or dihydropteridine reductase (DHPR) deficiency. Since 2008, Biopten® has been further approved to decrease serum phenylalanine values in HPA due to BH_4-responsive hyperphenylalaninemia (BH_4-responsive HPA).

Postmarketing surveillance of Biopten® for BH_4 deficiency has been underway in Japan for the last 16 years. In this study, we assessed the efficacy and safety of this agent in 19 patients with BH_4 deficiency in whom treatment was initiated before the age of 4 years.

2. Patients and methods

Among 19 HPA patients detected by neonatal PKU screening in Japan (Table 1), 17 were diagnosed with 6-pyruvoyl tetrahydropterin synthase (PTPS) deficiency based on pteridine analysis of urine or serum, whereas two were diagnosed with DHPR deficiency, based on Guthrie test results. All 19 patients were diagnosed with BH_4 deficiency and received treatment with BH_4, L-dopa, and 5-hydroxytryptophan (5-HTP), initiated before the age of 4 years, between 1982 and 2008.

This study was performed as a post-marketing surveillance study at 10 medical centers in Japan, between April 1992 and December 2008. During this period, the doctors in charge reported annually on the patients' heights, weights, serum phenylalanine concentrations, BH_4 dosages, concomitant medications, and provided information on drug effectiveness and safety in accordance with regulated survey slips. Similar information was retrospectively collected from clinical records available between 1982 and 1991.

Serum phenylalanine concentrations were determined using an automated amino acid analyzer (L-8800; Hitachi, Tokyo, Japan). Serum pteridine was measured by high-performance liquid chromatography (LC-10; Shimazu, Kyoto, Japan) after iodine oxidation. DHPR activity was measured using Guthrie card specimens, as described previously [4].

Table 1
Baseline characteristics.

Parameter	*n* (%)
Type	
PTPS deficiency	17 (89.5)
DHPR deficiency	2 (10.5)
Sex (M/F)	10/9 (52.6/47.4)
Hospitalization	
Outpatients	11 (57.9)
In ↔ out	8 (42.1)
Age at initiation of drug administration (years)	
0	16 (84.2)
1	2 (10.5)
2	0 (0.0)
3	1 (5.3)
Age at end of follow-up (years)	
0	1 (5.6)
1– < 4	4 (22.2)
4– < 10	2 (11.1)
10– < 16	1 (5.6)
⩾16	10 (55.6)
BH_4 dosage at start of therapy (mg/kg/day)	
<5	8 (42.1)
5– < 10	7 (36.8)
⩾10	4 (21.1)
BH_4 dosage at end of follow-up (mg/kg/day)	
<5	7 (36.8)
5– < 10	4 (21.0)
⩾10	8 (42.1)
L-dopa use during follow-up	
Yes	19 (100.0)
No	0 (0.0)
5-HTP use during follow-up	
Yes	19 (100.0)
No	0 (0.0)
Phenylalanine-restricted diet during follow-up	
Yes	12 (63.2)
No	6 (31.6)
Unknown	1 (5.3)
Blood phenylalanine level at neonatal mass screening (mg/dL)*	
Mean	14.2
Range	6.0–48.9

* Data available for 13 patients.

3. Results

Patients' background clinical characteristics are shown in Table 1. Seventeen patients were diagnosed with PTPS deficiency and two with DHPR deficiency; the mean age at end of follow-up was 14.6 years, and the mean age at initiation of BH_4 supplementation was 3.6 months. The mean duration of BH_4 therapy was 13.2 years; more than half the patients received BH_4 continuously for more than 10 years. The longest treatment duration was 28 years ($n = 1$). The mean daily dosage was 5 mg/kg; eight patients received less than 5 mg/kg and 11 patients received more than 10 mg/kg.

Changes in BH_4 dosage with age are shown in Fig. 1. The mean dosage in patients with PTPS deficiency increased over the initial few years of treatment, then remained stable thereafter. As of 2008, the average daily dose in 15 patients with PTPS deficiency was 7.9 mg/kg (Fig. 1). Of the two patients with DHPR deficiency, one was controlled by a stable dose, while the other required a high dose to control their serum phenylalanine level (Fig. 2).

Serum phenylalanine values in 13 of the 19 cases for whom data were available at the time of newborn screening are shown in Table 1. Changes in serum phenylalanine levels in patients with PTPS deficiency are shown in Fig. 2a. Serum phenylalanine was high (10 mg/dL) at the start of drug administration, but decreased to less than 2 mg/dL following Biopten® administration, with good phenylalaninemic control being maintained thereafter (Fig. 2a).

However, one patient with deficiency of DHPR, an enzyme responsible for BH_4 recycling, struggled to con-

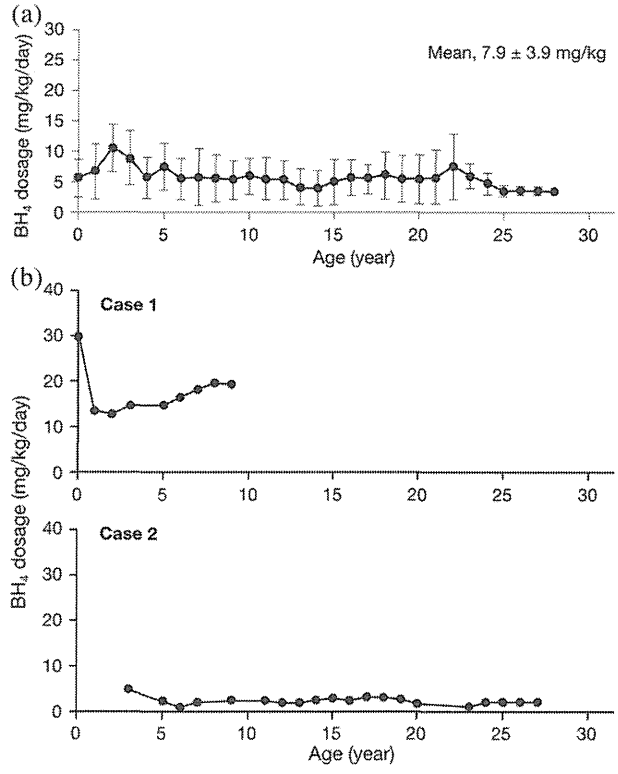

Fig. 1. Time courses of BH_4 dosage in patients with (a) PTPS deficiency ($n = 17$) and (b) DHPR deficiency (cases 1 and 2).

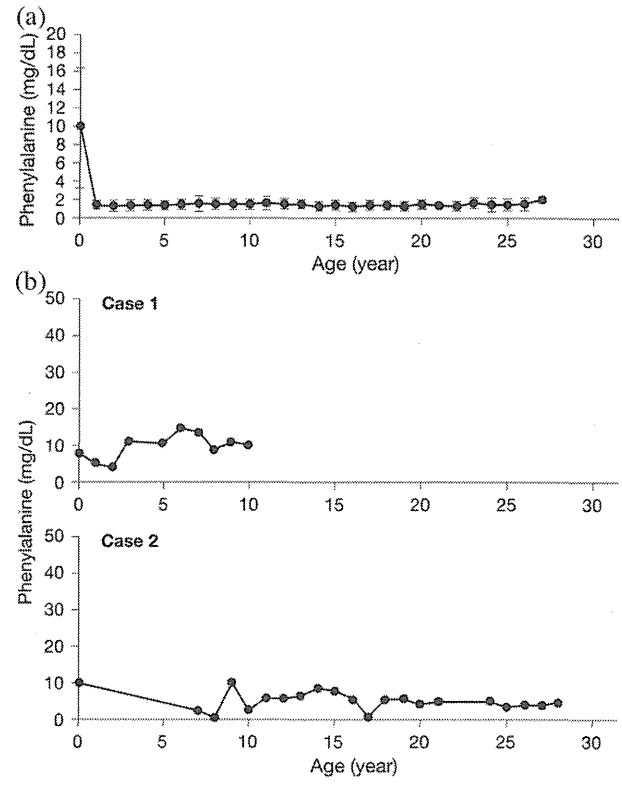

Fig. 2. Changes in serum phenylalanine levels in patients with (a) PTPS deficiency ($n = 17$) and (b) DHPR deficiency (cases 1 and 2).

trol serum phenylalanine levels despite the fact that the value at the start of treatment was slightly lower (9.1 mg/dL) than that in patients with PTPS deficiency. Serum phenylalanine levels fluctuated in this individual from the time of treatment initiation until the start-of-school age (Fig. 2b; case 1). However, another patient with DHPR deficiency exhibited stable long-term phenylalanine values (4.6 mg/dL at end of follow-up), within the age-specific reference range (Fig. 2b; case 2). The patients' heights and body weights by sex are shown in Fig. 3.

The mean height by age for male patients with PTPS deficiency ($n = 9$) was similar to that for the normal healthy population, but their body weight was lower by −1 standard deviation (SD), compared with healthy subjects (Fig. 3a). Female patients ($n = 8$), however, had greater mean height (+1 SD) and similar body weight to age-matched healthy females, except for one female patient with PTPS deficiency who had severe familial obesity (Fig. 3b). The two patients with DHPR deficiency (one male and one female) showed almost normal growth in terms of both height and body weight.

3.1. Combination therapy

Seventeen patients had evaluable data at the end of the survey period. L-dopa was used together with BH_4 in all patients, and 5-HTP was used in 13 patients. The doses of both L-dopa and 5-HTP tended to increase with age, probably consistent with normal weight gain, because the dosages/body weight remained roughly the same, at approximately 10 mg/kg per day of L-dopa (Fig. 4a) and 3–5 mg/kg per day of 5-HTP (Fig. 4b) throughout the study period. All patients with PTPS deficiency and one patient with DHPR deficiency showed good control of serum phenylalanine levels with BH_4 alone, with no need for restrictive diet therapy, indicating that BH_4 therapy could improve the patient's quality of life. However, one patient with DHPR deficiency received a phenylalanine-restricted diet in combination with BH_4 therapy, because BH_4 alone was unable to maintain serum phenylalanine levels within the normal reference range.

3.2. Safety and efficacy

No patients failed to respond to BH_4 therapy. One patient with PTPS deficiency who started BH_4 therapy soon after birth died at the age of 3 years, while not receiving BH_4 therapy. BH_4 therapy was considered to be effective in decreasing serum phenylalanine levels in all the remaining 18 patients with BH_4 deficiency.

Nervous system disorders were reported in two patients (10.5%); one patient experienced seizure and

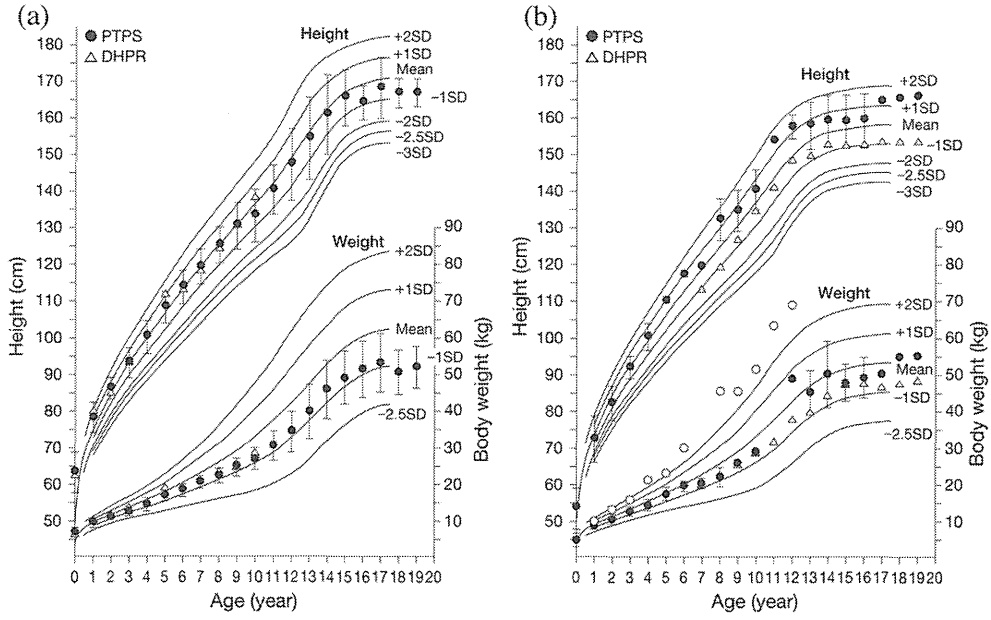

Fig. 3. Time courses of height and body weight changes in (a) male and (b) female patients. Although no undue abnormalities in height or body weight were noted in either sex, one female patient with PTPS deficiency had severe familial obesity (see text). ●, PTPS deficiency; △, DHPR deficiency; ○, outlier ($n = 1$).

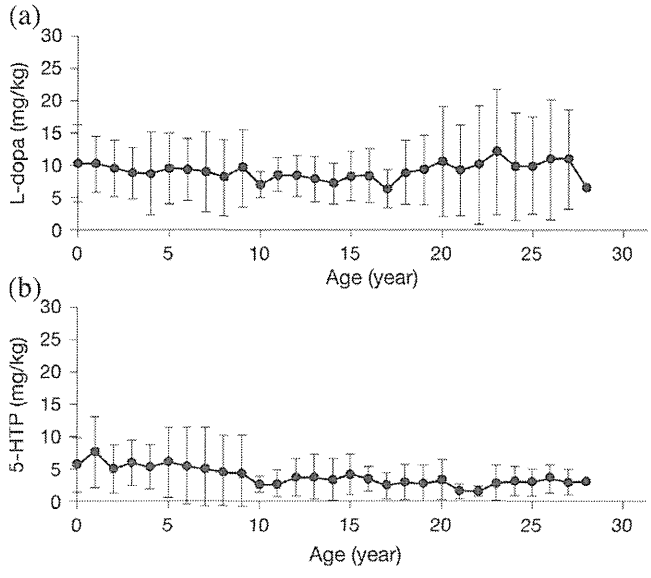

Fig. 4. Time courses of mean daily dosage of (a) L-dopa and (b) 5-hydroxytryptophan (5-HTP).

one developed increased muscle tone. These symptoms were deemed to have resulted from insufficient L-dopa doses, rather than being caused by BH_4. No other side effects were reported and no patient discontinued treatment because of side effects.

4. Discussion

BH_4 deficiency was first reported first by Smith et al. [5], while DHPR deficiency was the cause of BH_4 deficiency was recognized by Kaufman et al. [6]. Danks et al. [7] found no effect of oral BH_4 administration on DHPR deficiency, because of poor absorption from the intestinal tract. However, Schaub et al. [8] reported that oral administration of BH_4 was effective in a patient with DHBS deficiency, after which oral BH_4 was used to treat BH_4 deficiency. The form of BH_4 used initially was a mixture of the 6R and 6S isomers, because 6S form of BH_4 was inactive, but in 1982, Suntory Limited (Japan) succeeded in synthesizing 6R BH_4, after which 100% 6R BH_4, as Biopten® (sapropterin dihydrochloride), has been used in Japan. The first patient who was given Biopten® at that time now represents the longest-term usage of 28 years. Biopten® was approved for BH_4 deficiency in 1992, and over half the patients since then have been continuously administered BH_4 for more than 10 years. All 19 patients in this study also received treatment with L-dopa and 5-HTP, combined with BH_4, from before they were 4 years old, between 1982 and 2008. And, this study summarizes those patients, which represents a unique, long-term follow-up of a patient with BH_4 deficiency.

Control of serum phenylalanine levels by BH_4 appears to be easy in patients with PTPS deficiency [6], but more difficult in patients with DHPR deficiency, although levels can be still maintained within the range of age-specific reference values. Unlike the situation in PTPS deficiency, BH_4 cannot be recycled in DHPR deficiency. However, the sex-specific heights and body weights of patients with both PTPS and DHPR deficiencies were almost normal, or within 1SD. BH_4 administration in patients with BH_4 deficiency is intended to strictly limit serum phenylalanine

levels, though a phenylalanine-restricted diet, in combination with BH_4 therapy, may be necessary if BH_4 alone is unable to maintain levels within the normal range. The mental development of patients with BH_4 deficiency depends not only on the control of serum phenylalanine levels, but also on the treatment of neurotransmitter deficiencies, and combination therapy with a phenylalanine-restricted diet/BH_4, as well as supplementation with the neurotransmitter precursors, L-dopa and 5-HTP, is required. Because BH_4 does not pass the blood–brain barrier, L-dopa and 5-HTP are essential for the treatment of the central nervous system, and were used together with BH_4 in all patients. Therefore, although one patient started BH_4 administration after 3 years old, she developed normally because L-dopa and 5-HTP therapy with a phenylalanine-restricted diet had started already during infancy. The doses of both L-dopa and 5-HTP tended to increase with age, which was considered to be associated with weight gain. 5-HTP has not been approved as a drug in Japan, and although the 5-HTP sold as a supplement was used once, patients now buy 5-HTP as a supplement from overseas via the internet, because this represents a much cheaper option.

Nervous system side effects were reported in two patients (seizure and increased muscle tone, respectively), with an incidence of 10.5% (2/19). These symptoms were deemed to have occurred as a result of insufficient L-dopa, and were thought to be unlikely to be associated with BH_4. No other particular side effects were noted and no patients discontinued treatment because of side effects, suggesting that this drug showed a high degree of safety.

Women with PKU can have healthy children, as long as they maintain strict adherence to a low-phenylalanine diet throughout their pregnancy. One patient treated with sapropterin dihydrochloride for 28 years continued to take BH_4 during pregnancy to achieve adequate control of her serum phenylalanine. Apart from discontinuing BH_4 for a few days because of morning sickness, she recovered and resumed BH_4 therapy. She delivered a baby in October 2008, representing the first successful pregnancy of a patient while taking BH_4 in Japan. Both mother and baby were healthy, and the baby was confirmed as normal. The same patient is pregnant again, and continues to take BH_4 without morning sickness.

Sapropterin was originally found in Japan, and was approved for BH_4-responsive HPA in the US in 2007, and after a year EU approved for both BH_4-deficiency and BH_4-responsive HPA in 2008 (bland name in US and EU is Kuvan®). Then, more than 1000 patients of PKU were continuously administered sapropterin in each US and EU, and currently about 40 patients were taking in Japan as well. However, clinical trials were performed in patients older than 4 years in US and EU. The treatment of BH_4 deficiency requires lifelong administration of sapropterin, therefore it necessitates a high degree of safety. This was confirmed by the results of this survey.

5. Conclusions

BH_4 therapy is effective in controlling serum phenylalanine levels within the normal range in patients with BH_4 deficiency, with excellent long-term safety and no unwarranted side effects. Nineteen patients in Japan with BH_4 deficiency treated with BH_4 from before the age of 4 years have been followed for up to 28 years, representing a unique long-term follow-up of individuals with BH_4 deficiency. BH_4 deficiency requires lifelong drug therapy, and a high degree of drug safety is therefore required. The results of our survey suggest that BH_4 therapy is associated with a high level of safety in the setting of early treatment.

References

[1] Blau N, Thöny B, Cotton RGH, Hyland K. Disorders of tetrahydrobiopterin and related biogenic amines. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Kinzier KW, Vogelstein B, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. p. 1725–76.
[2] Shintaku H. Disorders of tetrahydrobiopterin metabolism and their treatment. Curr Drug Metab 2002;3:123–31.
[3] Shintaku H, Ichinose H. Hyperphenylalaninemia. In: Lang F, editor. Encyclopedia of molecular mechanisms of disease. New York: Springer; 2009. p. 923–5.
[4] Shintaku H, Kure S, Ohura T, Okano Y, Ohwada M, Sugiyama N, et al. Long-term treatment and diagnosis of tetrahydrobiopterin-responsive hyperphenylalaninemia with a mutant phenylalanine hydroxylase gene. Pediatr Res 2004;55:425–30.
[5] Smith I, Clayton BE, Wolff OH. New variant of phenylketonuria with progressive neurological illness unresponsive to phenylalanine restriction. Lancet 1975;1:1108–11.
[6] Kaufman S, Holtzman NA, Milstien S, Butler IJ, Krumholz A. Phenylketonuria due to a deficiency of dihydropteridine reductase. N Engl J Med 1975;293:785–90.
[7] Danks DM, Cotton RG, Schlesinger P. Letter: tetrahydrobiopterin treatment of variant form of phenylketonuria. Lancet 1975;2:1043.
[8] Schaub J, Däumling S, Curtius HC, Niederwieser A, Bartholome K, Viscontini M, et al. Tetrahydrobiopterin therapy of atypical phenylketonuria due to defective dihydrobiopterin biosynthesis. Arch Dis Child 1978;53:674–6.

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Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans

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Neural tube defects (NTDs), including spina bifida and anencephaly, are common birth defects of the central nervous system. The complex multigenic causation of human NTDs, together with the large number of possible candidate genes, has hampered efforts to delineate their molecular basis. Function of folate one-carbon metabolism (FOCM) has been implicated as a key determinant of susceptibility to NTDs. The glycine cleavage system (GCS) is a multi-enzyme component of mitochondrial folate metabolism, and GCS-encoding genes therefore represent candidates for involvement in NTDs. To investigate this possibility, we sequenced the coding regions of the GCS genes: *AMT*, *GCSH* and *GLDC* in NTD patients and controls. Two unique non-synonymous changes were identified in the *AMT* gene that were absent from controls. We also identified a splice acceptor site mutation and five different non-synonymous variants in *GLDC*, which were found to significantly impair enzymatic activity and represent putative causative mutations. In order to functionally test the requirement for GCS activity in neural tube closure, we generated mice that lack GCS activity, through mutation of *AMT*. Homozygous $Amt^{-/-}$ mice developed NTDs at high frequency. Although these NTDs were not preventable by supplemental folic acid, there was a partial rescue by methionine. Overall, our findings suggest that loss-of-function mutations in GCS genes predispose to NTDs in mice and humans. These data highlight the importance of adequate function of mitochondrial folate metabolism in neural tube closure.

INTRODUCTION

Neural tube defects (NTDs), such as spina bifida and anencephaly, are severe birth defects that result from failure of closure of the neural folds during embryonic development (1). Although NTDs are among the commonest birth defects in humans, the causes are still not well understood. This is most likely due to their complex, multifactorial causation

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which is thought to involve contributions from both genetic and environmental factors (2–4). The potential complexity of NTD genetics is illustrated by the fact that more than 200 different genes give rise to NTDs when mutated in mice (5,6). Moreover, inheritance patterns in humans suggest a multigenic model in which an affected individual may carry two or more risk alleles, which by themselves may be insufficient to cause NTDs (2).

Folate one-carbon metabolism (FOCM) is strongly implicated as a determinant of susceptibility to NTDs since suboptimal maternal folate status and/or elevated homocysteine are established risk factors, whereas periconceptional maternal folic acid supplementation can reduce the occurrence and recurrence of NTDs (7,8). Nevertheless, the precise mechanism by which folate status influences NTD risk remains elusive (7,9). FOCM comprises a network of enzymatic reactions required for synthesis of purines and thymidylate for DNA synthesis, and methionine, which is required for methylation of biomolecules (Fig. 1A) (9). In addition to the cytosol, FOCM also operates in mitochondria, supplying extra one-carbon units to the cytosolic FOCM as formate (Fig. 1A) (10).

Genes that are functionally related to folate metabolism have been subjected to intensive genetic analysis in relation to NTD causation, principally through association studies (reviewed in 3,4,11). In the most extensively studied gene, *MTHFR*, the c.677C>T SNP is associated with NTDs in some, but not all, populations. However, other FOCM-related genes have largely shown non-significant or only mild associations. Given the apparently complex inheritance of the majority of human NTDs, many association studies have been hampered by limitations on sample size. Moreover, although positive associations have been noted for other genes including *DHFR*, *MTHFD1*, *MTRR* and *TYMS* (12,13), these have not been replicated in all populations, and additional studies are required. The hypothesis that genetically determined abnormalities of folate metabolism may contribute to NTD susceptibility is supported by the observation of defects of thymidylate biosynthesis in a proportion of primary cell lines derived from NTDs (14). However, these defects do not correspond with known polymorphisms in FOCM-related genes. Overall, it appears likely that genetic influences on folate metabolism remain to be identified in many NTDs.

A potential link between mitochondrial FOCM and NTDs was suggested by the finding of an association of increased NTD risk with an intronic polymorphism in *MTHFD1L* (15). Another component of mitochondrial FOCM, the glycine cleavage system (GCS), acts to break down glycine to donate one-carbon units to tetrahydrofolate (THF), generating 5,10-methylenetetrahydrofolate (methylene-THF; Fig. 1B) (16,17). The GCS consists of four enzyme components, each of which is required for the glycine cleavage reaction (18,19). The components—glycine dehydrogenase (decarboxylating) (GLDC; P-protein), aminomethlyltransferase (AMT; T-protein), glycine cleavage system protein H (GCSH; H-protein) and dihydrolipoamide dehydrogenase (DLD; L-protein)—are encoded by distinct genes: *GLDC*, *AMT*, *GCSH* and *DLD*, respectively. The functions of *GLDC*, *AMT* and *GCSH* are specific to the GCS, whereas *DLD* encodes a housekeeping enzyme. GCS components have been found to be abundantly expressed in the neuroepithelium during embryogenesis in the rat (20).

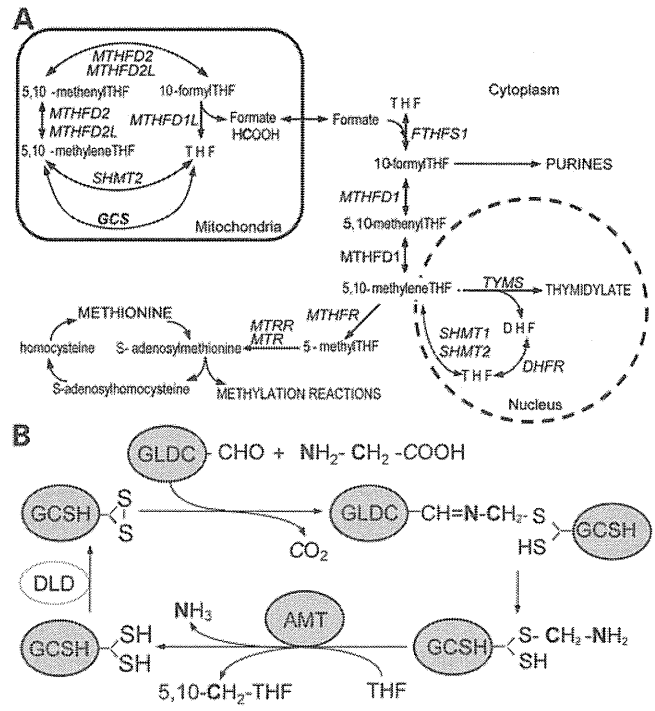

Figure 1. Schematic diagrams summarizing the key reactions of folate-mediated one-carbon metabolism and the GCS. (**A**) Folates donate and accept one-carbon units in the synthesis of purines, thymidylate and methionine. Mitochondrial FOCM supplies one-carbon units to the cytoplasm via formate. The GCS is a key component of mitochondrial FOCM that breaks down glycine and generates 5,10-methylene-THF from THF. Genes encoding enzymes for each reaction are indicated in italics. DHF, dihydrofolate; THF, tetrahydrofolate. (**B**) Summary of the GCS. The glycine cleavage reaction is catalysed by the sequential action of four individual enzymes: GLDC, GCSH, AMT and DLD. The first three of these (shaded grey) are specific to the GCS. Glycine is broken down into CO_2 and NH_3, and donates a one-carbon unit (indicated in bold) to THF, generating 5,10-methylene-THF. The other carbon in glycine (indicated in italics) enters CO_2.

We hypothesized that modulation of GCS activity has the potential to influence efficacy of cellular FOCM during the period of neural tube closure and, hence, susceptibility to NTDs. Therefore, in the current study, we screened genes encoding GCS components for possible mutations in NTD patients and controls. We tested variant proteins for loss of function by enzymatic assay and mice lacking GCS function were generated, to test the effect on embryonic development.

RESULTS

The hypothesis that genes of the GCS represent candidates for involvement in NTDs prompted us to screen for potential mutations in patient samples. Coding exons of *AMT* (9 exons), *GCSH* (5 exons) and *GLDC* (25 exons) were sequenced in a total of 258 NTD patients comprising cohorts from Japan, the UK and Sweden. Each of the major categories of NTDs was represented among study samples, including anencephaly ($n = 38$), spina bifida ($n = 198$) and craniorachischisis ($n = 22$).

Table 1. Nucleotide changes in NTD patients and controls identified by exon sequencing of AMT, GLDC and GCSH

Location	Nucleotide change	Effect	Number of mutation carriers in UK cohorts		Number of mutation carriers in the Japanese cohort		Number of mutation carriers in the Swedish cohort		Variant GLDC enzyme activity[a]
			NTD group (type[b]) ($n = 166$)[c]	Control group ($n = 189$)[c]	NTD group (type[b]) ($n = 14$)[c]	Control group ($n = 36$)[c]	NTD group (type[b]) ($n = 76$)[c]	Control group ($n = 145$)[c]	
AMT									
Exon 2	c.103A>C	p.R35R	0	1	0	0	0	—	
	c.214A>G	p.T72A	0	0	0	1	0	—	
Exon 6	c.623C>A	p.A208D	0	2	0	0	0	—	
	c.631G>A	p.E211K[d]	2 (SBA)	0	0	0	1	—	
	c.589G>C	p.D197H	0	0	1 (An)	0	0	—	
Exon 7	c.825T>A	p.N275K	0	1	0	0	0	—	
	c.850G>C	p.V284L	1 (SBA)	0	0	0	0	—	
GLDC									
Exon 1	c.52G>T	p.G18C	2 (SBO/SBA)	2	0	0	2 (SBA)	2	84%
Exon 5	c.668C>G	p.P223R	0	0	0	1	0	—	92%
Exon 12	c.1508A>C	p.E503A	1 (SBA)	0	0	0	0	0	—
	c.1512G>C	p.E504D	1 (SBA)	0	0	0	0	0	99%
	c.1519G>C	p.G507R	1 (An)	0	0	0	0	0	17%
	c.1525C>G	p.P509A[e]	1 (An)	0	0	0	0	0	41%
	c.1550G>C	p.S517T	0	0	0	0	1 (SBA)	0	—
	c.1570G>C	p.V524L	1 (SBA)	0	0	0	0	0	34%
Exon 14	c.1705G>A	p.A569T[f]	3 (SBA/SBO/SBO)	1	0	0	1 (SBA)	0	40%
Exon 17	c.1953T>C	p.H651H	0	1	0	0	0	—	—
Exon 19	c.2203G>T	p.V735L	0	2	0	0	0	—	81%
Intron 19	c.2316-1G>A	splice	1 (SBA)	0	0	0	0	—	
Exon 20	c.2380G>A	p.A794T	2 (SBASBA)	0	0	0	2 (SBA)	2	88%
	c.2406G>A	p.A802A	1 (An)	0	0	0	0	0	—
Exon 21	c.2474G>A	p.G825D	0	0	1 (An)	0	0	—	24%
	c.2487C>T	p.A829A	0	1	0	0	0	—	—
	c.2565A>C	p.A855A	1 (An)	0	0	0	0	—	—
Exon 23	c.2746C>T	p.L916L	1 (Crn)	0	0	0	0	—	—
Exon 25	c.2964G>A	p.R988R	0	0	0	0	1 (SBA)	0	—
	c.2965A>G	p.I989V	0	1	0	0	0	0	130%
GCSH									
Exon 1	c.53C>T	p.A18V	1 (An)	1	0	0	—	—	

All nucleotide changes were found in heterozygous form. One individual carried c.52G>T and c.1705G>A in *GLDC*, whereas no other individuals carried more than one of the nucleotide changes listed here. Eight silent polymorphisms and four missense variants present in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) are not listed in this table and include: *AMT*: c.954G>A (p.R318R, rs11715915); *GLDC*: c.249G>A (p.G83G, rs12341698), c.438G>A (p.T146T, rs13289273), c.501G>A (p.E167E, rs13289273), c.660C>T (p.L220L, rs2228095), c.666T>C (p.D222D, rs12004164), c.671G>A (p.R224H, rs28617412) and c.1384C>G (p.L462V, rs73400312); and for *GCSH*: c.62T>C (p.S21L, rs8052579), c.90C>G (p.P30P, rs8177847), c.159C>T (p.F53F, rs177876), c.218A>G (N73S, rs8177876), c.252T>C (Y84Y, rs8177907) and c.261C>G (L87L, rs8177908). Grey shading indicates loss-of-function mutations, based on enzymatic activity in the *in vitro* expression study or splicing defect.

[a]Residual enzymatic activity of GLDC mutant protein is expressed as %activity of the wild-type enzyme (Fig. 2).

[b]SBA, spina bifida aperta; SBO, spina bifida occulta; An, anencephaly; Crn, craniorachischisis.

[c]Total number of UK, Japanese or Swedish NTD patients.

[d]This variant was previously established as likely to be a non-functional polymorphism by segregation in an NKH family (21).

[e]A biochemical test of folate metabolism, the dU suppression test, was previously performed on primary fibroblasts derived from this patient and showed a defect of thymidylate biosynthesis to be present (14).

[f]p.A569T has previously been reported as a pathogenic mutation in a patient with typical NKH (21).

In *AMT*, we identified two novel sequence variants predicted to result in non-synonymous missense changes, c.589G>C (D197H) and c.850G>C (V284L), in anencephaly and spina bifida patients, respectively, from the UK cohort (Table 1). Neither variant was present in 526 UK or 36 Japanese control subjects or in the SNP databases dbSNP and 1000 Genomes. An additional missense variant, E211K, was also identified in three spina bifida patients, two from the UK and one from Sweden. Causative mutations in *AMT* have been found previously in an autosomal recessive inborn error of metabolism, non-ketotic hyperglycaemia (NKH) (17). The E211K variant had previously been identified in an NKH family but was established as likely to be a non-functional polymorphism by segregation (21). Therefore, this variant is considered unlikely to be causally related to NTDs.

Exon sequencing of *GCSH* revealed eight single-base substitutions, one of which (c.53C>T, p.A18V) was a novel change found in both an NTD and a single control (Table 1). The others all corresponded to known SNPs, which did not suggest a role for *GCSH* in NTDs.

Next we turned our attention to *GLDC*, in which we identified 27 single-base substitutions (Table 1), including 11 silent nucleotide changes, 15 non-synonymous changes and a splicing acceptor variant of intron 19 (c.2316-1G>A). The

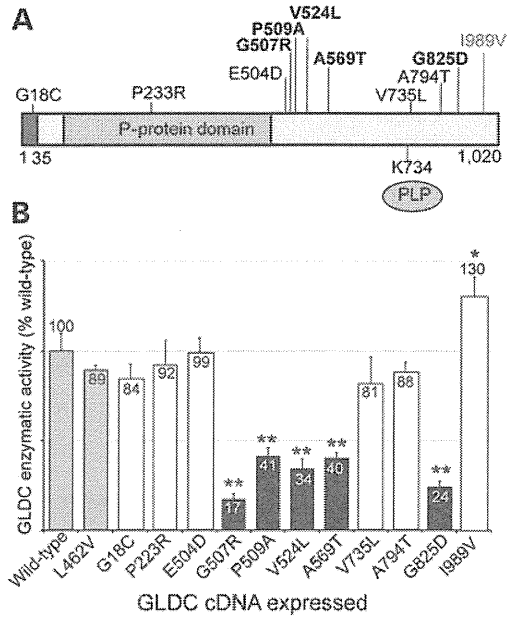

Figure 2. Characterization of *GLDC* missense mutations identified through DNA sequence analysis. (**A**) The schematic represents the 1020 amino acid residue GLDC polypeptide with the positions of the identified missense variants indicated. Mutations conferring significantly reduced activity (**B**) are indicated in bold. The leader peptide for mitochondrial import (shaded black) and the lysine 754-binding site for the co-factor pyridoxal phosphate (PLP) are indicated (49). (B) Enzymatic activity of GLDC missense variants. Expression vectors with wild-type and mutant *GLDC* cDNAs were transfected into COS7 cells for the evaluation of GLDC activity, which is expressed as relative activity (%) of cells expressing wild-type cDNA (shaded grey). The L462V GLDC enzyme (shaded grey) was tested as an example of a normally occurring variant (rs73400312). Variant proteins whose activities were significantly diminished compared with wild-type are indicated by black shading. The I989V variant, identified in a control parent, showed significantly elevated activity. Values are given as mean ± SD of triplicate experiments ($^{*}P < 0.05$; $^{**}P < 0.01$, compared with wild-type).

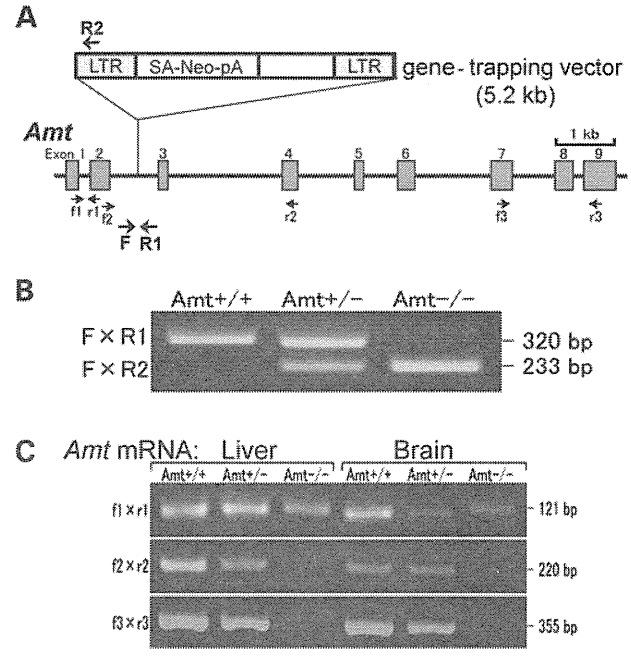

Figure 3. Generation of *Amt* knockout mouse by gene trapping. (**A**) The location of the gene-trap vector in *Amt* intron 2 in the ES cell line OST181110 was determined by inverse PCR. Mice carrying this mutation were generated using standard methods of blastocyst microinjection with OST181110 ES cells to generate chimeras, and germ-line transmission. LTR, long terminal repeats; SA, splicing acceptor site; Neo, neomycin phosphotransferase gene; pA, polyadenylation sequence. (**B**) For genotyping, mouse genomic DNA was subjected to allele-specific amplification with F, R1 and R2 primers (Supplementary Material, Table S1). A genomic fragment of 320 bp was amplified from the wild-type allele, whereas a 233 bp fragment was amplified from the *Amt*-mutant allele. (**C**) RT-PCR analysis of *Amt* mRNA expressed in the brain and liver of *Amt*-mutant mice. Primers in exon 1–2 generated a 121 bp band irrespective of mouse genotypes. RT-PCR in which either one (f2-r2) or both (f3-r3) primers were located in exons 3′ to the insertion site produced 220 and 355 bp cDNA fragments, respectively, in $Amt^{+/+}$ and $Amt^{+/-}$ mice, but not in $Amt^{-/-}$. The *Amt* mRNA in mice carrying the trap vector was, therefore, aberrantly spliced at the end of exon 2, resulting in truncation of *Amt* mRNA in $Amt^{-/-}$ mice.

latter is deduced to abolish normal splicing of the *GLDC* mRNA, with predicted skipping of exon 19 resulting in loss of the reading frame. Among the 15 missense variants identified in *GLDC*, 5 were unique to the NTD group, being absent from all 562 control individuals as well as from the SNP databases. A further three novel variants were found only in controls, whereas the remainder were found in both NTDs and controls, and included previously reported SNPs.

We investigated the possible functional effects of *GLDC* missense variants by expressing wild-type and mutant cDNA constructs in COS7 cells, followed by enzymatic assay of GLDC activity involving a decarboxylation reaction using [1-^{14}C]glycine (22). Twelve GLDC variants were tested, including those that were unique to NTD patients and, therefore, hypothesized to be potentially pathogenic (Fig. 2). The L462V variant, which corresponds to a known SNP (rs73400312), was included as an example of a known normally occurring form. Five of the missense changes, G507R, P509A, V524L, A569T and G825D, resulted in a significant reduction in GLDC activity compared with the wild-type protein ($P < 0.001$). Notably, all five of these deleterious variants were present solely in NTD cases, whereas none of the variants that were unique to controls (P223R, V735L and I989V) showed loss of enzymatic function. In the case of G18C and A794T, which occurred in both NTDs and controls, there was no significant loss of enzymatic activity, suggesting that these are unlikely to be causative mutations.

Having identified putative mutations in *AMT* and *GLDC* in NTD patients, we hypothesized that loss of GCS function could predispose to development of NTDs. In order to directly test the functional requirement for GCS activity in neural tube closure, we generated mice that lacked GCS activity, using a gene trap (OmniBank, OST181110) of the *Amt* gene. The vector was located in intron 2, resulting in a truncated transcript that lacked exons 3–9 (Fig. 3). The efficacy of the gene-trap vector in trapping expression of *Amt* (Amt^{-}) was confirmed by RT-PCR analysis (Fig. 3). Heterozygous $Amt^{+/-}$ mice were viable and fertile and exhibited no obvious malformations. Homozygous $Amt^{-/-}$ mice were not observed among post-natal litters from heterozygote intercrosses, and so fetuses were examined at embryonic day (E) 17.5. Strikingly, 87% of $Amt^{-/-}$ fetuses (34 out of 39) exhibited NTDs, whereas no malformations were observed in $Amt^{+/+}$ ($n = 33$) or $Amt^{+/-}$

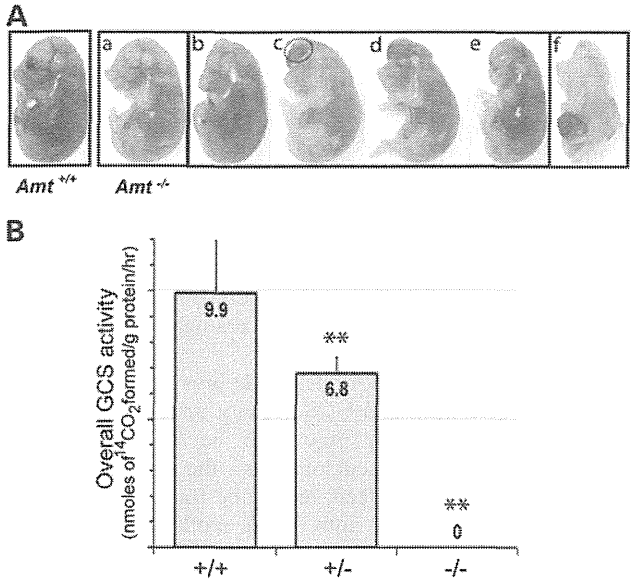

Figure 4. Mice lacking GCS activity exhibit NTDs. (**A**) Phenotypes of *Amt* mutant mice. NTDs were evident in the majority (88%) of $Amt^{-/-}$ fetuses (examples shown are at E17.5). Various types of NTDs were observed in $Amt^{-/-}$ fetuses, which principally affected the cranial region; a, no NTDs; b, small exencephaly (dotted circle); c–e, large exencephaly; f, craniorachischisis. (**B**) Enzymatic activity of the GCS in *Amt* knockout mice. $Amt^{+/-}$ and $Amt^{-/-}$ fetuses had significantly lower GCS activity in the liver than $Amt^{+/+}$ fetuses, with activity in $Amt^{-/-}$ samples below the level of detection ($^{**}P < 0.01$ compared with $Amt^{+/+}$).

($n = 66$) fetuses. Defects mainly comprised exencephaly (82%), in which the cranial neural folds persistently failed to close (Fig. 4). There was also a low frequency of the more severe condition, craniorachischisis (5%), in which the neural tube remains open from the mid- and hindbrain, and throughout the spinal region (Fig. 4). Fetal liver samples were subjected to enzyme assay to determine overall activity of the GCS. In $Amt^{-/-}$ mice, overall GCS activity was effectively ablated being below the detection level of the assay (0.01 nmoles of $^{14}CO_2$ formed/gram protein/h), consistent with the Amt^{-} allele being a functional null (22) (Fig. 4). These findings confirm that AMT function is essential for GCS activity, and that the latter is necessary for successful neural tube closure.

Given that GCS is a component of FOCM (Fig. 1), we evaluated the possible prevention of NTDs by folate-related metabolites. Maternal supplementation was performed with folic acid, thymidine monophosphate (TMP), methionine or methionine plus TMP (23). Neither folic acid nor TMP significantly affected the frequency of NTDs among the homozygous $Amt^{-/-}$ offspring. However, we observed a significant protective effect of maternal supplementation with methionine or methionine plus TMP, compared with the non-treated group ($P < 0.05$; Fig. 5).

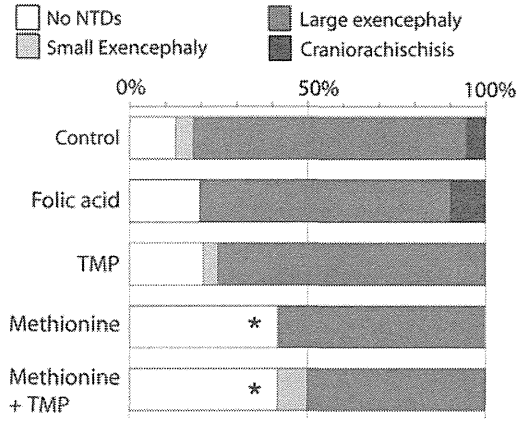

Figure 5. Maternal supplementation of *Amt* mutant embryos with folic acid, TMP or methionine. Maternal treatment with folic acid ($n = 10$ homozygous mutant fetuses) or TMP ($n = 12$) had no significant effect on NTD frequency, whereas the frequency of unaffected embryos was significantly increased following treatment with methionine ($n = 12$) or methionine plus TMP group ($n = 12$). The asterisk indicates significant difference compared with non-treated group ($P < 0.05$).

DISCUSSION

NTDs remain among the commonest human birth defects and understanding their genetic basis presents a considerable challenge owing to their multigenic inheritance and the potential influence of environmental factors, either predisposing or ameliorating. Several lines of evidence indicate a requirement for FOCM in neural tube closure and, therefore, GCS-encoding genes provide excellent candidates for possible involvement in NTD susceptibility. We identified putative mutations in *AMT* and *GLDC* which include a splice acceptor mutation and a number of non-synonymous variants that were absent from a large group of population-matched controls, as well as from public SNP databases. In the case of *GLDC*, enzymatic assay confirmed that several mutations resulted in significant loss of enzyme activity. Finally, *in vivo* functional evidence of a requirement for GCS function in neural tube closure was provided by the occurrence of NTDs in $Amt^{-/-}$ mice lacking GCS activity. Together these findings indicate that mutations in *GLDC* and *AMT* predispose to NTDs in both mice and humans.

Where parental samples were available (6 of the 11 NTD cases that involved putative mutations in *GLDC*), we demonstrated parent-to-child transmission (Supplementary Material, Table S2). Six were instances of maternal transmission and one involved paternal transmission. We hypothesize that absence of an overt NTD phenotype in parents who carry a deficient *GLDC* allele may result from incomplete penetrance, or lack of additional genetic or environmental factors which are predicted to be necessary for NTDs owing to their multifactorial aetiology. We also note that partial penetrance is a feature of numerous mouse models of NTDs (5,8).

Inherited GCS deficiency, owing to mutation of *AMT* and/or *GLDC*, has been shown to cause NKH in humans (17). NKH is a rare, autosomal recessive, inborn error of metabolism, characterized by accumulation of glycine and encephalopathy-like neurological signs, including coma and convulsive seizures in neonates. GCS activity is greatly diminished in NKH patients and they would, therefore, be predicted to be at increased risk of NTDs. It is possible that NTDs may occur in combination with NKH but as anencephaly is a lethal condition, co-existing

NKH would go undetected. Lack of NTDs in NKH patients may also reflect the multigenic nature of NTDs, which require the presence of additional risk alleles in non-GCS genes. NKH is a relatively rare condition, with a prevalence of 1/63 000 births in British Columbia (24) and 1/250 000 in the USA (25). It is therefore possible that an increased risk of NTDs among carriers of GCS mutations in NKH families may not have been noted and this possibility is worthy of investigation. Based on estimated carrier frequency and the incidence of mutations among NTD patients, we predict that NTDs might be expected among 1/150 of the siblings of NKH patients (see Supplementary Material, Table S3 for estimate calculation). One case report of an NKH patient with a *GLDC* mutation describes the additional presence of spinal cord hydromyelia (19). This condition is often associated with low spinal defects (involving secondary neurulation), but it is also possible that the expanded spinal canal was also present at a higher level and might indicate a limited defect in primary neurulation.

The mutations described in the current study were all present in heterozygous form and, therefore, are hypothesized to be insufficient to cause NKH while predisposing to NTDs. For example, in the current study we found four NTD patients and one control individual to be heterozygous for the A569T mutation, which is shown to result in reduced enzyme activity. This mutation was previously identified in a Caucasian patient with typical NKH, in combination with a second mutation, P765S (26), confirming that it is deleterious *in vivo*. Hence, we predict that, depending on the co-existing genetic milieu, the A569T variant may cause NKH, predispose to NTDs or be compatible with normal development.

The high incidence of NTDs in *AMT* mutant mice is particularly notable as NTDs have not previously been found to be a common feature of mouse models deficient for folate-metabolizing enzymes. This includes null mutants that have been reported for eight other genes that encode enzymes in FOCM (Fig. 1A) (27). Four have normal morphology at birth (*Cbs*, *Mthfd1*, *Mthfr* and *Shmt1*) (28–31), *Mthfd2* null embryos die by E15.5 but neural tube closure is complete (32) and null mutants for *Mtr*, *Mtrr* and *Mthfs* die before E9.5, prior to neural tube closure (33–35). Although analysis of mouse mutants has not supported a role for single-gene mutations in FOCM as major causes of NTDs, a requirement for cellular uptake of folate for neural tube closure has been demonstrated in *Folr1* null embryos, in which NTDs occur when rescued from early lethality by folic acid supplementation (36). There is also considerable evidence for possible involvement of gene–environment and/or gene–gene interactions in NTDs. For example, in *Pax3* mutant (*splotch*) embryos, which exhibit a defect of thymidylate biosynthesis, dietary folate-deficiency increases the frequency of cranial NTDs (23,37). Similarly, a diet deficient in folate and choline causes NTDs in *Shmt1* mutant embryos, whereas *Shmt1* and *Pax3* mutations exhibit genetic interaction (38).

Regarding the mechanisms by which GCS mutations affect neural tube closure, a key question is whether NTDs are caused by impairment of FOCM or by another cause such as glycine accumulation. Modelling of hepatic FOCM, based on biochemical properties of folate-metabolizing enzymes (39), predicts that loss of the mitochondrial GCS reaction would reduce the efflux rate of formate to the cytosol by ~50%. This results in reduced synthesis of purines and thymidylate, which are essential for the rapid cell division in the closing neural folds. Interestingly, a UK patient with anencephaly who was found to carry the *GLDC* loss-of-function mutation P509A in the current study (Table 1) was previously found to have impaired thymidylate biosynthesis, assayed in cultured fibroblasts (14). These findings support the hypothetical link between diminished GLDC function, reduced thymidylate biosynthesis and development of NTDs. Reduced thymidylate biosynthesis and diminished cellular proliferation are proposed to underlie folate-related cranial NTDs in *splotch* (*Pax3*) mouse mutants (37,38).

As well as impairment of nucleotide biosynthesis, the predicted effect of diminished GCS activity in reducing production of methionine (39) may also be of relevance as methionine is the precursor for the methyl donor *S*-adenosylmethionine. Indeed, metabolic tracing experiments suggest that ~80% of 1C units in the methylation cycle are generated within mitochondrial FOCM (40). Impairment of the methylation cycle and/or DNA methylation is known to cause NTDs in mice (41) and is proposed as a possible cause of human NTDs (7,42). It was therefore notable that we found a preventive effect of methionine supplementation in $Amt^{-/-}$ mice. Together, these findings suggest that FOCM, required for both thymidylate biosynthesis and methylation reactions that are essential for neural tube closure, may be functionally deficient in individuals who have mutations in *GLDC* or *AMT*.

MATERIALS AND METHODS

Patient cohorts and sequencing

Mutation analysis by DNA sequencing was performed on all exons of *AMT*, *GCSH* and *GLDC* as described (26). Cases comprised Japanese patients with anencephaly ($n = 14$) and two separate cohorts of UK patients with a diagnosis of anencephaly (combined $n = 24$), spina bifida ($n = 122$) or craniorachischisis ($n = 22$). In addition, the exons of *AMT*, *GCSH* and *GLDC* were sequenced in 76 Swedish patients with spina bifida. Unaffected controls, completely sequenced for these genes, comprised 36 Japanese and 189 unrelated UK subjects. Exons found to contain missense mutations were also sequenced in a further cohort of 192 well-characterized UK controls (43) and in 145 Swedish controls. This study was approved by the Ethical Committees of Tohoku University School of Medicine, UCL Institute of Child Health, Newcastle University and the Karolinska Institute.

Enzymatic assay of GCS activity and GLDC activity

GCS activity was measured in mouse liver samples by a decarboxylation reaction using [1-^{14}C]glycine as described (22). For analysis of GLDC activity, wild-type and mutant *GLDC* cDNAs were cloned into pCAG expression vector, kindly provided by Professor Jun-ichi Miyazaki (Osaka University, Japan) (44). Constructs were transfected into COS7 cells, which were harvested as described previously and cell pellets stored at −80°C prior to analysis (45). GLDC

enzymatic activity was determined, in triplicate, by exchange reaction between carbon dioxide and glycine using $NaH^{14}CO_3$ in the presence of excess recombinant bovine GCSH protein as described (22). An expression system of lipoylated bovine GCSH protein in *Escherichia coli* was kindly provided by Dr Kazuko Fujiwara (Tokushima University, Japan) (46). Statistical analysis was performed using SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA).

Knockout of Amt by insertion of a gene-trap vector

Mice carrying a gene-trap allele of *Amt* (here denoted Amt^{-}) were generated at Lexicon Genetics, Inc. (Houston, TX, USA) using the OST181110 ES cell line. The genomic insertion site of the gene-trap vector was determined by inverse PCR and localized to intron 2 (Supplementary Material, Fig. S1). Total RNA was prepared from the mouse liver and brain at E18 for RT-PCR analysis (Supplementary Material, Fig. S1 and Table S1). $Amt^{+/-}$ mice were backcrossed with wild-type C57BL/6 mice for nine generations to generate a congenic line of mice on the C57BL/6 background, for use in biochemical and histological analyses. This study was approved by the Animal Experiment Committee of Tohoku University.

Maternal supplementation with folic acid and related metabolites

Dams were treated with folic acid (25 mg/kg), thymidine-1-phosphate (TMP; 30 mg/kg) or L-methionine (70 mg/kg) by intra-peritoneal injection, 2 h prior to mating and daily from E7.5–10.5. Doses were based on previous studies (23,47,48).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

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REFERENCES

1. Greene, N.D. and Copp, A.J. (2009) Development of the vertebrate central nervous system: formation of the neural tube. *Prenatal Diag.*, **29**, 303–311.
2. Harris, M.J. and Juriloff, D.M. (2007) Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects. *Birth Defects Res. A Clin. Mol. Teratol.*, **79**, 187–210.
3. Greene, N.D.E., Stanier, P. and Copp, A.J. (2009) Genetics of human neural tube defects. *Hum. Mol. Genet.*, **18**, R113–R129.
4. Au, K.S., Ashley-Koch, A. and Northrup, H. (2010) Epidemiologic and genetic aspects of spina bifida and other neural tube defects. *Dev. Disabil. Res. Rev.*, **16**, 6–15.
5. Harris, M.J. and Juriloff, D.M. (2010) An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth Defects Res. A Clin. Mol. Teratol.*, **88**, 653–669.
6. Copp, A.J. and Greene, N.D.E. (2010) Genetics and development of neural tube defects. *J. Pathol.*, **220**, 217–230.
7. Blom, H.J., Shaw, G.M., Den Heijer, M. and Finnell, R.H. (2006) Neural tube defects and folate: case far from closed. *Nat. Rev. Neurosci.*, **7**, 724–731.
8. Molloy, A.M., Brody, L.C., Mills, J.L., Scott, J.M. and Kirke, P.N. (2009) The search for genetic polymorphisms in the homocysteine/folate pathway that contribute to the etiology of human neural tube defects. *Birth Defects Res. A Clin. Mol. Teratol.*, **85**, 285–294.
9. Beaudin, A.E. and Stover, P.J. (2009) Insights into metabolic mechanisms underlying folate-responsive neural tube defects: a mini review. *Birth Defects Res. A Clin. Mol. Teratol.*, **85**, 274–284.
10. Tibbetts, A.S. and Appling, D.R. (2010) Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annu. Rev. Nutr.*, **30**, 57–81.
11. Boyles, A.L., Hammock, P. and Speer, M.C. (2005) Candidate gene analysis in human neural tube defects. *Am. J. Med. Genet. C Semin. Med. Genet.*, **135**, 9–23.
12. Shaw, G.M., Lu, W., Zhu, H., Yang, W., Briggs, F.B., Carmichael, S.L., Barcellos, L.F., Lammer, E.J. and Finnell, R.H. (2009) 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. *BMC Med. Genet.*, **10**, 49.
13. Martinez, C.A., Northrup, H., Lin, J.I., Morrison, A.C., Fletcher, J.M., Tyerman, G.H. and Au, K.S. (2009) Genetic association study of putative functional single nucleotide polymorphisms of genes in folate metabolism and spina bifida. *Am. J. Obstet. Gynecol.*, **201**, 394–411.
14. Dunlevy, L.P.E., Chitty, L.S., Doudney, K., Burren, K.A., Stojilkovic-Mikic, T., Stanier, P., Scott, R., Copp, A.J. and Greene, N.D.E. (2007) Abnormal folate metabolism in foetuses affected by neural tube defects. *Brain*, **130**, 1043–1049.
15. Parle-McDermott, A., Pangilinan, F., O'Brien, K.K., Mills, J.L., Magee, A.M., Troendle, J., Sutton, M., Scott, J.M., Kirke, P.N., Molloy, A.M. and Brody, L.C. (2009) A common variant in MTHFD1L is associated with neural tube defects and mRNA splicing efficiency. *Hum. Mutat.*, **30**, 1650–1656.
16. Kikuchi, G. (1973) The glycine cleavage system: composition, reaction mechanism, and physiological significance. *Mol. Cell. Biochem.*, **1**, 169–187.
17. Kure, S., Tada, K. and Narisawa, K. (1997) Nonketotic hyperglycinemia: biochemical, molecular, and neurological aspects. *Jpn J. Hum. Genet*, **42**, 13–22.
18. Kure, S., Narisawa, K. and Tada, K. (1992) Enzymatic diagnosis of nonketotic hyperglycinemia with lymphoblasts. *J. Pediatr.*, **120**, 95–98.
19. Hayasaka, K., Tada, K., Kikuchi, G., Winter, S. and Nyhan, W.L. (1983) Nonketotic hyperglycinemia: two patients with primary defects of P-protein and T-protein, respectively, in the glycine cleavage system. *Pediatr. Res.*, **17**, 967–970.
20. Ichinohe, A., Kure, S., Mikawa, S., Ueki, T., Kojima, K., Fujiwara, K., Iinuma, K., Matsubara, Y. and Sato, K. (2004) Glycine cleavage system in neurogenic regions. *Eur. J. Neurosci.*, **19**, 2365–2370.
21. Toone, J.R., Applegarth, D.A., Kure, S., Coulter-Mackie, M.B., Sazegar, P., Kojima, K. and Ichinohe, A. (2002) Novel mutations in the P-protein (glycine decarboxylase) gene in patients with glycine encephalopathy (non-ketotic hyperglycinemia). *Mol. Genet. Metab.*, **76**, 243–249.
22. Sakata, Y., Owada, Y., Sato, K., Kojima, K., Hisanaga, K., Shinka, T., Suzuki, Y., Aoki, Y., Satoh, J., Kondo, H. *et al.* (2001) Structure and expression of the glycine cleavage system in rat central nervous system. *Brain Res. Mol. Brain Res.*, **94**, 119–130.
23. Fleming, A. and Copp, A.J. (1998) Embryonic folate metabolism and mouse neural tube defects. *Science*, **280**, 2107–2109.

24. Applegarth, D.A., Toone, J.R. and Lowry, R.B. (2000) Incidence of inborn errors of metabolism in British Columbia, 1969–1996. *Pediatrics*, **105**, e10.
25. Nyhan, W.L. (1989) Nonketotic hyperglycinemia. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic Basis of Inherited Disease*. McGraw-Hill, Inc., New York, 743–753.
26. Kure, S., Kato, K., Dinopoulos, A., Gail, C., DeGrauw, T.J., Christodoulou, J., Bzduch, V., Kalmanchey, R., Fekete, G., Trojovsky, A. *et al.* (2006) Comprehensive mutation analysis of GLDC, AMT, and GCSH in nonketotic hyperglycinemia. *Hum. Mutat.*, **27**, 343–352.
27. Harris, M.J. (2008) Insights into prevention of human neural tube defects by folic acid arising from consideration of mouse mutants. *Birth Defects Res. A Clin. Mol. Teratol.*, **85**, 331–339.
28. Watanabe, M., Osada, J., Aratani, Y., Kluckman, K., Reddick, R., Malinow, M.R. and Maeda, N. (1995) Mice deficient in cystathionine β-synthase: animal models for mild and severe homocyst(e)inemia. *Proc. Natl Acad. Sci. USA*, **92**, 1585–1589.
29. Champion, K.M., Cook, R.J., Tollaksen, S.L. and Giometti, C.S. (1994) Identification of a heritable deficiency of the folate-dependent enzyme 10-formyltetrahydrofolate dehydrogenase in mice. *Proc. Natl Acad. Sci. USA*, **91**, 11338–11342.
30. Chen, Z., Karaplis, A.C., Ackerman, S.L., Pogribny, I.P., Melnyk, S., Lussier-Cacan, S., Chen, M.F., Pai, A., John, S.W., Smith, R.S. *et al.* (2001) Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. *Hum. Mol. Genet.*, **10**, 433–443.
31. MacFarlane, A.J., Liu, X., Perry, C.A., Flodby, P., Allen, R.H., Stabler, S.P. and Stover, P.J. (2008) Cytoplasmic serine hydroxymethyltransferase regulates the metabolic partitioning of methylenetetrahydrofolate but is not essential in mice. *J. Biol. Chem.*, **283**, 25846–25853.
32. Di, P.E., Sirois, J., Tremblay, M.L. and Mackenzie, R.E. (2002) Mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase is essential for embryonic development. *Mol. Cell. Biol.*, **22**, 4158–4166.
33. Swanson, D.A., Liu, M.L., Baker, P.J., Garrett, L., Stitzel, M., Wu, J.M., Harris, M., Banerjee, R., Shane, B. and Brody, L.C. (2001) Targeted disruption of the methionine synthase gene in mice. *Mol. Cell. Biol.*, **21**, 1058–1065.
34. Elmore, C.L., Wu, X., Leclerc, D., Watson, E.D., Bottiglieri, T., Krupenko, N.I., Krupenko, S.A., Cross, J.C., Rozen, R., Gravel, R.A. and Matthews, R.G. (2007) Metabolic derangement of methionine and folate metabolism in mice deficient in methionine synthase reductase. *Mol. Genet. Metab.*, **91**, 85–97.
35. Field, M.S., Anderson, D.D. and Stover, P.J. Mthfs is an essential gene in mice and a component of the purinosome. *Front. Genet.* http://www.frontiersin.org/nutrigenomics/10.3389/fgene.2011.00036/abstract.
36. Spiegelstein, O., Mitchell, L.E., Merriweather, M.Y., Wicker, N.J., Zhang, Q., Lammer, E.J. and Finnell, R.H. (2004) Embryonic development of folate binding protein-1 (Folbp1) knockout mice: effects of the chemical form, dose, and timing of maternal folate supplementation. *Dev. Dyn.*, **231**, 221–231.
37. Burren, K.A., Savery, D., Massa, V., Kok, R.M., Scott, J.M., Blom, H.J., Copp, A.J. and Greene, N.D.E. (2008) Gene-environment interactions in the causation of neural tube defects: folate deficiency increases susceptibility conferred by loss of *Pax3* function. *Hum. Mol. Genet.*, **17**, 3675–3685.
38. Beaudin, A.E., Abarinov, E.V., Noden, D.M., Perry, C.A., Chu, S., Stabler, S.P., Allen, R.H. and Stover, P.J. (2011) Shmt1 and de novo thymidylate biosynthesis underlie folate-responsive neural tube defects in mice. *Am. J. Clin. Nutr.*, **93**, 789–798.
39. Nijhout, H.F., Reed, M.C., Lam, S.L., Shane, B., Gregory, J.F. III and Ulrich, C.M. (2006) In silico experimentation with a model of hepatic mitochondrial folate metabolism. *Theor. Biol. Med. Model.*, **3**, 40.
40. Pike, S.T., Rajendra, R., Artzt, K. and Appling, D.R. (2010) Mitochondrial C1-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitochondrial one-carbon units into the methyl cycle in embryos. *J. Biol. Chem.*, **285**, 4612–4620.
41. Dunlevy, L.P.E., Burren, K.A., Mills, K., Chitty, L.S., Copp, A.J. and Greene, N.D.E. (2006) Integrity of the methylation cycle is essential for mammalian neural tube closure. *Birth Defects Res. A*, **76**, 544–552.
42. Greene, N.D., Stanier, P. and Moore, G.E. (2011) The emerging role of epigenetic mechanisms in the aetiology of neural tube defects. *Epigenetics*, **6**, 875–893.
43. Apostolidou, S., Abu-Amero, S., O'Donoghue, K., Frost, J., Olafsdottir, O., Chavele, K.M., Whittaker, J.C., Loughna, P., Stanier, P. and Moore, G.E. (2007) Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. *J. Mol. Med.*, **85**, 379–387.
44. Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**, 193–199.
45. Oda, M., Kure, S., Sugawara, T., Yamaguchi, S., Kojima, K., Shinka, T., Sato, K., Narisawa, A., Aoki, Y., Matsubara, Y. *et al.* (2007) Direct correlation between ischemic injury and extracellular glycine concentration in mice with genetically altered activities of the glycine cleavage multienzyme system. *Stroke*, **38**, 2157–2164.
46. Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1991) Lipoylation of H-protein of the glycine cleavage system. The effect of site-directed mutagenesis of amino acid residues around the lipoyllysine residue on the lipoate attachment. *FEBS Lett.*, **293**, 115–118.
47. Wlodarczyk, B.J., Tang, L.S., Triplett, A., Aleman, F. and Finnell, R.H. (2006) Spontaneous neural tube defects in splotch mice supplemented with selected micronutrients. *Toxicol. Appl. Pharmacol.*, **213**, 55–63.
48. Essien, F.B. and Wannberg, S.L. (1993) Methionine but not folinic acid or vitamin B-12 alters the frequency of neural tube defects in *Axd* mutant mice. *J. Nutr.*, **123**, 27–34.
49. Nakai, T., Nakagawa, N., Maoka, N., Masui, R., Kuramitsu, S. and Kamiya, N. (2005) Structure of P-protein of the glycine cleavage system: implications for nonketotic hyperglycinemia. *EMBO J.*, **24**, 1523–1536.

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Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.

1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

Abbreviations: CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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Table 1
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

Primer/probe set	Mutation		Location	Nucleotide change	Effects of mutations	Allele frequency*[19]	References
A	Mutation [I]	:851del4	exon 9	c.851_854delGTAT	p.R284fs(286X)	33.2%	[1]
B	Mutation [II]	:g.IVS11+1G>A	intron 11	c.1019_1177del	p.340_392del	37.6%	[1]
C	Mutation [III]	:1638ins23	exon 16	c.1638_1660dup	p.A554fs(570X)	3.4%	[1]
D	Mutation [IV]	:S225X	exon 7	c.675C>A	p.S225X	5.3%	[1]
E	Mutation [V]	:g.IVS13+1G>A	intron 13	c.1231_1311del	p.411_437del	8.2%	[1]
F	Mutation [XIX]	:IVS16ins3kb	intron 16	c. aberrant RNA	p.A584fs(585X)	4.6%	[19]
G	Mutation [VI]	:1800ins1	exon 17	c.1799_1800insA	p.Y600X	1.3%	[10]
	Mutation [VII]	:R605X	exon 17	c.1813C>T	p.R605X	0.90%	[10]
	Mutation [VIII]	:E601X	exon 17	c.1801G>T	p.E601X	1.2%	[11]
	Mutation [IX]	:E601K	exon 17	c.1801G>A	p.E601K	0.30%	[11]
	Mutation [XXI]	:L598R	exon 17	c.1793T>G	p.L598R	0%	[15]
						Total 95.1%	

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

2. Methods

2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

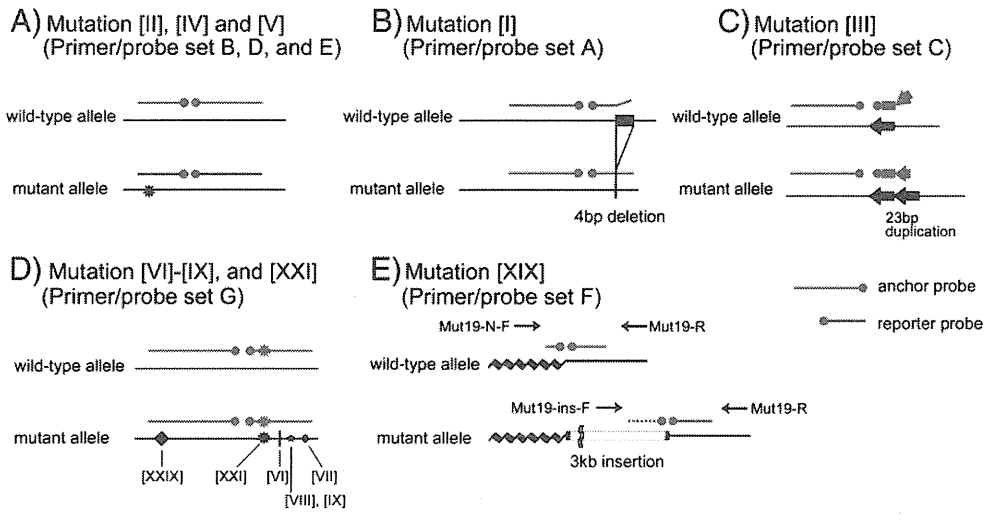

Fig. 1. Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.

specificity (i.e., binding of the probe to a perfectly matched sequence rather than to regions with sequence mismatches).

Seven primer/probe sets were designed for this study. Fig. 1 shows a schematic diagram of the strategy for mutation detection using these primer/probe sets. Tables 1 and 2 list the primer/probe sets and corresponding sequences and primer concentrations that were used to target the 11 mutations. Primer/probe sets A, B, C, D, E, and F were designed to detect mutations [I], [II], [III], [IV], [V], and [XIX], respectively. Primer/probe set G was designed to detect the five mutations clustered on exon 17: mutations [VI], [VII], [VIII], [IX], and [XXI] (Fig. 1D). All primers and probes were synthesized based on the NCBI reference SLC25A13 gene sequence (GenBank accession no. NM_014251) with the exception of mutation [XIX]:IVS16ins3kb, which was designed according to [19].

Real-time PCR and subsequent melting curve analyses were performed in a closed tube using a 20-μL mixture on a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The PCR mixture contained 2.0 μL of genomic DNA (10–50 ng), 0.5 μM of forward primer, 0.5 or 0.1 μM of reverse primer, 0.2 μM of each sensor and anchor probe, and 10 μL of Premix ExTaq™ (Perfect Real Time) reagent (TaKaRa Bio Inc., Otsu, Japan).

The thermal profile conditions were identical for all seven assays and consisted of an initial denaturation step (30 s at 95 °C), followed by 45 amplification cycles with the following conditions: denaturation for 5 s at 95 °C and annealing and extension for 20 s at 60 °C. The transition rate between all steps was 20 °C/s. After amplification, the samples were held at 37 °C for 1 min, followed by the melting curve acquisition at a ramp rate of 0.15 °C/s extending to 80 °C with continuous fluorescence acquisition.

Table 2
Primers, probes and target amplicon sequences, target mutation sites, and primer concentrations.

Primer/probe set	Name	Sequences of PCR products, primer locations, probe sequences, and mutation sites (5′ to 3′)	Concentration (μmol/L)
A		GGCTATACTGAAATATGAGAAatgaaaaaagggatgtttttaaattttataatgtaaattgtaataaattggtatatttgttgcttgtgtttgtttttcccctacagac	
		gtatgaccttagcagacattgaacggattgctcctctggaagagggaactctgccCTTTAACTTGGCTGAGG (181 bp)	
	Mut1-F	GGCTATACTGAAATATGAGAA	0.5
	Mut1-R	CCTCAGCCAAGTTAAAG	0.5
	Mut1-UP	ATGTAAATTGTAATAAATTGGTATATTTGTTGCTTGTGTT-FITC	
	Mut1-DW	LC Red640-GTTTTTCCCCTACAGA**CGACC**-P	
B		GAATGCAGAACCAACGAtcaactggctcttttgtgggagaactcatgtataaaaacagctttgactgttttaagaaagtgctacgctatgaaggcttctt	
		tggactgtatagag**gt**tagtgccacatgctcaatacctgttaggtgaaataacactcaaaggtttggtttctcatcttagtgcctGACATGAATTAGCAAGACTG (205 bp)	
	Mut2-F	GAATGCAGAACCAACGA	0.5
	Mut2-R	CAGTCTTGCTAATTCATGTC	0.1
	Mut2-UP	ACCTAACAGGTATTGAGCATGTG-FITC	
	MUt2-DW	LC Red640-CACTAA**C**CTCTATACAGTCCA-P	
C		GCAGTTCAAAGCACAGTTATTtttatatagtgagaatgtgaccagactgagatggtgttgtgtctctcctgcaggtatgcctgcagcatctttagtg	
		accctgctgatgttatcaagacgagattacaggtg	
		gctgcccggg(**gagatta**caggtggctgcccggg)ctggccaaaccaCTTACAGCGGAGTGATAGAC (175 bp)	
	Mut3-F	GCAGTTCAAAGCACAGTTATT	0.5
	Mut3-R	GTCTATCACTCCGCTGTAAG	0.5
	Mut3-UP	ACCCCTGCTGATGTTATCAAGACGAGATTACAGGT-FITC	
	Mut3-DW	LC Red640-GCTGCCCGGG**GAGATTA**-P	
D		TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctattttaatggatttaattc**g**ctcccttaacaac	
		atggaactcattagaaagatctatagcactc	
		tggctggcaccaggaaagatgttgaagtGACTAAGGGTGAGTGAGAA (164 bp)	
	Mut4-F	TCAATTTATTTGAGGCTGC	0.5
	Mut4-R	TTCTCACTCACCCTTAGTC	0.5
	Mut4-UP	AATGGATTTAATT**C**GCTCCTTAACA-FITC	
	Mut4-DW	LC Red640-ATGGAACTCATTAGAAAGATCTATAGCACTC-P	
E		TGCACAAAGATGGTTCGgtcccacttgcagcagaaattcttgctggaggctgc**g**taagtacctttgaagctctcttcattgaaaagacttgtttcac	
		atatatatcactaccatggtcaacaggtgtggactaaggcttctgttTAACCACAGATCCTGCA (162 bp)	
	Mut5-F	TGCACAAAGATGGTTCG	0.5
	Mut5-R	TGCAGGATCTGTGGTTA	0.5
	Mut5-UP	GTGAAACAAGTCTTTTCAATGAAGAGAGCTTC-FITC	
	Mut5-DW	LC Red640-AAGGTACTTA**C**GCAGCCTC-P	
F	normal allele	GGAGCTGGTGGTATGGAAataatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgtgatttctc	
		catttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaatttt	
		GGCTGCTGCTAATGCTC (244 bp)	
	insertion allele	CCATCTTCCTCCTCCCTTggcagccccgccccccgatttctccatttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgatttt	
		ggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (196 bp)	
	Mut19-N-F	GGAGCTGGTGGTATGGAA	0.5
	Mut19-ins-F	CCATCTTCCTCCTCCCTT	0.5
	Mut19-R	GAGCATTAGCAGCAGCC	0.5
	Mut19-UP	ACCAAACTGGGGTGAGGATCGAAATACACGAGCTTTAAAAAAATG-FITC	
	Mut19-N-DW	LC Red640-AGAAATCACAGATATAATTAGATATTT-P	
	Mut19-ins-DW	LC Red640-AGAAATCGGGGGGCGGGG-P	
G		TCTTAACTAACTCTTTGGTATCAGGTaaatttttaaaatatctaattatatctgtgatttctccatttttttaaagctcg	
		tgtatttcgatcctcaccccagtttggtgtaactttg**c**t**g**actt**a(a)cg**aattgctacag**c**ga	
		tggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (217 bp)	
	Mut6-9, 21-F	TCTTAACTAACTCTTTGGTATCAGGT	0.5
	Mut6-9, 21-R	GAGCATTAGCAGCAGCC	0.5
	Mut6-9, 21-UP	TGTATTTCGATCCTCACCCCAGTTTGGTGTAACTT-FITC	
	Mut6-9, 21-DW	LC Red640-GC**G**GACTT**ACG**AATTGCTACAG**C**GA-P	

Upper case and underlined letters indicate the locations of primers and probes, respectively. Inserted DNA is shown in parenthesis. Nucleotides in boldface were used for mutation detection.
F: forward, R: reverse, UP: upstream, DW: downstream, N: normal allele, ins: insertion allele, FITC: fluorescein isothiocyanate, P: phosphate.

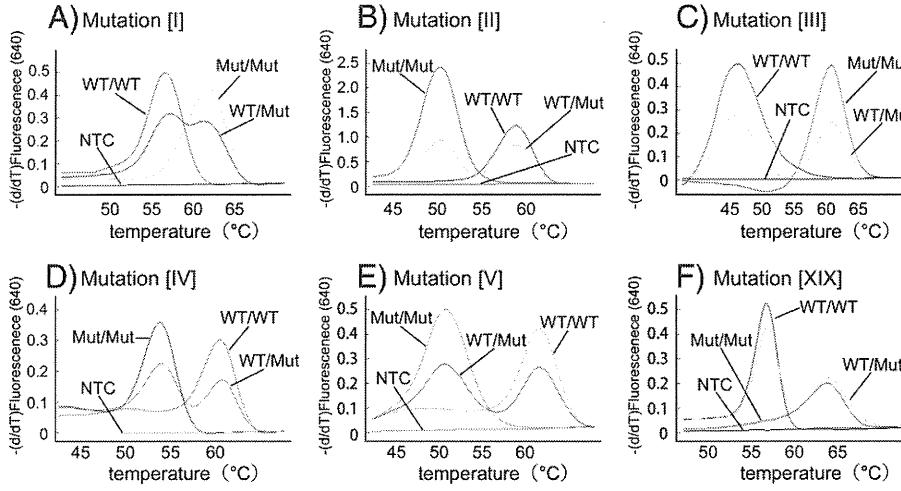

Fig. 2. Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

2.3. Validation of the mutation detection system

After establishing the protocol for detecting the 11 prevalent mutations, 50 DNA samples from patients' blood were sent from Kagoshima University to Tohoku University for the validation of this system in a single-blind manner. Similarly, 26 DNA samples purified from paper-filter blood samples were analyzed in the same manner as the blood DNA samples.

2.4. Estimation of the carrier frequency

For the estimation of the heterozygous carrier frequency, 420 genomic DNA samples from healthy volunteers were screened using the HybProbe analysis for the 11 prevalent mutations. All detected mutations were confirmed by direct sequencing.

2.5. Ethics

This study was approved by the Ethical Committees of Tohoku University School of Medicine and Kagoshima University. Written informed consent was obtained from all participants or their guardians.

3. Results

3.1. Development of the mutation detection system

In primer/probe sets B, D, and E, the reporter probes were designed to be complimentary to the wild-type allele (Fig. 1A). To allow for an improved detection of the mutations, primer/probe sets A and C were designed to be complementary to the mutant allele (Figs. 1B, C). In the primer/probe set F, two forward PCR primers, which were specific to the wild-type and the mutant alleles, were used with a common reverse primer for the co-amplification of the wild-type and 3-kb insertion alleles (Fig. 1E). Two reporter probes, which had a common anchor probe, were used for the detection of the wild-type and mutant alleles. Because the two reporter probes had different melting temperatures, we were able to identify the allele that was amplified. Fig. 2 shows representative results of the melting curve analyses using the primer/probe sets A–F, in which all of the mutant alleles generated distinct peaks corresponding to the wild-type alleles.

In the primer/probe set G, we used a reporter probe that was complementary to the mutant [XXI] allele (Fig. 1D). All five mutations in exon 17 were successfully differentiated from the wild-type allele (Figs. 3A–E). The [XXIX] mutation is an additional mutation in exon

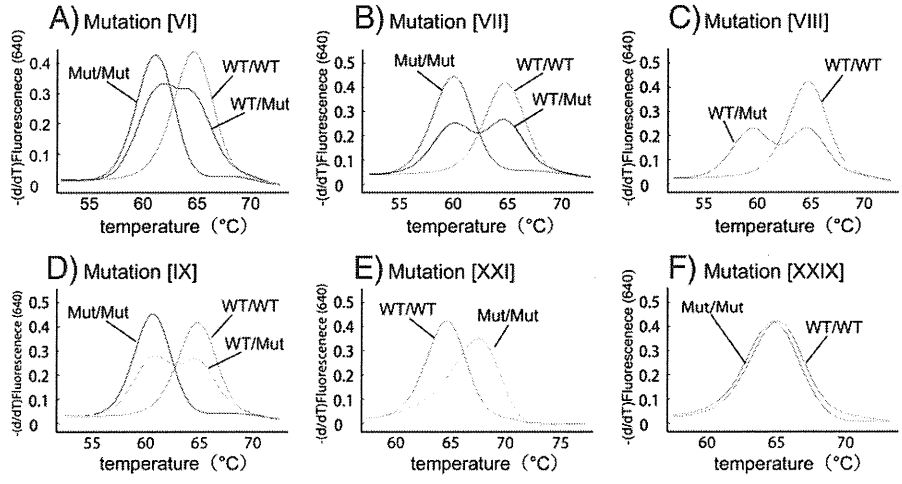

Fig. 3. Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor probe. WT: wild-type allele, Mut: mutant allele.

17 that is not listed in Table 1. The [XXIX] mutation is located in the anchor-probe binding site and not on the reporter-probe binding site (Fig. 1D). To examine the effect of mutations on the anchor probe, we genotyped a patient with a heterozygous [XXIX] mutation using primer/probe set G (Fig. 3F). We found no change in the melting curves between the wild-type allele and the [XXIX] allele, thereby suggesting that point mutations within the anchor probe sequence have little effect on the melting curve analysis.

3.2. Validation

The genotypes determined at Tohoku University using the proposed method and those determined at Kagoshima University using a previously published method were identical for the 11 common mutations (Table S1 in supplementary material). We performed a similar test using DNA samples purified from filter-paper blood samples to determine if this method could be used for newborn screening. The genotypes determined in both laboratories were identical for all 26 DNA samples (Table S2 in supplementary material).

3.3. Frequency of eleven prevalent mutations

We found four heterozygous carriers of mutation [I], three of mutation [II], and two of mutation [V]. In addition, primer/probe set G detected one heterozygous mutation, which was confirmed as mutation [VIII] by direct sequencing. Altogether, 10 mutations were detected in 420 Japanese healthy controls.

4. Discussion

We developed a simple and rapid genetic test using real-time PCR combined with the HybProbe system for the 11 prevalent mutations in *SLC25A13*: mutations [I], [II], [III], [IV], [V], [VI], [VII], [VIII], [IX], [XIX], and [XXI]. This genetic test is a closed-tube assay in which no post-PCR handling of the samples is required. In addition, the genotyping is completed within 1 h. This test can utilize DNA samples purified from both peripheral blood and filter-paper blood. The reliability of the test was confirmed by genotyping 76 blind DNA samples from patients with citrin deficiency, including 50 peripheral blood and 26 filter-paper blood DNA samples. Because screening for the 11 targeted mutations would identify 95% of mutant alleles in the Japanese population [19], both, one, and no mutant alleles are expected to be identified in 90.4%, 9.3%, and less than 0.3% of patients, respectively. This genetic test would be useful not only in Japan but also other East Asian countries, including China, Korea, Taiwan and Vietnam, in which the same mutations are prevalent. Our test is expected to detect 76–87% of the mutant alleles in the Chinese population [12,19,25], 95–100% in the Korean population [12,19,26], 60–68% in the Taiwanese population [27,28], and 100% in the Vietnamese population [12,19]. If we were to prepare a primer/probe set for mutation [X]:g.IVS6+5G>A [12], which is prevalent in Taiwan, the estimated sensitivity would exceed 90% in the Taiwanese population [27,28].

Recently, the high resolution melting (HRM) method was reported to be suitable for the screening of mutations in the diagnosis of citrin deficiency [28]. HRM analysis is a closed-tube assay that screens for any base changes in the amplicons. The presence of SNPs anywhere on the amplicons can affect the melting curve, thereby suggesting that HRM is not suitable for screening for known mutations, but rather, is best suited to screening for unknown mutations. When we detected one heterozygous prevalent mutation, we performed HRM screening for all 17 exons of *SLC25A13*. After HRM screening, only the HRM-positive exons were subjected to direct sequencing analysis. Several mutant alleles were identified using this approach.

The frequency of homozygotes, including compound heterozygotes, presenting *SLC25A13* mutations in the population at Kagoshima (a prefecture in the southern part of Japan) has been calculated to be 1/17,000 based on the carrier rate (1/65) [19]. The prevalence of NICCD has been also reported to be 1/17,000–34,000 [29]. In this study, the carrier rate in Miyagi (a prefecture in northern Japan) was 1/42 (95% confidential interval, 1/108–1/26), thereby yielding an estimated frequency of patients with citrin deficiency of 1/7,100. Our result, together with the previous report [19], suggests that a substantial fraction of the homozygotes or compound heterozygotes of SLC25A13 mutations was asymptomatic during the neonatal period.

The early and definitive diagnosis of citrin deficiency may be beneficial for patients with citrin deficiency by encouraging specific dietary habits and avoiding iatrogenic worsening of brain edema by glycerol infusion when patients develop encephalopathy [30,31]. Because the screening of blood citrulline levels by tandem mass analysis at birth does not detect all patients with citrin deficiency, the development of a genetic test would be welcomed. In this study, we demonstrated that genomic DNA extracted from filter paper blood samples was correctly genotyped, thereby indicating the feasibility of newborn screening using this genetic test. If 100,000 babies in the northern part of Japan were screened by this method, we would detect 14 homozygotes or compound heterozygotes with *SLC25A13* mutations and 2400 heterozygous carriers. In 2400 heterozygous carriers, we would expect to observe only 1 to 2 compound heterozygotes with one target and one non-target mutation. The estimated frequency of babies with two non-target mutations is 0.04/100,000. Our genetic method would therefore allow us to screen newborn babies efficiently. If we performed this genetic test in a high-throughput real-time PCR system, such as a 384- or 1,536-well format, the cost per sample could be lowered.

In conclusion, we have established a rapid and simple detection system using the HybProbe assay for the 11 prevalent mutations in *SLC25A13*. This system could be used to screen newborns for citrin deficiency and may facilitate the genetic diagnosis of citrin deficiency, especially in East Asian populations.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2011.12.024.

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References

[1] K. Kobayashi, D.S. Sinasac, M. Iijima, A.P. Boright, L. Begum, J.R. Lee, T. Yasuda, S. Ikeda, R. Hirano, H. Terazono, M.A. Crackower, I. Kondo, L.C. Tsui, S.W. Scherer, T. Saheki, The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein, Nat. Genet. 22 (1999) 159–163.
[2] T. Ohura, K. Kobayashi, Y. Tazawa, I. Nishi, D. Abukawa, O. Sakamoto, K. Iinuma, T. Saheki, Neonatal presentation of adult-onset type II citrullinemia, Hum. Genet. 108 (2001) 87–90.
[3] Y. Tazawa, K. Kobayashi, T. Ohura, D. Abukawa, F. Nishinomiya, Y. Hosoda, M. Yamashita, I. Nagata, Y. Kono, T. Yasuda, N. Yamaguchi, T. Saheki, Infantile cholestatic jaundice associated with adult-onset type II citrullinemia, J. Pediatr. 138 (2001) 735–740.
[4] T. Tomomasa, K. Kobayashi, H. Kaneko, H. Shimura, T. Fukusato, M. Tabata, Y. Inoue, S. Ohwada, M. Kasahara, Y. Morishita, M. Kimura, T. Saheki, A. Morikawa, Possible clinical and histologic manifestations of adult-onset type II citrullinemia in early infancy, J. Pediatr. 138 (2001) 741–743.
[5] T. Shigeta, M. Kasahara, T. Kimura, A. Fukuda, K. Sasaki, K. Arai, A. Nakagawa, S. Nakagawa, K. Kobayashi, S. Soneda, H. Kitagawa, Liver transplantation for an

infant with neonatal intrahepatic cholestasis caused by citrin deficiency using heterozygote living donor, Pediatr. Transplant. 14 (2009) E86–88.
[6] M. Kasahara, S. Ohwada, T. Takeichi, H. Kaneko, T. Tomomasa, A. Morikawa, K. Yonemura, K. Asonuma, K. Tanaka, K. Kobayashi, T. Saheki, I. Takeyoshi, Y. Morishita, Living-related liver transplantation for type II citrullinemia using a graft from heterozygote donor, Transplantation 71 (2001) 157–159.
[7] Y. Takashima, M. Koide, H. Fukunaga, M. Iwai, M. Miura, R. Yoneda, T. Fukuda, K. Kobayashi, T. Saheki, Recovery from marked altered consciousness in a patient with adult-onset type II citrullinemia diagnosed by DNA analysis and treated with a living related partial liver transplantation, Intern. Med. 41 (2002) 555–560.
[8] A. Tamamori, Y. Okano, H. Ozaki, A. Fujimoto, M. Kajiwara, K. Fukuda, K. Kobayashi, T. Saheki, Y. Tagami, T. Yamano, Neonatal intrahepatic cholestasis caused by citrin deficiency: severe hepatic dysfunction in an infant requiring liver transplantation, Eur. J. Pediatr. 161 (2002) 609–613.
[9] T. Ohura, K. Kobayashi, Y. Tazawa, D. Abukawa, O. Sakamoto, S. Tsuchiya, T. Saheki, Clinical pictures of 75 patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), J. Inherit. Metab. Dis. 30 (2007) 139–144.
[10] T. Yasuda, N. Yamaguchi, K. Kobayashi, I. Nishi, H. Horinouchi, M.A. Jalil, M.X. Li, M. Ushikai, M. Iijima, I. Kondo, T. Saheki, Identification of two novel mutations in the SLC25A13 gene and detection of seven mutations in 102 patients with adult-onset type II citrullinemia, Hum. Genet. 107 (2000) 537–545.
[11] N. Yamaguchi, K. Kobayashi, T. Yasuda, I. Nishi, M. Iijima, M. Nakagawa, M. Osame, I. Kondo, T. Saheki, Screening of SLC25A13 mutations in early and late onset patients with citrin deficiency and in the Japanese population: identification of two novel mutations and establishment of multiple DNA diagnosis methods for nine mutations, Hum. Mutat. 19 (2002) 122–130.
[12] Y.B. Lu, K. Kobayashi, M. Ushikai, A. Tabata, M. Iijima, M.X. Li, L. Lei, K. Kawabe, S. Taura, Y. Yang, T.-T. Liu, S.-H. Chiang, K.-J. Hsiao, Y.-L. Lau, L.-C. Tsui, D.H. Lee, T. Saheki, Frequency and distribution in East Asia of 12 mutations identified in the SLC25A13 gene of Japanese patients with citrin deficiency, J. Hum. Genet. 50 (2005) 338–346.
[13] E. Ben-Shalom, K. Kobayashi, A. Shaag, T. Yasuda, H.-Z. Gao, T. Saheki, C. Bachmann, O. Elpeleg, Infantile citrullinemia caused by citrin deficiency with increased dibasic amino acids, Mol. Genet. Metab. 77 (2002) 202–208.
[14] J. Takaya, K. Kobayashi, A. Ohashi, M. Ushikai, A. Tabata, S. Fujimoto, F. Yamato, T. Saheki, Y. Kobayashi, Variant clinical courses of 2 patients with neonatal intrahepatic cholestasis who have a novel mutation of SLC25A13, Metab. Clin. Exp. 54 (2005) 1615–1619.
[15] A. Luder, A. Tabata, M. Iijima, K. Kobayashi, H. Mandel, Citrullinaemia type 2 outside East Asia: Israeli experience, J. Inherit. Metab. Dis. 29 (2006) 59.
[16] T. Hutchin, M. Preece, K. Kobayashi, T. Saheki, R. Brown, D. Kelly, P. McKiernan, A. Green, U. Baumann, Neonatal intrahepatic cholestasis caused by citirn deficiency (NICCD) in a European patient, J. Inherit. Metab. Dis. 29 (2006) 112.
[17] J.-S. Sheng, M. Ushikai, M. Iijima, S. Packman, K. Weisiger, M. Martin, M. McCracken, T. Saheki, K. Kobayashi, Identification of a novel mutation in a Taiwanese patient with citrin deficiency, J. Inherit. Metab. Dis. 29 (2006) 163.
[18] J.M. Ko, G.-H. Kim, J.-H. Kim, J.Y. Kim, J.-H. Choi, M. Ushikai, T. Saheki, K. Kobayashi, H.-W. Yoo, Six cases of citrin deficiency in Korea, Int. J. Mol. Med. 20 (2007) 809–815.
[19] A. Tabata, J.-S. Sheng, M. Ushikai, Y.-Z. Song, H.-Z. Gao, Y.-B. Lu, F. Okumura, M. Iijima, K. Mutoh, S. Kishida, T. Saheki, K. Kobayashi, Identification of 13 novel mutations including a retrotransposal insertion in SLC25A13 gene and frequency of 30 mutations found in patients with citrin deficiency, J. Hum. Genet. 53 (2008) 534–545.
[20] P.S. Bernard, R.S. Ajioka, J.P. Kushner, C.T. Wittwer, Homogeneous multiplex genotyping of hemochromatosis mutations with fluorescent hybridization probes, Am. J. Pathol. 153 (1998) 1055–1061.
[21] C.N. Gundry, P.S. Bernard, M.G. Herrmann, G.H. Reed, C.T. Wittwer, Rapid F508del and F508C assay using fluorescent hybridization probes, Genet. Test. 3 (1999) 365–370.
[22] T. Saheki, K. Kobayashi, I. Inoue, Hereditary disorders of the urea cycle in man: biochemical and molecular approaches, Rev. Physiol. Biochem. Pharmacol. 108 (1987) 21–68.
[23] K. Kobayashi, M. Horiuchi, T. Saheki, Pancreatic secretory trypsin inhibitor as a diagnostic marker for adult-onset type II citrullinemia, Hepatology 25 (1997) 1160–1165.
[24] Y. Tazawa, K. Kobayashi, D. Abukawa, I. Nagata, S. Maisawa, R. Sumazaki, T. Iizuka, Y. Hosoda, M. Okamoto, J. Murakami, S. Kaji, A. Tabata, Y.B. Lu, O. Sakamoto, A. Matsui, S. Kanzaki, G. Takada, T. Saheki, K. Iinuma, T. Ohura, Clinical heterogeneity of neonatal intrahepatic cholestasis caused by citrin deficiency: case reports from 16 patients, Mol. Genet. Metab. 83 (2004) 213–219.
[25] H.Y. Fu, S.R. Zhang, X.H. Wang, T. Saheki, K. Kobayashi, J.S. Wang, The mutation spectrum of the SLC25A13 gene in Chinese infants with intrahepatic cholestasis and aminoacidemia, J. Gastroenterol. 46 (2011) 510–518.
[26] K. Kobayashi, Y.B. Lu, M.X. Li, I. Nishi, K.-J. Hsiao, K. Choeh, Y. Yang, W.-L. Hwu, J.K.V. Reichardt, F. Palmieri, Y. Okano, T. Saheki, Screening of nine SLC25A13 mutations: their frequency in patients with citrin deficiency and high carrier rates in Asian populations, Mol. Genet. Metab. 80 (2003) 356–359.
[27] T. Saheki, K. Kobayashi, M. Iijima, M. Horiuchi, L. Begum, M.A. Jalil, M.X. Li, Y.B. Lu, M. Ushikai, A. Tabata, M. Moriyama, K.-J. Hsiao, Y. Yang, Adult-onset type II citrullinemia and idiopathic neonatal hepatitis caused by citrin deficiency: involvement of the aspartate glutamate carrier for urea synthesis and maintenance of the urea cycle, Mol. Genet. Metab. 81 (Suppl 1) (2004) S20–S26.
[28] J.T. Lin, K.J. Hsiao, C.Y. Chen, C.C. Wu, S.J. Lin, Y.Y. Chou, S.C. Shiesh, High resolution melting analysis for the detection of SLC25A13 gene mutations in Taiwan, Clin. Chim. Acta 412 (2011) 460–465.
[29] Y. Shigematsu, S. Hirano, I. Hata, Y. Tanaka, M. Sudo, N. Sakura, T. Tajima, S. Yamaguchi, Newborn mass screening and selective screening using electrospray tandem mass spectrometry in Japan, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 776 (2002) 39–48.
[30] M. Yazaki, Y.-i. Takei, K. Kobayashi, T. Saheki, S.-I. Ikeda, Risk of worsened encephalopathy after intravenous glycerol therapy in patients with adult-onset type II citrullinemia (CTLN2), Intern. Med. 44 (2005) 188–195.
[31] H. Takahashi, T. Kagawa, K. Kobayashi, H. Hirabayashi, M. Yui, L. Begum, T. Mine, S. Takagi, T. Saheki, Y. Shinohara, A case of adult-onset type II citrullinemia—deterioration of clinical course after infusion of hyperosmotic and high sugar solutions, Med. Sci. Monit. 12 (2006) CS13–CS15.

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Two Novel Mutations in the Lactase Gene in a Japanese Infant with Congenital Lactase Deficiency

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Intestinal lactase is required for the hydrolysis of lactose that is the most essential carbohydrate in milk and the primary diet source of newborn. Congenital lactase deficiency [CLD (MIM 223000)] is a severe gastrointestinal disorder and is characterized by watery diarrhea due to an extremely low or the lack of lactase activity in the intestinal wall from birth. CLD is a rare disease and occurs more frequently in Finland. Recent studies have shown that mutations in the coding region of the lactase (*LCT*) gene underlie CLD in patients from Finland and other European countries. Here, we report two novel mutations in the *LCT* gene in a Japanese female infant with clinical features consistent with those of CLD. She suffered from severe watery diarrhea from the age of 2 days on breast milk/lactose containing cow's milk formula. With the lactose-free hydrolyzed cow's milk formula, diarrhea was stopped, and she has now developed well on a lactose-free diet. She shows a lactose-intolerance pattern on the lactose challenge test. Sequence analysis revealed the two mutations in her *LCT* gene: c.4419C>G (p.Y1473X) in exon 10 transmitted from her mother and c.5387delA (p.D1796fs) in exon 16 transmitted from her father. Both mutations cause premature truncation of lactase polypeptide and are supposed to be responsible for CLD. To our knowledge, this is the first report on mutations in the *LCT* gene in Japan. We suggest that an increased awareness is required regarding CLD.

Keywords: congenital lactase deficiency; cow's milk allergy; *lactase* gene; oral lactose challenge test; watery diarrhea
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Intestinal lactase is required for the hydrolysis of lactose, which is the most essential carbohydrate in milk and the primary diet source of newborn. Congenital lactase deficiency [CLD (MIM 223000)] is a severe gastrointestinal disorder characterized by watery diarrhea due to an extremely low or the lack of activity of lactase in the intestinal wall from birth. Affected infants are suffered from severe watery diarrhea shortly after the first feed with breast milk or lactose-containing formulas (Savilahti et al. 1983). Despite adequate feeding, they are dehydrated and have poor weight gain, because they are unable to hydrolyze lactose that accounts for 40% of energy ingested among infants. This disease is a rare autosomal disorder and occurs more frequently in Finland. Recently, mutations in the coding region of the lactase (*LCT*) gene were revealed to be the underlying cause of CLD and the molecular background is being identified. The *LCT* gene consists of 17 exons encoding 1927 amino acids comprising four homologous domains, I - IV. Domain IV harbors lactase activity. One mutation, c4170T>A (p.Y1390X) in exon 9, is enriched in Finnish population, and 84% of Finnish patients were homozygous for this mutation. Y1390X is located in domain IV, and results in a truncation of lactase (Kuokkanen et al. 2006; Behrendt et al. 2009).

Here, we report a Japanese female infant with clinical features consistent with those of CLD who has two novel mutations in the *LCT* gene in a heterozygous form: c.4419C>G (p.Y1473X) in exon 10 and c.5387delA (p.D1796fs) in exon 16. Both of the mutations are located in the domain IV and supposed to be causative of CLD. To our knowledge, this is the first report on mutations in the *LCT* gene in Japan, and our findings suggest that an increased awareness is required regarding CLD.

Clinical Report

The patient is the first child of healthy nonconsanguineous Japanese parents. She was born at term after an uneventful pregnancy with a birth weight of 3,124 g. When the patient was fed breast milk and lactose-containing formula, she developed watery diarrhea at the age of 2 days. At the age of 4 days, she was admitted to the department of pediatrics because of poor weight gain and dehydration.

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On admission, her weight was 2,722 g, and the serum levels of blood urea nitrogen, creatinine, sodium, and potassium were 29.4 mg/dL, 0.4 mg/dL, 157 mEq/L, and 5.4 mEq/L, respectively. Dehydration resolved after intravenous infusion was initiated, but severe watery diarrhea continued to be observed. The result of a stool culture was negative; in addition, rapid antigen tests for rotavirus and adenovirus were negative. Watery diarrhea promptly disappeared when the oral intake was discontinued at the age of 9 days and did not recur when she was administered a diet with lactose-free hydrolyzed cow's milk formula at the age of 10 days. She showed a remarkable improvement in weight gain, and the intravenous infusion was discontinued at the age of 12 days. She was discharged from the hospital at the age of 17 days, showed good body weight gain, and was free from gastrointestinal symptoms. She was suspected to have CLD or cow's milk protein allergy, and was admitted to our hospital for a lactose challenge test at the age of 4 months. The oral lactose challenge test with 2 g/kg of lactose showed no increase in the blood glucose level within 120 min and was followed by watery diarrhea within a few hours (Fig. 1). The serum level of total immunoglobulin E (IgE) antibody was 3 IU/mL and that of serum-specific IgE antibody to whole cow's milk measured using a chemiluminescent enzyme immunoassay was 0.07 lumicount; class 0 (MAST33; SRL, Tokyo, Japan). Lymphocyte stimulation tests for α-lactalbumin, β-lactoglobulin, α-casein, β-casein, and κ-casein were negative (stimulation indices were 0.7,

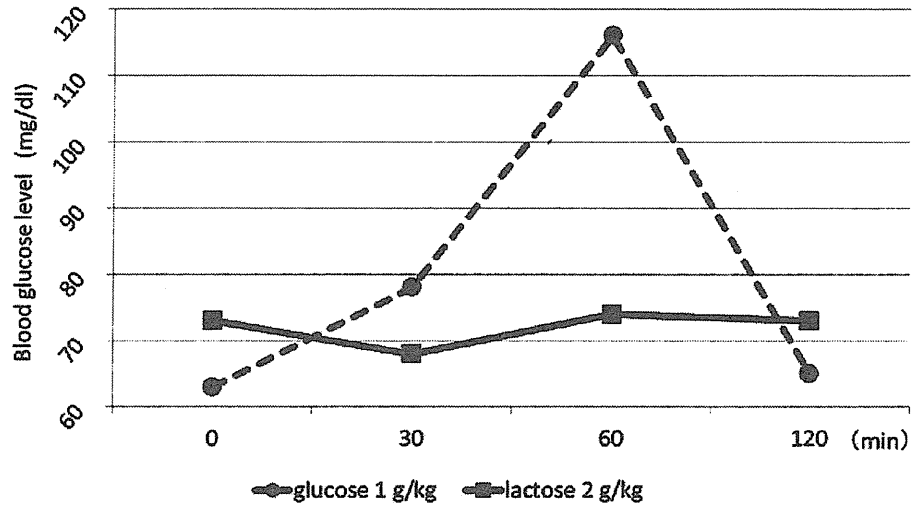

Fig. 1. Oral lactose challenge test at the age of 4 months.
After oral administration of 2 g/kg of lactose, no increase was observed in the blood glucose within 120 min and watery diarrhea developed. In contrast, a marked increase was observed in the blood glucose level after drinking 1 g/kg of glucose and no gastrointestinal symptoms were observed.

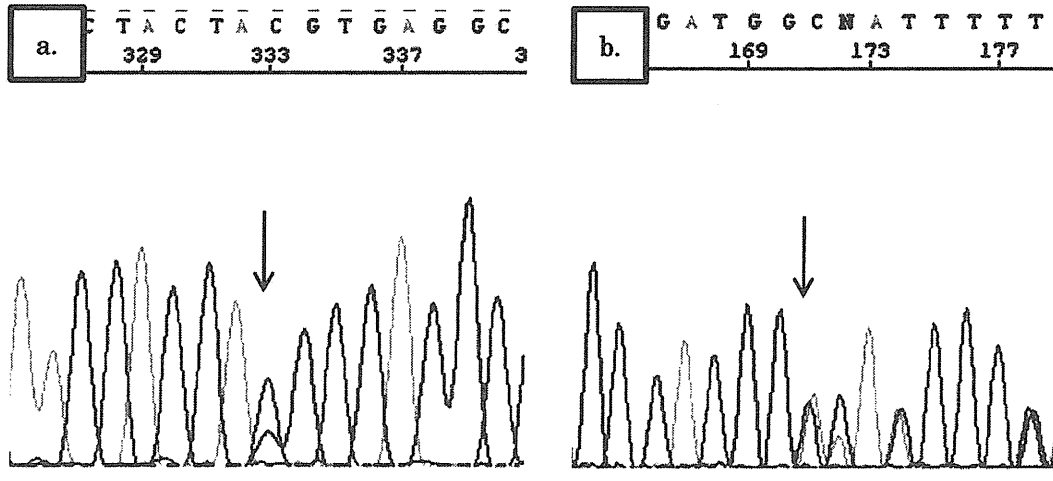

Fig. 2. Sequence analysis of the *LCT* gene.
a. LCT Ex10 c.4419C>G (p.Y1473X). b. LCT Ex16 c.5387delA (p.D1796fsX)