC25A13* mutations in the general Chinese population, based previous investigations[22, 32] and the collection of 3 million NBS variants from our center. This assay has also been validated by the genotyping of 50 clinical positive samples, with a detection rate of 100%. The designed panel is therefore suitable for large-scale screening as it covers all the important *SLC25A13* mutations in the Chinese population.

In this study, the iPLEX assay was not exclusively used for first-tier screening and was coupled with MS/MS for NICCD NBS, based on various considerations. Firstly, MS/MS is still the main tool used in NICCD NBS, with many rare inherited metabolic disorders screened through MS/MS[34]. Secondly, the combined screening strategy could effectively reduce the running cost. Thirdly, we speculate that combined screening could identify more NICCD cases, since the citrulline levels of most NICCD-affected patients should be higher than the normal population, despite the uncertainty regarding the relation of citrulline concentration and genotype. Thus far all NICCD cases from our center could be detected through this strategy, despite the risk of missing some cases. We therefore believed the combined screening mode to be a reasonable strategy and a promising practice. The question of how much the citrulline cutoff value should be reduced to, in order to achieve the optimal benefit, remains unclear. Further larger scale and more cost-effective analyses are necessary to optimize the screening protocols. If newborn genetic screening was not performed, all five NICCD patients with citrulline levels between the $^{1/2}$cutoff and cutoff values of upper limit, would have escaped

detection by reflecting negative NBS results, and only 4 NICCD patients with elevated citrulline levels were identified. Conversely, these five patients were successfully identified when we applied the combined large-scale screening strategy to current NBS, thus the total frequency of NICCD in our population was 1:26,403. This affords these patients the opportunity to receive timely therapeutic intervention, improving prognosis. By comparison, we identified 7 missed NICCD cases from our 3 million NBS database. Two of these cases presented with impaired liver function due to delayed diagnosis.

Combining NICCD genetic screening with NBS may have various benefits. Elevations of citrulline concentration are not entirely specific to NICCD and are also increased in citrullinemia type 1 (CTLN1) and argininosuccinate lyase deficiency (ASLD). Genetic screening may contribute to the early and definitive diagnosis of NICCD and is valuable in providing pre-symptomatic treatment and ensuring an optimal outcome. Genetic screening also helps clarify the etiology of NICCD and eliminates the need for repeated biochemical tests, especially in borderline cases that may be increasingly unnecessary to parents. Finally, the mutation spectrum and carrier prevalence of *SLC25A13* in target populations could be elucidated through genetic screening.

This is the first large-scale study on newborn genetic screening for NICCD. The carrier rate of NICCD in our population was 1/45. This is similar to 1/42-1/65 in Japanese populations, and higher than 1/90 in Thai populations and 1/112 in Korean populations[2, 25, 35]. However, we should be mindful of the differences in genetic background, sample size and detected mutations

of the tested population. In this study, 69.8% of the subjects were from southern China and as many as 28 mutations were detected. Most previous studies screened fewer mutations[2, 25, 35]. Previous investigations showed a remarkable difference in carrier rates in China, between south (1/48) and north (1/940) of the Yangtze River[2]. The mutations detected in this study were mainly targeted at the Japanese population. Some high-frequency mutations in Chinese populations, including the second-most common mutation IVS16ins3kb, were not included in the test and the sample size from northern China was limited. The results are therefore likely biased, with a potential underestimation of the low carrier rate in the north. In addition, some patient-based studies have shown differences in the regional distribution of *SLC25A13* mutations of Chinese patients. It is noteworthy that only a small number of patients came from northern China, thus the distribution difference may be due, in part, to the geographic location of the hospitals[31, 32]. This study revealed that there may be no differences in carrier rate distributions in the six regions of China. Notably, the carrier rate of Jining was lower than that of Quanzhou, but higher than that of other southern and border cities. Also, the carrier rate of Jining was as high as 1/43, which is significantly higher than the reported carrier rate of 1/940 in northern China[2]. The high carrier rate of Quanzhou is consistent with reports on healthy Taiwanese subjects (1/28), which might be due to the close geographic distribution of Taiwan and Quanzhou[24].

In summary, we have established a high throughput iPLEX assay for NICCD genotyping that could identify an additional subgroup of patients undetectable by MS/MS-based NBS. The

performance of NICCD NBS can be greatly improved by combining newborn metabolic screening with genetic screening, demonstrating the practicality of incorporating genetic screening into the current NBS program.

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References

1. Tabata A, Sheng JS, Ushikai M, Song YZ, Gao HZ, Lu YB, Okumura F, Iijima M, Mutoh K, Kishida S et al. Identification of 13 novel mutations including a retrotransposal insertion in SLC25A13 gene and frequency of 30 mutations found in patients with citrin deficiency. *J Hum Genet* 2008, 53(6):534-545.
2. Lu YB, Kobayashi K, Ushikai M, Tabata A, Iijima M, Li MX, Lei L, Kawabe K, Taura S, Yang Y et al. Frequency and distribution in East Asia of 12 mutations identified in the SLC25A13 gene of Japanese patients with citrin deficiency. *J Hum Genet* 2005, 50(7):338-346.
3. Saheki T, Kobayashi K. Mitochondrial aspartate glutamate carrier (citrin) deficiency as the cause of adult-onset type II citrullinemia (CTLN2) and idiopathic neonatal hepatitis (NICCD). *J Hum Genet* 2002, 47(7):333-341.
4. Dimmock D, Maranda B, Dionisi-Vici C, Wang J, Kleppe S, Fiermonte G, Bai R, Hainline B, Hamosh A, O'Brien WE et al. Citrin deficiency, a perplexing global disorder. *Mol Genet Metab* 2009, 96(1):44-49.
5. Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, Yasuda T, Ikeda S, Hirano R, Terazono H et al. The gene mutated in adult-onset type II citrullinaemia encodes

a putative mitochondrial carrier protein. *Nat Genet* 1999, 22(2):159-163.

6. Saheki T, Kobayashi K, Iijima M, Nishi I, Yasuda T, Yamaguchi N, Gao HZ, Jalil MA, Begum L, Li MX. Pathogenesis and pathophysiology of citrin (a mitochondrial aspartate glutamate carrier) deficiency. *Metab Brain Dis* 2002, 17(4):335-346.
7. Tazawa Y, Kobayashi K, Ohura T, Abukawa D, Nishinomiya F, Hosoda Y, Yamashita M, Nagata I, Kono Y, Yasuda T et al. Infantile cholestatic jaundice associated with adult-onset type II citrullinemia. *J Pediatr* 2001, 138(5):735-740.
8. Ohura T, Kobayashi K, Tazawa Y, Nishi I, Abukawa D, Sakamoto O, Iinuma K, Saheki T. Neonatal presentation of adult-onset type II citrullinemia. *Hum Genet* 2001, 108(2):87-90.
9. Song YZ, Guo L, Yang YL, Han LS, Kobayashi K, Saheki T. [Failure to thrive and dyslipidemia caused by citrin deficiency: a novel clinical phenotype]. *Zhongguo Dang Dai Er Ke Za Zhi* 2009, 11(5):328-332.
10. Song YZ, Deng M, Chen FP, Wen F, Guo L, Cao SL, Gong J, Xu H, Jiang GY, Zhong L et al. Genotypic and phenotypic features of citrin deficiency: five-year experience in a Chinese pediatric center. *Int J Mol Med* 2011, 28(1):33-40.
11. Ohura T, Kobayashi K, Tazawa Y, Abukawa D, Sakamoto O, Tsuchiya S, Saheki T. Clinical pictures of 75 patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). *J Inherit Metab Dis* 2007, 30(2):139-144.
12. Hirayama S, Nagasaka H, Honda A, Komatsu H, Kodama T, Inui A, Morioka I, Kaji S, Ueno T, Ihara K et al. Cholesterol Metabolism Is Enhanced in the Liver and Brain of

Children With Citrin Deficiency. *J Clin Endocrinol Metab* 2018, 103(7):2488-2497.

13. Abuduxikuer K, Chen R, Wang ZL, Wang JS. Risk factors associated with mortality in neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and clinical implications. *BMC Pediatr* 2019, 19(1):18.
14. Chong SC, Lo P, Chow CW, Yuen L, Chu WCW, Leung TY, Hui J, Scaglia F. Molecular and clinical characterization of citrin deficiency in a cohort of Chinese patients in Hong Kong. *Mol Genet Metab Rep* 2018, 17:3-8.
15. Dimmock D, Kobayashi K, Iijima M, Tabata A, Wong LJ, Saheki T, Lee B, Scaglia F. Citrin deficiency: a novel cause of failure to thrive that responds to a high-protein, low-carbohydrate diet. *Pediatrics* 2007, 119(3):e773-777.
16. Okano Y, Ohura T, Sakamoto O, Inui A. Current treatment for citrin deficiency during NICCD and adaptation/compensation stages: Strategy to prevent CTLN2. *Mol Genet Metab* 2019.
17. Komatsu M, Yazaki M, Tanaka N, Sano K, Hashimoto E, Takei Y, Song YZ, Tanaka E, Kiyosawa K, Saheki T et al. Citrin deficiency as a cause of chronic liver disorder mimicking non-alcoholic fatty liver disease. *J Hepatol* 2008, 49(5):810-820.
18. Tamamori A, Fujimoto A, Okano Y, Kobayashi K, Saheki T, Tagami Y, Takei H, Shigematsu Y, Hata I, Ozaki H et al. Effects of citrin deficiency in the perinatal period: feasibility of newborn mass screening for citrin deficiency. *Pediatr Res* 2004, 56(4):608-614.

19. Estrella J, Wilcken B, Carpenter K, Bhattacharya K, Tchan M, Wiley V. Expanded newborn screening in New South Wales: missed cases. *J Inherit Metab Dis* 2014, 37(6):881-887.

20. Yang CH, Chen CY, Chou YY, Chiu HC, Tsai WL, Shiesh SC. Bile acid profiles in neonatal intrahepatic cholestasis caused by citrin deficiency. *Clin Chim Acta* 2017, 475:28-35.

21. Wang LY, Chen NI, Chen PW, Chiang SC, Hwu WL, Lee NC, Chien YH. Newborn screening for citrin deficiency and carnitine uptake defect using second-tier molecular tests. *BMC Med Genet* 2013, 14:24.

22. Song YZ, Zhang ZH, Lin WX, Zhao XJ, Deng M, Ma YL, Guo L, Chen FP, Long XL, He XL et al. SLC25A13 gene analysis in citrin deficiency: sixteen novel mutations in East Asian patients, and the mutation distribution in a large pediatric cohort in China. *PLoS One* 2013, 8(9):e74544.

23. Yamaguchi N, Kobayashi K, Yasuda T, Nishi I, Iijima M, Nakagawa M, Osame M, Kondo I, Saheki T. Screening of SLC25A13 mutations in early and late onset patients with citrin deficiency and in the Japanese population: Identification of two novel mutations and establishment of multiple DNA diagnosis methods for nine mutations. *Hum Mutat* 2002, 19(2):122-130.

24. Lin JT, Hsiao KJ, Chen CY, Wu CC, Lin SJ, Chou YY, Shiesh SC. High resolution melting analysis for the detection of SLC25A13 gene mutations in Taiwan. *Clin Chim Acta* 2011,

412(5-6):460-465.

25. Kikuchi A, Arai-Ichinoi N, Sakamoto O, Matsubara Y, Saheki T, Kobayashi K, Ohura T, Kure S. Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in SLC25A13. *Mol Genet Metab* 2012, 105(4):553-558.
26. Chen ST, Su YN, Ni YH, Hwu WL, Lee NC, Chien YH, Chang CC, Chen HL, Chang MH. Diagnosis of neonatal intrahepatic cholestasis caused by citrin deficiency using high-resolution melting analysis and a clinical scoring system. *J Pediatr* 2012, 161(4):626-631 e622.
27. Gabriel S, Ziaugra L, Tabbaa D. SNP genotyping using the Sequenom MassARRAY iPLEX platform. *Curr Protoc Hum Genet* 2009, Chapter 2:Unit 2 12.
28. Jurinke C, Oeth P, van den Boom D. MALDI-TOF mass spectrometry: a versatile tool for high-performance DNA analysis. *Mol Biotechnol* 2004, 26(2):147-164.
29. Lu YH, Huang PH, Wang LY, Hsu TR, Li HY, Lee PC, Hsieh YP, Hung SC, Wang YC, Chang SK et al. Improvement in the sensitivity of newborn screening for Fabry disease among females through the use of a high-throughput and cost-effective method, DNA mass spectrometry. *J Hum Genet* 2018, 63(1):1-8.
30. Lee SH, Li CF, Lin HY, Lin CH, Liu HC, Tsai SF, Niu DM. High-throughput detection of common sequence variations of Fabry disease in Taiwan using DNA mass spectrometry. *Mol Genet Metab* 2014, 111(4):507-512.
31. Chen R, Wang XH, Fu HY, Zhang SR, Abudouxikuer K, Saheki T, Wang JS. Different

regional distribution of SLC25A13 mutations in Chinese patients with neonatal intrahepatic cholestasis. *World J Gastroenterol* 2013, 19(28):4545-4551.

32. Lin WX, Zeng HS, Zhang ZH, Mao M, Zheng QQ, Zhao ST, Cheng Y, Chen FP, Wen WR, Song YZ. Molecular diagnosis of pediatric patients with citrin deficiency in China: SLC25A13 mutation spectrum and the geographic distribution. *Sci Rep* 2016, 6:29732.
33. Chen JL, Zhang ZH, Li BX, Cai Z, Zhou QH. Bioinformatic and functional analysis of promoter region of human SLC25A13 gene. *Gene* 2019, 693:69-75.
34. McHugh D, Cameron CA, Abdenur JE, Abdulrahman M, Adair O, Al Nuaimi SA, Ahlman H, Allen JJ, Antonozzi I, Archer S et al. Clinical validation of cutoff target ranges in newborn screening of metabolic disorders by tandem mass spectrometry: a worldwide collaborative project. *Genet Med* 2011, 13(3):230-254.
35. Wongkittichote P, Sukasem C, Kikuchi A, Aekplakorn W, Jensen LT, Kure S, Wattanasirichaigoon D. Screening of SLC25A13 mutation in the Thai population. *World J Gastroenterol* 2013, 19(43):7735-7742.

Figure Legends

Figure 1. The analytical workflow of newborn genetic screening for neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). NBS indicates newborn screening; MS/MS, tandem mass spectrometry.

Figure 2. Mass spectra of five NICCD-affected positive patients. Red vertical dotted lines on the left indicate unextended primer mass. Red and blue vertical dotted lines on the right indicate extended primer mass. The blue WT indicates wild type, and the blue MUT/DEL/IVS indicates the corresponding mutations. (Het) indicates heterozygous mutations and (homo) indicates homozygous mutations.

Figure 3. Pedigree verification of *SLC25A13* mutations in five NICCD-affected positive patients and their parents, through Sanger sequencing, to establish segregation. Red arrows indicate identified mutations. P1 to P4 were compound heterozygotes for c.851_854del4bp and other *SLC25A13* mutations. P5 was homozygous for c.851_854del4bp.

Table 1. Genotypes of 50 clinical patients with NICCD detected by the iPLEX assay

No.	Genotype	iPLEX NICCD assay	Sanger sequencing
1	c.851_854del/c.851_854del	14	14
2	c.851_854del/IVS16ins3kb	6	6
3	c.851_854del/IVS6+5G>A	2	2
4	c.851_854del/c.1638_1660dup	2	2
5	c.851_854del/c.1399C>T	3	3
6	c.851_854del/c.135G>C	1	1
7	c.851_854del/c.401T>A	1	1
8	c.851_854del/c.640C>T	1	1
9	c.851_854del/c.889G>T	1	1
10	c.851_854del/c.1078C>T	1	1
11	c.851_854del/IVS11+1G>A	1	1
12	c.851_854del/c.1750+2T>C	1	1
13	c.1638_1660dup/IVS16ins3kb	2	2
14	c.1638_1660dup/IVS6+5G>A	1	1
15	c.1638_1660dup/c.550C>T	1	1
16	c.1638_1660dup/IVS11+1G>A	1	1
17	IVS6+5G>A/IVS6+5G>A	2	2
18	IVS6+5G>A/c.1399C>T	1	1
19	IVS16ins3kb/ IVS16ins3kb	2	2
20	IVS16ins3kb/c.1399C>T	1	1
21	c.1399C>T/c.135G>C	1	1
22	c.1399C>T/c.475C>T	1	1
23	c.15G>A/c.15G>A	1	1
24	c.1177G>A/c.124delA	1	1
25	c.1762C>T/c.1063C>G	1	1
Total		50	50

Table 2. Carriers and frequency of *SLC25A13* mutations in population study

No.	Mutations	Southern regions			Border regions		Northern region	Total (29,364)	Relative frequency (%)
		Zhejiang (n=14,684)	Quanzhou (n=2917)	Huaihua (n=2905)	Yancheng (2965)	Hefei (n=2940)	Jining (n=2951)		
1	c.851_854del4bp	141	36	35	9	19	22	262	39.82
2	IVS16ins3kb	50	24	15	18	7	23	137	20.82
3	IVS6+5G>A	42	25	3	8	7	7	92	13.98
4	c.1638_1660dup	25	6	2	5	3	3	44	6.69
5	c.1231G>A	16	1	2	0	0	1	20	3.04
6	IVS11+1G>A	3	0	0	7	4	4	18	2.74
7	c.1622C>A	10	4	1	0	3	0	18	2.74
8	c.1399C>T	9	1	0	2	0	2	14	2.13
9	c.775C>T	8	1	1	1	1	0	12	1.82
10	c.1095delT	3	0	0	2	3	0	8	1.22
11	c.955C>T	4	1	1	1	0	1	8	1.22
12	c.1177G>A	4	0	0	0	1	2	7	1.06
13	c.1064G>A	0	3	0	3	0	0	6	0.91
14	c.550C>T	1	0	1	0	1	1	4	0.61
15	c.2T>C	2	0	1	0	0	0	3	0.46
16	c.754G>A	1	2	0	0	0	0	3	0.46
17	c.1063C>G	0	0	0	0	0	1	1	0.15
18	c.265delG	0	0	0	0	0	1	1	0.15
	Total	319	104	62	56	49	68	658	100.00
	Carrier rate	1/46	1/28	1/47	1/53	1/60	1/43	1/45	

Table 3. Biochemical and genetic findings of five patients with NICCD

	P1	P2	P3	P4	P5	Reference range
Regions of Residence	Zhejiang	Zhejiang	Quanzhou	Jining	Huaihua	-
DBS Citrulline at first screening (μmol/L)	36.07	24.94	17.85	26.56	29.20	-
DBS Citrulline at diagnosis (μmol/L)	143.78	97.03	223.54	176.53	201.60	-
Current Reference Value (μmol/L)[a]	7.1-37.0	7.1-37.0	6.0-34.0	6.8-35.0	6.2-34.0	-
Genotype	c.851_854del/ IVS16ins3kb	c.851_854del/ IVS16ins3kb	c.851_854del/ IVS6+5G>A	c.851_854del/ IVS11+1G>A	c.851_854del/ c.851_854del	-
ALT (IU/L)	45	78	30	67	40	<50
AST (IU/L)	45	67	36	69	70	15-60
γ-GT (IU/L)	20	47	35	24	34	2-30
Total Protein (g/dL)	45.7	34.6	46.1	22.9	28.1	60-81
ALB (g/dL)	23.9	33.6	32.4	18.7	20.7	35-55
Total Bilirubin (mg/dL)	120.6	234.9	132.2	140.7	165.5	3.4-17.1
Direct Bilirubin (mg/dL)	96.5	128.3	105.6	112.3	99.8	0-3.4
Ammonia (μmol/Lol/L)	46	55	34	68	59	<30
Lactate (mmol/L)	1.9	1.8	2.1	2	1.8	<2.2
Hemoglobin (g/L)	96	92	104	97	99	110-116
AFP (ng/mL)	29167.00	46563.00	70567.40	50786.60	49887.30	0-25

[a]The cutoff values of citrulline were set on the 99.5th (0.05th) percentile in each laboratory. P1 to P5: Patient 1 to 5. DBS, dried blood spot; ALT, alanine transaminase; AST, aspartate transaminase; γ-GT, gamma-glutamyl transpeptidase; ALB, albumin; AFP, alpha fetoprotein.