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ORIGINAL ARTICLE

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Frequency and distribution in East Asia of 12 mutations identified in the *SLC25A13* gene of Japanese patients with citrin deficiency

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Abstract Deficiency of citrin, a liver-type mitochondrial aspartate-glutamate carrier (AGC), encoded by the *SLC25A13* gene on chromosome 7q21.3, causes autosomal recessive disorders: adult-onset type II citrullinemia (CTLN2) and neonatal hepatitis associated with intrahepatic cholestasis (NICCD). So far, we have described 12 *SLC25A13* mutations: 11 were from Japan and one from Israel. Three mutations found in Chinese and Vietnamese patients were the same as those in Japanese patients. In the present study, we identified a novel mutation IVS6+1G>C in a Japanese CTLN2 patient and widely screened 12 *SLC25A13* mutations found in Japanese patients in control individuals from East Asia to confirm our preliminary results that the carrier frequency was high in Asian populations. Mutations 851–854del and 1638–1660dup were found in all Asian countries tested, and 851–854del associated with 290-haplotype in microsatellite marker *D7S1812* was especially frequent. Other mutations frequently detected were IVS11+1G>A in Japanese and Korean, S225X in Japanese, and IVS6+5G>A in Chinese populations. We found a remarkable difference in carrier rates in China (including Taiwan) between north (1/940) and south (1/48) of the Yangtze River. We detected many carriers in Chinese (64/4169 = 1/65), Japanese (20/1372 = 1/69) and Korean (22/2455 = 1/112) populations, suggesting that over 80,000 East Asians are homozygotes with two mutated *SLC25A13* alleles.

Keywords Adult-onset type II citrullinemia (CTLN2) · Aspartate-glutamate carrier (AGC) · Citrin · Carrier frequency · *SLC25A13* · *D7S1812*

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Introduction

By using homozygosity mapping and positional cloning, *SLC25A13* was identified on chromosome 7q21.3 as the gene responsible for adult-onset type II citrullinemia (CTLN2; MIM#603471) (Kobayashi et al. 1999). As shown in Fig. 1, the gene consists of 18 exons and encodes a calcium-binding mitochondrial solute carrier protein, designated citrin (675 amino acids, 74 kDa), containing four EF-hand Ca^{2+}-binding motifs in the N-terminus and six mitochondrial transmembrane domains in the C-terminal half (Kobayashi et al. 1999, 2000; Sinasac et al.

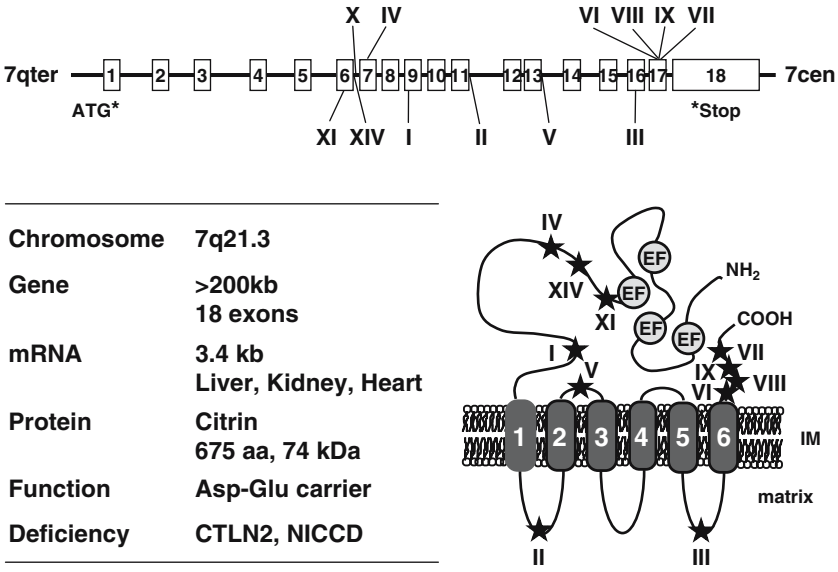

Fig. 1 The *SLC25A13* gene structure (*top*), characteristic features of transcripts (*left*), and locations of mutations in the predicted topographic of citrin protein (*right*). The locations of 12 mutations [I]–[XI] and [XIV] (see Table 1) are shown in the gene structure (*top*). *Asp-Glu carrier* aspartate-glutamate carrier, *CTLN2* adult-onset type II citrullinemia, *NICCD* neonatal intrahepatic cholestasis caused by citrin deficiency, *EF* calcium-binding EF-hand motif, *TM* (1–6 in *right*) mitochondrial transmembrane domains, *IM* inner membrane. Most mutations cause truncations of the protein ([I], [III], [IV], [VI]–[VIII], [XI] and [XIV]) or deletions of a loop between the TM domains ([II] and [V]). Sites of 11 mutations, except [X], resulting in RNA-negative or extremely low levels of transcripts, are shown in the predicted citrin structure (*lower right*)

1999). Citrin is expressed mainly in the liver (Kobayashi et al. 1999) and functions as a Ca^{2+}-stimulated aspartate-glutamate carrier (AGC) together with aralar, which is mainly expressed in the brain and skeletal muscle (Palmieri et al. 2001). Thus, citrin is a liver-type AGC, and aralar is a brain/muscle-type AGC. Although aralar deficiency is not known, citrin deficiency, an autosomal recessive disorder, causes not only CTLN2 in adults (Kobayashi et al. 1999, 2000; Yasuda et al. 2000) but also idiopathic neonatal hepatitis associated with jaundice in neonates/infantiles (Ohura et al. 2001; Tazawa et al. 2001; Tomomasa et al. 2001). As the symptoms in the neonates were different from those found in CTLN2, the neonatal disease was named neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; MIM#605814) (Yamaguchi et al. 2002; Saheki and Kobayashi 2002).

The CTLN2 is characterized by episodes of neurological symptoms associated with hyperammonemia involving disorientation, abnormal behavior (aggression, irritability, and hyperactivity), seizures, coma, and potentially death from brain edema (Saheki et al. 1987; Kobayashi et al. 2000; Kobayashi and Saheki 2003). For CTLN2 patients with poor prognosis and high mortality, there is no effective treatment at the present except liver transplantation (Ikeda et al. 2001; Kasahara et al. 2001; Yazaki et al. 2004). On the other hand, NICCD shows multiple metabolic abnormalities, including an aminoacidemia (involving citrulline, threonine, methionine, tyrosine, and arginine), galactosemia, hypoproteinemia, hypoglycemia, cholestasis, and fatty liver (Saheki and Kobayashi 2002; Kobayashi and Saheki 2003; Ohura et al. 2003; Tazawa et al. 2004; Hachisu et al. 2005). The NICCD is usually not severe and can be managed using nutritional manipulation; symptoms usually disappear within a year. A few NICCD patients, however, have a severe form of the disorder with liver damage associated with tyrosinemia and require liver transplantation (Tamamori et al. 2002). Some neonatal NICCD patients develop severe CTLN2 more than a decade or several decades later (Kasahara et al. 2001; Tomomasa et al. 2001; Saheki and Kobayashi 2002).

Citrin deficiency was thought at first to be restricted to the Japanese population. However, recently, several cases have been found in other countries. In Israel, we have detected seven NICCD patients in four families with mutations different from those found in Japanese patients (Ben-Shalom et al. 2002; Elpeleg et al. Mandel et al. and Luder et al. unpublished), indicating a wide distribution of citrin deficiency. In Asian populations, we have reported four patients with the same mutations as Japanese: two mutations, 851–854del (previous name: 851del4) [I] and/or 1638–1660dup (1638ins23) [III], were found in three Chinese CTLN2 patients from Taiwan (Hwu et al. 2001) and Beijing (Yang et al. 2003) and a Vietnamese NICCD patient who lives in Australia (Lee et al. 2002). From preliminary population analysis of the known 11 mutations identified in Japanese patients, we have shown that the carrier rates are frequent in China, Taiwan, and Korea (Kobayashi et al. 2003; Saheki et al. 2004) as well as Japan (Saheki and Kobayashi 2002).

In the present study, to investigate more precisely the frequency and distribution of the *SLC25A13* mutations, we identified a novel mutation in the *SLC25A13* gene of two Japanese patients with citrin deficiency and performed population analysis on 12 *SLC25A13* mutations found in Japanese patients in an increased number of

controls from all over East Asia. We found a remarkable geographical difference in the distribution of carriers between areas of China north and south of the Yangtze River. We also analyzed a microsatellite marker, *D7S1812*, located in intron 15 of the *SLC25A13* gene, which we used in order to haplotype mutation alleles, as described previously (Kobayashi et al. 1999), and we discuss the migration of mutation [I] allele found frequently in all East Asians tested and mutation IVS11 + 1G > A [II] allele found in Japanese and Koreans but not Chinese.

Materials and methods

Patients and control individuals

In addition to 129 CTLN2 and 103 NICCD Japanese (Kobayashi et al. 2003; Saheki et al. 2004), three CTLN2 Chinese (Hwu et al. 2001; Yang et al. 2003) and a NICCD Vietnamese (Lee et al. 2002) patients, genomic DNA samples from CTLN2 and NICCD cases (17 CTLN2 and 44 NICCD Japanese, four NICCD Chinese in Taiwan or in USA, and a NICCD Vietnamese in USA) and their family members were used in the present study with written informed consent. The diagnosis of CTLN2 was based on well-established criteria, including symptoms and laboratory findings such as high blood ammonia, increased plasma citrulline, arginine, ratio of threonine to serine and serum pancreatic secretory trypsin inhibitor (PSTI) levels, and decreased hepatic argininosuccinate synthetase (ASS) activity/protein levels (Saheki et al. 1987; Kobayashi et al. 1997, 2000; Yasuda et al. 2000; Kobayashi and Saheki 2003). The NICCD patients were found by newborn mass screening in association with galactosemia, methioninemia, and/or phenylalaninemia, or among those who suffered from persistent cholestatic jaundice, discolored stools, failure to thrive, and multiple aminoacidemia, including elevated citrulline, threonine, methionine, tyrosine, and arginine levels at 1–4 months of age. Other features shown in NICCD patients are hypoproteinemia, hypoglycemia, hemolytic anemia, α-fetoproteinemia, fatty liver, and/or mild liver dysfunction. The clinical data from CTLN2 or NICCD patients have been described (Ikeda et al. 2004; Yazaki et al. 2004, 2005; Tazawa et al. 2004; Hachisu et al. 2005) and will be reported elsewhere.

In addition to DNA samples from 1,372 Japanese, 1,008 Chinese, 1,314 Taiwanese, and 201 Korean controls (Kobayashi et al. 2003; Saheki et al. 2004), the dried spot blood specimens collected at newborn screening centers (54 in Liaoning, 302 in Shandong, 175 in Henan, 200 in Hubei, 638 in Guangdong, and 2,254 in Korea) were anonymized and used as controls without selection. The DNA was extracted from dried blood spots with the ReadyAmp purification system (Promega Corporation, Madison, WI, USA). Anonymous DNA samples were from the Hong Kong Red Cross (478 unrelated individuals). Samples were obtained with the approval of the Institutional Ethics Review Board in each country and area. This study was performed in accordance with the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Kagoshima University Faculty of Medicine.

Mutation detection

To identify unknown *SLC25A13* mutations, we mainly used sequencing analysis of the DNA fragments amplified by genomic DNA-PCR and/or RT-PCR, as described previously (Kobayashi et al. 1999; Yasuda et al. 2000; Yamaguchi et al. 2002). For genomic DNA analysis, regions (average size about 500 bp) each containing 18 exons were individually amplified using oligonucleotides derived from intronic sequences flanking each exon as PCR primers. For mRNA analysis, the first strand of cDNA was synthesized with total RNA extracted from the liver or fibroblast cells by using oligo-$dT_{12–18}$ and M-MuLV reverse transcriptase, and the entire coding portion of *SLC25A13* mRNA was amplified with suitable primer sets. The amplified PCR products of genomic DNA and/or cDNA 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).

DNA diagnosis

To detect simultaneously three mutations identified in exon 6 (R184X [XI]) and intron 6 (IVS6 + 5G > A [X] and IVS6 + 1G > C [XIV]), we established a multiple DNA diagnosis method using SNaPshot (see below) in addition to the PCR-RFLP method. The conditions in PCR-RFLP method are amplification using primers IVS5NF (5′-TGAGGGCTTGTTAGATCAAGAT-3′) and IVS6NB (5′-TTACCCAGACAACAAATTAACCT-3′), digestion by *Tas* I (Fermentas, Hanover, MD, USA) for [X] or *Dde* I (BioLabs, Beverly, MA, USA) for [XI] and [XIV], and electrophoresis on a 2.5% Low Range Ultra agarose gel (Bio-Rad, Hercules, CA, USA).

The nine known *SLC25A13* mutations ([I]–[IX]) were detected by PCR-RFLP and/or multiple GeneScan/SNaPshot methods, as described previously (Kobayashi et al. 1999; Yasuda et al. 2000; Yamaguchi et al. 2002). For simultaneous GeneScan detection of mutations [I], [III], and 1800–1801insA (1800ins1) [VI], PCR was performed in a mixture containing fluorescent-labeled [R110]-dUTP, dNTP and three sets of primers. After amplification, the size of each [R110]-labeled PCR-product was resolved using [ROX]-labeled GeneScan-500 as a size standard with GeneScan software on an ABI-310 genetic analyzer (Yamaguchi et al. 2002).

The SNaPshot method used for simultaneous detection of single-base substitution is based on the dideoxy single nucleotide extension of an unlabeled oligonu-

cleotide primer. An ABI Prime SNaPshot Multiplex Kit (PE Applied Biosystems) containing four kinds of fluorescent-labeled ddNTP was used for multiple-DNA diagnosis of mutations [X], [XI], and [XIV], as well as known mutations [II], S225X [IV], and IVS13 + 1G > A [V], and mutations R605X [VII], E601X [VIII], and E601K [IX] (Yamaguchi et al. 2002). The PCR primers (IVS5NF and IVS6NB) in the first PCR and the anneal primers (GSmXB: 5′-CACTTCATTAGGGCAAGTTACAA-3′, GSmXIB: 5′-ACATGGGGGCGGATGGTGACCATGATGTCTC-3′ and GSmXIVF2: 5′-TCCTTTTGTAGAAGAATGTCTAGTAGCT-3′) in the second PCR were used for multiple detection of mutations [X], [XI], and [XIV]. Shrimp alkaline phosphatase and exonuclease I (USB Corporation, Cleveland, OH, USA) were used to remove dNTP, ddNTP, and primers.

Haplotype analysis of *D7S1812*

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).

Statistical analysis

Carrier frequency in the three areas of China and frequencies of 290-haplotype or 281-haplotype between controls and carriers with mutations [I] or [II], respectively, were assessed using a 2×2 chi-square (χ^2) test.

Results and discussion

Identification and diagnosis of a novel *SLC25A13* mutation

By direct sequencing of PCR fragments (467 bp) using genomic DNA with primers IVS5NF and IVS6NB, two *SLC25A13* mutations, IVS6 + 5G > A [X] (a G to A substitution at the fifth position of 5′-end in intron 6) and R184X [XI] (a C to T substitution at position 550 in exon 6) were identified in an allele of Japanese patients with NICCD and CTLN2, respectively, (Saheki et al. 2004). The PCR-RFLP method to detect two mutations established was used for DNA diagnosis of the patients and/or the parents and for population analysis in control individuals. DNA diagnosis of the parents revealed that the NICCD patient (Tazawa et al. 2004) is a compound heterozygote with two different mutations: paternal [VIII] and maternal [X]. No extra mutation except [VIII] and [X] was found in PCR-products containing genomic DNA of this patient. However, no abnormal mRNA molecule from the allele with mutation [X] was detected by RT-PCR and sequencing analysis. As shown in Table 1, the mutation [X] was found in two NICCD patients from Taiwan (Hwu et al. and Lee et al. unpublished). Western blot analysis with anti-citrin antibody revealed that citrin protein was not detected in the cultured fibroblast cells from the Chinese NICCD patient who is a compound heterozygote with maternal [I] and paternal [X] mutations. These results

Table 1 Frequency and distribution of 12 *SLC25A13* mutations in East Asians

		Patients[a]		Controls		
		CTLN2 J (C)	NICCD J (C) (V)	Japanese[b]	Korean	Chinese
Tested patients		146 (3)	147 (4) (2)			
Numbers of mutated alleles detected						
[I]	851–854del	94 (4)	86 (5) (4)	4 (20%)	11 (50%)	45 (70%)
[II]	IVS11 + 1G > A	109	113	9 (45%)	8 (36%)	0
[III]	1638–1660dup	6 (2)	15	1 (5%)	1 (5%)	3 (5%)
[IV]	S225X	23	11	5 (25%)	0	0
[V]	IVS13 + 1G > A	16	21	1 (5%)	0	0
[VI]	1800–1801insA	3	7	0	0	0
[VII]	R605X	4	2	0	2 (9%)	0
[VIII]	E601X	2	5	0	0	0
[IX]	E601K	1	1	0	0	0
[X]	IVS6 + 5G > A	0	1 (2)	0	0	15 (23%)
[XI]	R184X	1	0	0	0	1 (2%)
[XIV]	IVS6 + 1G > C	2	1	0	0	0
Others[c]		7	19			
Unknown		24	12 (1)			
Tested control individuals				1,372	2,455	4,169
Carriers detected				20	22	64
Rate of carriers				1/69	1/112	1/65
Frequency of homozygotes[d]				1/19,000	1/50,000	1/17,000

[a]Patients (*J* Japanese, *C* Chinese, and *V* Vietnamese) diagnosed genetically at Kagoshima University to date (January 2005): 149 CTLN2 patients diagnosed by biochemical and enzymatic studies and 153 NICCD patients with at least one mutated *SLC25A13* gene

[b]Data reported by Saheki and Kobayashi (2002)

[c]Others involve five novel mutations identified in CTLN2 and NICCD patients (Tabata et al. in preparation; Takaya et al. submitted)

[d]Values were calculated from each carrier rate

suggest that the transcripts from mutant [X] allele may be unstable or easily degraded.

The mutation [XI] in exon 6 changes an arginine to a stop codon at position 184 and results in truncation at the N-terminal region (near fourth EF-hand) of citrin protein (Fig. 1). During DNA diagnosis for the mutation [XI] using the PCR-RFLP method, a novel mutation IVS6 + 1G > C [XIV] was detected in a CTLN2 patient (Iwasaki et al. unpublished). Sequencing analysis of the PCR products (467 bp) containing exon 6 of genomic DNA from this patient using primer sets of IVS5NF and IVS6NB revealed that the mutation [XIV] represents a G–C substitution at the 5′-end in intron 6. By RT-PCR and sequencing analyses, we found that mutation [XIV] results in abnormal splicing and inserts a whole sequence of intron 6 (1,789 bases) in mRNA (data not shown). This adds six new amino acids and introduces a stop codon at position 212, leading to premature truncation of the protein (Fig. 1). To date, mutation [XI] has only been found in an allele of a CTLN2 patient (Saheki et al. 2004), and mutation [XIV] found homozygously in the CTLN2 patient was detected in an allele of a NICCD patient (Table 1).

Frequency of 12 mutations in the patients with citrin deficiency

To date (January 2005), we have analyzed the *SLC25A13* gene of 146 Japanese CTLN2 patients who had been diagnosed by clinical, biochemical, and enzymatic studies. The mutations were found in two alleles of 128 cases and one allele of 12 cases. In the case of NICCD, 135 patients with known mutations in two alleles and 12 patients with known mutations in one allele are listed in Table 1: the NICCD shows various and transient symptoms, and the criteria of clinical and biochemical diagnosis are in the process of being established. As shown in Table 1, mutations [I] and [II] are found most frequently in the tested Japanese alleles of CTLN2 (70%) and NICCD (68%), respectively. Furthermore, we have diagnosed three CTLN2 Chinese with mutation [I] and/or [III], four NICCD Chinese with [I] and/or [X], and two NICCD Vietnamese with mutation [I] homozygously (Table 1).

Population analysis of 12 mutations

We have calculated that the frequency of homozygotes (including compound heterozygotes) with *SLC25A13* mutations in Japan is 1 in 19,000 as a minimal estimate from the rate of carriers, 1 in 69 (Saheki and Kobayashi 2002). In preliminary population analysis for the known 11 mutations, we have found that carriers (heterozygotes with one mutated gene out of known *SLC25A13* mutations in an allele) exist in China, Taiwan, and Korea at similar frequencies to Japan (Kobayashi et al. 2003; Saheki et al. 2004). In the present study, we screened 12 mutations in the increased number of control individuals and areas in East Asia by using established DNA diagnosis methods with PCR-RFLP and/or GeneScan/SNaPshot (see Materials and Methods). As shown in Table 1, 1,372 Japanese, 2,455 Koreans, and 4,169 Chinese including Taiwanese were tested, and 20, 22, and 64 carriers were detected, respectively. The mutations detected in the carriers were [I] (20%), [II] (45%), [III] (5%), [IV] (25%), and [V] (5%) in Japanese; [I] (50%), [II] (36%), [III] (5%), and [VII] (9%) in Korean; and [I] (70%), [III] (5%), [X] (23%), and [XI] (2%) in Chinese. In the Oriental populations, mutations [I] and [III] were found in all three national groups, suggesting that the distribution of these mutations may be due to the founder effect and genetic drift. Mutations [II] in Japanese and Korean, [IV] in Japanese, [VII] in Korean, and [X] in Chinese may have arisen after racial divergence. However, since all 12 mutations screened were identified in Japanese patients with citrin deficiency, it is necessary to conduct more detailed analyses of the other Asian populations to specify the origin of each mutation.

The frequencies of homozygotes, calculated to be 1 in 50,000 (Korean) and 1 in 17,000 (Chinese), are almost the same as in Japanese (1 in 19,000), suggesting that many CTLN2 and NICCD patients exist in East Asia. Considering the total population of East Asia (about 1.5×10^9), more than 80,000 people may be homozygotes with *SLC25A13* mutations. Further detailed search is needed in other Asian populations because two Vietnamese NICCD patients were detected in Australia and USA (Table 1).

Distribution of Chinese carriers with mutated *SLC25A13* gene and comparison of the carrier frequency among three areas in China

By cluster analysis of immunoglobulin Gm allotype, Zhao and Lee (1989) assumed that the modern Chinese derive from two distinct populations: one originating in the Yellow River valley and the other originating in the Yangtze River valley during early Neolithic times (3,000–7,000 years ago), and that the most likely boundary between northern and southern Chinese was drawn at a latitude of 30°N (see Fig. 2). In the present study, we screened 4,169 Chinese controls from two cities (Beijing and Shanghai), eight provinces (Liaoning, Hebei, Shandong, Henan, Hubei, Hunan, Guangxi, and Guangdong), the Hong Kong area, and Taiwan. Since the Yangtze River is considered a historically significant border, as mentioned above, the distribution of four mutations was investigated north, south, and on the border. Liaoning, Beijing, Hebei, Shandong, and Henan are north of the Yangtze River; Hunan, Guangxi, Guangdong, and Hong Kong are south; and Shanghai and Hubei are intermediate (Fig. 2). Taiwan was included in the south because most Taiwanese came from southern China in early times.

As shown in Table 2, only one carrier with mutation [I] was detected in Beijing; no other carrier was found in the northern area. In the intermediate area near the

Fig. 2 Map of East Asia, areas used in population analysis, and location of the Yangtze River. As shown in Table 2, the cities, provinces, and areas in China, including Taiwan, were separated by the Yangtze River into three parts (north: Liaoning, Beijing, Hebei, Shandong and Henan; border: Shanghai and Hubei; and south: Hunan, Guangxi, Guangdong, Hong Kong and Taiwan). *Close circles* represent the areas where carriers were detected, *open circles* show areas where no carrier was found, and a *dotted line* indicates the latitude 30°N

Table 2 Distribution of four *SLC25A13* mutations detected in Chinese populations, and comparison of carrier frequencies among the areas

	Mutations				Control individuals		Rates of carriers
	I	III	X	XI	Carriers	Tested	
Total	45	3	15	1	64	4,169	1/65
North	1				1	940	1/940
Liaoning	0	0	0	0	0	54	0/54
Beijing	1	0	0	0	1	208	1/208
Hebei	0	0	0	0	0	201	0/201
Shandong	0	0	0	0	0	302	0/302
Henan	0	0	0	0	0	175	0/175
Border	2				2	296	1/148[a]
Shanghai	0	0	0	0	0	96	0/96
Hubei	2	0	0	0	2	200	1/100
South	42	3	15	1	61	2,933	1/48[b,c]
Hunan	7	0	2	1	10	401	1/40
Guangxi	2	0	0	0	2	102	1/51
Guangdong	12	1	3	0	16	638	1/40
Hong Kong	7	1	2	0	10	478	1/48
Taiwan	14	1	8	0	23	1,314	1/57

Mutations [I], [III], [X], and [XI] are 851–854del, 1638–1660dup, IVS6 + 5G > A, and R184X, respectively. Chinese control individuals were divided in three areas (north, border, and south) of the Yangtze River in China (see Fig. 2), and carrier rates were compared

[a] $\chi^2 = 1.1$ ($P = 0.29$) with north
[b] $\chi^2 = 16.4$ ($P < 0.0001$) with north
[c] $\chi^2 = 2.1$ ($P = 0.15$) with border

Yangtze River, two carriers with mutation [I] were found. In contrast, 61 carriers were detected in the southern area. Forty-two carriers with mutation [I] were found in all five areas, three carriers with mutation [III] in three areas, 15 carriers with mutation [X] in four areas, and a carrier with mutation [XI] in Hunan. These results show a clear regional difference in carrier frequency and *SLC25A13* mutation pattern between areas north and south of the Yangtze River in China: the rate of carriers was high in the south (1/48) and low in the north (1/940). The frequency of homozygotes with mutated *SLC25A13* gene was calculated to be 1/9,200 in the south and 1/3,500,000 in the north, suggesting that many patients with citrin deficiency may exist south of the Yangtze River.

The origin of mutation [I] or [II] alleles in East Asia postulated from haplotype analysis of *D7S1812*

Mutation [I] was frequently found in southern China (1/70) but quite rare in northern China (1/940) while it was more frequent in Korea (1/223) and Japan (1/343), as shown in Tables 1 and 2. On the other hand, mutation [II] was frequently found in Japanese and Koreans but none of Chinese tested (Table 1). During homozygosity mapping analysis using Japanese CTLN2 patients, we found that mutations [I] and [II] were associated with '290-bp' *D7S1812* allele (290-haplotype) and '281-bp' *D7S1812* allele (281-haplotype), respectively (Kobayashi et al. 1999). In the present study, to define the origin and/or migration of mutation [I] or [II] alleles, we per-

Table 3 Haplotyping of a microsatellite marker *D7S1812*[a]

	Controls		Carriers with mutation [I]		
	Japanese	Chinese	Japanese	Chinese	Korean
Tested individuals	116	88	85	38	9
Haplotype					
272/287		1 (1.1)			
272/290		1 (1.1)		1 (2.6)	
275/281		1 (1.1)			
275/287	2 (1.7)	2 (2.3)			
275/290	1 (0.9)	2 (2.3)			
281	11 (9.5)	13 (14.8)			
281/284	9 (7.8)	1 (1.1)		1 (2.6)	1 (11.1)
281/287	13 (11.2)	6 (6.8)			
281/290	32 (27.6)	26 (29.6)	26 (30.6)	17 (44.8)	5 (55.6)
281/293	3 (2.6)	1 (1.1)			
284		4 (4.6)			
284/290	4 (3.4)	3 (3.4)	7 (8.2)	3 (7.9)	
284/296				1 (2.6)	
287	3 (2.6)	2 (2.3)			
287/290	12 (10.3)	4 (4.6)	9 (10.6)	3 (7.9)	
290	24 (20.7)	20 (22.7)	40 (47.1)	12 (31.6)	3 (33.3)
290/293	2 (1.7)		3 (3.5)		
293		1 (1.1)			
Allele no.					
Tested	232	176	170	76	18
290-haplotype	99 (42.7%)	76 (43.2%)	125 (73.5%)[b]	48 (63.2%)[c]	11 (61.1%)

[a]Haplotype of microsatellite marker *D7S1812* were analyzed by using GeneScan method in control individuals from Japan (Kobayashi et al. 1999) and China-Hunan, and carriers with mutation [I] from Japanese, Chinese, and Koreans. Frequency of 290-haplotype was compared

[b]$\chi^2 = 36.6$ ($P < 0.0001$) with Japanese controls
[c]$\chi^2 = 7.7$ ($P = 0.0055$) with Chinese controls

formed haplotype analysis of *D7S1812* in intron 15 of the *SLC25A13* gene in controls and carriers. As shown in Table 3, the rate of 290-haplotype in the carriers with mutation [I] was significantly higher in Japanese (73.5%, $P < 0.0001$) and Chinese (63.2%, $P = 0.0055$) than in control Japanese (42.7%) and Chinese in Hunan (43.2%), respectively. We also found homozygotes of 290-haplotype in many homozygotes with mutation [I] in Japanese (24 of 24), Chinese (two of three), and Vietnamese (two of two). On the other hand, 281-haplotype was homozygously detected in all eight Japanese homozygotes with mutation [II] tested. The rate of 281-haplotype in the carriers with mutation [II] was significantly high in Japanese (61.8%, $P < 0.0001$) and Koreans (83.3%, $P = 0.0016$) compared with the rate of 281-haplotype in Japanese controls (34.1%).

Studies of the peopling of East Asia can be classified into two major models: a southeast Asian origin followed by a northward migration (Chu et al. 1998; Su et al. 1999). and a multidirectional route through central Asia and southeast Asia (Karafet et al. 2001). Su et al. (1999) reported that the first entry of modern humans into the southern part of eastern Asia was about 60,000 years ago, followed by a northward migration coinciding with glaciers receding in that area. The Jomon people arrived in Japan more than 12,000 years ago, and the Yayoi people, originally from northeast Asia, started migrating to Japan from the Korean peninsula about 2,300 years ago bringing with them the rice paddy. The distribution pattern of the Y-chromosomal DNA variation have shown that modern Japanese populations have resulted from distinctive genetic contributions involving the ancient Jomon people and Yayoi immigrants from Korea or mainland China (Hammer and Horai 1995), and that the complex origin of the Koreans resulted from genetic contributions involving the northern Asian settlement and expansions mostly from southern to northern China (Jin et al. 2003). The studies on the genetic marker of the human immunoglobulin G have revealed the distribution and migration pattern of Mongoloids in East Asia (Matsumoto 1988). One of two subgroups, the northern Mongoloid population, might have originated in the Baikal area and then spread to north China, Korea, and Japan through subsequent migrations. The southern Mongoloid population seems to have originated around the Guangxi and Yunnan areas and spread mainly to south China and southeast Asia.

The distribution patterns of the *SLC25A13* mutations detected in the present study are partly compatible with the presumed migration pattern of the Mongoloids. Superimposing our data onto these observations, it is likely that mutation [I] associated with the 290-haplotype occurred in the southern Mongoloid population. The fact that the mutation [I] 290-hapotype is found relatively frequently in Japanese, Korean, southern Chinese, and Vietnamese but very rarely in northern Chinese suggests that the ancestors of the Jomonese came from

southeast Asia along the east coast to Korea and Japan about 30,000 years ago when the Korean peninsula and Japanese Archipelago were contiguous land masses and were then cut off from the Asian mainland when sea levels rose about 12,000 years ago (Normile 1999). On the other hand, it is reasonable to think that mutation [II] associated with the 281-haplotype occurred just before the divergence into Korean and Japanese: it may come from northern Mongolia via Manchuria (Matsumoto 1988; Normile 1999; Jin et al. 2003). However, it is necessary to analyze the frequency of the mutation [II] 281-haplotype in Manchurians, Mongolians, and the people living in southeastern Siberia.

Frequency and incidence of homozygotes with mutated *SLC25A13* gene in two alleles

Considering the frequencies of homozygotes calculated from carrier rate, it is predicted that there are many patients with citrin deficiency (homozygotes or compound heterozygotes) in East Asia: approximate predictions are 80 in Beijing (1.4×10^7 and 1/173,000), 1,500 in Hubei (6×10^7 and 1/40,000), 28,700 in four areas of the southern part (Hunan, Guangxi, Guangdong, and Hong Kong: total 2×10^8 and 1/6,400 to 1/10,000), and 1,690 in Taiwan (2.2×10^7 and 1/13,000). However, the number of patients with citrin deficiency that we have genetically diagnosed to date is very small: one CTLN2 in Beijing (Yang et al. 2003), two CTLN2 in Taiwan (Hwu et al. 2001), three NICCD in Taiwan, and one NICCD Chinese who lives in the USA (Table 1).

In Japan, 40% of NICCD patients are detected based on high galactose, methionine, and/or phenylalanine concentrations on newborn mass screening. Since almost all NICCD patients tested show increased citrulline levels in the blood at 1 month of age (Tamamori et al. 2004), tandem mass measurement of amino acids, including citrulline, helps to diagnose NICCD patients (Shigematsu et al. 2002). The frequency of homozygotes in Japan (1/19,000) calculated from the carrier rate (Saheki and Kobayashi 2002) is almost the same as the NICCD incidence (Shigematsu et al. 2002) but quite different from CTLN2 incidence (1/100,000 to 1/230,000) (Nagata et al. 1991; Kobayashi et al. 1993). We do not know now whether the penetrance of citrin deficiency is complete or incomplete. In one patient, the first CTLN2 episode occurred at 79 years of age (Yasuda et al. 2000). Furthermore, many CTLN2 patients, because of their presenting symptoms, have been incorrectly diagnosed as suffering from epilepsy, schizophrenia, or depression (Kobayashi and Saheki 2003). On the other hand, the homozygotes may be diagnosed as having other diseases, such as pancreatitis, hyperlipidemia, or hepatoma (Kobayashi et al. 2000; Kobayashi and Saheki 2003). Further investigations are needed (1) to search patients with citrin deficiency worldwide, especially in southern China; (2) to find whether non-Japanese homozygotes with *SLC25A13* mutations show any symptoms; and (3) to identify genetic and/or environmental factors that trigger severe CTLN2.

Many patients with citrin deficiency show a fondness for protein/lipid-rich foods, such as peanuts, soybeans, egg, cheese, milk, meat, and fish, and they dislike to eat carbohydrates and sweets (Kobayashi and Saheki 2003; Saheki et al. 2004). This peculiar fondness is noted from their early childhood (Hachisu et al. 2005). Infusions of large amounts of carbohydrate including glycerol may be harmful for patients with citrin deficiency, and a high carbohydrate diet may trigger CTLN2 (Imamura et al. 2003; Saheki et al. 2004; Yazaki et al. 2005). Discrepancy in CTLN2 incidence may be related to variations in food customs. We are now analyzing nutrient intake quantitatively in Japanese patients. It may be important to compare food intake (nutrients) of homozygotes between Japan and southern China.

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