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.

Homozygous c.14576G>A variant of RNF213 predicts early-onset and severe form of moyamoya disease

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Supplemental data at www.neurology.org

Supplemental Data

ABSTRACT

Objective: RNF213 was recently reported as a susceptibility gene for moyamoya disease (MMD). Our aim was to clarify the correlation between the RNF213 genotype and MMD phenotype.

Methods: The entire coding region of the RNF213 gene was sequenced in 204 patients with MMD, and corresponding variants were checked in 62 pairs of parents, 13 mothers and 4 fathers of the patients, and 283 normal controls. Clinical information was collected. Genotype-phenotype correlations were statistically analyzed.

Results: The c.14576G>A variant was identified in 95.1% of patients with familial MMD, 79.2% of patients with sporadic MMD, and 1.8% of controls, thus confirming its association with MMD, with an odds ratio of 259 and $p < 0.001$ for either heterozygotes or homozygotes. Homozygous c.14576G>A was observed in 15 patients but not in the controls and unaffected parents. The incidence rate for homozygotes was calculated to be >78%. Homozygotes had a significantly earlier age at onset compared with heterozygotes or wild types (median age at onset 3, 7, and 8 years, respectively). Of homozygotes, 60% were diagnosed with MMD before age 4, and all had infarctions as the first symptom. Infarctions at initial presentation and involvement of posterior cerebral arteries, both known as poor prognostic factors for MMD, were of significantly higher frequency in homozygotes than in heterozygotes and wild types. Variants other than c.14576G>A were not associated with clinical phenotypes.

Conclusions: The homozygous c.14576G>A variant in RNF213 could be a good DNA biomarker for predicting the severe type of MMD, for which early medical/surgical intervention is recommended, and may provide a better monitoring and prevention strategy. *Neurology* 2012;78:803-810

GLOSSARY

CI = confidence interval; **HRM** = high-resolution melting; **MMD** = moyamoya disease; **OR** = odds ratio; **PCA** = posterior cerebral artery.

Moyamoya disease (MMD) is a cerebrovascular disease, which is now a relatively common cause of pediatric strokes.[1,2] Annual incidence is estimated to be 0.35–0.54 per 100,000 person-years in Japan[3,4] and about one tenth of that in Europe.[5,6] MMD can lead to devastating neurologic deficits and intellectual impairments if left untreated.

Although MMD is a progressive disease, its natural history varies from slow progression to rapid neurologic decline.[7] Preoperative infarctions, early age at onset, intellectual impairment, seizure, and progressive posterior cerebral artery (PCA) stenosis are known prognostic factors.[8-11] Surgical revascularization can improve the cerebrovascular hemodynamics and prevent subsequent attacks in the ischemic type of MMD.[8] Thus, early diagnosis and surgical intervention are very important.

Genetic factors underlying MMD are of clinical relevance. Epidemiologic studies have shown that about 15% of patients had a family history.[12] Anticipation of the disease is

Table 1	Sample demographics of patients with moyamoya disease and controls[a]		
Clinical features	No. of patients (%)		No. of patients without data
MMD	204		
Gender (M/F)	68 (33.5)/135 (66.5)		1
Distribution of age at onset	0–58 y		7
Frequency of childhood onset (<15 y)	143 (72.6)		
Frequency of childhood onset (<4 y)	36 (18.3)		
Clinical manifestation			11
Infarction	87 (45.1)		
TIA	77 (39.9)		
ICH/IVH	17 (8.8)		
Others	12 (6.2)		
With family history	41 (20.1)		0
With intellectual impairment	33 (17.7)		18
With epilepsy	33 (17.6)		16
PCA involvement			52
Unilateral	31 (20.4)		
Bilateral	43 (28.3)		
Total	74 (48.7)		
Bilateral MMD	148 (96.1)		50
Controls	283		
Gender (M/F)	140 (51.5):132 (48.5)		11

Abbreviations: ICH/IVH = intracranial hemorrhage/intraventricular hemorrhage; MMD = moyamoya disease; PCA = posterior cerebral artery; TIA = transient ischemic attack.
[a] Numbers of patients (%) in each feature are shown, except for the distribution of ages at onset for all patients.

also observed in familial MMD.[13] Recently, the important MMD susceptibility gene, *RNF213*, was identified.[14,15] However, its clinical relevance remains unknown. For this investigation, we conducted a comprehensive genetic study of *RNF213* as well as a clinical phenotype analysis of MMD.

METHODS **Study subjects.** Blood samples from 204 Japanese patients with MMD were obtained consecutively between January 2008 and February 2011. There were no sample overlaps between ours and those in the previous studies.[14,15] MMD was diagnosed as either definite (bilateral) or probable (unilateral) according to published guidelines.[16] Six patients with probable MMD were female adults and 5 of them had sporadic MMD. The medical charts were completed by the clinicians who were blinded to the genotype of the patients. Sample demographics are shown in table 1. We also obtained either blood or saliva samples from 62 pairs of

parents, as well as 4 fathers and 13 mothers whose partners were unavailable. As many as 94 to 283 samples from healthy Japanese individuals were tested as normal controls for each sequence variant found.

Standard protocol approvals, registrations, and patient consents. Experimental protocols were approved by the Committee for Ethical Issue at Yokohama City University School of Medicine. Written informed consents were obtained from all the patients or their parents.

Mutation screening. Genomic DNA was obtained from peripheral blood leukocytes using QuickGene-610L (Fujifilm, Tokyo, Japan) or from saliva using Oragene DNA (DNA Genotek, Kanata, Canada). DNA was amplified using GenomiPhi version 2 (GE Healthcare, Buckinghamshire, UK). Mutation analysis of exons and exon-intron borders covering the coding region of *RNF213* (GenBank accession number, NM_020914.4), except for exon 61, was performed in all MMD patient samples by high-resolution melting (HRM) analysis on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland). Primer sequences, PCR conditions, and HRM settings are available on request. HRM analysis with and without the spike-in method was performed for the detection of homozygous mutations.[17] If samples showed any aberrant melting curve patterns, direct sequencing was performed using an ABI Genetic Analyzer 3100 or 3500xL (Applied Biosystems, Foster City, CA) and analyzed with sequence analysis software version 5.1.1 (Applied Biosystems) and Sequencher 4.10 build 5828 (GeneCodes Corporation, Ann Arbor, MI). For exon 61, which bears the c.14576G>A variant, direct sequencing was performed for all patients with MMD and their parental samples. Additional screenings by HRM analysis were performed for the confirmed mutations in up to 283 normal control Japanese individuals. All variants were confirmed with PCR direct sequencing using either genomic DNA or another DNA, amplified with GenomiPhi separately. No discrepancy was seen in the data between the 2 different conditions of DNA.

Statistical analysis. Patients without information for each clinical feature (listed below) were excluded from the analyses (table 1 and tables e-2 and e-3 on the *Neurology*® Web site at www.neurology.org). All statistical analyses were performed using SPSS Statistics 19 (IBM, New York, NY) software. χ^2 tests were applied to compare each categorical phenotype variable between different genotypes: clinical symptom at onset, with/without family history, intellectual impairment, epilepsy, and the unilateral/bilateral distribution of vasculopathy. Nonnormally distributed continuous variables, such as age at onset and the number of steno-occlusive PCA arteries were compared using the Mann-Whitney U test and Kruskal-Wallis test between different genotypes. $p < 0.05$ was considered statistically significant. A Kaplan-Meier curve was used to assess the cumulative incidence with the log-rank test. The Cox regression model was used to test which variables were associated with age at onset. The exact 95% confidence interval (CI) of the incidence rate of MMD was calculated according to the binomial distribution. The comparisons of clinical features between parent-offspring pairs or sibling pairs were performed using the Wilcoxon signed-rank test and McNemar test.

RESULTS **Identification of *RNF213* variants.** c.14576G>A was identified in 39 of 41 patients with familial MMD (95.1%), in 129 of 163 patients with nonfamilial MMD (79.2%), and in 5 of

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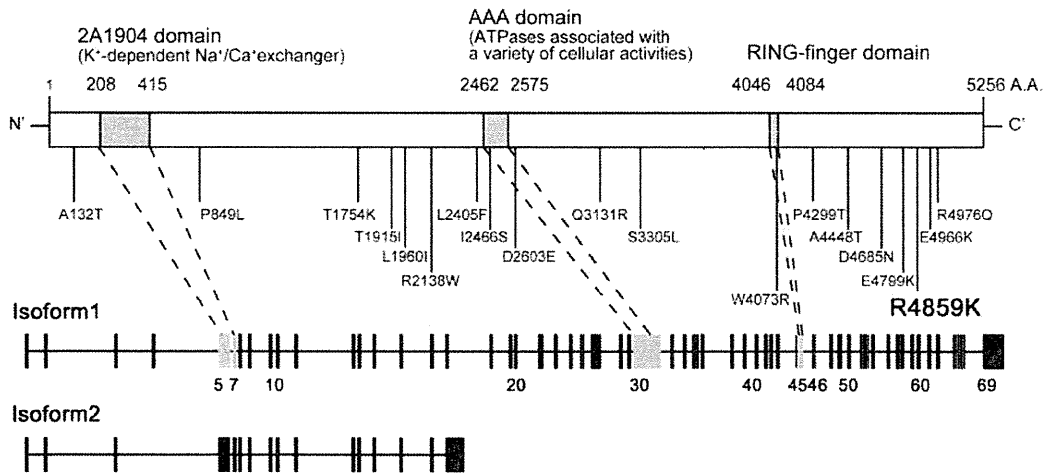|		c.14576G>A genotype			
	Total	Wild-type: G/G (%)	Heterozygous: G/A (%)	Homozygous: A/A (%)	GA + A/A (%)
Patients with MMD	204	36 (17.6)	153 (75.0)	15 (7.4)	168 (82.4)
Sporadic	163	34 (20.8)	117 (71.8)	12 (7.4)	129 (79.2)
With no other variant	137	20 (14.6)	105 (76.6)	12 (8.8)	117 (85.4)
With one other variant	25	13 (52)	12 (48)	0	12 (48)
With one other homozygous variants	1	1 (100)	0	0	0
Familial	41	2 (4.9)	36 (87.8)	3 (7.3)	39 (95.1)
With no other variant	36	0	33 (91.7)	3 (8.3)	36 (100)
With one other variant	3	0	3 (100)	0	3 (100)
With 2 other compound heterozygous variants	2	2 (100)	0	0	0
Parents of patients with MMD	141	77 (54.6)	63 (44.7)	1 (0.7)	64 (45.4)
Affected	9	0	8 (88.9)	1 (11.1)	9 (100)
Unaffected	132	77 (58.3)	55 (41.7)	0	55 (41.7)
Normal controls	283	278 (98.2)	5 (1.8)	0	5 (1.8)
OR (patients with MMD vs normal control)			236	ND	259
95% CI			91-615		100-674
p (Fisher exact test)			<0.001	<0.001	<0.001

Abbreviations: CI = confidence interval; MMD = moyamoya disease; ND = not determined; OR = odds ratio.
[a] Numbers of patients in each category (%) are shown.

283 normal control Japanese individuals (1.8%) (table 2 and table e-1). Sixty-two pairs of parents were also tested for the c.14576G>A genotype, with the conclusion that the c.14576G>A variant allele was inherited from either or both parents in all patients tested. Among 168 patients with the c.14576G>A variant, 15 had a homozygous change, whereas none of the controls and unaffected parents did. We conclude that the heterozygous c.14576G>A variant increases the risk for MMD with an odds ratio (OR) of 236 (95% CI 91–615, $p < 0.001$). Because no homozygous mutation was detected in the control samples or unaffected family members, the OR for the homozygote could not be calculated (∞), suggesting its strong effect. The incidence rate of MMD was calculated to be extremely high with a 95% CI of 0.78–1.00 with the homozygous mutation. Eighteen other genetic variants beside c.14576G>A were also identified in RNF213 (figure 1, table e-1). Sixteen of them were novel, which had not been reported in the previous studies.[14,15] Two of the variants were also found in the previous study[14]; however, they were thought to be common single nucleotide polymorphisms because they were also found in the normal controls without the significant difference of frequency. Other genetic variants showed a relatively small OR without any significance (table e-1). Thirty-one patients had these individual variants (table 2). Fifteen of them also had the heterozygous

c.14576G>A, and 4 of 5 patients whose parents' samples were available had these 2 variants existing as compound heterozygotes (for example, one variant from the father and the other from the mother). In the other 16 patients having no c.14576G>A, 1 had a homozygous c.13342G>A variant, and 2 had 2 variants: c.13342G>A and c.14053G>A as a compound heterozygote. Of the novel 16 variants, 11 of them were not found in 188 normal control Japanese individuals and were all private mutations (only once in one family).

Correlation between the c.14576G>A genotype and clinical phenotype. We compared the clinical features of patients with MMD according to the c.14576G>A genotype, the wild type (genotype GG, as group GG), the heterozygote (genotype GA, as group GA), or the mutant homozygote (genotype AA, as group AA). Age at onset was lower in AA than in GA or GG ($p = 0.002$ or $p = 0.007$) (figure 2A and table e-2). Median age at onset was 3 years in AA, 7 years in GA, and 8 years in GG. Among those with childhood onset (age at onset <15 years), in whom the effect of secondary vascular changes in later life could be ignored and therefore a pure genetic effect could be expected, the association between earlier onset age and the homozygous c.14576G>A genotype was clearly replicated (table e-2). Although the clinical manifestation is different

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A schematic presentation of the RNF213 protein with 3 conserved domains, the genetic variants we have identified, and the genomic structures of 2 RNF213 isoforms (shown from top to bottom). All missense changes, including R4859K (c.14576G>A as larger characters) are indicated. A.A. = amino acids; AAA = ATPases associated with a variety of cellular activities; RING = really interesting new gene. (Based on National Center for Biotechnology Information Reference sequence, NP_065965.4.)

between the childhood-onset group and the adult-onset group, the rates of the patients with this variant, 83.2% (119 of 143 patients) and 79.6% (43 of 54 patients), respectively, were not significantly different. Among adult patients, there was no significant difference in the rate of having this variant between those with familial history (84.6%, 11 of 13 patients) and those without (78.0%, 32 of 41 patients). The univariate Cox regression analysis showed that only the c.14576G>A genotype was the significant predictive variable for age at onset (table e-4). The cumulative incidence of MMD was higher in AA than GA or GG at almost all age distributions (figure 2B), but this tendency was more apparent in the childhood-onset group. Further investigation with more AA and GG patients is necessary for better statistical accuracy. More AA patients were affected before age 4, compared with GA and GG patients ($p < 0.001$) (figure 3A). All AA patients had infarctions at initial presentation.
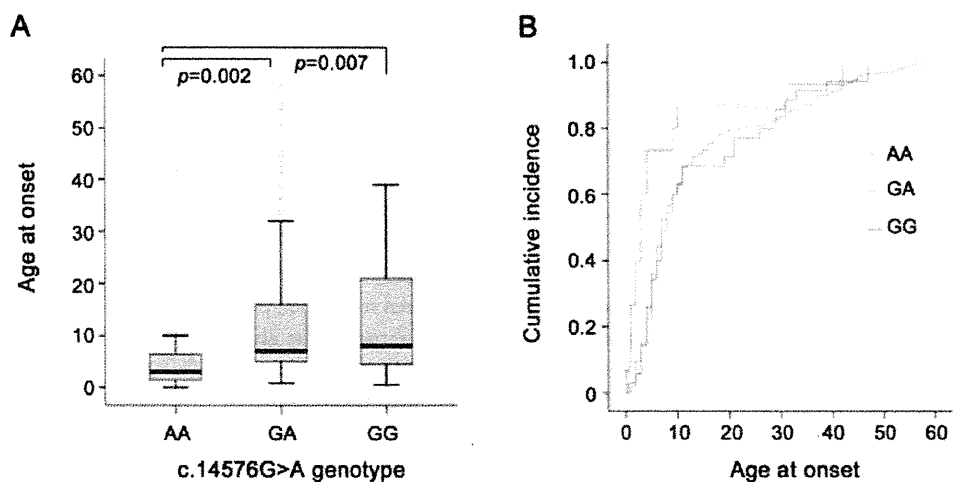
(A) A box plot of age at onset between 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant. O indicates mild outliers; △ indicates extreme outliners. (B) Cumulative incidence curve of the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant.

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Figure 3 Correlation between c.14576G>A variant and clinical features
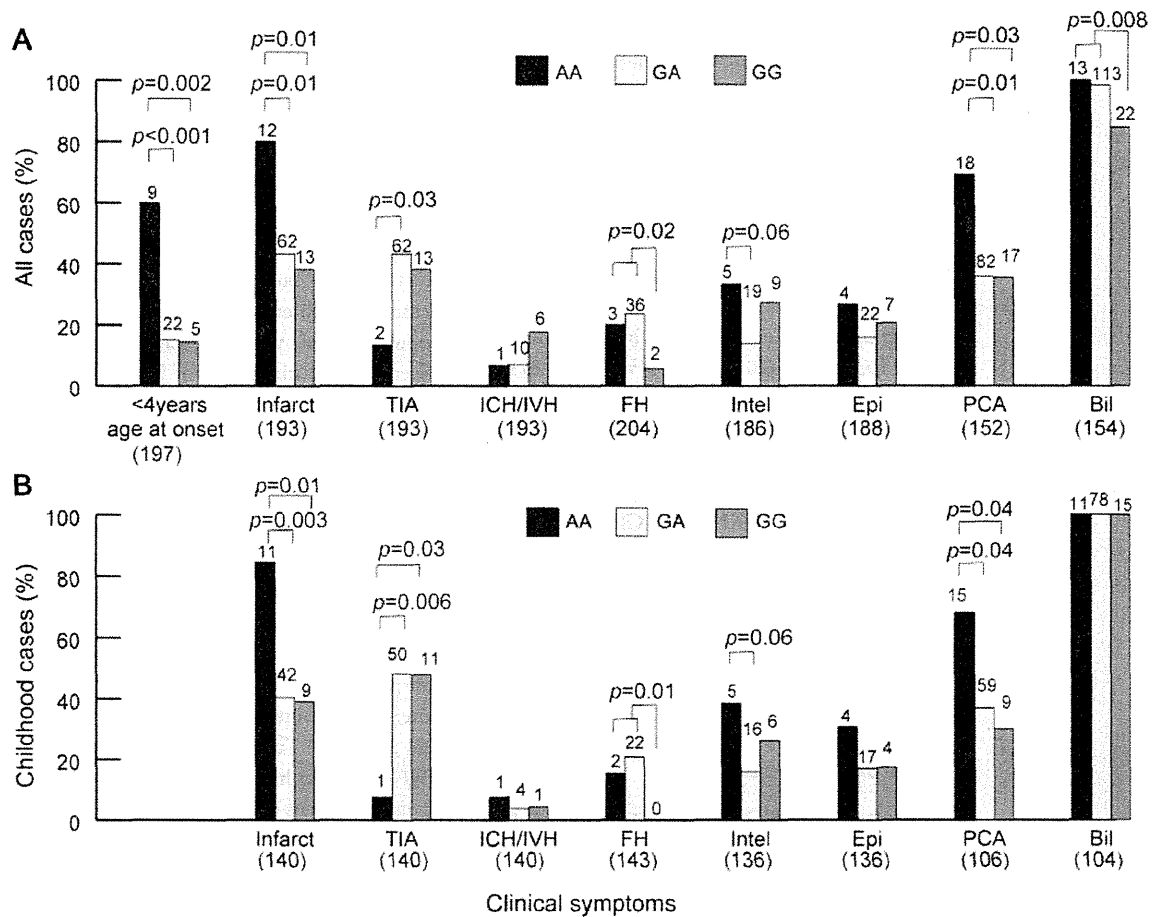
(A) The clinical characteristics of MMD for the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant (204 patients). The numbers of total patients with clinical records regarding either the presence or absence of each characteristic are indicated below the bars, and the numbers of patients in each group are indicated above the respective bars. (B) Clinical characteristics of MMD for the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant among those with age at onset younger than 15 years. The numbers of total patients with clinical records regarding either the presence or absence of each characteristic are indicated below the bars, and the numbers of patients in each group are indicated above the respective bars. Bil = bilateral vasculopathy; Epi = epilepsy; FH = with family history; ICH/IVH = intracranial hemorrhage/intraventricular hemorrhage, Infarct = infarction; Intel = intellectual impairment; PCA = posterior cerebral artery involvement.

The frequencies of other clinical features of MMD in AA, GA, and GG were also compared (figure 3A and table e-2). As the clinical manifestation at diagnosis, infarction was more common in AA than in GA or GG ($p = 0.01$, OR 5.3, 95% CI 1.43–19.56 or $p = 0.01$, OR 6.5, 95%CI 1.53–27.32); TIA was less common in AA than in GA ($p = 0.03$; OR 0.20; 95% CI 0.04–0.94). Bilateral MMD and family history of the disease were more frequent in AA and GA than in GG ($p = 0.008$, OR 11, 95% CI 1.98–66.36 and $p = 0.02$, OR 5.1, 95% CI 1.18–22.36). The number of steno-occlusive PCAs was larger in AA than in GA ($p = 0.01$) (counted as 2 arteries per person). Seventy-four of the 152 patients (48.6%) had PCA lesions, and infarctions and intel-

lectual impairment were more frequent in those with PCA involvement than those without (infarctions 68.9% vs 30.8%, $p < 0.001$; intellectual impairment 26.8% vs 5.3%, $p < 0.001$). Intellectual impairment and epilepsy tended to be a more common complication in AA than in GA, with and without marginal significance. We also compared these clinical features in AA, GA, and GG, excluding 6 patients with unilateral MMD, but the results were not changed (data not shown). Among childhood-onset cases (age at onset <15 years), the associations between the c.14576G>A genotype and these clinical features were generally similar, except for bilateral vasculopathy (all genotypes in childhood-onset cases showed bilateral involvement) (figure 3B).

Correlation between variants other than c.14576G>A and clinical phenotype. We also compared the clinical features of patients with MMD with the other variants, except the c.14576G>A variant, with those without (table e-3). Interestingly, none of the c.14576G>A homozygotes had any other variants. The other patients were categorized into 4 groups, who showed at least one of any individual variants without c.14576G>A (as group GG1), no other variant without c.14576G>A (as group GG0), at least one of any other variants with heterozygous c.14576G>A (as group GA1), and no other variant with heterozygous c.14576G>A (as group GA0). Although there were no differences in age at onset between GG1 and GG0 patients, it was lower in GA0 patients than GA1 patients ($p = 0.03$). Median age at onset was 7 years for GA0 and 12 years for GA1. The frequency of infarctions was lower and that of intracerebral hemorrhage was higher in GA1 than in GA0 ($p = 0.02$, OR 0.19, 95% CI 0.04–0.90 and $p = 0.009$, OR 8.3, 95% CI 2.00–34.19). However, when patients with MMD with another variant, which was predicted to be pathogenic by PolyPhen-2[18] or SIFT[19] algorithms, were compared with those without, consistently no differences in any of these clinical features were observed (data not shown). Further analyses with larger numbers of patients are needed to validate this effect.

Anticipation of MMD. In addition, statistical comparisons of clinical features between 5 parent-offspring pairs having the same *RNF213* genotype (heterozygous c.14576G>A) were performed (table e-5). Age at onset was lower in the second generation than in the first generation ($p = 0.04$). Median age at onset was 5 and 37 years, respectively. This result may support the anticipation of MMD as reported previously.[13] Conversely, age at onset was not different between 6 sibling pairs having the same *RNF213* genotype ($p = 0.67$). Median age at onset was 8 years for the older siblings and 12.5 years for the younger ones. There were no differences in other clinical symptoms among patients from the same pedigree.

DISCUSSION We confirmed a strong association between c.14576G>A in *RNF213* and MMD with the larger number of Japanese patients different from those of the previous studies.[14,15] More importantly, this is the first report showing the significant phenotype-genotype correlation. The OR for the heterozygous c.14576G>A was 236 ($p < 0.001$) and could not be exactly calculated for the homozygote (∞). With the assumption that the effects of both heterozygous and homozygous changes on MMD onset were similar, the homozygous

c.14576G>A variant would increase the risk with an OR of 259 (95% CI 100–674, $p < 0.001$). However, the effect of the homozygous variant on MMD onset was expected to be much larger than that of the heterozygote because no homozygote was found in a total of 283 normal controls and 132 unaffected family members in this study and 429 normal controls and 28 unaffected family members in the previous study.[14] We also showed that the risk of being diagnosed with MMD with the homozygous variant was more than 78%. Although this variant does not exactly fit the pure Mendelian inheritance pattern because it is observed to some extent in the normal population, this variant might have a much larger effect on the pathogenesis of MMD than the common variants of complex diseases, considering its extremely high OR. This rare variant could be an example of missing heritability, that is, the majority of heritability of complex traits that are unexplained by common variants with a small effect size.[20,21] Thus, this variant should not be considered as one of common variants contributing to common diseases.

The c.14576G>A variant has not been found among the total number of 55 Caucasian patients in the previous studies on *RNF213*.[14,15] However, 4 other rare variants were identified in 4 of 50 Caucasian patients.[15] The overall variant detection rate for *RNF213* was as high as 90.2% for our Japanese patients, in contrast to 8% for the Caucasian patients in the previous study.[15] Importantly, 82.4% of our patients were accounted for by the c.14576G>A variant. It was reported that c.14576G>A variant was identified in 90% of Japanese patients, 79% of Korean patients, and 23% of Chinese patients.[15] The founder effect widely distributed in some areas of east Asia was likely to be expected, and this variant could explain the difference of prevalence of MMD between Asian and non-Asian populations.

RNF213 is a RING (really interesting new gene) finger protein containing an AAA (ATPases associated with variety of a cellular activities) domain, indicating that it has E3 ubiquitin ligase activity and energy-dependent unfoldase activity.[14,22] Knockdown of *RNF213* in zebrafish leads to the abnormal sprouting and irregular diameter of intracranial vessels, suggesting its possible contribution to vascular formation.[15] More research on its contribution to MMD pathogenesis will be necessary.

Although the number of adult-onset cases was relatively small, the similar rates of the cases with this variant between childhood-onset patients and sporadic adult-onset patients might suggest that the variant apparently contributes to both groups. Either a heterozygous or homozygous c.14576G>A variant increased the risk for adult-onset MMD (OR 217,

95% CI 72–656, $p < 0.001$) compared with that in adult normal controls.

Whether bilateral and unilateral MMD belong to a single entity is a very important question. Of the 6 patients with unilateral MMD, 2 were heterozygotes and the others were wild types, which indicated a lower frequency of heterozygotes than that in the previous study.[14] Because we showed a significant difference in the frequency of bilateral vasculopathy between GG and other genotypes, we speculate that to some extent patients with unilateral MMD share a genetic background, but there could be different genetic backgrounds in these groups. Further investigation is needed to confirm these findings with larger numbers of patients with unilateral MMD.

The recent spread of brain check-up has increased the opportunity to encounter patients with asymptomatic MMD.[23] Whereas our patients in this study all had symptomatic MMD, it is necessary to further examine the RNF213 variant in the asymptomatic group.

The homozygous c.14576G>A variant carriers showed significantly earlier age at onset, more frequent occurrence of infarctions at initial presentation, and PCA involvement. The association of PCA involvement and infarction or intellectual impairment in our data were compatible with the previous report.[11] These features indicate that c.14576G>A homozygotes have more severe and wider vasculopathy in the brain. The other poor prognostic factors, such as intellectual impairment and epilepsy,[8] were probably more frequent in homozygotes but did not reach statistical significance. We speculated that these conditions might be modified or prevented by early diagnosis and by surgical and medical interventions.

Early surgery for young patients with MMD (<3–4 years of age) has been recommended previously,[24] because they often demonstrate a more severe clinical course.[9,10,24] Approximately 80% of these patients had infarction at initial presentation and had subsequent preoperative infarctions more frequently than patients with older age at onset.[24,25] In our study, 77.1% of the patients diagnosed before age 4 had infarctions at diagnosis, whereas 38% of those diagnosed after age 4 had infarctions ($p < 0.001$), results similar to the previous data.

Conversely, it was demonstrated that young age at onset of symptoms did not always herald a poor later outcome. Instead, neurologic deficits due to infarctions at the time of surgery held the most prognostic value.[7,26] It was recently reported that an irreversible infarction was the greatest risk for an unfavorable outcome by multivariate logistic regression analysis.[8] Specific biomarkers, which might be strongly associated with infarction, would be of invaluable clinical importance to provide the appropriate timing for an operation. In our study, 60% of homozygous c.14576G>A individuals were diagnosed with MMD before age 4, and all of them had infarctions at initial presentation. Thus, the homozygous c.14576G>A variant may be a more specific predictor, which would discriminate those with poor prognosis from those with relatively favorable prognosis among patients with young-onset MMD.

We therefore propose that the homozygous c.14576G>A genotype could be an efficient DNA marker predicting the severe type of MMD with a poor prognosis and a strong biomarker for patients requiring early operation. c.14576G>A genotyping could also be useful to predict the actual risk of severe initial infarctions. Careful follow-up of these high-risk homozygotes could make it possible to undertake intervention before the first infarctions and prevent the irreversible neurologic deficits that can occur in these patients. Thus, the homozygous c.14576G>A variants may provide a better monitoring and prevention strategy. Furthermore, this variant could be very useful in genetic counseling.

REFERENCES

1. Bigi S, Fischer U, Wehrli E, et al. Acute ischemic stroke in children versus young adults. Ann Neurol 2011;70:245–254.
2. Mackay MT, Wiznitzer M, Benedict SL, Lee KJ, Deveber GA, Ganesan V. Arterial ischemic stroke risk factors: the International Pediatric Stroke Study. Ann Neurol 2011;69:130–140.
3. Wakai K, Tamakoshi A, Ikezaki K, et al. Epidemiological features of moyamoya disease in Japan: findings from a nationwide survey. Clin Neurol Neurosurg 1997;99(suppl 2):S1–S5.
4. Kuriyama S, Kusaka Y, Fujimura M, et al. Prevalence and clinicoepidemiological features of moyamoya disease in Japan: findings from a nationwide epidemiological survey. Stroke 2008;39:42–47.
5. Yonekawa Y, Ogata N, Kaku Y, Taub E, Imhof HG. Moyamoya disease in Europe, past and present status. Clin Neurol Neurosurg 1997;99(suppl 2):S58–S60.
6. Kuroda S, Houkin K. Moyamoya disease: current concepts and future perspectives. Lancet Neurol 2008;7:1056–1066.
7. Scott RM, Smith ER. Moyamoya disease and moyamoya syndrome. N Engl J Med 2009;360:1226–1237.
8. Kim SK, Cho BK, Phi JH, et al. Pediatric moyamoya disease: an analysis of 410 consecutive cases. Ann Neurol 2010;68:92–101.
9. Karasawa J, Touho H, Ohnishi H, Miyamoto S, Kikuchi H. Long-term follow-up study after extracranial-intracranial bypass surgery for anterior circulation ischemia in childhood moyamoya disease. J Neurosurg 1992;77:84–89.
10. Kurokawa T, Tomita S, Ueda K, et al. Prognosis of occlusive disease of the circle of Willis (moyamoya disease) in children. Pediatr Neurol 1985;1:274–277.
11. Yamada I, Himeno Y, Suzuki S, Matsushima Y. Posterior circulation in moyamoya disease: angiographic study. Radiology 1995;197:239–246.
12. Yamauchi T, Houkin K, Tada M, Abe H. Familial occurrence of moyamoya disease. Clin Neurol Neurosurg 1997; 99(suppl 2):S162–S167.
13. Nanba R, Kuroda S, Tada M, Ishikawa T, Houkin K, Iwasaki Y. Clinical features of familial moyamoya disease. Childs Nerv Syst 2006;22:258–262.
14. Kamada F, Aoki Y, Narisawa A, et al. A genome-wide association study identifies RNF213 as the first moyamoya disease gene. J Hum Genet 2011;56:34–40.
15. Liu W, Morito D, Takashima S, et al. Identification of RNF213 as a susceptibility gene for moyamoya disease and its possible role in vascular development. PLoS One 2011; 6:e22542.
16. Fukui M. Guidelines for the diagnosis and treatment of spontaneous occlusion of the circle of Willis ('moyamoya' disease): Research Committee on Spontaneous Occlusion of the Circle of Willis (Moyamoya Disease) of the Ministry of Health and Welfare, Japan. Clin Neurol Neurosurg 1997;99(suppl 2):S238–S240.
17. Garritano S, Gemignani F, Voegele C, et al. Determining the effectiveness of high resolution melting analysis for SNP genotyping and mutation scanning at the TP53 locus. BMC Genet 2009;10:5.
18. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248–249.
19. Ng PC, Henikoff S. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res 2003;31: 3812–3814.
20. Eichler EE, Flint J, Gibson G, et al. Missing heritability and strategies for finding the underlying causes of complex disease. Nat Rev Genet 2010;11:446–450.
21. Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. Nature 2009;461: 747–753.
22. Lupas AN, Martin J. AAA proteins. Curr Opin Struct Biol 2002;12:746–753.
23. Ikeda K, Iwasaki Y, Kashihara H, et al. Adult moyamoya disease in the asymptomatic Japanese population. J Clin Neurosci 2006;13:334–338.
24. Kim SK, Seol HJ, Cho BK, Hwang YS, Lee DS, Wang KC. Moyamoya disease among young patients: its aggressive clinical course and the role of active surgical treatment. Neurosurgery 2004;54:840–844.
25. Mugikura S, Higano S, Shirane R, Fujimura M, Shimanuki Y, Takahashi S. Posterior circulation and high prevalence of ischemic stroke among young pediatric patients with Moyamoya disease: evidence of angiography-based differences by age at diagnosis. AJNR Am J Neuroradiol 2011;32:192–198.
26. Scott RM, Smith JL, Robertson RL, Madsen JR, Soriano SG, Rockoff MA. Long-term outcome in children with moyamoya syndrome after cranial revascularization by pial synangiosis. J Neurosurg 2004;100:142–149.

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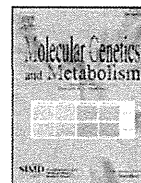
Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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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.

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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

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

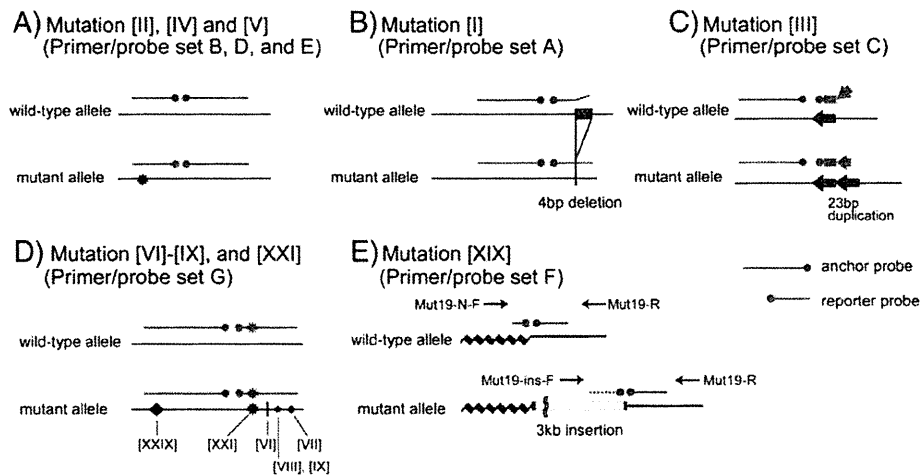**Fig. 1.** Principle of SLC25A13 mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type 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-GTTTTTCCCCTACAGAC**GACC**-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-CACTAACCTCTATACAGTCCA-P	
C		GCAGTTCAAAGCACAGTTATTtttatatagtgagaatgtgaccagactgagatggtgttgtgtctctcctgcaggtatgcctgcagcatctttagtg acccctgctgatgttatcaagacgagattacaggtg 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		TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctattttaatggatttaatt**c**gctccttaacaac atggaactcattagaaagatctatagcactc tggctggcaccaggaaagatgttgaagtGACTAAGGGTGAGTGAGAA (164 bp)	
	Mut4-F	TCAATTTATTTGAGGCTGC	0.5
	Mut4-R	TTCTCACTCACCCTTAGTC	0.5
	Mut4-UP	AATGGATTTAATTCGCTCCTTAACA-FITC	
	Mut4-DW	LC Red640-ATGGAACTCATTAGAAAGATCTATAGCACTC-P	
E		TGCACAAAGATGGTTCGgtcccacttgcagcagaaattcttgctggaggctgc**gt**aagtaccttttgaagctctcttcattgaaaagacttgtttcac atatatatcactaccatggtcaacaggtgtggactaaggcttctgttTAACCACAGATCCTGCA (162 bp)	
	Mut5-F	TGCACAAAGATGGTTCG	0.5
	Mut5-R	TGCAGGATCTGTGGTTA	0.5
	Mut5-UP	GTGAAACAAGTCTTTTCAATGAAGAGAGCTTC-FITC	
	Mut5-DW	LC Red640-AAGGTACTTACGCAGCCTC-P	
F	normal allele	GGAGCTGGTGGTATGGAAataatgtgttcttaactaactctttggtatcaggtaaattttaaaatatctaattatatctgtgatttctc catttttttaaagctcgtgtatttcgatcctcaccccagtttggt gtaactttgctgacttacgaattgctacagcgatggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaatttt GGCTGCTGCTAATGCTC (244 bp)	
	insertion allele	CCATCTTCCTCCTCCCTTggcagccccgcccccgatttctccattttttaaagctcgtgtatttcgatcctcaccccagtttggt gtaactttgctgacttacgaattgctacagcgatggttctacattgatttt ggaggagtgtaagtatcatgctaaatctgctgctaaatttGGCTGCTGCTAATGCTC (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		TCTTAACTAACTCTTTGGTATCAGGTaaattttaaaatatctaattatatctgtgatttctccatttttttaaagctcg tgtatttcgatcctcaccccagtttggtgtaactttgctgactta(**a**)**c**gaattgctacagcga 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-GCGGACTTA**C**G**A**ATTGCTACAGCGA-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.
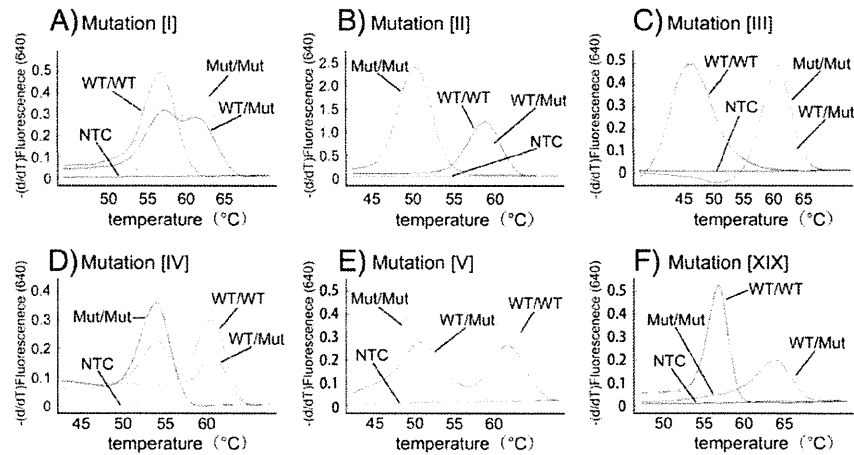
Fig. 2. Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

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
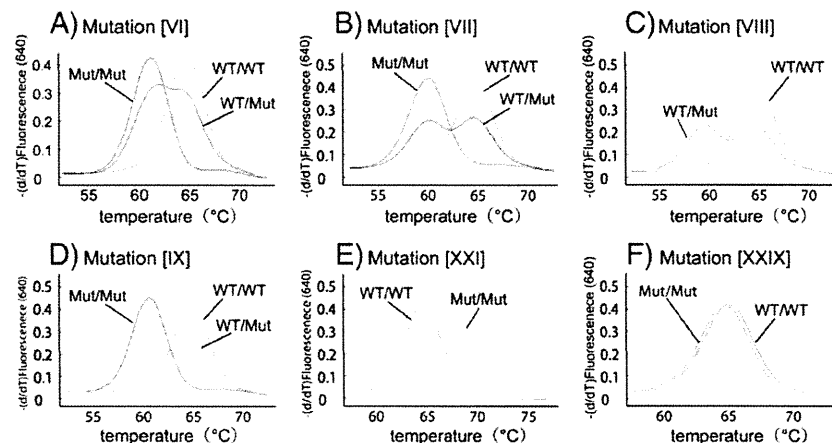

Fig. 3. Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor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

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.

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.

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,
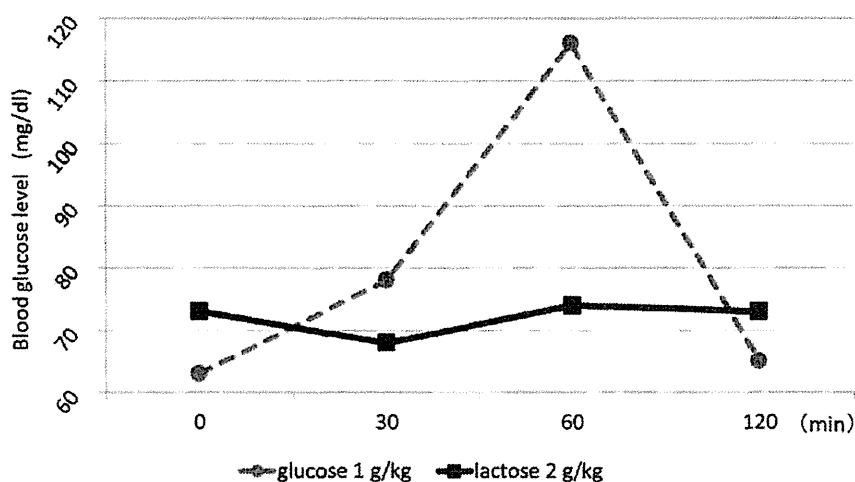

Fig. 1. Oral lactose challenge test at the age of 4 months.
　　　After oral administration of 2 g/kg of lactose, no increase was observed in the blood glucose within 120 min and watery diarrhea developed. In contrast, a marked increase was observed in the blood glucose level after drinking 1 g/kg of glucose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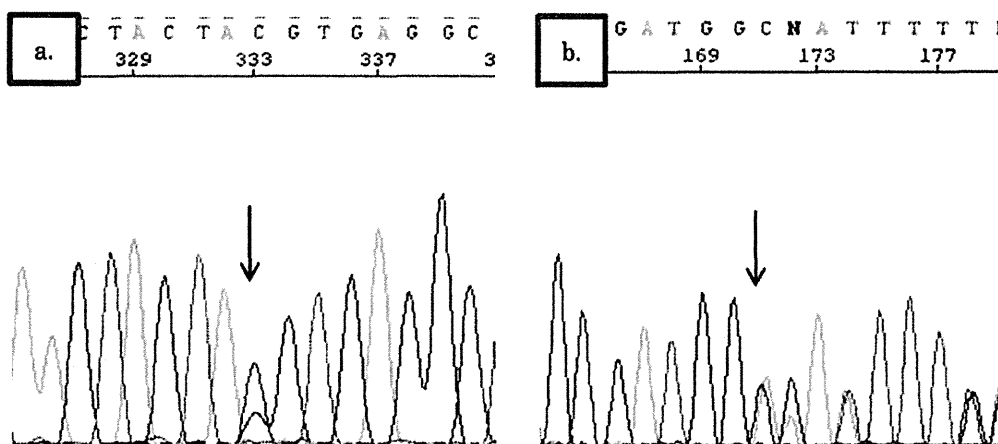

Fig. 2. Sequence analysis of the LCT gene.
　　　a. LCT Ex10 c.4419C>G (p.Y1473X). b. LCT Ex16 c.5387delA (p.D1796fsX)

0.9, 1.1, 1.3, and 1.0, respectively). The oral lactose challenge test was repeated at the age of 5 months, and the patient continued to show a lactose-intolerance pattern (no increase in the blood glucose level).

After experiencing acute gastroenteritis, she passed blood-tinged stools for a few weeks and underwent sigmoid colonoscopy with biopsy. The colonoscopy showed lymphoid nodular hyperplasia with patchy erythema, and histological examination indicated a relatively high number of eosinophils (about 10 per high power field) and a small number of neutrophils in the lamina propria. Although these findings were consistent with those of mild proctocolitis, they were not sufficiently strong to confirm the presence of a food allergy.

To confirm whether lactose intolerance was primary or secondary, we performed sequence analysis of 17 exons of the LCT gene after the patient's parents provided written informed consent. The Ethics Committee of the Tohoku University School of Medicine approved the present study. The result showed two novel mutations: c.4419C>G (p.1473X) in exon 10 and c.5387delA (p.D1796fs) in exon 16 (Fig. 2). The p.Y1473X mutation was transmitted from her mother, and the other mutation (p.D1796fs) was transmitted from her father.

The patient is now administered a lactose-free diet, and her psychomotor development was appropriate for her age at the latest examination at the age of 11 months.

Discussion

CLD is one of the rare autosomal disorders commonly occurring in the Finnish population because of a founder effect and genetic drift. A few cases of CLD in patients with different ethnic origins have also been reported. The incidence of CLD was estimated to be 1:60 000 newborns in Finland on the basis of the number of patients who had been diagnosed until 1998 (Järvelä et al. 1998). After the molecular background of CLD was confirmed, the number of patients newly diagnosed with CLD in Finland increased, and the novel LCT mutations were reported in the CLD patients with different ethnic origins (Torniainen et al. 2009). In Japan, only few cases of CLD have been reported since Akabane and Arakawa published the first case in 1965 (Akabane 1965; Yabuuchi et al. 1966; Nose et al. 1979). Infants who develop severe watery diarrhea after consuming breast milk/lactose-containing formula are unlikely to be suspected of having CLD because this disease is thought to be very rare.

The nascent lactase polypeptide comprises four homologous domains, I-IV. After posttranslational processing, the mature lactase contains only domains III and IV. Domain IV comprises lactase activity, and domains I-III act as intramolecular chaperone which is critical for the maturation during lactase-folding process (Kuokkanen et al. 2006; Behrendt et al. 2009). To date, nine mutations are known to underlie CLD and there are quite evenly distributed covering both the pro-region and the mature lactase

(Kuokkanen et al. 2006; Torniainen et al. 2009). Five of them result in a premature stop codon. One of the missense mutations, G1363S, located in the domain III, leads to defective lactase activity and impaired trafficking of mutant lactase polypeptide to the cell surface at physiological temperature (Behrendt et al. 2009).

In the case of our patient, she was suspected of having CLD or cow's milk protein allergy in the early neonatal period, because her symptoms improved with a change in her diet from breast milk/lactose-containing formula to hydrolyzed cow's milk formula, which is lactose-free. Small bowel biopsy would be useful to distinguish CLD from cow's milk protein allergy (Heyman 2006), but it is an invasive procedure and requires excellent technical skills. Accordingly, since it would need to have been performed in a 4-month old baby, we performed sequence analysis as the diagnostic examination. Sequence analysis revealed that she has one nonsense mutation and one frame-shift mutation in domain IV. These mutations lead to premature truncation of lactase protein being causative of CLD. Sequence analysis would be useful for the diagnosis if a CLD patient also has cow's milk protein allergy.

Our findings suggest that CLD is possibly more common in Japan than it was thought to be. CLD patients may be treated as patients with cow's milk protein allergy, using lactose-free hydrolyzed cow's milk formula. Sequence analysis is useful for diagnosing CLD, which is sometimes difficult to distinguish from cow's milk protein allergy. Pediatricians should have an increased awareness regarding CLD.

Conflict of Interest

The authors have no conflict of interest associated with this article.

References

Akabane, J. (1965) Lactose Intorelance. The Jounal of Pediatric Practice, 28, 697-703 (in Japanese).
Behrendt, M., Keiser, M., Hoch, M. & Naim, H.Y. (2009) Impaired trafficking and subcellular localization of a mutant lactase associated with congenital lactase deficiency. Gastroenterology, 136, 2295-2303.
Heyman, M.B. (2006) Committee on Nutrition. Lactose intolerance in infants, children, and adolescents. Pediatrics, 118, 1279-1286.
Järvelä, I., Enattah, N.S., Kokkonen, J., Varilo, T., Savilahti, E. & Peltonen, L. (1998) Assignment of the locus for congenital lactase deficiency to 2q21, in the vicinity of but separate from the lactase-phlorizin hydrolase gene. Am. J. Hum. Genet., 63, 1078-1085.
Kuokkanen, M., Kokkonen, J., Enattah, N.S., Ylisaukko-Oja, T., Komu, H., Varilo, T., Peltonen, L., Savilahti, E. & Järvelä, I. (2006) Mutations in the translated region of the lactase gene (LCT) underlie congenital lactase deficiency. Am. J. Hum. Genet., 78, 339-344.
Nose, O., Iida, Y., Kai, H., Harada, T., Ogawa, M. & Yabuuchi, H. (1979) Breath hydrogen test for detecting lactose malabsorption in infants and children. Prevalence of lactose malabsorption in Japanese children and adults. Arch. Dis. Child., 54, 436-440.

Savilahti, E., Launiala, K. & Kuitunen, P. (1983) Congenital lactase deficiency. A clinical study on 16 patients. *Arch. Dis. Child.*, **58**, 246-252.

Torniainen, S., Freddara, R., Routi, T., Gijsbers, C., Catassi, C., Höglund, P., Savilahti, E. & Järvelä, I. (2009) Four novel mutations in the lactase gene (LCT) underlying congenital lactase deficiency (CLD). *BMC Gastroenterol.*, **9**, 8.

Yabuuchi, H., Ishii, T. & Iida, Y. (1966) A case report of lactase insufficiency. *The Journal of Pediatric Practice*, **29**, 1058-1063 (in Japanese).

PostScript

LETTER

Identification of novel missense CTRC variants in Japanese patients with chronic pancreatitis

We read with great interest the article by Paliwal et al[1] reporting the identification of novel missense variants in the chymotrypsin C (CTRC) gene in patients with tropical pancreatitis and idiopathic chronic pancreatitis (CP) in India. Because there might be geographical differences in the genetics of pancreatitis, we conducted screening of the CTRC gene in Japanese patients with CP. All the eight exons in the CTRC gene were directly sequenced in 506 patients with CP (244 alcoholic, 206 idiopathic, 35 hereditary and 21 familial) and 274 healthy controls as previously described.[1] This study was approved by the ethics committee of Tohoku University School of Medicine (article #2012-1-158). We identified five missense variants in patients with CP and one in controls (table 1).

Four variants were located in exon 7, 1 in exon 2, and 1 in exon 6. All missense variants were in a heterozygous form. Five of these six variants were novel ones. The c.86G>A (p.R29Q) and c.716C>G (p.S239C) variants were found in patients with alcoholic CP, whereas the c.715T>G (p.S239A) was found in a patient with idiopathic CP. The previously reported c.760C>T (p.R254W) variant was found in a patient with idiopathic CP, who also had the p.N34S variant in the serine protease inhibitor Kazal type I (SPINK1) gene in a heterozygous form. The c.739A>G (p.K247E) variant was found in a patient with alcoholic CP and two patients with idiopathic CP. The combined frequency of the missense CTRC variants was not statistically different between patients with CP (7/506) and controls (1/274) (p=0.27, Fisher's exact test). In addition to these missense variants, two synonymous variants, c.180C>T and c.285C>T, were found both in patients with CP and controls at the similar frequencies. Previous reports have shown an association of the CTRC variants with CP in Europe[2 3] and India.[1] In European cohorts, the microdeletion variant c.738_761del24 (p.K247_R254del) and p.R254W, both located in exon 7, are the most common CTRC variants. In India, these variants are rare, and the c.217G>A (p.A73T) variant in exon 3 and the c.703G>A (p.V251I) variant are the most common ones.[1] These common variants in Europe and India were very rare in Japanese subjects, and only one patient with idiopathic CP had the p.R254W variant. Our results show that the spectrum and distribution of the CTRC variants in Japanese subjects with CP were different from those reported from Europe and India. In addition, the synonymous variant c.180C>T was common and was found in 32.7% and 27.2% of the patients with CP in India[1] and France,[3] respectively. This variant was significantly associated with CP (32.7% in patients vs 15.2% in controls) in India.[1] However, the c.180C>T variant is very rare in the Japanese population, further suggesting geographical differences in the distribution of the CTRC variants. It would be of interest to see the functional consequence of the missense variants identified in this study. Beer et al[4] have identified three different loss-of-function mechanisms caused by the CTRC variants: reduced secretion with associated endoplasmic reticulum stress, decreased catalytic activity, and degradation by trypsin. Obviously, further studies are required to establish the role of the novel missense variants in the pathogenesis of pancreatitis.

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REFERENCES
1. **Paliwal S,** Bhaskar S, Mani KR, et al. Comprehensive screening of chymotrypsin C (CTRC) gene in tropical calcific pancreatitis identifies novel variants. Gut Published Online First: 12 May 2012. doi:10.1136/gutjnl-2012-302448.
2. **Rosendahl J,** Witt H, Szmola R, et al. Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis. Nat Genet 2008;**40**:78–82.
3. **Masson E,** Chen JM, Scotet V, et al. Association of rare chymotrypsinogen C (CTRC) gene variations in patients with idiopathic chronic pancreatitis. Hum Genet 2008;**123**:83–91.
4. **Beer S,** Zhou J, Szabó A, et al. Comprehensive functional analysis of chymotrypsin C (CTRC) variants reveals distinct loss-of-function mechanisms associated with pancreatitis risk. Gut Published Online First: 1 September 2012. doi:10.1136/gutjnl-2012-303090.

Table 1 CTRC variants in the exons identified in this study

CTRC Variants	Total CP (%)	Alcoholic CP (%)	Non-alcoholic CP (%)	Controls (%)	p Value (Total CP vs Controls)
Missense variants					
Exon 2					
c.86G>A (p.R29Q)	1/506 (0.2)	1/244 (0.4)	0/262 (0)	0/274 (0)	>0.99
Exon 6					
c.627C>G (p.I209M)	0/506 (0)	0/244 (0)	0/262 (0)	1/274 (0.4)	0.35
Exon 7					
c.715T>G (p.S239A)	1/506 (0.2)	0/244 (0)	1/262 (0.4)	0/274 (0)	>0.99
c.716C>G (p.S239C)	1/506 (0.2)	0/244 (0)	1/262 (0.4)	0/274 (0)	>0.99
c.739A>G (p.K247E)	3/506 (0.6)	1/244 (0.4)	2/262 (0.8)	0/274 (0)	0.56
c.760C>T (p.R254W)	1/506 (0.2)	0/244 (0)	1/262 (0.4)	0/274 (0)	>0.99
CTRC missense variants total	7/506 (1.4)	2/244 (0.8)	5/262 (2.0)	1/274 (0.4)	0.27
Synonymous variants					
c.180C>T (p.(=))	1/506 (0.2)	1/244 (0.4)	0/262 (0)	0/274 (0)	>0.99
c.285C>T (p.(=))	51/506 (10.1)	23 /244 (9.4)	28/262 (10.7)	24/274 (8.8)	0.61

Nomenclature follows the guidelines recommended by the Human Genome Variation Society (http://www.hgvs.org/): cDNA-based numbering with A of the ATG translational initiation codon ascribed +1. GenBank accessions NM_007272.2 and NC_000001.10 were used as the reference mRNA and genomic DNA sequences, respectively.
CP, chronic pancreatitis.

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