

***SLC25A13* cDNA cloning analysis using peripheral blood lymphocytes facilitates the identification of a large deletion mutation: Molecular diagnosis of an infant with neonatal intrahepatic cholestasis caused by citrin deficiency**

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Abstract. Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) is an autosomal recessive disorder resulting from biallelic mutations of the *SLC25A13* gene. Due to the lack of well-recognized clinical or biochemical diagnostic criteria, the definitive diagnosis of this disease relies on the genetic analysis of *SLC25A13* at present. As novel large deletion/insertion mutations of the *SLC25A13* gene are difficult to detect using routine DNA analytic approaches, the timely diagnosis of patients with these types of mutations remains a challenge. The present study aimed to examine *SLC25A13* mutations in an infant with a suspected diagnosis of NICCD. DNA was extracted from blood samples, and *SLC25A13* mutations were examined by screening for

high-frequency mutations and Sanger sequencing. Reverse transcription-polymerase chain reaction and cDNA cloning analyses were then performed using peripheral blood lymphocytes (PBLs) to identify the obscure mutation. The results demonstrated that the infant was heterozygous for a paternally-inherited mutation, c.851_854del4, and a maternally-inherited large deletion, c.1019_1177+893del, which has not been reported previously. A positive diagnosis of NICCD was made, and the infant responded favorably to a galactose-free and medium-chain triglyceride-enriched formula. The present study confirmed the effectiveness of this formula in NICCD therapy, enriched the *SLC25A13* mutational spectrum and supported the feasibility of cDNA cloning analysis using PBLs as a molecular tool for facilitating the identification of large *SLC25A13* deletions.

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Abbreviations: CD, citrin deficiency; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; AGC2, aspartate/glutamate carrier isoform 2; PBLs, peripheral blood lymphocytes; PCR, polymerase chain reaction; LA-PCR, long and accurate-PCR; RT-PCR, reverse transcription-PCR; ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ -glutamyl transpeptidase; TBA, total bile acid; Tbil, total bilirubin; Dbil, direct bilirubin; Ibil, indirect bilirubin; MCT, medium-chain triglyceride; GC-MS, gas chromatography-mass spectrometry; MS-MS, tandem mass spectrometry; ASVs, alternative splice variants; CMV, cytomegalovirus; NADH, reduced form of nicotinamide-adenine dinucleotide

Key words: *SLC25A13*, citrin deficiency, peripheral blood lymphocytes, cDNA cloning

Introduction

Citrin deficiency (CD) is an autosomal recessive disorder caused by biallelic mutations in the *SLC25A13* gene, which encodes citrin, the aspartate/glutamate carrier isoform 2 (AGC2) (1-3). In 1999, Kobayashi *et al* (4) cloned the *SLC25A13* gene, providing a foundation for subsequent investigations of CD. An increasing number of patients have been diagnosed with CD, not only in Asia (5-16), but also in Europe (17-20) and North America (21-24). Currently, CD has been recognized as a worldwide panethnic disease.

Neonatal intrahepatic cholestasis caused by CD (NICCD) is a CD phenotype found in neonates and infants, and its diagnosis relies on the genetic analysis of *SLC25A13* (2). However, conventional DNA analytic approaches, including PCR and Sanger sequencing, cannot identify all *SLC25A13* mutations (25). Obscure mutations are usually large insertions/deletions (12,24,26), and the early diagnoses of patients with these types of mutations require time-consuming and expensive experimental techniques, which presents a constant challenge. In the present study, a novel large *SLC25A13* deletion was identified in a patient with NICCD using a diverse

range of tools, including cDNA cloning analysis of the *SLC25A13* gene in the peripheral blood lymphocytes (PBLs) of the infant. The present study reports on the molecular and clinical findings.

Materials and methods

Subjects and ethical statement. A 6-month-old male infant who was referred to Department of Pediatrics (First Affiliated Hospital, Jinan University, Gangzhou, China) on May 20, 2014 and his parents were enrolled as research subjects. The clinical findings were described as a case report. The data were predominantly collected from the Department of Pediatrics and biochemical analysis was performed in the Department of Laboratory Science. However, partial biochemical or imaging results from the Children's Hospital Affiliated to the Capital Institute of Pediatrics (Beijing, China) were provided by the parents upon request. Written informed consent was obtained from parents prior to the investigation, which was approved by the Committee for Medical Ethics of the First Affiliated Hospital, Jinan University (Guangzhou, China).

Detection of high-frequency mutations and Sanger sequencing. As described previously (10-12,16), peripheral blood samples were collected from the subjects, and DNA was extracted according to the instructions of the genomic DNA extraction kit (Simgen, Hangzhou, China). The four high-frequency *SLC25A13* mutations c.851_854del4, c.1638_1660dup, c.615+5G>A and IVS16ins3 kb, were screened using polymerase chain reaction (PCR)/long and accurate (LA)-PCR and PCR-restriction fragment length polymorphism procedures. All 18 exons and their flanking sequences in the *SLC25A13* gene were then sequenced to identify the possible mutations. All PCR amplification in the present study was conducted by using a Mastercycler nexus PCR instrument (Eppendorf, Hamburg, Germany) and Sanger sequencing was performed using a 96-capillary ABI 3730xl DNA Analyzer with BigDye Terminator version 3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol.

Reverse transcription (RT)-PCR and cDNA cloning analysis. The cDNA cloning was performed as in our previous study (12,27). Briefly, PBLs were isolated from the heparinized blood samples of the infant using lymphocyte separation medium (ICN Biomedicals, Santa Ana, CA, USA). RNAiso Plus (Takara Bio, Inc., Otsu, Japan) was then used to extract the total RNA. Following this procedure, RT-nested PCR was performed. The cDNAs were synthesized from 2 µg of total RNA in the presence of primer UP-dT (5'-CGGCAGTGGTATCAACGCAGAGTAC(T)₁₈-3') and 200U MMLV reverse transcriptase (Promega, Beijing, China). The first PCR was carried out in a 50 µl volume containing 10 µl of 5 x PrimeSTAR buffer (Mg²⁺ plus), 0.5 µl of PrimeSTAR HS DNA polymerase (2.5 U/µl, Takara Bio, Inc., Dalian, Liaoning, China), 1 µl of cDNA, 1 µl of each primer RAS2 (5'-AACGCACGCTGCCTGCCGTATC-3') and RACEA1 (5'-CCACCTTCACAAATTCATGCGCC-3'; 20 µM), 4 µl of dNTP mixture (10 mM) and 32.5 µl PCR-grade water. Following initial denaturation at 94°C for 3 min, 20 cycles of DNA amplification were performed

(98°C for 10 sec, 60°C for 15 sec, 72°C for 4 min), followed by a terminal extension at 72°C for 7 min. Subsequently, 1 µl of the first PCR product was subjected to the second PCR for 30 cycles in 50 µl mixture including 10 µl of 5 x PrimeSTAR buffer (Mg²⁺ plus), 0.5 µl of PrimeSTAR HS DNA polymerase (2.5 U/µL, Takara Bio, Inc), 4 µl of dNTP mixture (10 mM), 1 µl of each primer RAS3 (5'-GCCGCCGGGACTAGAAAGTGAGC-3') and Ex18R (5'-TGCTTCATTCCCAGGAGGGA-3'; 20 µM), and 32.5 µl PCR-grade water. The PCR thermocycling conditions were: 94°C for 3 min followed by 30 cycles of 98°C for 10 sec, 60°C for 5 sec, 72°C for 2.5 min and a final extension step at 72°C for 10 min. The products were then purified by a gel extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and cloned into the pMD™ 18-T Vector (Takara Bio, Inc.), following which they were transformed into DH5α *Escherichia coli* competent cells by means of heat shock and cultured for 12-16 h at 37°C. Positive clones were subsequently selected and the *SLC25A13* transcripts were sequenced. The sequencing results were aligned with the *SLC25A13* cDNA sequence, which was available at <http://www.ensembl.org/> by using a DNAMAN software version 7.212 (Lynnon Corporation, QC, Canada).

LA-PCR approach. Based upon the findings of the cDNA cloning and sequencing, the c.851_854del4 mutation was used as a marker for identifying the obscure mutation in the DNA samples. For LA-PCR, three sets of primers (Fig. 1) were designed and the PCR kit was purchased from Takara Bio, Inc. Every 50 µl LA-PCR mixture comprised 0.5 µl LA Taq (5 U/µl), 5 µl 10 x LA Buffer (Mg²⁺ plus), 1 µl DNA, 6 µl dNTP mixture (2.5 mM), and 35.5 µl PCR-grade water and the relevant primer pair (Set A, B and C in Fig. 1, respectively, with 1 µl of each primer in 20 µM). The temperature profile was set at 94°C for 4 min, followed by 35 cycles at 98°C for 11 sec, 62°C for 40 sec, 68°C for 3 min and a final extension step at 68°C for 10 min. The amplified products were then separated using electrophoresis, purified and sequenced, respectively. The electrophoresis was conducted in an 1.5% agarose gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the target products were purified by a gel extraction kit (Omega Bio-Tek, Inc.).

Results

Case report. A male infant, aged 2.6 months, was admitted to the Children's Hospital Affiliated to the Capital Institute of Pediatrics due to prolonged jaundice for >2 months. As the first child of a non-consanguineous couple, the infant was born at the gestational age of 38 weeks with a birth weight of 2.35 kg [-2.1 standard deviations (SD)] and a body length of 48 cm (-0.9 SD). The father and grandmother had hypercholesterolemia. There was no family history of any other genetic disease.

Physical examination revealed a body weight of 4.7 kg (-2.0 SD), body length of 55 cm (-2.2 SD) and a head circumference of 37.5 cm (-1.7 SD). Jaundiced skin and sclera were observed. No positive signs were found in the two lungs or the heart. The liver was palpable with a soft edge 3 cm below the right costal margin. Biochemical analysis showed increased levels of serum γ-glutamyl transpeptidase, total

Table I. Biochemical alterations over time in the patient with neonatal intrahepatic cholestasis caused by citrin deficiency.

Biochemical index	Normal range	Age (months)									
		2.6 ^a	3.1	3.3 ^b	3.5 ^c	4	4.4	7.3	9.4	12	18
ALT (U/l)	5-40	66.0	26.00	113.0	75.7	24.0	42.0	71.0	47.00	46.0	27
AST (U/l)	5-40	132.0	52.00	578.0	134.3	47.0	58.0	63.0	48.00	36.0	47
GGT (U/l)	8-50	171.0	152.00	215.0	165.3	139.0	58.0	22.0	25.00	21.0	21
ALP (U/l)	20-500	810.0	396.00	395.0	-	412.0	285.0	241.0	198.0	208.0	240
TP (g/l)	60.0-83.0	45.8	52.00	48.2	-	54.1	56.3	57.8	68.20	67.9	73.4
Alb (g/l)	35.0-55.0	32.2	35.30	38.2	-	39.1	40.1	45.8	50.30	50.8	50.3
Glb (g/l)	20.0-30.0	13.6	16.70	10.0	-	15.0	16.2	12.0	17.90	17.1	23.1
Tbil (μ mol/l)	2-19	174.7	134.90	192.8	126.2	44.9	13.3	11.4	5.90	7.0	8.0
Dbil (μ mol/l)	0-6	99.7	60.40	83.0	66.7	18.5	4.5	2.0	1.30	1.5	2.2
Ibil (μ mol/l)	2.56-20.9	75.0	74.50	109.8	59.5	25.4	8.8	9.4	4.60	5.5	5.8
TBA (μ mol/l)	0-10	210.2	168.83	170.9	123.2	64.5	25.6	6.9	7.36	7.3	0.5

^aAge at which lactose-restricted milk was introduced; ^bage at which laparoscopic surgery was performed and a T tube drain was positioned in the gallbladder bed; ^cage at which the T tube was removed and lactose-free/MCT-enriched therapeutic formula was introduced.

Table II. *SLC25A13* ASVs detected by cDNA analysis using peripheral blood lymphocytes of the patient with neonatal intrahepatic cholestasis caused by citrin deficiency.

Allele	Name	ASV	Annotation	Clone (n)	Percentage
Maternally-inherited	M-01	<i>r.213_468del; r.1019_1177del</i>	Exon 4, 5 and 11 skipping	1	16.7
	M-02	<i>r.213_328del; r.1019_1177del</i>	Exon 4 and 11 skipping	1	16.7
	M-03	<i>r.1019_1177del</i>	Exon 11 skipping	2	33.3
	M-04	<i>r.213_328del; r.1019_1177del; r.1311_1312ins1311+102_1311+176</i>	Exon 4 and 11 skipping	1	16.7
	M-05	<i>r.213_468del; r.755_933del; r.1019_1177del</i>	Exon 4, 5, 8, 9 and 11 skipping	1	16.7
Paternally-inherited	F-01	<i>r.213_328del; r.851_854del4</i>	Exon 4 skipping	1	100

ASV, alternative splice variant.

bilirubin, direct bilirubin and total bile acids, as shown in Table I, indicating the presence of cholestasis. The levels of cytomegalovirus (CMV) DNA in the urine and breast milk were 5.57×10^3 copies/ml and 1.07×10^4 copies/ml, respectively.

According to the above findings, the patient was suspected to have CMV hepatitis, and breastfeeding was terminated followed by the commencement of galactose-restricted milk and ganciclovir treatment. As a result, the urinary CMV DNA levels became negative; however, the infant remained jaundiced and there was no marked improvement in biochemistry (Table I). Laparoscopy and cholangiography were then performed at 3.3 months (in a hospital in Beijing, China), revealing normal bile ducts, however, liver biopsy showed microvesicular steatosis, mildly hepatocellular and canalicular cholestasis, moderate inflammatory cell infiltration in the portal tracts and perisinusoidal fibrosis. Marginally elevated levels of citrulline, ornithine and methionine were found on tandem mass spectrometry (MS-MS) analysis of the serum

amino acids; whereas a high level of 4-hydroxyphenyllactate was identified in the urinary gas chromatography (GC)-MS assay (both previously performed at a hospital in Beijing, China).

In view of the above findings, the patient was suspected to have NICCD, and was switched to a galactose-free, MCT-enriched formula at the age of 3.5 months. His jaundice subsided rapidly, and the biochemical abnormalities improved markedly within half a month. At the 12-month follow-up, the patient had a body weight of 10.0 kg (-0.1 SD), body length of 74 cm (-1.2 SD) and head circumference of 46 cm (-0.4 SD), and cholestatic indices were no longer detected on biochemical assessment. The patient (aged, 6 months) was referred to our hospital for *SLC25A13* genetic analysis.

Results of DNA sequencing and cDNA cloning analysis. Screening of high-frequency mutations and Sanger sequencing of the *SLC25A13* gene in the infant revealed a

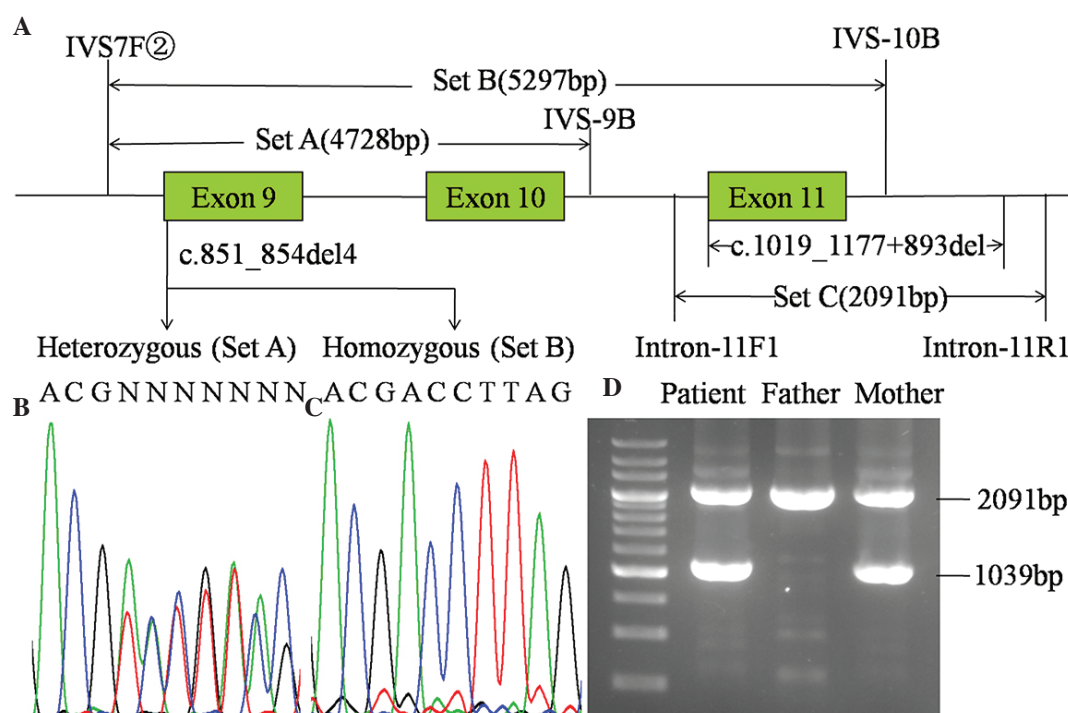


Figure 1. Identification of the large deletion, c.1019_1177+893del. (A) Positions of the primer sets are shown in the upper lane. Primer sequences were Intron11-F1, 5'-TGAGGTTGGGCTGTAGCAATGTG-3'; Intron11-R1, 5'-GAAC TGAAACGGCAAGAGTGGAAG-3'; IVS7F②, 5'-GGTATATTTGTTGCTTGTGTTT-3'; IVS-9B, 5'-CCTGTCTTTGGAA GGCCTGA-3' and IVS-10B, 5'-CTAGATGCCTCCAGCCTACT-3', respectively. The c.851_854del mutation was (B) heterozygous when amplified using primer set A and homozygous when amplified using (C) primer set B. (D) Long and accurate polymerase chain reaction analysis using primer set C yielded an unexpected band of ~1 kb inherited from the mother, in addition to the expected 2,091 bp product from the father.

5'-tgagggtgggctgtagcaatgtgccttaattaaatctatgacatcctttattaggggatactgaatgaaaacccctcaat
tgtttataaagataaagaagtagtagctgttttaaactctgtatttaaattcaaacacatttctcagctctgtcctgacgt
taactacctcttcttctgacatcttcttctgagataatgataattgtaatttttag **CTGTTGGAGCCACTGCT**
GTGTAATCTCTATCGATCTTGTA AAAAC TCGAATGCAGAACCAACGATCAACTGGCTC
TTTTGTGGGAGAACTCATGTATA AAAAC AGCTTTGAC TGTTTTAAGAAAGTGC TACG
CTATGAAGGCTTCTTTGGACTGTATAGAGgttagtgccacatgctcaatacctgttaggtgaataa
cactcaaggtttgttctcatctagtgcctgacatgaattagcaagctgcgttaaatggaaatacagcagattt
caattatccatgctaagggagtaggctggaggcatctaggcttggtcaatcaaacagccataatctgaaatt
atttatagtgctctgtgtattttcttaaaataataaattttttataagcatcttccgtgaattttgaaggctcac
attgttgcaccttgaagtactctattctccagctaacagggaagggtattctgccaccaggacaagcctactcag
gattcatgatttctggaatacaccacaaaaagataacgaagggttcagtagagctctctttgctgctcatcctg
aattaaagtgtcccttttctcatctgactctcaaccatggagttgattcacacacccctctagccaaggtttccc
ccaactgagacagcctgaaacaggtaactcttcttccaccttttcttaaccatctaaggttcattaagcaatggaa
gtagtagcagagagtagtcatcactgtaggaaggatccacagaccctgactgctttccactggcattggccg
aaggagactctcaactgttcttgcgggattgcttctacgagaaggaaacaggtttctgcatcagttaaatacag
tatcacaaggcagttcaactcagctgttataattggcagcggcaagctggcagagtttccctctctccct
actaatgctctctagatgaagaaaacagtaatttagtactctgctgaggtggacggtgaaa ctggcagacatggaaa
cctgtgctctctgggctcttgcacatctgactctgtctcaatttaataccacagtaaaaaaaacaaaaggataaac
ctttctgaacacctcacccttagaagccagaaagtctcaaacctcagagaagttcattttgcagggtcctattgttaggt
agcccccatcaaggcctttgatcttgcctgctccctcttctatccctgctcttcttaaatggaaggctttgacctcagg
tgattgatgatgaccttccaggtcactgaccttcaattgaaatgcttttctccctgatggcaacatttgcgaatgaaatggct
ccaagtagtaacaggtaatgtcacagttcctatctgcagtgagtcaggcaaaattggccgcttaacctgtccatattgggtgt
tttgaagggaatctgagctcaagctgggagacatctgagcattgtctcataaactattttgggcttttctgttattagat
taaatgtcaaggatgctttagaattgtaagaatgttcccaactgctcagtgctttaaattgtctaagttgatttttattgtatc
cttttctgactttagaccatacctggcaggctgattttgttgcgtctataattccctttgcaacctgtactgttggcaactagaa
gtgtgcctgtagtctgagctaatgtggcctatgcaagaggctccactctgcgtttca-3'

Figure 2. Illustration of the nucleotide sequences of the LA-PCR products. The large deletion, c.1019_1177+893del (represented in bold brown, 1,052 bp in size) involved the entire exon 11 (upper case letters) and partial sequences of intron 11 (lower case letters). Underlined regions represent the positions of the Intron11-F1 and Intron11-R1 primers for LA-PCR, which yielded the expected band of 2,091 bp, and an unexpected band of 1,039 bp resulting from the large deletion. The lower-case letters in green represent the undeleted sequences in introns 10 and 11, respectively. LA-PCR, long and accurate polymerase chain reaction.

paternally-inherited mutation, c.851_854del4. Following *SLC25A13* cDNA cloning analysis using PBLs, five alternative splice variants (ASVs) were identified from the maternally-inherited *SLC25A13* allele, with the common feature of exon 11 skipping (r.1019_1177del), as shown in Table II. This suggested the existence of a large insertion or deletion within the DNA fragment spanning the region between intron 10 and intron 11 in the *SLC25A13* gene.

Identification of the large deletion. The targeted DNA span between intron 10 and intron 11, described above, was then investigated to identify the obscure mutation of maternal origin. As shown in Fig. 1, the c.851_854del4 mutation was heterozygous in the PCR product using primer set A, and was homozygous with primer set B. These results led to the design of primer set C, with which the LA-PCR amplification procedure yielded an unexpected product of ~1.0 kb in the infant and the mother in addition to the expected 2,091 bp band in all family members. As shown in Fig. 2, direct sequencing of the unexpected product revealed a large deletion, c.1019_1177+893del, with a length of 1,052 bp, entirely involving exon 11 and partially involving intron 11.

Discussion

The intro- and extra-uterine growth retardation, prolonged jaundice, liver enlargement and biochemical alterations, which were exhibited by the infant in the present study were all non-pathognomonic. However, the metabolome and hepatopathologic findings were consistent with the relevant results in cases of NICCD reported previously (10,28), warranting the genetic analysis of *SLC25A13* in the present study. Based on the findings of cDNA cloning analysis using PBLs and the subsequent LA-PCR analysis and Sanger sequencing procedures, the maternally-inherited novel deletion, c.1019_1177+893del, was identified in the patient. To the best of our knowledge, this mutation has not been reported previously. The identification of this large deletion, in addition to the paternally-inherited c.851_854del4 mutation identified, constituted reliable evidence for a definitive diagnosis of NICCD in the infant.

The novel large deletion caused the production of the r.1019_1177del (exon 11 skipping) ASV, which predictively led to the loss of 53 amino acid residues (codons 340-392) in the citrin protein. According to the functional domains of citrin (4), the loss of these residues produces a truncated citrin molecule without the first and second transmembrane domains. Consequently, the AGC2 function was impaired in all hepatocytes, resulting in the laboratory and clinical manifestations observed in the infant with NICCD. Of note, this pathogenic process, resulting from the novel large deletion, is consistent with that underlying the IVS11+1G>A mutation reported previously, which also resulted in exon 11 skipping (r. 1019_1177del) during the splicing of pre-mRNA (4).

Accumulating clinical evidence has suggested the therapeutic effectiveness of galactose-free and MCT-enriched formulas in patients with NICCD (9,16,29,30), and the clinical findings in the present study further supported this. A key biochemical alteration of citrin deficiency is the increased cytosolic NADH/NAD⁺ ratio in hepatocytes,

and a malate-citrate shuttle may be a form of compensation for the increased ratio (1). The pathophysiology of CD may be associated with an energy shortage in the liver caused by impairment of glycolysis caused by the increased NADH/NAD⁺ ratio (29,31). The metabolism of galactose in hepatocytes further increases this ratio and reduces the production of mitochondrial acetyl-CoA, a component of the malate-citrate shuttle. By contrast, MCT supplementation can produce an excess of acetyl-CoA in the mitochondria of hepatocytes, providing energy and improving the cytosolic NADH/NAD⁺ ratio via the malate-citrate shuttle (29,31). These mechanisms explain the clinical and laboratory improvements of the patient examined in the present study shortly following the introduction of the galactose-free and MCT-enriched formula.

In conclusion, the present study reported on clinical and molecular investigations of an infant with NICCD, who was confirmed to be a compound heterozygote of the c.851_854del4 mutation and a novel large deletion c.1019_1177+893del in the *SLC25A13* gene. These findings confirmed the effectiveness of the galactose-free and MCT-enriched formula on NICCD therapy, enriched the *SLC25A13* mutational spectrum, and supported the feasibility of *SLC25A13* cDNA cloning analysis using PBLs as a molecular tool to facilitate the identification of large *SLC25A13* deletion.

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