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MINIREVIEW

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Mitochondrial aspartate glutamate carrier (citrin) deficiency as the cause of adult-onset type II citrullinemia (CTLN2) and idiopathic neonatal hepatitis (NICCD)

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Abstract By using homozygosity mapping and positional cloning, we have shown that adult-onset type II citrullinemia (CTLN2) is caused by mutations of the *SLC25A13* gene, which is localized on chromosome 7q21.3 and encodes a mitochondrial solute carrier protein named citrin. So far, we have reported nine mutations, most of which cause loss of citrin, and we have established several methods for DNA diagnosis. These methods have shown that more than 90% of the patients diagnosed as suffering from CTLN2 by enzymatic analysis carry *SLC25A13* mutations in both alleles, indicating that CTLN2 is caused by citrin deficiency. Furthermore, by using the same DNA diagnosis methods, we discovered that 70 neonates or infants suffering from a particular type of neonatal hepatitis carry the same *SLC25A13* mutations. Since the symptoms of the neonates are different from those of the more severe CTLN2 and usually ameliorate without special treatment, we designated the neonatal disease neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). We conclude that citrin deficiency causes NICCD in neonates and CTLN2 in adults through the additional effects of genetic or environmental modifiers. Since the function of citrin, together with that of an isoform, aralar, was found to be as a mitochondrial aspartate glutamate carrier, the various symptoms of NICCD and CTLN2 may be understood as caused by defective aspartate export from the mitochondria to the cytosol and defects in the malate aspartate shuttle. It is, however, still difficult to understand the cause of the hepatic deficiency of argininosuccinate synthetase protein in CTLN2.

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Introduction

Citrullinemia (OMIM #215700) (McKusick 1998) is characterized by the accumulation of citrulline in the body fluid and hyperammonemia caused by a deficiency of argininosuccinate synthetase (ASS), the third enzyme of the urea cycle, which catalyzes the formation of argininosuccinate from citrulline and aspartate at the expense of ATP breakdown. Citrullinemia was first described by McMurray et al. (1962). Since then, many neonatal and infantile cases have been reported. Miyakoshi et al. (1968) reported that blood citrulline levels were increased in adult hyperammonemic patients with a specific type of chronic recurrent hepatocerebral degeneration described by Inose (1952). Later, Saheki et al. (1980, 1981) reported that the enzyme abnormalities of citrullinemia can be classified as qualitative (type I) or quantitative (type II), and that in type I, ASS is affected not only in the liver but also in the kidney and in cultured fibroblast cells, and is kinetically abnormal, while in type II, ASS is affected only in the liver (Saheki et al. 1982, 1983a), where it is deficient but kinetically normal and has the same specific activity as the control. Further research showed that type I citrullinemia, together with type III, in which ASS is almost completely absent in every cell where the *ASS* gene is expressed (Saheki et al. 1985a, 1987a,b; Imamura et al. 1987), is caused by mutations in the *ASS* gene (Kobayashi et al. 1987, 1990, 1991, 1994, 1995a; Kakinoki et al. 1997; Vilaseca et al. 2001). Kinetically abnormal mutant ASS is found in patients with type I citrullinemia, and mainly splicing mutations, but not missense mutations, cause type III citrullinemia (Kobayashi et al. 1987, 1994, 1995a). Citrullinemia caused by mutations in the *ASS* gene is now named CTLN1 (OMIM #215700), and the other form is named adult-onset type II citrullinemia, or CTLN2 (OMIM #603471).

Clinical features of CTLN2

Patients with CTLN2 show hyperammonemia and neuropsychiatric symptoms such as disorientation, delirium, aberrant behavior, delusion, and disturbance of consciousness, often leading to rapid death (Saheki et al. 1987b; Kobayashi et al. 2000). The onset is sudden, usually between the ages of 20 and 40. Patients diagnosed were from 11 to 79 years old with a mean age of 34.4 ± 12.6 (n = 102) (Kobayashi et al. 1997, 2000; Yasuda et al. 2000). Although the prognosis is bad, liver transplantation is remarkably effective (Todo et al. 1992; Yazaki et al. 1996; Kawamoto et al. 1997; Onuki et al. 2000; Takenaka et al. 2000; Kasahara et al. 2001; Ikeda et al. 2001). Symptoms are often provoked after medication, infection, or alcohol intake. Many patients have a peculiar fondness for beans and peanuts. They also like to eat high-protein diets such as eggs, fish, and meat, and dislike carbohydrates such as rice and sweets. Most patients are thin, more than 90% showing a body mass index less than 20, and about 40% show an index less than 17. Pancreatitis, hyperlipidemia, and hepatoma are the major complications of CTLN2 (Kobayashi et al. 2000; Ikeda et al. 2001; Tsujii et al. 1976).

Liver-specific ASS deficiency

Metabolically, CTLN2 is characterized by serum arginine levels higher than those in the controls, whereas patients with CTLN1 are arginine-deficient (Saheki et al. 1985b, 1986). Arginine is mainly synthesized in the small intestine in neonates and in the kidney in adults via citrulline formed in the small intestine (Featherston et al. 1973; Windmueller and Spaeth 1981; Funahashi et al. 1981; Hurwitz and Kretchmer 1986). Therefore, ASS in the small intestine and kidney plays a role in arginine synthesis. In the case of CTLN1, a generalized ASS deficiency results in arginine deficiency, while the citrulline accumulated as a result of the liver-specific ASS deficiency in CTLN2 becomes a good substrate for normal kidney or small intestine ASS. Even the product of the deficient ASS, argininosuccinate, is increased in the urine of CTLN2 patients (Saheki et al. 1987c). This is in accordance with the liver-specific ASS deficiency in CTLN2, which contrasts with the generalized ASS deficiency in CTLN1.

SLC25A13 and citrin

CTLN2 is characterized by a decrease in ASS activity and protein in the liver, but there are no abnormalities in ASS mRNA in the liver of CTLN2 patients with respect to amount, translational activity, or gross structure (Kobayashi et al. 1986, 1993; Sase et al. 1985). We have found that about 20% of CTLN2 patients are from consanguineous parents and that siblings are sometimes affected, suggesting that CTLN2 is an autosomal recessive disease. Restriction fragment length polymorphism (RFLP) analysis of 16 patients with CTLN2 from consanguineous marriages showed that the frequency of the heterozygous haplotype is not different from that in the controls, suggesting that the abnormality is not within the *ASS* gene locus (Kobayashi et al. 1993).

Since 1977, we have analyzed over 150 CTLN2 patients. During our studies, we have collected DNA samples from CTLN2 patients from 18 consanguineous families, which has allowed us to perform homozygosity mapping to delimit the critical region for the disease. *SLC25A13* on chromosome 7q21.3, which encodes a putative calcium-binding mitochondrial solute carrier protein, was identified as the disease-causing gene for CTLN2 by positional cloning and a mutation search (Kobayashi et al. 1999). As shown in Fig. 1, the human *SLC25A13* gene spans 160 kb of genomic DNA organized into 18 exons (Kobayashi et al. 1999; Sinasac et al. 1999). We have named the protein, which has a molecular weight of 74 kDa and is encoded by *SLC25A13*, citrin (Kobayashi et al. 1999).

Citrin consists of 675 amino acid residues, and, like other mitochondrial solute carriers such as ATP/ADP translocase, an ornithine transporter and phosphate carrier, it has six transmembrane domains on the C-terminal half. It also has four EF-hands known to bind calcium ions on the long N-terminal extension, which is characteristic of both citrin and an analogue, aralar (del Arco and Satrústegui, 1998). Aralar, encoded by *SLC25A12* (Crackower et al. 1999), has been found to be a calcium-binding mitochondrial solute carrier, too, and is 77.8% identical to citrin in its amino acid sequence. Q21153 of *Caenorhabditis elegans* is another analogue, 53.7% identical to citrin (Kobayashi et al. 1999).

We first identified five mutations in *SLC25A13* in 18 consanguineous CTLN2 patients (Kobayashi et al. 1999), and then four other mutations (Yasuda et al. 2000; Yamaguchi et al. 2002) as listed in Table 1. Except for the ninth mutation, E601K, all mutations caused truncation of the citrin protein by nonsense or frameshift mutations, or disruption of the membrane structure by splicing mutations (Fig. 1). No cross-reactive immune material with anti-human citrin antibody was detected in the livers of CTLN2 patients with the first seven mutations (Yasuda et al. 2000). We have established DNA diagnosis methods for the nine mutations by using polymerase chain reaction (PCR) analysis and gel electrophoresis (Kobayashi et al. 1999; Yasuda et al. 2000) and multiple DNA diagnosis methods by using a genetic analyzer with GeneScan software and a single primer extension procedure (SNaPshot) (Yamaguchi et al. 2002).

Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD)

Establishment of a DNA diagnosis method for citrin deficiency revealed that *SLC25A13* mutations are the cause of a type of neonatal hepatitis. Ohura et al. (2001) and Tazawa

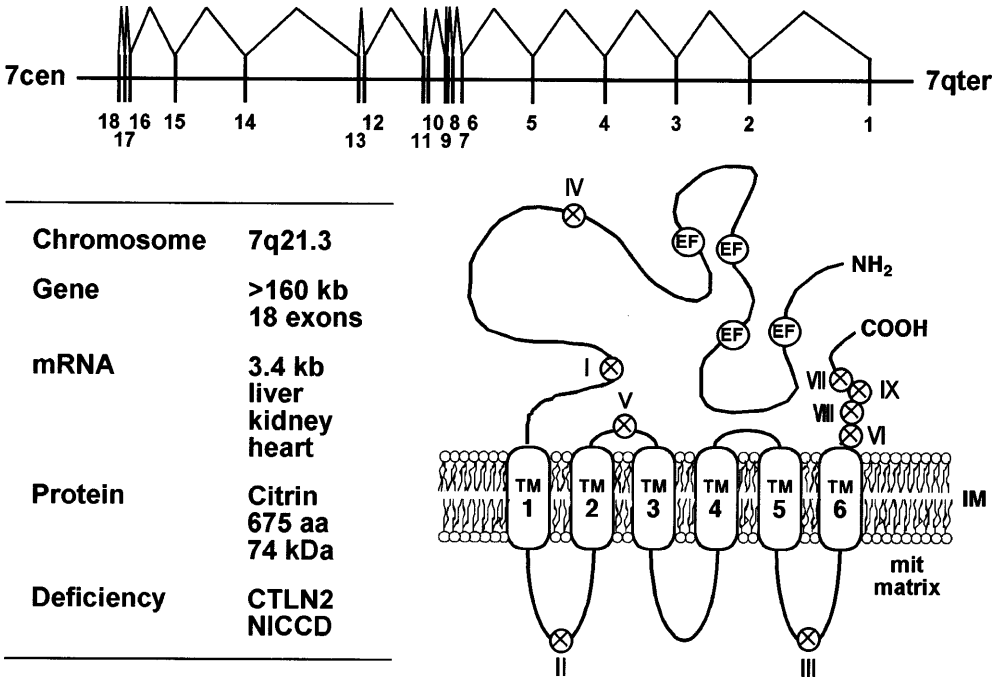

Fig. 1. Structure of *SLC25A13* gene (*top*) and its product, citrin (*bottom* and *right*). *CTLN2* and *NICCD* denote adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency, respectively. Sites of nine mutations, [I] to [IX] (see Table 1), are shown in the predicted citrin structure (*bottom right*). *aa*, amino acids; *EF*, calcium-binding EF hand motif; *TM*, mitochondrial (*mit*) transmembrane spanner; *IM*, inner membrane. The figure is based on data reported by Kobayashi et al. (1999), Sinasac et al. (1999), Yasuda et al. (2000), and Yamaguchi et al. (2002)

Table 1. Screening of SLC25A13 mutations

Mutation	Alleles (%)[a]		
	CTLN2	NICCD	Control
[I] 851del4	82 (38)	39 (31)	4 (20)
[II] IVS11+1G>A	95 (44)	62 (49)	9 (45)
[III] 1638ins23	5 (2)	4 (3)	1 (5)
[IV] S225X	13 (6)	3 (3)	5 (25)*
[V] IVS13+1G>A	13 (6)	11 (9)	1 (5)
[VI] 1800ins1	3	3	0
[VII] R605X	2	0	0
[VIII] E601X	1	3	0
[IX] E601K	0	1	0
Other mutations[c]	4	1	0
Total mutated alleles	218 (91)[b]	127 (91)[b]	20
Total tested alleles	240	140	2744

Nine mutations, [I] to [IX], have been reported previously (Kobayashi et al. 1999; Yasuda et al. 2000; Yamaguchi et al. 2002)
CTLN2, adult-onset type II citrullinemia; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency
* CTLN2/control and NICCD/control ($P < 0.01$)
[a] ratio to mutated alleles
[b] ratio to total alleles
[c] Four novel mutations identified will be published elsewhere

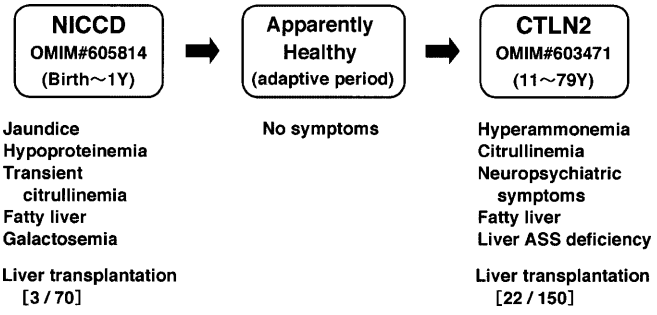

Fig. 2. Adaptation and decompensation in citrin deficiency. The figure is slightly modified from Saheki et al. (2002), since numbers (70 *NICCD* and 150 *CTLN2*) of patients diagnosed in our laboratory have increased. To date (January 2002), 3 *NICCD* and 22 *CTLN2* patients have had liver transplantation therapy. *ASS*, argininosuccinate synthetase; *Y*, year(s)

et al. (2001) have found mutations in neonates suffering from cholestasis and multiple aminoacidemia including citrulline, threonine, methionine, and tyrosine. Tomomasa et al. (2001) reported that a 16-year-old patient who received liver transplantation after diagnosis with CTLN2 had shown transient hypoproteinemia and jaundice in early infancy, and they also diagnosed a 2-month-old baby who developed symptoms very similar to the infantile symptoms of the former patient with CTLN2 as having an *SLC25A13* gene mutation. So far (January 2002), 70 neonates or infants have been diagnosed as having *SLC25A13* gene mutations. They suffer from a variety of symptoms, such as multiple aminoacidemia as described above, cholestasis, hypoproteinemia, galactosemia, and hypoglycemia (Ohura et al. 2001; Tazawa et al. 2001; Tomomasa et al. 2001; Naito et al. 2002). We now designate this disease as NICCD (neonatal intrahepatic cholestasis caused by citrin deficiency; OMIM #605814) (Yamaguchi et al. 2002; Ohura et al. 2001). Most NICCD patients showed ameliorated symptoms by 12 months without special treatment other than feeding programs (Ohura et al. 2001; Tazawa et al. 2001); formulas containing middle-chain triglyceride or lactose-free formulas and supplementation with fat-soluble vitamins have been used. As described previously (Tomomasa et al. 2001; Saheki et al. 2002) and summarized in Fig. 2, however, more than 10 years or even several decades later, some of these patients developed CTLN2, although we do not know

which NICCD patients or what percentage of them developed CTLN2.

Frequency

We have analyzed the frequency of the nine *SLC25A13* mutations among 120 Japanese patients with CTLN2, 70 with NICCD, and 1372 Japanese controls. As shown in Table 1, mutations [I] and [II] are most frequent, between them accounting for 82% and 80% of CTLN2 and NICCD, respectively. There were about twice as many male as female CTLN2 patients (Kobayashi et al. 1997, 2000; Yasuda et al. 2000), but there was no such sex difference among NICCD patients (Yamaguchi et al. 2002), suggesting that sex hormones may affect the onset of CTLN2. Twenty out of 1372 controls had a mutation in one allele of the *SLC25A13* gene, which implies that the rate of heterozygosity is approximately 1 in 70 in the Japanese population. Thus, the frequency of homozygotes with *SLC25A13* mutations is calculated to be 1 in 20,000 as a minimal estimate from the carrier rate (Yasuda et al. 2000; Yamaguchi et al. 2002; this paper). This frequency of homozygotes with mutated *SLC25A13* is higher than the incidence of CTLN2 (1/100,000) calculated from consanguinity (Kobayashi et al. 1993). The discrepancy between the two is difficult to explain. Patients homozygous for *SLC25A13* mutations may be classified at any given time into one of the following groups: (1) diagnosed as suffering from CTLN2; (2) diagnosed as having another disease such as hepatitis, hepatoma, pancreatitis, or hyperlipidemia; (3) misdiagnosed as having a psychosis such as schizophrenia, epilepsy, or depression; and (4) apparently healthy. We have already detected several noncitrullinemic individuals with *SLC25A13* mutations in both alleles (Onuki et al. 2000; Imamura et al. unpublished data). Others are those who recovered from NICCD (Ohura et al. 2001; Tazawa et al. 2001). It is important to predict whether those who recovered from NICCD will suffer from CTLN2. We postulate that the probability is not very high because the sex ratio is different between CTLN2 and NICCD patients (Yamaguchi et al. 2002).

Until recently, CTLN2 had been found mostly among Japanese, and there were no reports of CTLN2 cases from other countries except for in one Chinese from Singapore, who was clinically diagnosed as suffering from CTLN2 (Chow et al. 1996). However, we recently found two Chinese CTLN2 patients from Taiwan (Hwu et al. 2001) and a Vietnamese infant suffering from NICCD in Australia (Wilcken et al. unpublished data). It is interesting that these patients had the same *SLC25A13* mutations as those identified in Japanese patients. These results suggest that the common mutations are old enough to go back to a common ancestor and prevail at least in East Asia. Most recently, we found a Palestinian patient with NICCD but with a novel mutation (Elpeleg et al. unpublished data), suggesting a wide distribution of citrin deficiency among races.

Citrin and aralar as aspartate glutamate carriers

In collaboration with two other groups of researchers, we have found that citrin and aralar are isoforms of aspartate glutamate carriers (AGCs) (Palmieri et al. 2001). Recombinant citrin and aralar reconstituted into liposomes showed electrogenic exchange of aspartate for glutamate and H^+. The substrate specificity and kinetic parameters were essentially identical to those determined by using natural AGCs (Dierks and Krämer, 1988; Dierks et al. 1988; Bisaccia et al. 1992). The transport activity of citrin and aralar are entirely accounted for by their C-terminal domains (Palmieri et al. 2001). The turnover number of citrin is about four times greater than that of aralar. The activity of citrin and aralar transfected into mammalian cells is stimulated by calcium ions on the external side of the inner mitochondrial membrane, where the calcium ion-binding domains of these proteins are located (Palmieri et al. 2001). These results strongly suggest that calcium ion activation of the malate aspartate shuttle (Leverve et al. 1986; Sugano et al. 1988; Scaduto 1994) is based on the calcium-binding property of citrin and aralar as AGCs and that AGCs are one of the most important targets of calcium-mediating hormones.

CTLN2 and NICCD as a citrin deficiency

As shown in Fig. 3, citrin, as an AGC, plays a role (1) in the supply of aspartate from the mitochondria to the cytosol and (2) as a member of the malate aspartate shuttle. The supply of aspartate formed in the mitochondria to the cytosol via AGCs is very important for urea synthesis from ammonia (Williamson 1976) and also from alanine, because oxaloacetate can be formed only in the mitochondria without reduced nicotinamide adenine dinucleotide (NADH) formation in the cytosol (Fig. 3A). Lack of aspartate for the ASS reaction probably causes accumulation of citrulline. Aspartate is low in the plasma and is much less taken up by the liver, as judged from the rate of gluconeogenesis (Ross et al. 1967), suggesting that the requirement of aspartate in the cytosol largely depends upon the supply from the mitochondria. Therefore, citrin deficiency probably causes a deficiency of aspartate in the cytosol of the liver, followed by inhibition of protein and nucleotide synthesis, resulting in hypoproteinemia in NICCD. Beans and peanuts are the highest dietary sources of aspartate and asparagine. This may be the reason why CTLN2 patients show an extraordinary liking for beans and peanuts.

Citrin, as an AGC, is a member of the malate aspartate shuttle, which plays a role in the transport of the cytosolic reducing equivalent into the mitochondria (Fig. 3B). Citrin deficiency blocks the malate aspartate shuttle, which may increase the cytosolic NADH to oxidized nicotinamide adenine dinucleotide ratio ($NADH/NAD^+$). The increased $NADH/NAD^+$ ratio inhibits glycolysis and makes alcohol metabolism difficult. This may be the reason why CTLN2 patients dislike carbohydrates and cannot drink alcohol,

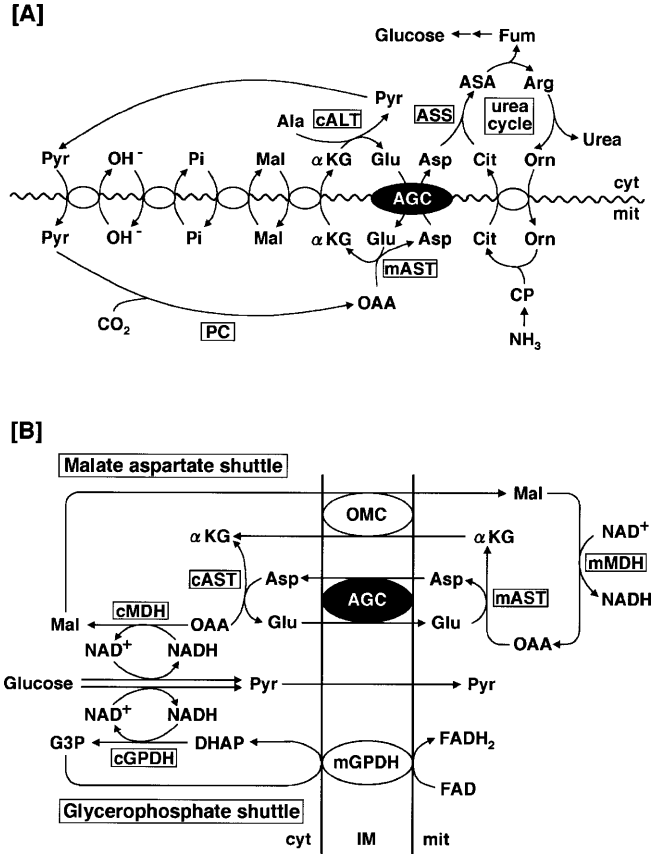

Fig. 3A,B. Role of citrin as an aspartate glutamate carrier (AGC). **A** Gluconeogenesis from alanine and urea synthesis. **B** Malate aspartate shuttle and glycerophosphate shuttle. *Ala*, alanine; *αKG*, α-ketoglutarate; *cALT*, cytosolic alanine aminotransferase; *Glu*, glutamate; *Pyr*, pyruvate; *PC*, pyruvate carboxylase; *OAA*, oxaloacetate; *Asp*, aspartate; *Cit*, citrulline; *ASS*, argininosuccinate synthetase; *ASA*, argininosuccinate; *Fum*, fumarate; *Arg*, arginine; *Orn*, ornithine; *CP*, carbamoyl phosphate; *mAST* and *cAST*, mitochondrial and cytosolic aspartate aminotransferase; *Mal*, malate; *cMDH* and *mMDH*, cytosolic and mitochondrial malate dehydrogenase; *OMC*, oxoglutarate malate transporter; *cGPDH* and *mGPDH*, cytosolic and mitochondrial glycerophosphate dehydrogenase; *G3P*, glycerol-3-phosphate; *DHAP*, dihydroxyacetone phosphate; *cyt*, cytosol; *IM*, inner membrane; *mit*, mitochondria

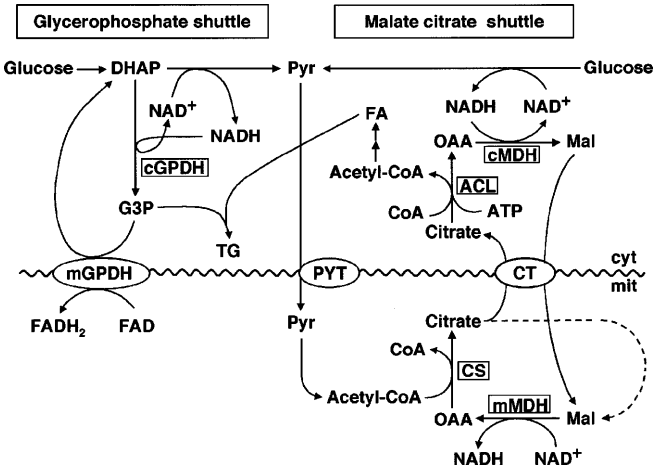

Fig. 4. Predicted metabolic pathway for compensation in citrin deficiency. *TG*, triglyceride; *FA*, fatty acid; *ACL*, ATP citrate lyase; *PYT*, pyruvate transporter; *CT*, citrate transporter; *CS*, citrate synthase

and why alcohol often causes psychiatric symptoms in CTLN2 patients. An increased NADH/NAD^+ ratio also inhibits gluconeogenesis from reduced substrates such as lactate, glycerol, and sorbitol (Krebs et al. 1967). This, together with the difficulty of alanine metabolization to urea and glucose under a citrin deficiency, may cause hypoglycemia in NICCD patients. Although NICCD patients suffer from galactosemia, sometimes even leading to cataracts, no abnormality in the enzymes for galactose metabolism has been found (Ohura et al. 2001; Naito et al. 2002). Since uridine diphosphate (UDP)-glucose epimerase requiring NAD as a cofactor is strongly inhibited by NADH (Langer and Glaser 1974), galactosemia in NICCD may represent a high NADH level in the cytosol of the liver.

Another shuttle system for the cytosolic reducing equivalent is the glycerophosphate shuttle (Figs. 3B and 4), which functions like the malate aspartate shuttle in rat (Sugano et al. 1988). In human liver, however, the glycerophosphate shuttle has not been described, except to note that human liver contains less than one-twentieth the mitochondrial glycerophosphate dehydrogenase (mGPDH) contained in rat or mouse liver (Sadava et al. 1987). The symptoms listed above caused by an elevated cytosolic NADH level suggest low activity of the glycerophosphate shuttle in human liver. The amelioration of NICCD symptoms in a year suggests some adaptation, compensation, or metabolic change during development (Fig. 2). One such system may be the glycerophosphate shuttle. The other possibility is that the malate citrate shuttle, which, as shown in Fig. 4, produces oxaloacetate from citrate in the cytosol by the catalytic activity of ATP citrate lyase (ACL), reduces oxaloacetate concomitantly with consumption of NADH and transports malate into the mitochondria. Mitochondrial malate is converted to oxaloacetate to produce NADH and is condensed with acetyl-coenzyme A (CoA) to form citrate, which is then transported to the cytosol. As a result, cytosolic NADH or the reducing equivalent is transported into the mitochondria. However, since this system plays a role in fatty acid synthesis, it also produces acetyl-CoA in the cytosol and may stimulate fatty acid synthesis. If the glycerophosphate shuttle is less active, triglyceride synthesis may be activated by supplying glycerol-3-phosphate, which is produced by cytosolic glycerophosphate dehydrogenase (cGPDH). All these promote fatty liver and hyperlipidemia. Actually, Imamura et al. (unpublished data) found a CTLN2 patient who showed a dramatic increase in triglycerides with a high-carbohydrate diet, which was normalized after the patient changed to a high-protein low-carbohydrate diet.

Increased cytosolic NADH may cause so-called reductive stress and subsequent oxidative stress (Khan and O'Brien 1995; Lieber 1997; Williamson et al. 1999), leading to liver damage. This hypothesis should be examined.

Possible therapy based on a new concept

CTLN2 patients have been treated by using two kinds of therapeutic procedures: (1) symptomatic therapy against hyperammonemia and (2) liver transplantation (reviewed by Takenaka et al. 2000). Low-protein diets are the first choice for hyperammonemia. Various medicines that decrease blood ammonia, such as lactulose, sodium benzoate, citrate, and arginine, have been used, but have failed to improve the long-term prognosis (Takenaka et al. 2000). On the other hand, liver transplantation has been shown to be very effective. Following two cases of orthotopic liver transplantation performed at Pittsburgh in 1988 (Todo et al. 1992) and at Brisbane in 1993 (Kawamoto et al. 1997), 20 cases of CTLN2 in Japan have been treated with living-related partial liver transplantation (Yazaki et al. 1996; Onuki et al. 2000; Takenaka et al. 2000; Kasahara et al. 2001; Ikeda et al. 2001). Liver transplantation normalizes most of the metabolic disturbances and symptoms, except those from brain atrophy already occurred. It is curious that liver transplantation is so effective even though citrin is not specifically distributed in the liver, but also in the kidney, the heart, and so on (Kobayashi et al. 1999; del Arco et al. 2000; Iijima et al. 2001; Begum et al. 2002). This is probably because the liver plays a major role in metabolism and is the only major organ that expresses citrin, but not aralar. Aralar may deputize for citrin as an AGC isoform. This treatment, however, raises the problems of financial support and liver donors in Japan.

Now, since we know the functions of citrin and the characteristics of NICCD and CTLN2, we are in a position to establish new therapeutic procedures based on the functions of citrin. There are five possible treatment procedures: (1) aspartate could be supplied in the cytosol; (2) the cytosolic NADH/NAD^+ ratio could be decreased; (3) dehydroepiandrosterone and peroxisome proliferators may be effective in activating an alternative NADH shuttle; (4) sex hormone therapy may have some effect on preventing or delaying the onset of CTLN2; and (5) gene therapy for hepatocytes may be effective.

Supplying aspartate or oxaloacetate in the cytosol may be effective, but, as described above, aspartate may be taken up into the hepatocytes only slowly, and oxaloacetate is very unstable. Asparagine may be substituted for them, although it should be noticed that asparagine loads additional nitrogen onto the urea cycle. Citrate has been used effectively in some cases for CTLN2 therapy (Yajima et al. 1981). Citrate supplies oxaloacetate via ATP citrate lyase, although it may also supply acetyl-CoA for the synthesis of fatty acids, resulting in fatty liver and hyperlipidemia. It is again important to note that CTLN2 patients have a special liking for beans and peanuts, and dislike carbohydrates. Beans and peanuts probably provide aspartate/asparagine, which may support the above-stated processes, but it is also important to note that they load the liver with excessive nitrogen. Arginine or ornithine should be supplied, although beans and peanuts contain much arginine. Care should be taken not to supply high-carbohydrate diets, which may lead to a high NADH/NAD^+ ratio in the liver. Alcohol, too, should be avoided.

Mitochondrial glycerophosphate dehydrogenase (mGPDH), a member of the glycerophosphate shuttle, is known to be induced by thyroid hormone (Lee and Lardy 1965), dehydroepiandrosterone (Su and Lardy 1991; Lardy et al. 1995), and peroxisome proliferators (Shoemaker and Yamazaki 1991). These may be effective in activating the glycerophosphate shuttle, and in decreasing the cytosolic NADH/NAD^+ ratio. To establish proper therapeutic procedures for CTLN2, animal models are essential.

Questions to be answered

Liver-specific ASS deficiency, the main characteristic of CTLN2, was found to be secondary to the citrin deficiency (Kobayashi et al. 1999; Yasuda et al. 2000). However, it is difficult to imagine how a citrin AGC deficiency causes a decrease in the ASS protein. The liver specificity of the ASS deficiency may derive from the fact that the hepatic AGC is composed only of citrin, and a citrin deficiency causes a complete loss of AGC activity in the liver. Purified ASS is stabilized by the substrates, citrulline and aspartate, and the product, argininosuccinate, but requires high concentrations (Saheki et al. 1977; Takada et al. 1979). It is also difficult to imagine direct protein–protein interaction between ASS and citrin, because they are localized in the cytosol and inner membrane of the mitochondria, respectively, being separated by the outer membrane. Further research is needed.

The remaining mechanisms to be clarified are the enhanced expression of the pancreatic secretory trypsin inhibitor (*PSTI*) gene in the liver (Kobayashi et al. 1995b, 1997) and the unique uneven distribution of the ASS protein in the liver lobulus (Saheki et al. 1983b, 1987b; Yagi et al. 1988) of CTLN2 patients. These phenomena are useful for diagnosis of CTLN2 (Kobayashi et al. 1997; Tsuboi et al. 2001; Ikeda et al. 2001; Maruyama et al. 2001; Oshiro et al. 2002) and to determine the prognosis of the patients (Yagi et al. 1988), and may be related to the finding that about 10% of the patients with CTLN2 suffer from hepatoma without liver cirrhosis at relatively young ages (Tsujii et al. 1976; Kobayashi et al. 2000).

Concluding remarks

The discovery of the disease gene, *SLC25A13*, for CTLN2 has revealed that a neonatal disease, NICCD, is caused by the same gene mutations. Usually, NICCD is not as severe a disease as CTLN2 except in some cases, and the patients generally recover apparently healthy states. But more than 10 years or even several decades later, some of the individuals carrying *SLC25A13* gene mutations in both alleles may develop CTLN2 with neuropsychiatric symptoms. It is now important and urgent to find the genetic and/or environ-

mental factors that lead to the deterioration to CTLN2, because we have diagnosed 70 patients as suffering from or having previously suffered from NICCD, and have found siblings of CTLN2 patients to be citrin deficient but without CTLN2 symptoms. It is now much easier than before to study the pathophysiology of citrin deficiency and develop therapies, because we now know the functions of citrin, and we have available two kinds of citrin-deficient mice, which we are examining to see whether they are suitable as animal models for NICCD or CTLN2.

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