Clinical, Molecular and Functional Investigation on an Infant with Neonatal Intrahepatic Cholestasis Caused by Citrin Deficiency (NICCD)

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Abstract

**Background and Objective:** SLC25A13 analysis has provided reliable evidences for the definitive diagnosis of citrin deficiency (CD) in the past decade. Meanwhile, these studies generated some issues yet to be resolved, including the pathogenicity of SLC25A13 missense mutations and the mRNA product from the mutation c.615+5G>A. This study aims to investigate the effect of a novel missense mutation on the aspartate/glutamate carrier (AGC) function of citrin protein, and to explore the aberrant transcript from c.615+5G>A in the same CD infant.

**Methods and Results:** By means of screening for prevalent SLC25A13 mutations and exons sequencing, the patient proved a compound heterozygote of c.615+5G>A and a novel c.1064G>A (p.Arg355Gln) mutation. An aberrant transcript with retention of the entire intron 6, r[615+1,615+1789ins; 615+5 g->a] (GenBank accession number KJ128074), which was resulted from c.615+5G>A, was detected by RT-PCR and cDNA sequencing. After bioinformatic analyses of the novel missense mutation c.1064G>A, the growth abilities of three agc1Δ yeast strains were tested, which had been transformed with recombinant or empty vectors, respectively. Besides the bioinformatically pathogenic evidences, the growth ability of the agc1Δ strains transformed with mutant recombinant was the same as with empty vector, but significantly lower than that with normal control in functional analysis.

**Conclusions:** A CD infant was definitely diagnosed in this paper by a genetic, transcriptional and functional analysis of SLC25A13 gene. This study provided direct laboratory evidences supporting the splice-site nature of the c.615+5G>A mutation, and the novel c.1064G>A variation, which proved a pathogenic mutation bioinformatically and functionally, enriched the SLC25A13 mutation spectrum.

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Introduction

Human citrin deficiency (CD) is an autosomal recessive disease entity caused by SLC25A13 gene mutations [1]. As the liver-type aspartate-glutamate carrier isoform 2 (AGC2), citrin plays roles in the metabolic pathways of aerobic glycolysis, gluconeogenesis, urea cycle, and synthesis of proteins and nucleotides [2–6], and the deficiency of citrin leads to a variety of biochemical, metabolomics, medical imaging, hepatohistological and even behavioral alterations [7–11]. Up to now, at least three age-dependant clinical phenotypes have been described for this disease entity, i.e. Neonatal Intrahepatic Cholestasis caused by Citrin Deficiency (NICCD, OMIM#605814) in infants, adult-onset citrullinemia type II (CTLN2, OMIM#603471) in adolescents/adults, and Failure to Thrive and Dyslipidemia caused by Citrin Deficiency (FTTDCD), a novel CD phenotype between NICCD and CTLN2 stage which was proposed very recently [12–15].

The analyses of SLC25A13 gene and its transcriptional and/or translational products have provided reliable evidences for the definitive diagnoses of CD patients worldwide [16–20]. However, molecular diagnosis of CD patients in the past decade engendered some issues to be resolved. For example, the abnormal mRNA molecule from the mutation c.615+5G>A was not detected yet, making it obscure how this mutation causes formation of inactive citrin protein [17,21]. Furthermore, most of the reported SLC25A13 mutations are missense mutations [13,22], but their pathogenicity was just predicted on the basis of clinical and bioinformatics analysis, and functional study on SLC25A13 missense mutations still remains rather limited [23,24].

Very recently, we encountered a new NICCD child in our clinical practice, and SLC25A13 analysis uncovered a novel missense mutation along with the possible splice-site mutation c.615+5G>A. In this paper, by using an agc1Δ yeast model, we
investigated the functional effect of the novel mutation, and the aberrant transcript in peripheral blood lymphocytes (PBLs) due to the mutation c.615+5G>A was also explored. We herein reported the clinical, molecular and functional findings.

**Subjects and Methods**

**Subjects and Ethics Statement**

The research subjects in this study were a male patient (C0165) suspected to have NICCD and his parents as well. The clinical information was collected and described as a case report. This research was carried out with written informed consent from the parents, and has been approved by the Committee for Medical Ethics, the First Affiliated Hospital, Jinan University in China, adhering to the World Medical Association Declaration of Helsinki (WMADH 2008), which was adopted by the 59th WMA General Assembly, Seoul, in October 2008.

**SLC25A13 Mutation Analysis**

Genomic DNA was extracted from peripheral venous blood samples collected from the subjects. Four High-frequency mutations, i.e. c.851_854delGTAT, c.1638_1660dup, c.615+5G>A and IVS16ins3kb, were screened by PCR/LA-PCR and PCR-RFLP. To explore the possible novel mutation, all the 18 exons and their flanking sequences in SLC25A13 gene were amplified by PCR/LA-PCR, and the products were then sequenced, as described previously [8,12,16,25].

**Bioinformatic Analyses**

The conservative property of the amino acid affected by the novel missense mutation was surveyed by using the software ClustalX 2.0 (http://www.ebi.ac.uk/Tools/msa/clustalo/). The amino acid sequence of human citrin was comparatively aligned with those of the homologous proteins in various species including *H. sapiens* (NP_055066.1), *B. Taurus* (DAA30813.1), *X. tropicalis* (AAI59168.1), *A. aegypti* (XM_001651912.1), *S. cerevisiae* (NP_015346.1) and *C. elegans* (NM_064873.3), which were obtained from the NCBI database (www.ncbi.nlm.nih.gov).

To evaluate the structural effect on citrin protein caused by the amino acid change, a three-dimensional (3D) model of the transmembrane segment of citrin protein was constructed by homology modeling with the assistance of the online tool SWISS-MODEL [26,27] with the bovine mitochondrial ADP/ATP carrier monomers (2c5eA) [28] as a template. Structures were viewed using the software SWISS-Pdb Viewer 4.10.

The software PolyPhen-2 (Polymorphism Phenotyping version 2.2.2, available at http://genetics.bwh.harvard.edu/pph2/) was applied to predict the possible impact of the missense mutation on the structure and function of human protein [29]. If the probabilistic score of one mutation is above 0.85, it will be classified as “probably damaging”, while as “possibly damaging” with the score above 0.15 [29]. Moreover, MutationTaster (http://mutationtaster.org/MutationTaster/index.html) was also used to evaluate the disease-causing potential of the missense mutation. The probability value close to 1 indicates a high ‘severity’ of the prediction [30].

**Reverse Transcription-PCR (RT-PCR)**

The RT-PCR process was conducted as in our previous publications [13,16,25]. Briefly, two milliliters of EDTA anticoagulant peripheral blood was centrifuged over Lymphocyte Separation Medium (LSM, MP) immediately after sampling from the patient and his parents. Peripheral blood lymphocytes (PBLs) were collected according to the manufacturer’s instructions and then homogenized immediately in Trizol (Life Technologies) to extract total RNA following the manufacturer’s protocol. cDNAs were synthesized from 2 µg of total RNA in the presence of primer oligo-(dT)18. Using the cDNA above as template, PCR was performed with the primer set Ex5F and Ex8R (Table 1), and the temperature profile was 94°C for 3 min followed by 30 cycles of 90°C for 10 s, 60°C for 5 s, 72°C for 2.5 min and a final extension step at 72°C for 10 min. The RT-PCR products were then sequenced to explore aberrant transcript with c.615+5G>A.

**Yeast Strains and agc1 Gene Disruption**

The yeast strain *Saccharomyces cerevisiae* BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) was used to construct a caryokaryotic model by disruption of *agc1*, the gene highly homologous to *SLC25A13* in human, by means of the Cre-loxP recombination system with plasmid set of pUG6 and pSH47 [as illustrated in Figure 1]. All the primers in this process were listed in Table 1 and all culture media prepared as described previously [31,32]. The improved LiAc/SS-DNA/PEG procedure was used to transform the disruption cassette or plasmid to yeast cells [33]. The diploid *agc1*-disrupted strain with genotype *agc1::loxP*/*agc1::loxP* was designated as BYagc1Δ.

Two primer pairs were used to evaluate the knocking-out of *agc1* gene. The primers JMUS and JMDA were located in the upstream and downstream of the *agc1* gene sequence to be disrupted, while the primers AGC1A and AGC1B within the disrupted *agc1* sequences (Fig. 2, Table 1). Successful knocking-out of the *agc1* gene gave rise to a deduced 1789 bp product on PCR amplification with JMUS and JMMDA while no product was observed when amplified with the primer set AGC1A and AGC1B.

**Plasmid Construction and Transformation**

The normal citrin-coding sequence (NM_014251.2) was amplified with the primers PSLCF and PSLCR (Table 1), then the PCR product was recombined into expression plasmid pYX212 (Novagen) via EcoRI/HindIII, generating the vector of pYX212-CITRIN. The novel missense mutation was introduced into the wild type *SLC25A13* cDNA by overlap-extension PCR with the complementary primers V01F and V01R (Table 1) according to the literature [34]. Following mutagenesis, the generated variants were cloned into pYX212, constituting the vector pYX212-mutant. Then, transformation of BYagc1Δ strains with different plasmids including empty vector (pYX212), citrin control (pYX212-CITRIN) and the mutant plasmid (pYX212-mutant) were performed, respectively, by the improved lithium acetate method [33], and the positive clones were screened with the selection medium of SD-URA without uracil.

**Growth Ability and Statistics**

The growth abilities were tested for the yeast strains of BY4741 (WT), the *agc1*-abrogated strain (BYagc1Δ), and BYagc1Δ strains transformed with the empty vector, citrin control and mutant plasmids, respectively, by using acetate as the unique carbon source. Growth tests were initiated with late log precultures grown in 2 ml of minimal medium (MM) [0.67% yeast nitrogen base with ammonium sulphate (YNB, Difco), 0.2% glucose as carbon source and 30 µmol·L⁻¹ L-leucine, 20 µmol·L⁻¹ L-methionine, 10 µmol·L⁻¹ L-histidine, 20 µmol·L⁻¹ uracil]. The precultures were diluted with synthetic acetate (SA) medium containing 100 mM sodium acetate, pH 5 [24,35] until a final optical density of 5×10⁻³ at OD600 was reached. Growth tests were monitored by measuring the OD600 at an interval of 12 hours.
**Results**

**Clinical Findings**

A male infant at the age of 4.5 months was referred to our hospital due to prolonged jaundice over 4 months. Mild jaundice appeared at the second day after birth and lasted without any alleviation for 1.5 months of age. Then the infant was admitted to the local hospital where physical examination revealed mildly jaundiced skin and sclera, and slightly enlarged liver 1.5 cm below the right costal margin. On biochemical analysis, elevated serum levels of alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGT), total bilirubin (TBil), direct bilirubin (DBil), and total bile acid (TBA) were discovered (Table 2), indicating the existence of cholestatic liver disease. Urinary gas chromatography-mass spectrometry (GC-MS) analysis unveiled large quantity of 4-hydroxyphenyllactate (4HPL) and 4-hydroxyphenylpyruvate (4HPPV), while tandem mass spectrometry (MS-MS) analysis of serum amino acid revealed elevated levels of citrulline, threonine, hydroxyphenyllactate (4HPL) and 4-hydroxyphenylpyruvate. No evidences of specific pathogens such as cytomegavirus, Epstein-Barr virus and tuberculosis were found. The diagnosis was idiopathic infantile hepatitis, and breastfeeding was stopped with lactose-free formula. The infant was discharged when the TBil was lowered to the normal level of 116.2 \mu mol/L at the age of 2 months. However, due to persistent jaundice in the later 2.5 months, the infant was referred to our hospital for further investigation. As the third child of a non-consanguineous couple, the patient was born at the gestational age of 38\textsuperscript{th} weeks with the birth weight 2.6 kg. He had two healthy elder sisters and family history of any genetic disease was denied.

Physical examination in our hospital revealed the weight 5.9 kg (−2.0 SD), length 59 cm (−2.8 SD), and head circumference 40 cm (−1.8 SD). A chubby face and mildly-jaundiced skin and sclera were observed. The lungs were clear on auscultation, and no abnormal heart sound or murmur was heard. No abdominal distention, but a soft liver was palpable 3 cm below the costal margin on the right midclavicular line. Biochemical analysis still revealed elevated ALT, DBil, GGT and TBA (Table 2). Based on the history, physical findings and laboratory evidences, the infant was suspected to have NICCD, and SLC25A13 gene analysis was then performed to confirm the diagnosis. A lactose-free and MCT-enriched therapeutic formula was introduced. As a result, his physical and biochemical conditions were improved gradually, and on following-up at his age of 10 months, the chubby face, jaundice and liver enlargement were resolved, with the weight 10.3 kg (+0.6 SD), length 71.0 cm (−1.4 SD) and head circumference 46.5 cm (+0.5 SD), indicating a successful catch-up growth. No serum biochemical abnormalities were observed (Table 2).

**SLC25A13 Gene Mutations**

High-frequency mutation screening and direct sequencing revealed the patient a compound heterozygote of a maternally-inherited mutation c.615+5G>A and a novel missense mutation c.1064G>A (p.Arg355Gln), which was inherited from his father (Fig. 3). No carrier of this novel mutation was found in 50 control individuals, indicating a frequency less than 1%.

**Bioinformatic Analyses**

Comparative alignment of homologous proteins from yeast to human documented a conserved amino acid residue ARG355 in citrin protein (Fig. 4A). On structural model analysis, this residue was located within the first transmembrane-spanning helix of citrin protein (Fig. 4B), and the mutation p.Arg355Gln shortened the distance of the H-bond between residue 355 and LEU351 from 2.94 Å to 1.95 Å, and destructed the other two H-bonds between this residue and ASN356 and GLN359 (Fig. 4C–D). The hydrogen bond alterations predictively changed the spatial structure of the transmembrane-spanning helix, and thus affected the AGC function of citrin protein. Furthermore, functional prediction of the variant p.Arg355Gln by PolyPhen-2 produced a value of 1.000, and the probability value is >0.999 on

### Table 1. Primer sequences in this study.

| Primers | Sequences from 5’ to 3’ end | Purpose |
|---------|-----------------------------|---------|
| ExSF    | TTTGGACAGACCAATTCATC        | Amplification the transcript from the allele with c.615+5G>A |
| ExBR    | CCCCTTGCTGTTAATTAGCTG        |         |
| LS      | GTGGATAGCTCACTCAAGTCTAGATTTTTAGAATGACGTTTGG | Amplification the disruption cassette |
| LA      | CCTCTGCTTGGTTTGGTTCTTTGCTTTTGACGAGCGATAGAAAATAGTAGGTTG | Detection of the agc1 gene |
| AGC1A   | AGTATGACAGCTTTGGCTACCG | Evaluation of the disruption effect of agc1 gene |
| AGC1B   | CTTGATCGTATCCATCTGCC | Construction recombinant plasmids |
| JMDA    | AACCACGAATAGAACCCAGGAAGAGGGATGTTCTTTACTG | Site-directed mutagenesis for p.R355Q |
| PSLCF   | ACCCGGATCTG6CGGCGCGCCAAGGTG | Site-directed mutagenesis for p.R355Q |
| PSLCR   | ACCCGGATCTG6CGGCGCGCCAAGGTG | Site-directed mutagenesis for p.R355Q |
| V01F    | CTTGAAACTCAATATGCAGAACCCAAC | Site-directed mutagenesis for p.R355Q |
| V01R    | GGTTCGTGATTGAATTTTACAAAGAGGAGCGATTGCTGAGTACAGAAGGATGTTCTTTACTG | Site-directed mutagenesis for p.R355Q |

The nucleotides in italic in the primers PSLCF and PSLCR were introduced restriction sites, those in italic bold in V01F and V01R are introduced point mutations, and those in underlined bold in LS and LA are homologous sequences on both sides of the deletion region in agc1 gene.

The data were analyzed by means of one-way ANOVA followed by the Bonferroni method to compare the differences in the mean values among the different groups above, with P<0.05 as the significance criteria.
MutationTaster analysis, both suggesting a disease-causing mutation.

Transcripts from the SLC25A13 Allele Harboring the c.615+5G>A Mutation

On RT-PCR product electrophoresis, the patient and his mother both had two bands of 503 bp and 2292 bp, while his father just one band of 503 bp (Fig. 5A). The sequencing results demonstrated that the 503 bp product had expected normal
sequence (Fig. 5C), while the entire intron 6 (1789 bp) of SLC25A13 gene, along with the mutation c.615+5G>A, was retained in the 2292 bp product (Fig. 5B), strongly indicating that this variation was indeed a splice-site SLC25A13 mutation. This concept was confirmed by further studies on 5 additional NICCD patients having the same mutation (results not shown). The aberrant mRNA molecule with intron 6 retention, r.[615+1_615+1789ins; 615+5G>a] (GenBank accession number: KJ128074), caused a frame shift from codon 206, added 6 amino acids, and produced a premature termination (TAA from the inserted sequence) at codon 212, thus forming a truncated citrin molecule p.Ala206Valfs212X.

Knocking-out of the agc1 Gene in Yeast Strain

Using the Cre-loxP recombination system, the agc1 gene of Saccharomyces cerevisiae BY4741 was knocked out following two rounds of deletion procedures (Fig. 1). PCR detection of normal and agc1Δ strains were showed in Figure 2. The size of the PCR product amplified with the primers JMUS and JMDA were shortened from normal 2831 bp to 1789 bp after agc1 deletion. Meanwhile, PCR with the second primer set gave rise to an 852 bp band before but no product was detected after agc1 disruption. These results indicated that a fragment of agc1 gene was deleted successfully.

Functional Effect of the Novel Missense Mutation

The recombinant of pYX212 vector and the citrin variant with mutation c.1064G>A was named as pYX212-mutant. The growth abilities of the different yeast models were illustrated in Figure 6. BYagc1Δ cells had very poor growth ability with acetate as the unique carbon source, which could be restored after the transformation with the plasmid pYX212-CITRIN but not with the empty pYX212 vector (Fig. 6A). After growing for 96 hours, the cell density of the mutant group (Arg355Gln) did not demonstrate significant difference from that of the empty vector group, but both of them were significantly lower than that of the citrin control (Fig. 6B). These results indicated that the mutation p.Arg355Gln caused complete loss of the AGC function of citrin protein.

Discussion

The growth retardation, prolonged jaundice, liver enlargement and the biochemical alterations indicating cholestatic liver disease were all non-pathognomonic in this infant. As mentioned above, however, citrin plays important roles as the liver-type AGC2 in a

Table 2. Alterations of the serum biochemical indices in the patient.

| Ages (Months) | 1.5 M | 2 M* | 2.5 M | 3 M | 4 M | 4.5 M* | 5.5 M | 10 M |
|--------------|-------|------|-------|-----|-----|--------|-------|------|
| ALT (5–40 U/L) | 38    | 40   | 45    | 60  | 83  | 66     | 50    | 41   |
| AST (5–40 U/L) | 95    | 96   | 83    | 67  | 101 | 74     | 60    | 39   |
| GGT (8–50 U/L) | 242   | 233  | 320   | 222 | 217 | 176    | 238   | 23   |
| ALP (20–500 U/L) | 1480  | 1030 | –     | 632 | 638 | 759    | 595   | 338  |
| TP (60.0–83.0 g/L) | 44.3  | 41.5 | 37.4  | 39.5 | 43.1 | 42.9   | 52.9  | 61.6 |
| Alb (35.0–55.0 g/L) | 30.5  | 27.5 | 27.7  | 29.0 | 33.3 | 31.9   | 37.4  | 46.1 |
| Glb (20.0–30.0 g/L) | 13.8  | 14.0 | 9.7   | 10.5 | 9.8 | 11.0   | 15.5  | 15.5 |
| Tbil (2–19 μmol/L) | 150.3 | 116.2 | 96.9  | 72.6 | 66.9 | 50.4   | 15.3  | 2.9 |
| Dbil (0–6 μmol/L) | 66.4  | 65.3 | 60.1  | 54.0 | 49.2 | 37.1   | 11.5  | 1.2 |
| Ibil (2.56–20.9 μmol/L) | 83.9  | 50.9 | 36.8  | 18.6 | 17.7 | 13.3   | 3.8   | 1.7 |
| TBA (0–10 μmol/L) | 354   | 227.8| 230.4 | 153.8 | 66.4 | 39.9   | 40.6  | 2.6 |

ALT alanine transaminase; AST aspartate transaminase; GGT gamma-glutamyl transpeptidase; ALP alkaline phosphatase; TP total protein; Alb albumin; Glb globulin; Tbil total bilirubin; Dbil direct bilirubin; Ibil indirect bilirubin; TBA total bile acid.

*When breastfeeding was stopped, whist lactose-free formula introduced.

When lactose-free and MCT-enriched therapeutic formula was introduced.

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diversity of metabolic pathways such as aerobic glycolysis, gluconeogenesis and urea cycle, and thus it is not surprising that citrin deficiency could be reflected by the metabolic changes in NICCD. Actually, the metabolome findings in the infant in this paper, including the large excretion of 4HPL and 4-HPPV in the urine and the alterations of serum amino acids as well, provided valuable evidences strongly suggestive of NICCD [8]. SLC25A13 genetic analysis (Fig. 3) unveiled a compound heterozygote of c.615+5G>A and c.1064G>A (p.Arg355Gln). The former was a common SLC25A13 mutation, but the latter, so far as we know, has never been reported in any other references. Its frequency less than 1% indicated a novel mutation, and further bioinformatic analyses in this paper supported its pathogenicity by different tools including comparative alignment of homologous proteins, 3D structural analysis in silico and functional prediction using Polyphen-2 and MutationTaster.

Lactose-free and/or MCT-enriched therapeutic formulas have increasingly been reported to be effective on NICCD [7,8,36–38]. The clinical and biochemical improvement in this infant provided further evidences supporting this concept. Actually, galactitol and galactonate, the well-known metabolome markers for galactosemia, were degraded soon after transcription, and the derangement of galactose metabolism in such cases. Since galactitol was one of the substrates that caused the pathological abnormalities in classical galactosemia [39,40], the effectiveness of lactose restriction might be attributed to the alleviation of the toxicity of galactitol on hepatocytes. On the other hand, MCT could be better absorbed and transported via the portal vein, and then more rapidly oxidized in comparison with long chain triglycerides (LCTs) [41–43]. Since the absorption of fat is bile acid-dependent, the well absorption of MCTs could thus reduce the burden of the liver to synthesize and excrete bile salt into the gut. Moreover, the increased cytosolic NADH/NAD⁺ ratio in hepatocytes were a key pathophysiologic alteration in citrin deficiency [44,45]. This change could result in an energy shortage in the liver due to the impairment of glycolysis, and the therapeutic effect of MCT on NICCD might occur via a supply of acetyl-CoA, FADH2 and NADH to hepatic cells as energy sources [37].

Our study in this paper documented c.615+5G>A a real splice-site mutation that led to retention of the entire intron 6 in SLC25A13 cDNA (Fig. 5). Pre-mRNA splicing involving the recognition of exon-intron junctions by the spliceosome and intron excision through a two-step transesterification reaction [46,47], relying on conserved sequence elements at both ends of introns, termed splice sites [48,49]. The most conserved 5’ Splice sites (5’s) positions lies at the first two intronic nucleotides (GT or GU, +1 and +2), and the next most conserved 5’s positions (> 75% in humans) are −1G (the last exonic nucleotide) and +5G [48,50]. This could be the reasonable explanation for intron 6 retention in cDNA due to c.615+5G>A in SLC25A13 gene. The retention may lead to production of a premature termination codon that trigger nonsense-mediated mRNA decay (NMD), a surveillance mechanism that selectively degrades nonsense mRNAs [51–53], and thus the transcripts from the SLC25A13 allele with c.615+5G>A were degraded soon after transcription, making its detection rather difficult in liver specimens or fibroblast cells. In this paper, the finding of the aberrant SLC25A13 transcript r.[615+1_615+1789ins; 615+5g>a] (GenBank accession number: KJ128074) in PBLs of our NICCD infant might be partially attributed to the freshness of the blood samples for the RT-PCR process, and the difference of SLC25A13 expression between PBLs and other tissues might be another likely contributing factor. Actually, the alternative splice variants (ASVs) with r.213_328del took account for over half among all SLC25A13 transcripts in PBLs [16], constituting a transcriptional feature that had never been found in hepatocytes.

Missense mutations accounted for the largest proportion in the SLC25A13 mutation list. Actually, there were 32 missense variations out of the total 84 possibly pathogenic SLC25A13 variations identified in CD patients (Present study, [13,22]). However, the pathogenicity of most missense mutations remains an unresolved issue due to the lack of functionally analytic evidences. Since human AGC2 was capable of functionally replacing the yeast gene age1 [35], a viable and simple model using yeast age1Δ mutant for the functional analysis of human citrin variants was established very recently [24]. This model was utilized in our study to analyzed the effect of the novel c.1064G>A mutation on AGC2 function of citrin protein. As illustrated in Figure 6, c.1064G>A caused complete loss of AGC2 function of citrin protein, and therefore could be considered as a deleterious mutation. Besides the indirect bioinformatic evidences, this finding provided direct eukaryotic evidence for the pathogenicity evalu-
Figure 4. Comparative alignment of homologous proteins and structural alteration of citrin protein due to the novel mutation p.ARG355GLN. A. Comparative alignment of the homologous proteins in diverse species. The arrow showed the amino acid position affected by the novel mutation. Protein sequences were collected from Human (H. sapiens), Cow (B. Taurus), Xenopus tropicalis (X. tropicalis), Aedes aegypti (A. aegypti), Yeast (S. cerevisiae) and Caenorhabditis elegans (C. elegans), respectively. B. Ribbon model of the 6 transmembrane-spanning helices in citrin protein, which was constructed using bovine mitochondrial ADP/ATP carrier monomers (2c3eA) [28] as a template. The two views represented 180° rotation of the model. The ribbon in purple stood for the first transmembrane-spanning helix and the lines in red for the predicted β-turns. The residue in white represented the affected amino acid position. The ball-and-stick models in Figures C and D illustrated the backbone of the residues 348 to 359 with and without the mutation, with the short bars in white, red and blue standing for the carbon, oxygen and nitrogen atoms, respectively. Only the side-chain of residue 355 was shown. The dashed lines in green illustrated the potential hydrogen bonds from the residue 355, and the digits in green indicated the hydrogen bond distance (Å).
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Figure 5. Sequencing results of the RT-PCR products. A. Electrophoresis of the PCR products with cDNA from PBLs as the templates. L, 200 bp DNA ladder marker; P, Patient; F, Father of the patient; M, Mother of the patient. The 2292 bp and 503 bp products were sequenced, and the segmental sequencing results were illustrated in figures B and C, respectively. The 503 bp product had normal SLC25A13 cDNA sequence, while the 2292 bp product retained the entire intron 6 due to the c.615+5G>A mutation.

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Figure 6. Growth properties of different yeast strains. A. wild type BY4741 (WT), strains with agc1 gene disrupted (BYagc1Δ), BYagc1Δ strains transformed with empty plasmid pYX212 (vector) and normal control pYX212-CITRIN (citrin). The results were means ± SD of six repeated experiments. B. Growth abilities of the agc1Δ yeast strains. Strains of vector, citrin and Arg355Gln (BYagc1Δ strains transformed with mutant recombinant pYX212-mutant) was cultured in SA medium for 96 hours, and their growth abilities were monitored by measuring OD600 nm. The results were means ±SD of six repeated experiments, and different letters above the bars indicated statistically significant difference from each other (P < 0.05).

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ation of the missense mutation, and added further experience to the limited functional analysis of SLC25A13 variation in NICCD patients.

In conclusion, a NICCD infant was definitely diagnosed as a compound heterozygote of c.615+5G>A and a novel c.1064G>A mutation by means of a clinical, genetic, transcriptional and functional investigation. Our findings provided direct laboratory evidences supporting the splice-site nature of the c.615+5G>A mutation, and added further evidence to the pathogenic mutation bioinformatically and functionally, expanded the functional investigation. Our findings provided direct laboratory evidences supporting the splice-site nature of the c.615+5G>A mutation, and added further evidence to the pathogenic mutation bioinformatically and functionally, expanded the functional investigation. Our findings provided direct laboratory evidences supporting the splice-site nature of the c.615+5G>A mutation, and added further experience to the limited functional analysis of SLC25A13 variation in NICCD patients.

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Author Contributions

Conceived and designed the experiments: Y-ZS. Performed the experiments: Z-HZ MD S-TZ H-SZ H-SZ F-PC. Wrote the paper: Y-ZS Z-HZ W-XL.

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