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ORIGINAL ARTICLE

# Identification of 13 novel mutations including a retrotransposal insertion in *SLC25A13* gene and frequency of 30 mutations found in patients with citrin deficiency

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**Abstract** Deficiency of citrin, liver-type mitochondrial aspartate-glutamate carrier, is an autosomal recessive disorder caused by mutations of the *SLC25A13* gene on chromosome 7q21.3 and has two phenotypes: neonatal intrahepatic cholestatic hepatitis (NICCD) and adult-onset type II citrullinemia (CTLN2). So far, we have described 19 *SLC25A13* mutations. Here, we report 13 novel *SLC25A13* mutations (one insertion, two deletion, three splice site, two nonsense, and five missense) in patients with citrin deficiency from Japan, Israel, UK, and Czech Republic. Only R360X was detected in both Japanese and Caucasian. IVS16ins3kb identified in a Japanese CTLN2 family seems to be a retrotransposal insertion, as the inserted sequence (2,667-nt) showed an antisense strand of processed complementary DNA (cDNA) from a gene on chromosome 6 (*C6orf68*), and the repetitive sequence (17-nt) derived from *SLC25A13* was found at both ends of the insert. All together, 30 different mutations found in 334 Japanese, 47 Chinese, 11 Korean, four Vietnamese and seven non-East Asian families have been summarized. In Japan, IVS16ins3kb was relatively frequent in 22 families, in addition to known mutations IVS11 + 1G > A, 851del4, IVS13 + 1G > A, and S225X in 189, 173, 48 and 30 families, respectively; 851del4 and IVS16ins3kb were found in all East Asian patients tested, suggesting that these mutations may have occurred very early in some area of East Asia.

**Keywords** Adult-onset type II citrullinemia (CTLN2) · Aspartate-glutamate carrier (AGC) · Citrin · Malate-aspartate shuttle · Neonatal intrahepatic cholestatic hepatitis (NICCD) · Retrotransposal insertion · *SLC25A13*

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## Introduction

Citrin deficiency, which results from mutations in the *SLC25A13* gene, is a newly established autosomal recessive disease first reported in Japan and has two major clinical phenotypes: adult-onset type II citrullinemia (CTLN2; MIM #603471) and neonatal intrahepatic cholestatic hepatitis (NICCD; MIM#605814) (Kobayashi et al.

1999; Yasuda et al. 2000; Ohura et al. 2001; Tazawa et al. 2001; Tomomasa et al. 2001; Saheki and Kobayashi 2002; Yamaguchi et al. 2002). The *SLC25A13* gene identified by using homozygosity mapping and positional cloning is localized on chromosome 7q21.3, expresses mainly in the liver, and encodes a calcium-binding mitochondrial solute carrier protein with a molecular weight of 74 kDa (675 amino acids), designated citrin (Kobayashi et al. 1999). This citrin is localized in the mitochondrial inner membrane and functions as a liver-type calcium ($Ca^{2+}$)-stimulated aspartate–glutamate carrier (AGC) by the electrogenic exchange of mitochondrial aspartate for cytosolic glutamate and proton (Palmieri et al. 2001). AGC provides aspartate for the syntheses of urea, protein, and nucleotide in addition to participating in gluconeogenesis from lactate and transporting cytosolic nicotinamide adenine dinucleotide (NADH), reducing equivalents into mitochondria as part of the malate–aspartate shuttle; thus, the deficiency of citrin (liver-type AGC) shows various symptoms.

Patients with CTLN2 (11–79 years of age) suffer from recurring neuropsychiatric symptoms associated with hyperammonemia, including disorientation, delirium, seizures, and coma, that can lead to death from brain edema. Laboratory findings of CTLN2 patients show high blood ammonia; increased plasma citrulline, arginine, threonine to serine ratio, and serum pancreatic secretory trypsin inhibitor (PSTI) levels; and decreased hepatic argininosuccinate synthetase (ASS) activity/protein levels (Saheki et al. 1986, 1987; Kobayashi et al. 1997). No cross-reactive immune materials have been detected in the liver of CTLN2 patients by Western blot analysis with antihuman citrin antibody (Yasuda et al. 2000), indicating that CTLN2 is a citrin deficiency, although the mechanisms of secondary ASS deficiency and stimulated *PSTI* gene expression in the liver are still unknown. Very few clinical symptoms were known in the neonatal/infantile period of CTLN2 patients. DNA analyses of the *SLC25A13* gene have revealed that some neonatal/infantile patients with a type of hepatitis associated with galactosemia, multiple aminoacidemia including citrullinemia, hypoproteinemia, intrahepatic cholestasis, and prolonged jaundice, were homozygotes or compound heterozygotes for the same *SLC25A13* mutations as those found in CTLN2 patients (Ohura et al. 2001; Tazawa et al. 2001; Tomomasa et al. 2001). Therefore, we designated them neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) as a neonatal/infantile phenotype (Saheki and Kobayashi 2002; Yamaguchi et al. 2002).

Until recently, citrin deficiency was thought to be restricted to Japanese subjects. Recently, however, we have found several non-Japanese patients with *SLC25A13* mutation: a Palestinian family with duplication of exon 15 (Ben-Shalom et al. 2002), two Israeli families with L598R (Luder et al. 2006), and two Caucasian families: one in the UK with C489R (Hutchin et al. 2006), and one in the USA with deletion of exon 3 in messenger ribonucleic acid (mRNA) (Dimmock et al. 2007), indicating a wider distribution of citrin deficiency. Some of the 13 *SLC25A13* mutations identified in Japanese patients with citrin deficiency were found in Chinese, Korean, and Vietnamese patients from Taiwan, China, Korea, Australia, France, Czech Republic, and USA (Hwu et al. 2001; Kobayashi et al. 2003; Yang et al. 2003; Lu et al. 2005; Liu et al. 2006; Kim et al. 2006; Sheng et al. 2006; Lee et al. 2006; Yeh et al. 2006; Tsai et al. 2006; Song et al. 2006, 2007; Ko et al. 2007). These results suggest that the mutations go back to a common ancestor and prevail at least in East Asia. On the other hand, we have found that the frequency of carriers (heterozygotes with the mutated *SLC25A13* gene in one allele) is very high in the East Asian population: China (1/65), especially south of China (1/48); Japan (1/69), and Korea (1/112) (Saheki and Kobayashi 2002; Kobayashi et al. 2003; Lu et al. 2005).

So far, worldwide, we have identified 18 different mutations in the *SLC25A13* gene of patients with citrin deficiency (Kobayashi et al. 1999; Yasuda et al. 2000; Yamaguchi et al. 2002; Ben-Shalom et al. 2002; Lu et al. 2005; Takaya et al. 2005; Luder et al. 2006; Hutchin et al. 2006; Sheng et al. 2006; Ko et al. 2007) and a mutation-deleted exon 3 in mRNA but not identified yet in the *SLC25A13* gene (Dimmock et al. 2007). In this study, we report 13 novel mutations, including a retrotransposal insertion identified in the *SLC25A13* gene of CTLN2 and NICCD patients, and we summarize the frequency and distribution of all 30 *SLC25A13* mutations.

## Subjects and methods

### Subjects

CTLN2 patients have been diagnosed on the basis of well-established criteria, including symptoms and laboratory findings such as high blood ammonia, increased plasma/serum citrulline, arginine, ratio of threonine to serine and PSTI levels, and decreased hepatic ASS activity levels (Saheki et al. 1986, 1987; Kobayashi et al. 1997). NICCD patients with various transient symptoms have been found or suspected by newborn screening in association with galactosemia and/or methioninemia or among those who suffered from persistent infantile cholestatic jaundice and multiple aminoacidemias, including elevated citrulline, methionine, threonine, tyrosine, and arginine levels at 1–4 months of age (Tazawa et al. 2004; Ohura et al. 2007). Other features shown in NICCD patients are hypoproteinemia, growth retardation, hypoglycemia, fatty liver, mild

liver dysfunction, and/or high levels of plasma α-fetoprotein. Most NICCD patients show symptoms that ameliorate by 1 year of age, and more than 10 years or even several decades later, some patients develop CTLN2 (Kasahara et al. 2001; Tomomasa et al. 2001; Saheki and Kobayashi 2002). Many individuals with CTLN2 are fond of protein/lipid-rich foods and have an aversion to sugar/carbohydrate-rich foods. As we recently found that children show strong food preferences starting around 1–2 years of age after NICCD symptoms have disappeared, we use this as a diagnostic marker of citrin deficiency. The clinical data from CTLN2 or NICCD patients used for identifying novel *SLC25A13* mutations have been described (Oshiro et al. 2002; Waki et al. 2004; Ikeda et al. 2004; Yazaki et al. 2005) and will be reported elsewhere. Samples such as liver specimens and/or cultured fibroblast cells were obtained with the approval of the Institutional Ethics Review Board in each country or area.

## Mutation detection

To identify unknown *SLC25A13* mutations, we mainly used sequencing analysis of DNA fragments amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and/or genomic DNA-PCR, as described previously (Kobayashi et al. 1999; Yasuda et al. 2000; Yamaguchi et al. 2002; Ben-Shalom et al. 2002; Lu et al. 2005; Takaya et al. 2005). Total RNA was isolated from cultured fibroblast cells or autopsied/biopsied liver specimens, and the first-strand complementary DNA (cDNA) was synthesized using oligo-$(dT)_{12–18}$ and M-MuLV reverse transcriptase. The entire coding portion of *SLC25A13* mRNA was amplified with suitable primer sets as described previously (Kobayashi et al. 1999). For genomic DNA-PCR, regions (average size about 500 bp) containing 18 exons each were individually amplified using oligonucleotides derived from intronic sequences flanking each exon as PCR primers. The amplified PCR products of cDNA and/or genomic DNA were separated on agarose gels, extracted from gel with QIA Quick Gel Extraction Kit (Qiagen Inc., CA, USA), and sequenced by means of the Dye Terminator Cycle Sequencing Ready Reaction on an ABI-310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA).

To find the large insertion mutation in the P557 family with CTLN2, haplotyping using a microsatellite marker, *D7S1812*, located in intron 15 of the *SLC25A13* gene, was performed according to the GeneScan method, as described previously (Kobayashi et al. 1999). Two single nucleotide polymorphisms (SNPs), rs1016968 in intron 13 (SNP15) and rs2269781 in intron 14 (SNP22) of the *SLC25A13* gene (Fig. 1a), were detected by PCR/restriction fragment length polymorphism (RFLP) with primers 15F: 5′-TTTCCATCTTTTCCCTTCTTGG-3′ and 15R: 5′-GCGCTGCAGCCTACAGATCA-3′ and restriction enzyme *Tru*9I or *Ase*I, and by direct sequencing of PCR product with primers 22F: 5′-TCAACCTAGACTTGTATACCCT-3′ and 22R: 5′-TCTTGGCTTCCCCCAAGCAA-3′, respectively. Long-range PCR was performed according to the manufacturer's instructions using Takara LA Taq™ polymerase (Takara Bio, Shiga, Japan) with primer set "c" shown in Fig. 1b (IVS15F: 5′-CACAGTGGGTTTCTAAGCATTT-3′ and Ex18R: 5′-TGCTTCATTCCCAGGAGGGA-3′). DNA diagnosis for mutation [XIII]:P632L was performed PCR-RFLP with primers IVS17F: 5′-CTGATGAGAATGTATCAACTCC-3′ and Ex18Bm: 5′-GCCCCCAACGTGATCAGGAC-3′, modified base is underlined, and *Dde* I. To detect the antisense sequence of *C6orf68* (NM_138459) inserted in the cDNA of P557, RT-PCR was performed using a primer set "d" shown in Figs. 1b and 2: Ex14F: 5′-TCAGTGCTCTGTCTGTCGTG-3′ and MGC2331R: 5′-CTTCCCTCACTGCTGATTCTT-3′. The downstream nucleotide (nt) sequence of the mutated transcript with new exon 17 (see Figs. 2 and 3) was confirmed by using the 3′-RACE System (Gibco BRL, Tokyo, Japan) and *SLC25A13*-specific primer Ex14F. The 3′-RACE products were sequenced by using the method described above.

The scores of splice-site prediction were calculated by means of the software provided by http://www.fruitfly.org/cgi-bin/seq_tools/splice.pl for splice site prediction (SSP) and http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html for Maximum Entropy (MaxEnt). The amino acid sequence of human citrin (AAD38501) was aligned comparatively with homologous proteins from nine other eukaryotic species (chimpanzee: XP_527824, macaque: Q8HXW2, horse: XP_001494475, cattle: XP_872377, dog: XP_852644, mouse: AAF21426, rat: XP_001054092, chicken: NP_001012967 and *Xenopus*: AAI21260), with human aralar (NP_003696) and the homologous protein from six other species (orangutan: CAH90932, monkey: XP_001085208, cattle: NP_001094664, dog: XP_535962, mouse: NP_766024 and *Xenopus*: AAI23038), and with homologous protein from *C. elegans* (Q21153).

## DNA diagnosis

For East Asian patients, 13 known mutations were screened according to the methods described previously by using GeneScan/SNaPshot and/or PCR/gel running (Yamaguchi et al. 2002; Lu et al. 2005; Takaya et al. 2005). After identification of novel mutations, we established the DNA diagnosis methods for each mutation. The detection of 13 novel mutations identified in this study was performed using genomic DNA by a combination of PCR (or modified PCR) with or without restriction enzyme digestion, or by direct sequencing. DNA samples were obtained according

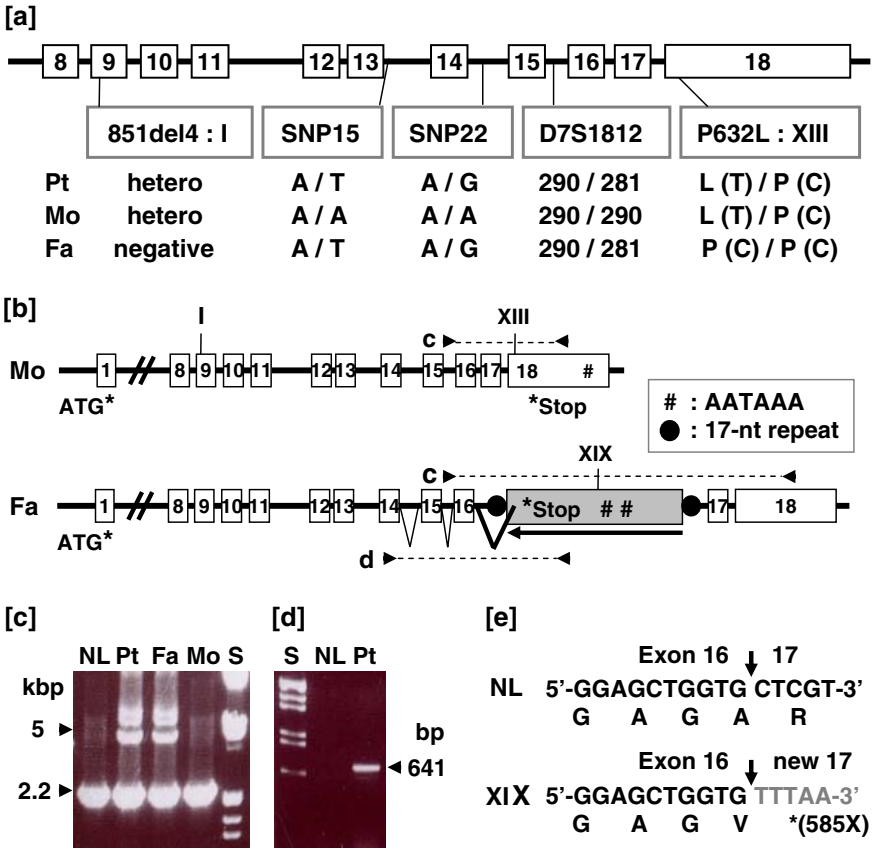


**Fig. 1** Identification of a novel retrotransposal insertion mutation, [XIX]:IVS16ins3kb, in an adult-onset type II citrullinemia (CTLN2) (P557) family. **a** DNA diagnosis of two mutations, [I]:851del4 and [XIII]:P632L, and haplotyping analysis of three polymorphisms, SNP15, SNP22 and microsatellite *D7S1812*. *Numbers in box 8–18* show exons in the *SLC25A13* gene. *Pt* patient, *Mo* mother, *Fa* father. **b** Schematic representation of the mutated alleles from the parents of P557. The *shaded box* in the paternal allele with the mutation [XIX]:IVS16ins3kb is the inserted antisense strand sequence of *C6orf68* cDNA (see Fig. 2), and the *lower arrow* shows the direction of insert. **c** Gel running of long and accurate polymerase chain reaction (LA-PCR) product using primer set "c" (IVS15F and Ex18R) shown in **b**, from intron 15 to exon 18 (2,201 bp: IVS15-1321_Ex18 + 261) of genomic DNA in a CTLN2 family (*Pt* patient, *Fa* father, *Mo* mother) and control (NL). *S* size marker of DNA. **d** Gel running of reverse transcriptase (RT)-PCR using primer set "d" (Ex14F and MGC2332R) shown in **b**, from exon 14 to inserted sequence (641 bp: see Fig. 2). **e** Predicted amino acid sequence of the allele with mutation [XIX]

to institutional guidelines in each country. DNA was prepared using standard methods from blood, cells, or tissues of patients and from blood of their family members and unrelated volunteer individuals as controls after informed consent was obtained. This study was approved by the Committee for Ethics of Kagoshima University Faculty of Medicine and was carried out in accordance with the principles of the Declaration of Helsinki.

## Results

### Identification of a novel retrotransposal insertion mutation ([XIX]:IVS16ins3kb)

A Japanese patient (P557, 16 years of age) was diagnosed as CTLN2 by clinical and biochemical studies and treated with partial liver transplantation from her mother (Waki et al. 2004). Before the operation, DNA diagnosis for known *SLC25A13* mutations revealed that she was a compound heterozygote with two different mutations, [I]:851del4 from the maternal allele and [?]:unknown from the paternal allele. By sequencing analysis of PCR product with 664 bp from intron 16 to exon 18 (IVS16-35_Ex18 + 261), a base substitution from C to T at 1895 ([XIII]:P632L) was detected in P557 homozygously and in the mother heterozygously but not in the father (data not shown). However, DNA diagnosis for the mutation [XIII] using a primer set of IVS17F and Ex18Bm after the detection of a novel mutation in the paternal allele showed in P557 heterozygously (Fig. 1a). The sequencing of RT-PCR product from exon 9 to exon 18 (871–2055) using hepatic cDNA of P557 showed homozygous for two mutations, [I] and [XIII] (data not shown), suggesting that

5′-GTATGCCTGCAGCATCT-(Exon 16)-TGGAAGGGAGCTGGTG▽gtatggaaa
taatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgt **gatttctccatttttt**
[*(3′)-ttttttttttttttttttag▼TT*TAA*tcatt**ttttatt***tcaagtgtatttaaaaaatcataaatggggtttcataa
*tccaaagttgaaacatttattcttcatagcttcagaatttaacaaccaattgtagaccatgctttccaaatccag
tcttctttgctatttttcaaaacttctgagatctagtattaaactgctccattctaaatgtatagttttagataagtatt
gtacacttgttgataagggttttctgaaagcagtctatcaaatataaagaatggtttctatct*aagaatcagca
*gtgagggaag*gaaatattaaacacctatcaagaaatcaattattcattttaaaaatacagaaccagtgctg
ctctctgtcataaaagagaacatgtaaaatttatttttatagactttggtaatattttattcccccacagaggcctt
caatcctacttaaagatattttacacacagtaaccatcaggatttactgagtaaaaatctcaggtattaacca
tgcccctaaaatgtgctattccaaagaggaacaggttacttttgaggaaaaaagctgccttggtaacttccc
tcaaatgtttatttta**AATAAA**aatggttgatggaaatatttttaaaagaactttgggtataatatggcatac
tgcccatcaaacaaaaaagga**AATAAA**actttctttccattt^at^aataagttttccacctttactatcaa
gattacaacttattgaccttttatgcttgcttggt--*(1740 bp)*--*C6orf68 cDNA--gcagcgcgacgg*
*ctttttatccgcccctgcggccggcgcgggccatcgctccgcgtccccccgccccccgagcccgaacccc*
*tttccactgccaacacctcacctcgccccgccgc**cat**cttcctcctccttggcagccccgccccc-(5′)*]
**gatttctccatttttt**aaag▽CTCGTGTATTTCG-(Exon 17)-ATTTTGGAGGAGT-3′

**Fig. 2** Sequence of exon16-intron16-exon17 in the *SLC25A13* gene and the inserted antisense strand complementary DNA (cDNA) processed from gene on chromosome 6 (*C6orf68*). The [XIX]:IVS16ins3kb found in paternal allele of an adult-onset type II citrullinemia (CTLN2) patient had an inserted antisense strand *C6orf68* cDNA (the sequence of 2,667-nt shown by *italic*) with 17-nucleotide (nt) repeat (*shaded boxes*), whose sequence is from −21 to −5 of intron 16 between +71 and −4 position of intron 16 in the *SLC25A13* gene, as shown in Fig. 1b. As a result, the new transcription termination (∧) and new 3′ side of splicing site (*closed arrowhead and dotted underline*) were created. The new messenger ribonucleic acid (mRNA) from the insertion mutation caused a frame shift from codon 584, leading to premature termination (*TAA* from the inserted sequence) at codon 585 in citrin protein, instead of mature termination at codon 675. The sequence of primer MGC2331R used in Fig. 1d is *underlined*. *Open arrowheads* are 5′ and 3′ side of splicing sites in intron 16 of the normal *SLC25A13* gene

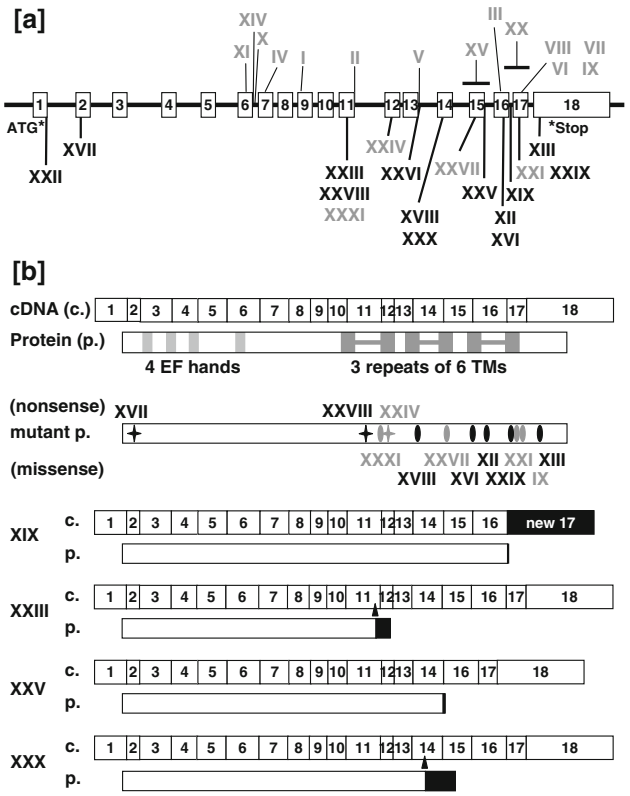


**Fig. 3** Summary and location of 30 mutations in the *SLC25A13* gene (**a**), and schematic structures of *SLC25A13* complementary DNA (cDNA) and citrin protein predicted from novel mutations (**b**). Characteristics of mutations [I]–[XXXI] are shown in Table 1. *Black and gray letters* show the novel and known mutations, respectively. *TM* mitochondrial transmembrane, *filled star* nonsense, *filled oval* missense, *filled triangle*, 1-bp deletion mutation. *Closed boxes* represent the regions of abnormal sequence in cDNA (*c*) and protein (*p*)

the unknown mutation from the paternal allele in the 577 family is located on the 3′ side of the *SLC25A13* gene. The finding that both [I] and [XIII] mutations are from the maternal allele (see Fig. 1b) indicates that [XIII]:P632L has no effect on the citrin function, because mutation [I]:851del4 produces a truncated form, R284fs(286X).

Haplotype analysis with SNP15, SNP22, and *D7S1812* revealed that P557 had alleles from both parents in the regions tested (Fig. 1a). To define the paternal mutation, we performed long-range PCR with genomic DNA of the 557 family members from intron 15 to exon 18 (primer set "c" in Fig. 1b), and detected a PCR product of larger size in P557 and her father but not in her mother or control (Fig. 1c). By sequencing, we found that the inserted sequence (2,667-nt) showed an antisense strand of processed cDNA from a gene on chromosome 6 (*C6orf68*: NM_138459) and that the repetitive sequence (17-nt) derived from the *SLC25A13* gene was found at both ends of the insert (Fig. 2), suggesting that the [XIX]:IVS16ins3kb (Table 1) is a retrotransposal insertional mutation. As shown in Figs. 1b and 2, the direction of the inserted *C6orf68* cDNA is reverse to the *SLC25A13* transcript, and two poly(A) additional signals, AATAAA, are present within the inserted sequence, indicating the termination of RNA synthesis in the insert. The new cryptic splice site (closed arrowhead in Fig. 2) is predicted in the insert from the high score (1.00) of the acceptor site predictions by the SSP method. We confirmed that the mutated mRNA molecule with new exon 17 (Figs. 2 and 3b) was transcribed in the liver of P557 by RT-PCR with primer set "d" (Figs. 1b,d). Furthermore, 3′-RACE revealed that the major transcription termination at 17-/19-nt downstream second AATAAA ("∧" in Fig. 2) resulted from the use of AATAAA located downstream out of two AATAAA sites (Fig. 2). On the other hand, Western blot analysis showed that the truncated protein with mutation [XIX], A584V(585X) as shown in Fig. 1e, was not detected in the liver of P557 ([I]/[XIX]) nor in another CTLN2 patient (P92) with homozygous [XIX] (data not shown).

After identifying mutation [XIX] in a Japanese family with CTLN2 (P557), to detect both [XIX]:IVS16ins3kb and [XX]:Ex16 + 74_IVS17-32del516 (Takaya et al. 2005),

**Table 1** *SLC25A13* mutations/variations found in patients with citrin deficiency

| Mutation (location) | | Nucleotide change | Amino acid change |
|---|---|---|---|
| I[a] | :851del4 (Ex9) | :g./c.851_854delGTAT | :p.R284fs(286X) |
| II[a] | :g.IVS11 + 1G > A | :c.1019_1177del | :p.340_392del |
| III[a] | :1638ins23 (Ex16) | :g./c.1638_1660dup | :p.A554fs(570X) |
| IV[a] | :S225X (Ex7) | :g./c.674C > A | :p.S225X |
| V[a] | :g.IVS13 + 1G > A | :c.1231_1311del | :p.411_437del |
| VI[b] | :1800ins1 (Ex17) | :g./c.1799–1800insA | :p.Y600X |
| VII[b] | :R605X (Ex17) | :g./c.1813C > T | :p.R605X |
| VIII[c] | :E601X (Ex17) | :g./(c.)1801G > T | :p.E601X |
| IX[c] | :E601K (Ex17) | :g./(c.)1801G > A | :p.E601K |
| X[d] | :g.IVS6 + 5G > A | :c. not detectable | : |
| XI[d] | :R184X (Ex6) | :g./(c.)550C > T | :p.R184X |
| **XII*** | **:T546M (Ex16)** | **:g./c.1637C > T** | **:p.T546M** |
| **XIII*** | **:P632L (Ex18)** | **:g./c.1895C > T** | **:p.P632L** |
| XIV[d] | :g.IVS6 + 1G > C | :c.IVS6(1789bp)ins | :p.A206fs(212X) |
| XV[e] | :g.Ex15dup (IVS14_15) | :c.1453_1591dup | :p.M532fs(560X) |
| **XVI*** | **:G531D (Ex16)** | **:g.1592G > A** | **:p.G531D** |
| **XVII*** | **:E16X (Ex2)** | **:g./c.46G > T** | **:p.E16X** |
| **XVIII*** | **:T446P (Ex14)** | **:g./c.1336A > C** | **:p.T446P** |
| **XIX*** | **:g.IVS16ins3kb** | **:c. aberrant RNA** | **:p.A584fs(585X)** |
| XX[f] | :g.Ex16 + 74_IVS17-32del516 | :(c. aberrant RNA) | :p.Q556fs(565X) |
| XXI[g] | :L598R (Ex17) | :g./c.1793T > G | :p.L598R |
| **XXII*** | **:g.Ex1-1G > A (Ex1)** | **:c. not detectable** | **:** |
| **XXIII*** | **:1146delA (Ex11)** | **:g./c.1146delA** | **:p.R383fs(407X)** |
| XXIV[h] | :Q397X (Ex12) | :g./c.1189C > T | :p.Q397X |
| **XXV*** | **:g.IVS15 + 1G > T** | **:c.1453_1591del** | **:p.G485fs(491X)** |
| **XXVI*** | **:g.IVS13 + 2T > G** | **:c. not detectable** | **:** |
| XXVII[i] | :C489R (Ex15) | :g./c.T1465C | :p.C489R |
| **XXVIII*** | **:R360X (Ex11)** | **:g./c.1078C > T** | **:p.R360X** |
| **XXIX*** | **:R588Q (Ex17)** | **:g./c.1763G > A** | **:p.R588Q** |
| **XXX*** | **:1374/5delG (Ex14)** | **:g./c.1374 or 1375delG** | **:p.A459fs(507X)** |
| XXXI[j] | :G393S (Ex11) | :g./(c.)1177G > A | :p.G393S |

[a–j] These mutations have been described previously by [a]Kobayashi et al. (1999), [b]Yasuda et al. (2000), [c]Yamaguchi et al. (2002), [d]Lu et al. (2005), [e]Ben-Shalom et al. (2002), [f]Takaya et al. (2005), [g]Luder et al. (2006), [h]Sheng et al. (2006), [i]Hutchin et al. (2006), [j]Ko et al. (2007)

* Novel mutations/variations found in this study are indicated by bold letters. Nucleotide numbering based on complementary DNA (cDNA) sequence (GenBank: NM_014251) begins with +1 as the A of the ATG initiation codon

we established a DNA diagnosis method using agarose gel electrophoresis of PCR product amplified with LA-Taq polymerase and primer set (Ex16F: 5′-GTATGCCTGC AGCATCTTTAG-3′ and Ex18-3′R: 5′-TGCTTCATTCCC AGGAGGGA-3′). This diagnostic method revealed that mutation [XIX] was found relatively frequent in East Asia including Japan, Korea, China, Taiwan, and Malaysia, as summarized in Table 2. Clinical data of some patients with mutation [XIX] have been described (Waki et al. 2004; Tazawa et al. 2004; Liu et al. 2006; Kim et al. 2006; Tokuhara et al. 2007; Ko et al. 2007) and will be reported (Y.-Z Song et al., D.-M Niu et al.; M.-K Thong et al., manuscript in preparation). On the other hand, we detected a heterozygous carrier with mutation [XIX] in 1,372 Japanese controls but no carrier with [XIX] in 2,641 Chinese or 2,228 Korean controls.

Three novel splice-site mutations ([XXII]:Ex1-1G > A, [XXV]:IVS15 + 1G > T, [XXVI]:IVS13 + 2T > G)

By DNA diagnosis for known mutations, we found that one Japanese CTLN2 patient (P857) was [II]/[?] (Ikeda et al. 2004; Yazaki et al. 2005). Western blotting of the liver specimens revealed that P857 is a citrin deficiency (data not shown). RT-PCR detected only one band of mRNA from an allele with mutation [II]:IVS11 + 1G > A

**Table 2** Frequency of the 30 *SLC25A13* mutations found in patients with citrin deficiency

| | CTLN2 | | | NICCD | | | | |
|---|---|---|---|---|---|---|---|---|
| | J | C | K | J | C* | K | V | O |
| | Numbers of family (numbers of allele) | | | | | | | |
| Tested total mutations | 151 (302) | 3 (6) | 3 (6) | 183 (366) | 44 (88) | 8 (16) | 4 (8) | 7 (14) |
| I | 74 (102) | 3 (4) | 2 (2) | 99 (120) | 38 (61) | 3 (4) | 4 (8) | – |
| II | 84 (118) | – | 1 (1) | 105 (133) | – | 2 (2) | – | – |
| III | 4 (6) | 2 (2) | – | 16 (17) | 7 (8) | – | – | – |
| IV | 19 (23) | – | – | 11 (12) | – | – | – | – |
| V | 17 (19) | – | 1 (1) | 31 (36) | – | 1 (1) | – | – |
| VI | 3 (3) | – | – | 5 (6) | – | – | – | – |
| VII | 3 (4) | – | – | 2 (2) | – | – | – | – |
| VIII | 3 (3) | – | – | 5 (5) | – | – | – | – |
| IX | 1 (1) | – | – | 1 (1) | – | – | – | – |
| X | – | – | – | 1 (1) | 9 (9) | – | – | – |
| XI | 1 (1) | – | – | – | – | – | – | – |
| **XII** | **1 (1)** | – | – | – | – | – | – | – |
| XIV | 1 (2) | – | – | 1 (1) | – | – | – | – |
| XV | – | – | – | – | – | – | – | 1 (2) |
| **XVI** | **3 (4)** | – | – | **4 (4)** | **3 (3)** | – | – | – |
| **XVII** | – | – | – | **2 (2)** | – | – | – | – |
| **XVIII** | – | – | – | – | – | – | – | **1 (1)** |
| **XIX** | **6 (8)** | – | **2 (2)** | **16 (16)** | **6 (6)** | **6 (8)** | – | – |
| XX | – | – | – | 5 (6) | – | – | – | – |
| XXI | – | – | – | – | – | – | – | 2 (4) |
| **XXII** | **5 (5)** | – | – | **2 (2)** | – | – | – | – |
| **XXIII** | **1 (1)** | – | – | – | – | – | – | – |
| XXIV | – | – | – | – | 1 (1) | – | – | – |
| **XXV** | **1 (1)** | – | – | – | – | – | – | – |
| **XXVI** | – | – | – | – | – | – | – | **1 (1)** |
| XXVII | – | – | – | – | – | – | – | 1 (1) |
| **XXVIII** | – | – | – | **2 (2)** | – | – | – | **1 (1)** |
| **XXIX** | – | – | – | – | – | – | – | **1 (2)** |
| **XXX** | – | – | – | – | – | – | – | **1 (1)** |
| XXXI | – | – | – | – | – | 1 (1) | – | – |

Of families listed, most of them were diagnosed at Kagoshima University in Japan, and 5 of 44 Chinese (C*) neonatal intrahepatic cholestatic hepatitis (NICCD) families were diagnosed in Taiwan (Yeh et al. 2006; Lee et al. 2006)

Novel mutations found in this study are indicated by bold letters. *CTLN2* adult-onset type II citrullinemia, *J* Japanese, *C* Chinese, *K* Korean, *V* Vietnamese, *O* others (non-East Asian)

(mRNA molecules deleted exon 11). Sequencing all 18 exons using genomic DNA, we found a base substitution from G to A at the 3′ end of exon 1 ([XXII]:Ex1-1G > A), but no change in amino acid sequences. However, the scores of predicted splicing sites, SSP and MaxEnt, decreased in mutation [XXII] from 0.95 to <0.40 and from 9.24 to 2.93, respectively.

[XXV]:IVS15 + 1G > T found in a Japanese CTLN2 patient (P474: [III]/[?]) produced a deletion of exon 15 (139 bp) in mRNA (data not shown), causing a frame shift and leading to a premature termination codon in exon 16 and a truncated form of the citrin protein G485fs(491X), as shown in Fig. 3b and Table 1. The SSP and MaxEnt scores decreased in mutation [XXV] from 0.98 to <0.40 and from 10.10 to 1.59, respectively. [XXVI]:IVS13 + 2T > G from paternal allele was found in genomic DNA of an Ashkenazi Jewish NICCD patient (P709) from Israel, but RT-PCR/sequencing of cDNA prepared from the cultured fibroblast cells of P709 showed homozygous [XVIII]:T446P mutation from maternal allele, suggesting

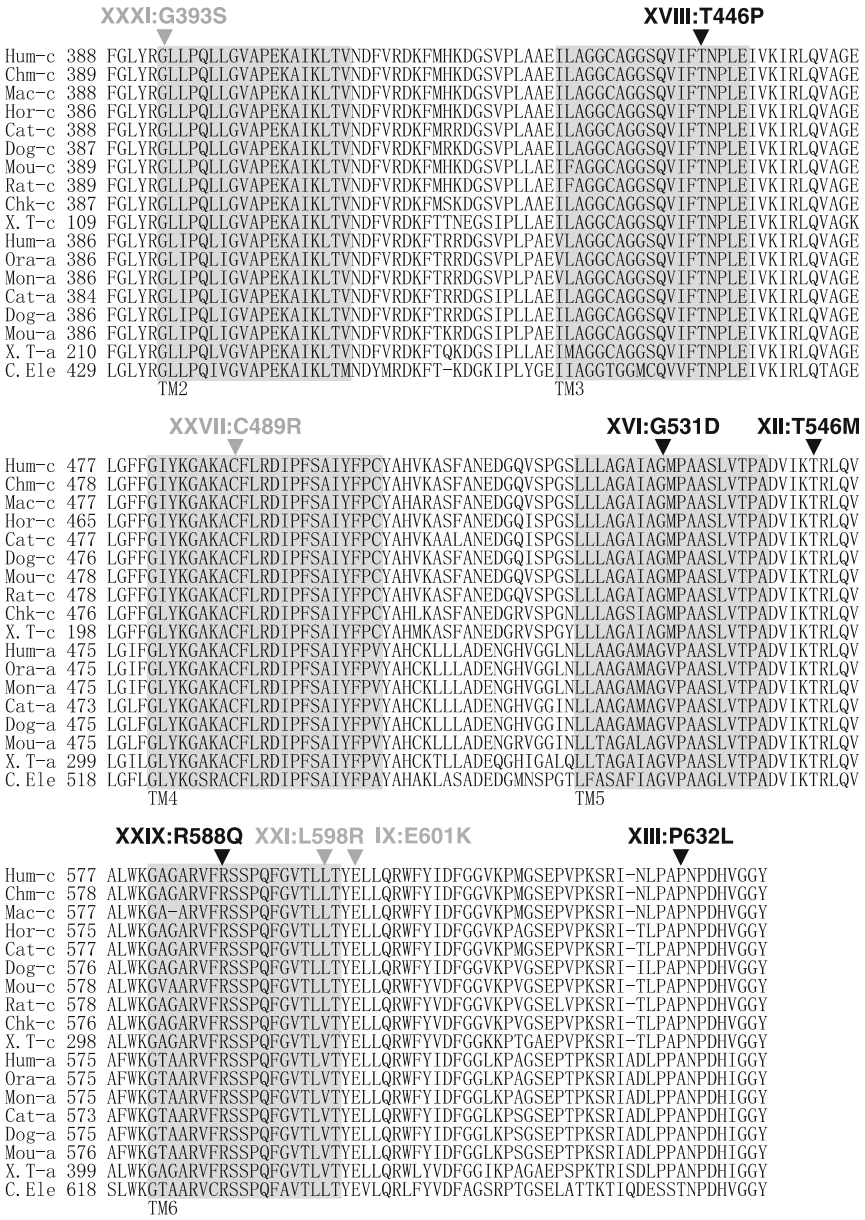


**Fig. 4** Alignment of human citrin (*hum-c*), aralar (*hum-a*), and their homologous protein in the mutated areas. *Chm* chimpanzee, *Mac* macaque, *Hor* horse, *Cat* cattle, *Mou* mouse, *Chk* chicken, *X.T Xenopus tropicalis*, *Ora* orangutan, *Mon* monkey, *C. Ele* Caenorhabditis elegans. *TM* denotes mitochondrial transmembrane (*shaded area*). The *black and gray letters* show the novel and known mutations, respectively

that the [XXVI]-mRNA may be less transcribed or unstable. The SSP and MaxEnt scores in mutation [XXVI] also decreased from 0.99 to <0.40 and from 9.73 to 2.09, respectively.

Novel nonsense and deletion mutations ([XVII]:E16X, [XXVIII]:R306X, [XXIII]:1146delA, [XXX]:1374/5delG)

We found a base substitution from G to T at 46 ([XVII]:E16X) in a Japanese NICCD patient (P758: [IV]/[?]) by direct sequencing of RT-PCR product from exon 1 to exon 18 (-87_2055). Our DNA diagnosis method using PCR-RFLP with *Pvu*II revealed mutation [XVII] in a maternal allele of another family (P919 with mutation [I] in the paternal allele). Genomic DNA analysis of a Japanese NICCD patient (P730 with mutation [II] in maternal allele) revealed a base substitution from C to T at 1078 [XXVIII]:R360X in the paternal allele. By RT-PCR/sequencing, the same [XXVIII] mutation was also found in a Caucasian NICCD patient (P1037) from the Czech Republic.

As shown in Fig. 3b and Table 1, we found a 1-bp deletion mutation at 1,146 in exon 11, [XXIII]:1146delA, in a Japanese CTLN2 patient (P549) with [XXII] from maternal allele and confirmed the [XXIII] mutation in her paternal allele by sequencing PCR products from genomic DNA (IVS9-313_IVS11 + 158). The [XXIII] mutation caused a frame shift, leading to a premature termination

and a truncated form of the citrin protein: R383fs(407X). Furthermore, by using RT-PCR/sequencing, we found a 1-bp deletion mutation at 1,374 or 1,375 in exon 14, [XXX]:1,374/5delG leading to A459fs(507X), in an allele of P1037. We confirmed by analysis with genomic DNA that P1037 was a compound heterozygote with [XXVIII]:R360X and [XXX]:1,374/5delG.

Missense mutations ([XII]:T546M, [XVI]:G531D, [XVIII]:T446P, [XXIX]:R588Q)

A base substitution from C to T at 1,637 in exon 16 ([XII]:T546M) was found by RT-PCR/sequencing of the whole coding sequence in the fibroblast RNA of a Japanese CTLN2 patient (P456) with mutation [I] from her maternal allele, who was diagnosed by clinical and biochemical studies (Oshiro et al. 2002). The mutation [XII] was confirmed in her paternal allele by DNA diagnosis. A base substitution from G to A at 1,592 in exon 16 ([XVI]:G531D) was identified by sequencing genomic DNA-PCR from a Japanese CTLN2 patient (P702) with mutation [VIII]:E601X in one allele. By sequencing RT-PCR/genomic DNA-PCR products, a base substitution from A to C at 1,336 in exon 14 ([XVIII]:T446P) was found in a maternal allele of an Israeli NICCD patient (P709) with mutation [XXVI]:IVS13 + 2T > G in the paternal allele. On the other hand, we found homozygously a base substitution from G to A at 1,763 in exon 17 ([XXIX]:R588Q) in both cDNA and genomic DNA of a Pakistani NICCD patient (P1221) in the UK.

Frequency and distribution of *SLC25A13* mutations

Thirteen novel mutations identified are shown in Table 1 and Fig. 3. These are one insertion, two deletion, three splice-site, two nonsense, and five missense mutations. Of these 13 novel mutations, 12 (all except G531D) were found in both cDNA and genomic DNA, and nine were confirmed in the patients' genomic DNA. These novel mutations were not detected in alleles from more than 50 Japanese control individuals. Western blot analysis showed no mutant citrin in the liver specimens or cultured fibroblast cells of the 11 non-T546M/G531D patients. The residues of the eight (four novel and four known) missense mutations (except P632L) were conserved within the three repeats of mitochondrial transmembrane (TM) spanners located on the 3′ side of citrin, aralar, and homologous proteins from human to *Caenorhabditis. elegance* (Fig. 4). These results strongly suggest that all mutations except P632L (which is located downstream of the same allele with mutation [I]:851del4) are pathogenic, although further confirmation by functional assay is necessary, especially in the missense mutations.

We also summarized the characteristics of all 30 mutations except [XIII]:P632L found in the *SLC25A13* gene involving those described previously (Kobayashi et al. 1999; Yasuda et a. 2000; Yamaguchi et al. 2002; Ben-Shalom et al. 2002; Lu et al. 2005; Takaya et al. 2005; Luder et al. 2006; Hutchin et al. 2006; Sheng et al. 2006; Ko et al. 2007) and assessed their frequency in 334 Japanese, 47 Chinese, 11 Korean, four Vietnamese, and seven non-East Asian families with citrin deficiency (Tables 1, 2). Out of 30 mutations, 12 (two nonsense, five missense, one duplication/insertion, two deletion, and two splice site) were private mutations each found in only one (Japanese, Israeli, Czech Republic, or UK) family. Three missense mutations were detected in more than one family from Japan (E601K and G531D) and in more than one family from Israel (L598R). As mentioned before (Kobayashi et al 2003; Lu et al. 2005), five mutations were found in East Asia: [I]:851del4 in 223 families (173 Japanese, 41 Chinese, five Korean, and four Vietnamese), [II]: IVS11 + 1G > A in 192 families (189 Japanese and three Korean), [III]:1638ins23 in 29 families (20 Japanese and nine Chinese), [V]:IVS13 + 1G > A in 50 families (48 Japanese and two Korean), and [X]:IVS6 + 5G > A in ten families (one Japanese and nine Chinese). In addition, two novel mutations identified in this study were also found in East Asia: [XVI]:G531D in ten families (seven Japanese and three Chinese), and [XIX]:IVS16ins3kb in 36 families (22 Japanese, six Chinese, and eight Korean).

## Discussion

In this study, we identified for the first time a large pathological insertion mutation [XIX]:IVS16ins3kb into the *SLC25A13* gene that was created upon a retrotransposal insertion of cDNA processed from a gene located on chromosome 6 (*C6orf68*). Babushok and Kazazian (2007) suggest that the integration of LINE-1, SINE elements, and other cellular transcripts by L1 retrotransposition machinery may occasionally cause disease by direct disruption of the target gene. As mutation [XIX]:IVS16ins3kb was found not only in Japanese (3.6% of mutated alleles) but also in other East Asians such as Chinese, including Taiwanese and Malaysian Chinese (6.4% of mutated alleles), and Koreans (45.5% of mutated alleles), this integration using LINE machinery or by infection of retrovirus in a founder may have occurred in a very early historical period in some region of East Asia. We have found that the frequency of mutation [XIX] in Korean patients with citrin deficiency is very high (Table 2) but found no carrier with mutation [XIX] in 2,228 Korean controls.

We have reported that the frequency of homozygotes with *SLC25A13* mutations in Japan is calculated to be

1:19,000 as a minimal estimate from the rate of carriers (1/69) (Saheki and Kobayashi 2002; Lu et al. 2005). In this study, we found a heterozygous carrier with mutation [XIX]:IVS16ins3kb in 1,372 Japanese controls, resulting in a carrier rate of 1:65, the same carrier frequency as in China (Lu et al. 2005). Considering the total population of East Asia (about $1.5 \times 10^9$), more than 100,000 individuals may be homozygotes with *SLC25A13* mutations, although further investigation is needed. On the other hand, we have found eight mutations (except [XXVIII]:R360X), which were not found in East Asian patients, in Israel (Ben-Shalom et al. 2002; Luder et al. 2006; this paper), USA (Dimmock et al. 2007), UK (Hutchin et al. 2006; this paper), and the Czech Republic (this paper), indicating that there is genetic heterogeneity among races in citrin deficiency, as described in other diseases such as classical citrullinemia (Gao et al. 2003). It is necessary to perform population analyses with sufficient numbers of controls after identifying novel mutations in each country.

In Japan, the frequency of homozygotes including compound heterozygotes (1/17,000) is almost the same as the incidence of NICCD: 1/17,000–34,000 (Shigematsu et al. 2002), but higher than the incidence of CTLN2: 1/100,000–230,000 (Kobayashi et al. 1993). From our observation of Japanese patients, we consider that most homozygotes suffer from NICCD. Following amelioration, some homozygotes may suffer from CTLN2 (10–80 years of age) or be diagnosed as suffering from other diseases, such as pancreatitis (Ikeda et al. 2004), hepatoma (Hagiwara et al. 2003; Tsai et al. 2006; Soeda et al. 2008), hyperlipidemia (Imamura et al. 2003; Terada et al. 2006), fatty liver such as nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD) (Takagi et al. 2006; Tanaka et al. 2007) or psychosis (Ikeda et al. 2001), but it is probable that many homozygotes remain healthy. To avoid misdiagnosis and mistreatment of patients with citrin deficiency, it is necessary to find them, to treat them properly, and to prevent severer outcomes.

We should realize that the outcome of NICCD patients is not always benign, because four NICCD patients showed severe phenotypes with liver dysfunction and were treated with liver transplantation at the age of 10–12 months (Tamamori et al. 2002). We do not know which NICCD patients, or how many, will go on to develop CTLN2. We need to follow NICCD patients carefully, even during infancy, to investigate their health and to detect the mechanisms and factors that cause severe NICCD and CTLN2. Recently, we described carbohydrate toxicity in patients with CTLN2 (Saheki et al. 2005; Yazaki et al. 2005; Takahashi et al. 2006) and in mouse models of citrin deficiency (Saheki et al. 2007). Intake or administration of large amounts of carbohydrates/sugars, which generate cytosolic NADH, should be avoided. Many CTLN2 patients have a peculiar fondness for protein/lipid-rich foods and aversion for sugar/carbohydrate-rich foods from childhood, probably from weaning, and their symptoms are often provoked by medication, infection, and/or alcohol intake. It is important to find the additional effects of genetic modifiers and/or environmental factors that lead to the deterioration to CTLN2, because we have diagnosed more than 300 cases to be suffering or have suffered from NICCD (220 Japanese, more than 40 Chinese, seven Korean, four Vietnamese, and 12 non-East Asian, including Caucasian) and several Japanese homozygotes and compound heterozygotes without CTLN2 symptoms (three fathers or two mothers of NICCD and 13 siblings of CTLN2 patients).

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