539delAC and 1159insA mutations cause a frame shift of the CD36 gene, resulting in the formation of a stop codon and a marked reduction in the CD36 messenger RNA level [8,21]. DNA was extracted from whole blood and amplified by polymerase chain reaction. The polymerase chain reaction products were digested with endonuclease, electrophoresed on a 4% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME), and stained with ethidium bromide for restriction fragment length polymorphism analysis.

When these common mutations were not detected, we directly determined the sequences covering all exons and exon-intron boundaries [22].

2.6. Statistical analysis

Values between groups were compared using the Mann-Whitney *U* test. Values at 2 time points within the group were compared using the 1-factor analysis of variance test. Changes in parameters (Δ values) between 2 time points among the groups were compared using the Mann-Whitney *U* test. All *P* values < .05 were considered significant.

3. Results

3.1. Prevalence of CD36 deficiency

Of 53 children with histories of episodic hypoglycemia, 2 were diagnosed with glycogen synthase and glutamate dehydrogenase deficiencies, respectively, which were confirmed by gene analyses (Fig. 1). The parents of the child with glycogen synthase deficiency were first cousins.

The remaining 51 children without any abnormalities in hormones, metabolic profiles, and muscle enzymes such as creatine kinase and aldolase were divided into 3 hypoglycemic groups: type I, type II, and wild-type groups. The numbers of type I, type II, and wild-type among these children were 6 (2 girls and 4 boys), 4 (2 girls and 2 boys), and 41 (18 girls and 23 boys), respectively. Accordingly, the prevalence of type I and II deficiencies were 11.8% and 7.8%, respectively.

Of the 49 healthy control children, 3 children (2 girls and 1 boy) exhibited the expression pattern of type II CD36 deficiency (6.1%); but no one showed that of type I deficiency.

3.2. Gene mutations of CD36 deficiency

Of the 6 children with type I CD36 deficiency, 5 were homozygous for the C478T mutation and 1 was compound heterozygous for C478T/1159insA. Of the 7 children with type II CD36 deficiency, 1 girl in the control group had a heterozygous T970C mutation (a substitution of C for T at nt 970 in exon 9) in CD36 gene reported by Hanawa et al (Fig. 1) [22].

3.3. Comparisons of clinical features

In general, the clinical features of children with type I CD36 deficiency were similar to those of control children as well as to those of other children with episodic hypoglycemia. However, the number of hypoglycemic episodes in the type I group was significantly greater than that in the other groups. It should also be noted that the wild-type CD36 hypoglycemic group showed lower body weight SD scores than the other 3 groups (Table 1).

3.4. Effects of extended fasting on glucose and FA metabolism

Blood glucose concentrations were always significantly lower in the type I CD36 group than in the other 2 hypoglycemic groups (type II CD36 deficiency and wild-type groups) and the control group (Fig. 2A, left panel).

Table 1
Characteristics of the hypoglycemic and control groups

Group	Hypoglycemic group (n = 51)				Controls (n = 49)
	Type I[a] (n = 6)	Type II[a] (n = 4)	Wild type[a] (n = 41)	Total (n = 51)	
Age, y	3.2 ± 0.7	3.8 ± 1.0	3.7 ± 0.6	3.6 ± 0.7	3.3 ± 0.3
Sex, F/M	2/4	2/2	18/23	22/29	22/27
No. of episodes, ranges	1.9 ± 0.5† (1–4)	1.0 ± 0 (1)	1.2 ± 0.3 (1–2)	1.3 ± 0.4	0
BW SD score	−0.4 ± 0.5	−0.1 ± 0.7	−0.9 ± 0.5*	−0.6 ± 0.7	0.2 ± 0.6
Ht SD score	0.1 ± 0.6	−0.2 ± 0.6	0.3 ± 0.6	0.2 ± 0.6	0.4 ± 0.6
Total protein, g/dL	6.8 ± 0.2	6.9 ± 0.3	6.6 ± 0.2	6.7 ± 0.2	6.8 ± 0.3
Albumin, g/dL	3.9 ± 0.2	4.0 ± 0.2	3.9 ± 0.2	3.9 ± 0.2	4.1 ± 0.2
AST, IU/L	19 ± 3	17 ± 4	16 ± 3	17 ± 3	16 ± 5

Values are mean ± SD. BW indicates body weight; Ht, height; AST, aspartate aminotransferase.

[a] Hypoglycemic group was classified into 3 subgroups according to CD36 phenotypes: type I CD36 deficiency (type I), type II CD36 deficiency (type II), and wild-type groups.

* $P < .05$ vs controls (Mann-Whitney *U* test).

† $P < .001$ vs controls (Mann-Whitney *U* test).

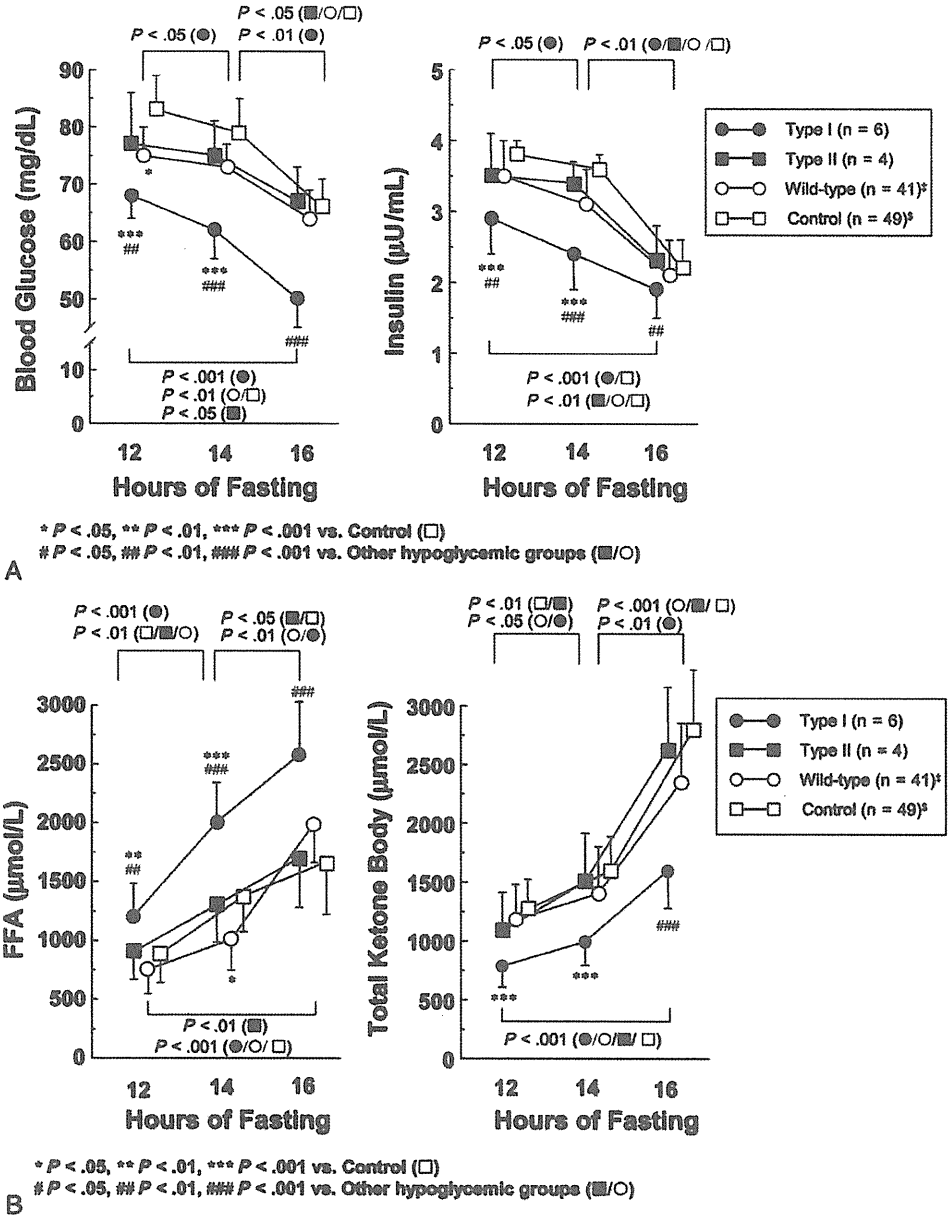

Fig. 2. Differences in BG, insulin, FFAs, and TKB concentrations at the 12-, 14-, and 16-hour fasting points. Children were not allowed to ingest any food or drink after supper on the day before blood sampling. #Data obtained from 24 of the 41 wild-type group children are presented. $Data obtained from 17 of the 49 control group children are presented.

After 16 hours of fasting, BG concentrations in the type I CD36 deficiency group were less than 50 mg/dL. Serum insulin concentrations showed results similar to those of BG concentrations (Fig. 2A, right panel).

The FFA concentrations were always significantly higher in the type I CD36 group than in the other 3 groups (Fig. 2B, left panel). The ΔFFA between 12 and 14 hours was larger in the type I CD36 deficiency group ($P < .001$) than in the other 3 groups ($P < .01$).

The TKB concentrations were always lower in the type I CD36 group than in the other 3 groups. The last 2 hours of fasting induced a great increase in TKB in all the groups. However, the ΔTKB was significantly smaller in the type I CD36 group ($P < .01$) than in the other groups ($P < .001$) (Fig. 2B, right panel).

Unlike type I CD36 group, these parameter levels in the type II CD36 group were consistently similar to those in the wild-type and control groups and did not show any significant differences.

At any fasting point, serum lipid concentrations were not significantly different among the groups. Triglyceride concentration but not other lipid concentrations exhibited

Table 2
Serum lipid concentrations at 12, 14, and 16 hours of fasting in the hypoglycemic and control groups

Group	Hypoglycemic group (n = 51)									Controls (n = 49)		
Subgroup	Type I (n = 6)			Type II (n = 4)			Wild type (n = 41)					
Hours of fasting	12	14	16	12	14	16	12	14	16[a]	12	14	16[b]
TC	152 ± 20	149 ± 22	144 ± 19	155 ± 17	151 ± 20	147 ± 22	142 ± 11	138 ± 12	137 ± 10	151 ± 13	149 ± 12	145 ± 8
TG	53 ± 15	49 ± 9	44 ± 12†	56 ± 17	49 ± 15	47 ± 15*	51 ± 12	47 ± 10	44 ± 10†	51 ± 10	47 ± 10	45 ± 8†
HDL-C	56 ± 12	56 ± 11	54 ± 9	55 ± 10	57 ± 9	55 ± 9	56 ± 10	55 ± 10	56 ± 11	56 ± 14	55 ± 13	55 ± 12
LDL-C	85 ± 13	82 ± 11	81 ± 11	89 ± 16	83 ± 14	81 ± 15	80 ± 19	77 ± 15	74 ± 15	80 ± 13	78 ± 11	77 ± 11

Values are mean ± SD (in milligrams per deciliter). At any fasting point, there were no significant differences in lipid concentrations between each 2 groups (Mann-Whitney *U* test). Triglyceride concentrations but not other lipid concentrations exhibited significant changes between the 12- and 16-hour fasting points in respective groups:*$P < .05$ and †$P < .01$ (1-factor analysis of variance test). There were no significant differences in changes in lipid concentrations (Δ values) between 2 fasting points among the groups (Mann-Whitney *U* test). TC indicates total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

[a] Data obtained from 24 of the 41 wild-type children were presented.

[b] Data obtained from 17 of the 49 controls were presented.

significant changes between the 12- and 16-hour fasting points in the respective groups (Table 2).

4. Discussion

The present study showed that fasting BG concentration was lower in preschool children of type I CD36 deficiency than in children of type II/wild-type CD36 hypoglycemic groups and control children.

Type I CD36 deficiency accounted for 11.8% of 51 children with histories of hypoglycemia but was not diagnosed in the 49 age-matched control children without histories of hypoglycemia. On the other hand, the prevalence of type II CD36 deficiency was not different between the hypoglycemic and control children. According to earlier reports, the prevalence of type I CD36 deficiency in the general Japanese population is 0.5% to 1.0% [9-10,15]. It is, therefore, plausible that this deficiency is highly prevalent among hypoglycemic children. However, the children groups enrolled in this study were too small to define the reliable prevalence. To gain more informative data, more extensive studies would be essential.

Besides fasting BG level, fasting insulin, TKB, and FFA concentrations in type I CD36 group were different from those in the other groups. We found that fasting insulin and TKB concentrations were significantly lower in the type I group than in the other groups, whereas the fasting FFA level was significantly higher in the type I group. These differences were more prominent as the fasting time was extended (Fig. 2A, B).

High FFA levels at the fasting state can probably be attributed to impaired FFA uptake by skeletal and heart muscles in type I CD36 deficiency. Free FA is used as an energy source in skeletal and heart muscles, as well as in the liver. Long-chain FAs enter cells either by passive diffusion or by transporter-dependent uptake. In humans, there are 3 transporters for LCFA: CD36, plasma membrane-associated FA binding protein (43 kd), and FA transport protein (FATP, 63 kd) [23–25]. Although plasma membrane-associated FA binding protein and FATP are expressed in the liver as well as skeletal and heart muscles, CD36 is expressed in skeletal and heart muscles, but not in the liver. Most serum LCFAs are bound to albumin. CD36 promotes the dissociation of LCFA from albumin, which results in increased passive diffusion of LCFA [24]. Furthermore, CD36 is coexpressed with FATP in skeletal and heart muscles [24]. This colocalization strongly suggests that the FATP-mediated uptake of LCFA is promoted by an interaction with CD36 in skeletal and heart muscles. In patients with type I CD36 deficiency, the radiolabeled LCFA analog ^{123}I-15-(*p*-idiophenyl)-(*R,S*)-methylpentadecanoic acid (BMIPP) does not accumulate in heart muscles; and BMIPP clearance from the circulation is delayed [5]. Furthermore, the oxidation rate of LCFA is reduced by 40% to 60% in the isolated hearts of CD36-deficient mice [25]. These observations agree well with our hypothesis.

In skeletal and heart muscles, impaired FA uptake in CD36 deficiency is probably compensated by shifting the energy supply from FA dominant to glucose dominant. In fact, CD36-deficient rats had high glucose oxidation rates and maintained normal myocardial functions [26]. If this is the case in humans, it would be a rationale for the increased susceptibility of CD36-deficient children to hypoglycemia in the fasting state. Similar to CD36-deficient children, CD36-deficient rats had higher serum FFA concentrations; and their BG concentrations were lower than those observed in control rats [27].

Nevertheless, the findings obtained from this study were not consistent with those in CD36-deficient adults described in earlier reports [10-13]. It has been shown that CD36 deficiency predisposes one to insulin resistance and the subsequent hyperglycemia. Most of the subjects described in such reports were older than 30 years, and young subjects have been scarcely examined. Yanai et al [14] previously reported increased insulin sensitivity in young adults with CD36 deficiency, suggesting different effects of CD36 deficiency on carbohydrate metabolism by age. To gain a

better understanding of the relationship between CD36 deficiency and insulin sensitivity, we will perform more extensive studies covering large groups of children and adolescents.

The energy demands of young children exhibiting rapid growth and development are quite high. Their glucose and FA metabolisms must be greatly activated. In fact, ketone body synthesis via FA β-oxidation is far greater in young children than in adults [19,28]. Therefore, impaired FA metabolism must have a more serious effect in children than in adults, although both have the same genetic disorder (CD36 deficiency).

The reason for the low TKB levels in type I CD36 deficiency remains to be elucidated. Ketone bodies are generated mainly in the liver and secreted into the blood circulation. The substrate of ketone bodies is acetyl–coenzyme A, which is a product of FA β-oxidation. From this context, we speculated that decreased FA β-oxidation in heart and muscles led to the limited synthesis of ketone body from acetyl–coenzyme A. In type I CD36 deficiency, hepatic uptake of BMIPP (an analog of LCFA) is nearly double [5], probably because CD36 is naturally absent in normal liver and the other transporters play significant roles in hepatic uptake of LCFA [23-25]. As a whole, ketone body synthesis along with FA β-oxidation in type I CD36 deficiency was substantially but not definitely reduced.

The results of this study suggested that type I CD36 deficiency predisposes preschool children to hypoglycemia. If this is true, affected children should avoid long-time fasting and excess exercise without sugar supplementation.

References

[1] Greenwalt DE, Lipsky RH, Ockenhouse CF, Ikeda H, Tandon NN, Jamieson GA. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction and transfusion medicine. Blood 1992; 80:1105-15.

[2] Ge Y, Eighetany MT. CD36: multiligand molecule. Lab Hematol 2005;11:31-7.

[3] Silverstein RL, Asch AS, Nachman RL. Glycoprotein IV mediated thrombospondin-dependent platelet-monocyte and platelet-U937 cell adhesion. J Clin Invest 1989:84:546-52.

[4] Nozaki S, Kashiwagi H, Yamashita S, et al. Reduced uptake of oxidized low-density lipoprotein in monocyte-derived macrophages from CD36-deficient subjects. J Clin Invest 1995;96:1859-65.

[5] Yoshizumi Y, Nozaki S, Fukuchi K, Yamazaki K, Fukuchi K, Maruyama T, et al. Pharmacokinetics and metabolism of 123I-BMIPP fatty acid analog in healthy and CD36-deficient subjects. J Nuclear Med 2000;41:1134-8.

[6] Yamamoto N, Akamatsu H, Sakuraba H, Yamazaki H, Tanoue K. Platelet glycoprotein IV (CD36) deficiency is associated with the absence (type I) or the presence (type II) of glycoprotein IV on monocytes. Blood 1994;83:392-7.

[7] Kashiwagi H, Tomiyama Y, Honda S, et al. Molecular basis of CD36 deficiency. Evidence that a 478C→T substitution (proline90→serine) in CD36 cDNA accounts for CD36 deficiency. J Clin Invest 1995;95: 1040-6.

[8] Kashiwagi H, Tomiyama Y, Nozaki S, et al. A single nucleotide insertion in codon 317 of the CD36 gene leads to CD36 deficiency. Arterioscler Thromb Vasc Biol 1996;16:1026-32.

[9] Yanai H, Chiba H, Fujiwara H, et al. Phenotype-genotype correlation in CD36 deficiency types I and II. Thromb Haemost 2000;84:436-41.

[10] Furuhashi M, Ura N, Nakata T, Shimamoto K. Insulin sensitivity and lipid metabolism in human CD36 deficiency. Diabetes Care 2003;26: 471-4.

[11] Aitman JA, Glazier AM, Wallace CA, et al. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. Nat Genet 1999;21: 76-83.

[12] Miyaoka K, Kuwasako T, Hirano K, Nozaki S, Yamashita S, Matsuzawa Y. CD36 deficiency with insulin resistance. Lancet 2001;357:686-7.

[13] Masuda D, Hirano K, Oku H, et al. Chylomicron remnants are increased in the postprandial state in CD36 deficiency. J Lipid Res 2009;50:999-1011.

[14] Yanai H, Chiba H, Morimoto M, et al. Metabolic changes in human CD36 deficiency displayed by glucose loading. Thromb Haemost 2001;86:995-9.

[15] Teraguchi M, Ikemoto Y, Unishi G, Ohkohchi H, Kobayashi Y. Influence of CD36 deficiency on heart disease in children. Circ J 2004; 68:435-8.

[16] Haworth JC, Coodin FJ. Idiopathic spontaneous hypoglycemia in children. Report of seven cases and review of the literature. Pediatrics 1960;25:748-65.

[17] Daly LP, Osterhoudt KC, Weinzimer SA. Presenting features of idiopathic ketotic hypoglycemia. J Emerg Med 2003;25:39-43.

[18] Pershad JMonroe K, Atchison J. Childhood hypoglycemia in an urban emergency department epidemiology and a diagnostic approach to the problem. Pediatr Emerg Care 1996;14:268-71.

[19] Roe CR, Ding JH. Mitochondria fatty acid oxidation disorders. In: Scriver CR, Baudet AL, Valle D, Sly WS, editors. The metabolic and molecular bases of inherited disease. 8th ed. New-York: McGraw-Hill; 2001. p. 2297-326.

[20] Shigematsu Y, Hata I, Kikawa Y, et al. Modifications in electrospray tandem mass spectrometry for a neonatal-screening pilot study in Japan. J Chromatgr B Biomed Sci Appl 1999;731:97-103.

[21] Kashiwagi H, Tomiyama Y, Kosugi S, et al. Identification of molecular defects in a subject with type I CD36 deficiency. Blood 1994;83: 3545-52.

[22] Hanawa H, Watanabe K, Nakamura T, et al. Identification of cryptic splice site, exon skipping, and novel point mutations in type I CD36 deficiency. J Med Genet 2002:39286-91.

[23] Storch J, Thumser AE. The fatty acid transporter function of fatty acid-binding proteins. Biochim Biophys Acta 2000;1486:28-44.

[24] Storch J, Veerkamp JH, Hsu KT. Similar mechanisms of fatty acid transfer from human anal rodent fatty acid-binding proteins to membranes: liver, intestine, heart muscle, and adipose tissue FABPs. Mol Cell Biochem 2002;239:25-33.

[25] Koonen DP, Glatz JF, Bonen A, Leiken JJ. Long-chain fatty acid uptake and FAT/CD36 translocation in heart and skeletal muscle. Biochim Biophys Acta 2005;1736:163-80.

[26] Kuang M, Febbraio M, Wagg C, Lopaschuk GD, Dyck JR. Fatty acid translocase/CD36 deficiency does not energetically or functionally compromise heart before or after ischemia. Circulation 2004;109: 1550-7.

[27] Febbraio M, Abumrad NA, Hajjar DP, et al. A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. J Biol Chem 1999;274:19055-62.

[28] Bonnefont JP, Specola NB, Vassault A, et al. The fasting test in paediatrics: application to the diagnosis of pathological hypo-and hyperketotic states. Eur J Pediatr 1990;150:80-5.

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Pediatric Diabetes

Original Article

Comprehensive molecular analysis of Japanese patients with pediatric-onset MODY-type diabetes mellitus

Yorifuji T, Fujimaru R, Hosokawa Y, Tamagawa N, Shiozaki M, Aizu K, Jinno K, Maruo Y, Nagasaka H, Tajima T, Kobayashi K, Urakami T. Comprehensive molecular analysis of Japanese patients with pediatric-onset MODY-type diabetes mellitus.
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Background: In Asians, mutations in the known maturity-onset diabetes of the young (MODY) genes have been identified in only <15% of patients. These results were obtained mostly through studies on adult patients.
Objective: To investigate the molecular basis of Japanese patients with pediatric-onset MODY-type diabetes.
Subjects: Eighty Japanese patients with pediatric-onset MODY-type diabetes.
Methods: Mitochondrial 3243A>G mutation was first tested by the polymerase chain reaction restriction fragment length polymorphism analysis for maternally inherited families. Then, all coding exons and exon–intron boundaries of the *HNF1A*, *HNF1B*, *GCK*, and *HNF4A* genes were amplified from genomic DNA and directly sequenced. Multiplex ligation-dependent probe amplification analysis was also performed to detect whole-exon deletions.
Results: After excluding one patient with a mitochondrial 3243A>G, mutations were identified in 38 (48.1%) patients; 18 had *GCK* mutations, 11 had *HNF1A* mutations, 3 had *HNF4A* mutations, and 6 had *HNF1B* mutations. In patients aged <8 yr, mutations were detected mostly in *GCK* at a higher frequency (63.6%). In patients >9 yr of age, mutations were identified less frequently (45.1%), with *HNF1A* mutations being the most frequent. A large fraction of mutation-negative patients showed elevated homeostasis model assessment (HOMA) insulin-resistance and normal HOMA-β indices. Most of the *HNF1B* mutations were large deletions, and, interestingly, renal cysts were undetectable in two patients with whole-gene deletion of *HNF1B*.
Conclusion: In Japanese patients with pediatric-onset MODY-type diabetes, mutations in known genes were identified at a much higher frequency than previously reported for adult Asians. A fraction of mutation-negative patients presented with insulin-resistance and normal insulin-secretory capacities resembling early-onset type 2 diabetes.

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Key words: Japanese – MODY – mutation – pediatric

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Maturity-onset diabetes of the young (MODY) has been defined as diabetes mellitus characterized by dominant inheritance, non-obesity [body mass index (BMI) <25], and early onset (<25 years of age). To date, at least six causative genes (*HNF4A, GCK, HNF1A, PDX1, HNF1B,* and *NEUROD1*) for MODY 1–6, respectively, have been identified. In addition, several other genes (*KLF11, CEL, PAX4,* and *INS*) have been shown to be associated with MODY-type diabetes (1–6). Since the term 'MODY' has been questioned by some investigators as inappropriate and obsolete (1), 'MODY-type diabetes' is used in this study to describe diabetes with above characteristics.

Previous studies have revealed that mutations in these genes, especially in the *HNF1A, GCK,* and *HNF4A* genes, account for 54–86% of MODY-type diabetes in Caucasians, whereas in Asians, only 7.5–10.3% of patients have mutations in these genes (7–13). In the Japanese, previous studies have focused mainly on single genes (14–16), and comprehensive molecular analysis is lacking. Taken together, however, these studies point to a similarly low prevalence of mutations (<15%) in the Japanese.

One possible explanation for this racial disparity is the presence of an unidentified major MODY-X gene in Asians. To date, a number of investigators have pursued this possibility without much success, only to identify genes with low prevalence (17–23). Alternatively, this racial difference could be due to the higher prevalence of early-onset type 2 diabetes mellitus (T2DM) in Asians (24). Japanese patients with T2DM reportedly have lower body mass indices (BMIs) than do Caucasian patients (25). Although, T2DM is based on insulin-resistance rather than on ?-cell dysfunction, clinical differentiation between these is not easy when the patients are not obese and the onset of diabetes is earlier, because both tend to aggregate within families and are autoantibody negative.

In this study, as part of an effort to address this issue, we performed a comprehensive mutational analysis on Japanese patients with pediatric-onset MODY-type diabetes mellitus because previous results were obtained mostly through studies on adult patients. This is also the most comprehensive molecular analysis of known MODY genes in Japan. Unexpectedly, our study revealed that the genetic background of MODY-type diabetes in the Japanese pediatric age group is similar to that reported for Caucasians. The results of this study, therefore, served to delineate the demographic and clinical characteristics of 'unknown MODY' in the Japanese.

Subjects and methods

Subjects

The study subjects were 80 Japanese patients who developed MODY-type diabetes before 20 yr of age. Other inclusion criteria were (i) negative autoantibodies, including anti-GAD and anti-IA2 antibodies; (ii) family history suggestive of dominant inheritance; and (iii) non-obesity in the patients or in affected family members. Patients who developed diabetes before 6 months of age were excluded because the genetic background of diabetes in this age group is known to differ from those in later age groups (26). In addition to the 77 patients who met the above criteria, 3 additional patients without a positive family history were included because of the known presence of renal cysts.

Out of these 80 patients, 56 were ascertained through the nationwide school urinalysis program performed annually in Japan, and 21 by incidentally identified glucosuria/hyperglycemia. The rest of the patients were ascertained after consulting primary care physicians for diabetes-related symptoms. The patients were then referred to local diabetes centers and blood samples were sent to our laboratory during 2005–2011 under a suspected diagnosis of MODY-type diabetes. The local diabetes centers were distributed throughout Japan without geographical biases and each center basically recruited all available patients for analysis.

The patients were enrolled after obtaining written informed consent, and the study protocol was approved

by the Institutional Review Board at Osaka City General Hospital (No. 742).

Mutational analysis

Genomic DNA was extracted from peripheral blood leukocytes with the QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany). In families with maternal transmission, the mitochondrial 3243A>G mutation was first tested by polymerase chain reaction (PCR)-restriction fragment length polymorphism as previously described (27). For patients negative for 3243A>G, all coding exons, exon–intron boundaries, and promoter regions of the *HNF1A*, *GCK*, *HNF4A*, and *HNF1B* genes were amplified from genomic DNA. For *HNF4A*, exon 1D and the P2 promoter approximately 50 kb upstream of exon 1A were also amplified because previous reports identified this region as a specific promoter for pancreatic expression of this gene (28). For *GCK*, the pancreatic form using exon 1A was sequenced (29). The primer sequences and amplification conditions are listed in Table S1 and Appendix S1. The amplified products were purified using the Wizard PCR Preps DNA Purification Kit (Promega, Madison, WI, USA) or the Agencourt AMPure XP purification system (Beckman Coulter Genomics, Danvers, MA, USA) and directly sequenced using the BIGDYE TERMINATOR v3.1 Cycle Sequencing Kit (Roche, Basel, Switzerland). They were then analyzed by the ABI PRISM 3100xl automated sequencer (Applied Biosystems, Foster City, CA, USA). After identification, co-segregation of the mutations with the diabetic phenotype was confirmed by sequencing analyses of other family members. In addition, whole-exon deletion/duplications that might escape the PCR-sequencing strategy described above were tested using multiplex ligation-dependent probe amplification (MLPA) analyses. Briefly, 200 ng of genomic DNA was used to perform the MLPA reactions for all exons in the *HNF1A*, *GCK*, *HNF4A*, and *HNF1B* genes. The reactions were performed using the SALSA MLPA kit P241 (MRC-Holland, Amsterdam, The Netherlands) as recommended by the manufacturer and analyzed using the ABI PRISM 3100xl automated sequencer and GENEMAPPER software (Applied Biosystems).

Results

Mitochondrial 3243A>G was found in one patient and the patient was excluded from further analysis. Of the remaining 79 patients, mutations were identified in 38 (48.1%), which is a much higher frequency than that previously reported for Asians. The demographic and clinical features of these 38 patients are listed in Table 1 together with the details of identified mutations.

Mutations of *HNF1A* were identified in 11, *GCK* in 18, *HNF4A* in 3, and *HNF1B* in 6 (Table 1). Most of these mutations have been previously reported, and no predominant mutation was identified. Novel missense mutations were not found in the public domain single nucleotide polymorphism databases, and the Sorting Intolerant from Tolerant (SIFT) (http://sift.bii.a-star.edu.sg/) or the POLYPHEN-2 (http://genetics.bwh.harvard.edu/pph2/) programs predicted the deleterious nature of these mutations. In addition, these mutations co-segregated with the diabetic phenotype in each family, suggesting that these are pathogenic mutations.

The majority of patients (five out of six) with *HNF1B* mutations had exon deletions. All these patients with deletions presented with a higher blood glucose level at onset, necessitating immediate insulin treatment. Interestingly, in two patients with whole-gene deletion (patients 32 and 34), renal cysts could not be identified by repeated abdominal magnetic resonance imaging or echography.

We also identified a whole-gene deletion of the *GCK* gene in one patient (patient 15). This patient was born after 39 weeks of gestation with a birth weight of 2418 g. Hyperglycemia was incidentally found at the age of 4 years, and the patient showed no diabetes-related symptoms. Fasting glucose and hemoglobin A1c were slightly elevated at 124 mg/dL (6.88 mmol/L) and 6.6%, respectively. All of these features were consistent with those of other GCK-MODY patients, and no specific features were observed for this patient.

When the patients were sorted by age at diagnosis (Fig. 1), *GCK* mutations were found to be predominant in patients <9 years of age, whereas detection of mutations in the *HNF1A* and *HNF4A* genes began after 8 years of age. After 9 years of age, *HNF1A* mutations were the most common. The frequency and spectrum of mutations did not change significantly during 9–13 years of age, and the identification rate appeared to decline thereafter.

BMI percentiles at diagnosis were obtained for 76 patients. For patients who lost weight before the initial presentation, the most recent height and weight data before weight loss were used for the calculation of BMI. Mutations were identified in 89% of the leanest patients with BMIs below the 10th percentile (Fig. 2). Between BMIs of the 10th–90th percentiles, mutations were identified at a relatively constant frequency, although the detection rate appeared to be lower (10%) for BMIs above the 90th percentile.

The homeostasis model assessment insulin-resistance (HOMA-IR) index as a marker of insulin resistance (normal range, <2.0) and the HOMA-β index as a marker of insulin-secreting capacity (normal range for Japanese, 40–60) were calculated for 57 patients (30). As shown in Table 2, a large

Table 1. Summary of patients with mutations

No.	Gene	Mutation		Previous report	Gender	Age (yr)	BMI percentile	FBS, mg/dL (mmol/L)	IRI, μU/mL (pmol/L)	HOMA-IR	HOMA-β
		cDNA	Protein								
1	*GCK*	c.1517C>T	p.T206M	Yes	F	3	95.2	82 (4.55)	1.5 (10.42)	0.30	28.4
2	*GCK*	c.130G>A	p.G44S	Yes	M	5	83.48	118 (6.55)	2.8 (19.45)	0.81	18.3
3	*GCK*	c.571C>T	p.R191W	Yes	M	11	80.45	144 (7.99)	20.4 (141.68)	7.25	90.7
4	*GCK*	c.1278_1286dup9	p.S426_R428dup	No	M	6	76.17	120 (6.66)	4.16 (28.89)	1.37	29.1
5	*GCK*	c.895G>C	p.G299R	Yes	M	9	58.08	122 (6.77)	4.6 (31.95)	1.39	28.1
6	*GCK*	c.1142T>G	p.M381R	No	M	14	57.13	139 (7.71)	5.8 (40.28)	1.99	27.5
7	*GCK*	c.571C>T	p.R191W	Yes	M	7	40.63	124 (6.88)	5.5 (38.2)	1.68	32.5
8	*GCK*	c.175C>T	p.P59S	No	M	5	32.61	118 (6.55)	2 (13.89)	0.58	13.1
9	*GCK*	c.781G>A	p.G261R	Yes	M	4	24.88	106 (5.88)	1.54 (10.7)	0.40	12.9
10	*GCK*	c.118G>A	p.E40K	Yes	M	1	23.18	129 (7.16)	2 (13.89)	0.64	10.9
11	*GCK*	c.575G>A	p.R191Q	Yes	M	5	21.5	118 (6.55)	7.1 (49.31)	2.07	46.5
12	*GCK*	c.182A>G	p.Y61C	No	F	8	20.52	118 (6.55)	1.82 (12.64)	0.53	11.9
13	*GCK*	c.234C>G	p.D38E	Yes	F	13	12.8	121 (6.72)	5.73 (39.79)	1.71	35.6
14	*GCK*	c.556C>T	p.R186X	Yes	F	12	11.52	135 (7.49)	10.3 (71.53)	3.43	51.5
15	*GCK*	All exon deletion		No	M	4	6.68	124 (6.88	3.42 (23.75)	1.05	20.2
16	*GCK*	c.437T>G	p.L146R	Yes	M	8	5.07	119 (6.6)	2.3 (15.97)	0.68	14.8
17	*GCK*	c.576G>T	p.G193W	No	M	12	1.92	118 (6.55)	6.36 (44.17)	1.85	41.6
18	*GCK*	c.500G>A	p.W167X	No	F	5	0.43	113 (6.27)	4 (27.78)	1.12	28.8
19	*HNF1A*	c.1043T>C	p.L348P	No	F	15	83.1	175 (9.71)	21.6 (150.01)	9.33	69.4
20	*HNF1A*	c.779C>T	p.T260M	Yes	F	12	80.53	124 (6.88)	10.6 (73.62)	3.25	62.6
21	*HNF1A*	c.391C>T	p.R131W	Yes	M	9	78.79	124 (6.88)	5.37 (37.29)	1.64	31.7
22	*HNF1A*	Exon 7-9 deletion		No	M	11	68.07	165 (9.16)	11.4 (79.17)	4.6	40.2
23	*HNF1A*	c.788G>A	p.R263H	Yes	M	8	48.33	84 (4.66)	3.29 (22.85)	0.68	56.4
24	*HNF1A*	c.872delC	p.P291fs	Yes	M	13	43.38	83 (4.61)	11.3 (78.48)	2.32	203.4
25	*HNF1A*	c.1181delC	p.P394fs	Yes	F	13	21.83	137 (7.6)	7.3 (50.7)	2.47	35.5
26	*HNF1A*	c.1054delT	p.S352fs	No	F	11	18.66	153 (8.49)	2.7 (18.75)	1.02	10.8
27	*HNF1A*	c.392G>A	p.R131Q	Yes	F	11	2.0	167 (9.27)	7.9 (54.87)	3.26	27.3
28	*HNF1A*	c.872_873insC	p.P291fs	Yes	F	13	1.65	98 (5.44)	7.18 (49.87)	1.74	73.9
29	*HNF1A*	c.598C>T	p.R200W	Yes	M	14	0.33	77 (4.27)	0.9 (6.25)	0.17	23.1
30	*HNF1B*	c.395A>C	p.H132P	No	M	7	70	111 (6.16)	10.3 (71.53)	2.82	77.3
31	*HNF1B*	Exon 1-4 deletion		No	M	1	62.16	NA	NA	NA	NA
32	*HNF1B*	All exon deletion		Yes	F	13	38.68	NA	NA	NA	NA
33	*HNF1B*	Exon 3-4 deletion		No	M	12	19.23	NA	NA	NA	NA
34	*HNF1B*	All exon deletion		Yes	F	12	8.42	NA	NA	NA	NA
35	*HNF1B*	Exon 3-4 deletion		No	F	10	NA	NA	NA	NA	NA
36	*HNF4A*	c.802C>T	p.Q268X	Yes	F	9	77.22	NA	NA	NA	NA
37	*HNF4A*	c.915_916insT	p.V306fs	No	F	11	55.28	105 (5.83)	7.2 (50)	1.87	61.7
38	*HNF4A*	c.970C>T	p.R324C	No	F	13	45.36	188 (10.43)	6.5 (45.14)	3.02	18.7

BMI, body mass index; F, female; FBS, fasting blood sugar; HOMA-β, homeostasis model assessment-β; HOMA-IR, homeostasis model assessment insulin resistance; IRI, immunoreactive insulin; M, male; NA, not available.

All sequence information is based on GenBank reference sequences NM_000162.3 (*GCK*), NM_000457.3 (*HNF4A*), NM_000545.5 (*HNF1A*), and NM_000458.2 (*HNF1B*). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the major start codon of exon 1 (*HNF1A* and *HNF1B*) and 1A (*GCK*), respectively. For *HNF4A*, conventional nucleotide numbering starting at the A of the Met9 of isoform b was used.

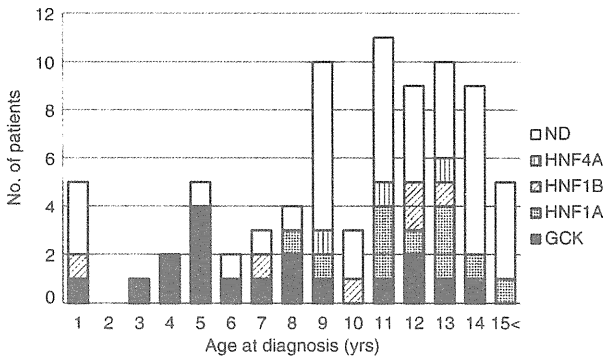

Fig. 1. Distribution of age at diagnosis. ND, not detected.

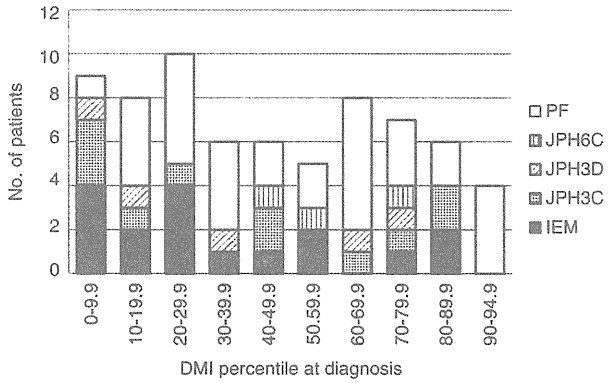

Fig. 2. Distribution of body mass index percentiles at diagnosis. ND, not detected.

fraction of mutation-negative patients showed elevated HOMA-IR or normal HOMA-β indices. Mutations were identified less frequently with increasing HOMA-IR or HOMA-β indices ($p = 0.0248$ and 0.002, respectively, by the Jonckheere's trend test). In the remaining 22 patients, these indices were not available because of the lack of fasting glucose and insulin data at diagnosis. Nineteen of these patients presented with a blood glucose level that was too high (>170 mg/dL, 9.44 mmol/L) at diagnosis to calculate these indices, and/or insulin therapy was initiated immediately before calculating these indices. In three patients, fasting glucose and insulin data were not recorded or simply not obtained. Of these 22 patients with missing HOMA-IR and HOMA-β data, mutations were identified in 6 (27.3%); 4 had *HNF1B* mutations and 2 had *HNF4A* mutations.

Discussion

Contrary to previous studies on Asians, the results of this study showed that the frequency of mutations in known genes is significantly higher in pediatric-onset Japanese patients with MODY-type diabetes. Even if we exclude the three patients with renal cysts and without positive family history, the mutation identification rate (46.1%) was still higher than previously reported. The overall frequency of mutation was only slightly lower than that reported for Caucasians (7–11), and the mutation spectrum did not differ much between Japanese and Caucasian patients. These results suggest that the genetic background of MODY-type diabetes in the pediatric age group is similar for these two ethnicities.

The cause of the difference in the frequencies of mutations between adult Asian and Caucasian MODY patients remains unclear. Assuming that the genetic background of pediatric-onset patients is similar, the racial disparity should be caused by a group of patients who develop diabetes at a later age (15–25 yr).

Among pediatric-onset patients, mutation-negative patients are likely to comprise a mixed population. There is a group of patients who fit the classical definition of MODY with normal insulin sensitivity and diminished insulin-secretory capacity. Presumably, some of these patients' conditions can be explained by mutations in the known MODY genes, including *PAX4* and *INS*, and the remaining was caused by mutations in true MODY-X genes. At present, there is no evidence of racial disparity in this group of patients.

The results of our study also showed that a large fraction of mutation-negative patients has elevated insulin-resistance and normal insulin-secretory capacities as measured by the HOMA-IR and HOMA-β indices. Although these patients' conditions might also be explained by an unknown monogenic trait, these clinical features are more consistent with those of early-onset T2DM, which is highly prevalent in Japan. Considering the known racial difference in the incidence of T2DM, the observed difference in the

Table 2. Distribution of HOMA-IR and HOMA-β indices

	HOMA-IR			HOMA-β					
	0–0.99	1–1.99	>2	10–19.9	20–29.9	30–39.9	40–49.9	50–59.9	>60
No. of patients	15	20	22	9	12	7	6	6	17
Mutation positive	10	12	10	7	8	4	3	3	7
Fraction mutation positive	0.67	0.6	0.45	0.78	0.67	0.57	0.5	0.5	0.41

HOMA-β, homeostasis model assessment-β; HOMA-IR, homeostasis model assessment insulin resistance.
HOMA-IR was calculated as fasting blood glucose (mg/dL) × fasting insulin (μU/mL)/405 (normal range, <2.0), and HOMA-β was calculated as 360 × fasting insulin (μU/mL)/fasting blood glucose (mg/dL) − 63 (normal range for Japanese, 40–60).

prevalence of MODY gene mutations in adult patients appears to be more easily explained by inclusion of early-onset T2DM in previous studies. Although adult T2DM patients in Japan are not heavier than non-diabetic controls (25), even in Japan, pediatric patients with T2DM are heavier than their peers (31). By focusing on non-obese pediatric patients, we may be able to minimize the influence of early-onset T2DM in our study. A similar explanation might also apply to other ethnic groups, such as Mexicans, known to have a higher prevalence of T2DM and lower prevalence of MODY gene mutations (32).

As previously reported for pediatric patients with *HNF1B* mutations, the majority of our HNF1B-MODY patients had deletions rather than point mutations (33, 34). This is in contrast to the approximately 30% prevalence of deletions in adult patients (35). It was previously proposed that deletions might lead to more severe loss of function, which leads to earlier onset of diabetes (34). In our series, patients with *HNF1B* deletions (patients 31–35) actually presented with higher blood glucose at onset compared with patient 30, who had a point mutation. It appears more likely, however, that interactions with other genetic or environmental factors play a more important role in determination of the phenotype of *HNF1B* mutations because none of the deletion patients reported by Ulinski et al. (33) had diabetes, and some patients with a whole-gene deletion do not have renal structural abnormalities (34), including the two patients in our series. Practically, it is important to include the analysis of *HNF1B* deletions as a part of routine mutation screening of MODY-type diabetes, even without signs of renal abnormalities.

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Conflict of interest

No conflict of interests declared.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Amplification conditions.

Table S1. Sequences of the primers used for amplification.

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References

1. Murphy R, Ellard S, Hattersley A. Clinical implications of a molecular genetic classification of monogenic β-cell diabetes. Nat Clin Pract Endocrinol Metab 2008: 4: 200–213.
2. Vaxillaire M, Froguel P. Monogenic diabetes in the young, pharmacogenetics and relevance to multifactorial forms of type 2 diabetes. Endocr Rev 2008: 29: 254–264.
3. Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V et al. Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. Proc Natl Acad Sci U S A 2005: 102: 4807–4812.
4. Raeder H, Johansson S, Holm PI et al. Mutations in the CEL VNTR cause a syndrome of diabetes and pancreatic exocrine dysfunction. Nat Genet 2006: 38: 54–62.
5. Shimajiri Y, Sanke T, Furuta H Y et al. A missense mutation of Pax4 gene (R121W) is associated with type 2 diabetes in Japanese. Diabetes 2001: 50: 2864–2869.
6. Molven A, Ringdal M, Nordbø AM et al. Mutations in the insulin gene can cause MODY and autoantibody-negative type 1 diabetes. Diabetes 2008: 57: 1131–1135.
7. Frayling TM, Evans JC, Bulman MP et al. Beta-cell genes and diabetes: molecular and clinical characterization of mutations in transcription factors. Diabetes 2001: 50 (Suppl. 1): S94–S100.
8. Froguel P, Zouali H, Vionnet N et al. Familial hyperglycemia due to mutations in glucokinase. Definition of a subtype of diabetes mellitus. N Engl J Med 1993: 328: 697–702.
9. Lindner TH, Cockburn BN, Bell GI. Molecular genetics of MODY in Germany. Diabetologia 1999: 42: 121–123.
10. Letho M, Wipemo C, Ivarsson SA et al. High frequency of mutations in MODY and mitochondrial genes in Scandinavian patients with familial early-onset diabetes. Diabetologia 1999: 42: 1131–1137.
11. Costa A, Bescos M, Velho G et al. Genetic and clinical characterization of maturity-onset diabetes of the young in Spanish families. Eur J Endocrinol 2000: 142: 380–386.
12. Hwang JS, Shin CH, Yang SW, Jung SY, Huh N. Genetic and clinical of Korean maturity-onset diabetes of the young (MODY) patients. Diabetes Res Clin Pract 2006: 74: 75–81.
13. Ng MC, Cockburn BN, Lindner TH et al. Molecular genetics of diabetes mellitus in Chinese subjects: identification of mutations in glucokinase and hepatocyte nuclear factor-1 alpha genes in patients with early-onset type 2 diabetes mellitus/MODY. Diabet Med 1999: 16: 956–963.
14. Tonooka N, Tomura H, Takahashi Y et al. High frequency of mutations in the HNF-1α gene (TCF1) in non-obese patients with diabetes of youth in Japanese and identification of a case of digenic inheritance. Diabetologia 2002: 45: 1709–1712.

15. Ikema T, Shimajiri Y, Komiya I et al. Identification of three new mutations of the HNF-1α gene in Japanese MODY families. Diabetologia 2002: 45: 1713–1718.
16. Iwasaki N, Ohgawara H, Nagahara H, Kawamura M, Bell GI, Omori Y. Characterization of Japanese families with early-onset type 2 (non-insulin dependent) diabetes mellitus and screening for mutations in the glucokinase and mitochondrial tRNA (Leu(UUR)) genes. Acta Diabetol 1995: 32: 17–22.
17. Yu L, Wei Q, Jin L et al. Genetic variation in the hepatocyte nuclear factor (HNF)-3alpha gene does not contribute to maturity-onset diabetes of the young in Japanese. Horm Metab Res 2001: 33: 163–166.
18. Shimomura H, Sanke T, Hanabusa T, Tsunoda K, Furuta H, Nanjo K. Nonsense mutation of islet-1 gene (Q310X) found in a type 2 diabetic patient with a strong family history. Diabetes 2000: 49: 1597–1600.
19. Horikawa Y, Horikawa Y, Cox NJ et al. Beta-cell transcription factors and diabetes: no evidence for diabetes-associated mutations in the gene encoding the basic helix-loop-helix transcription factor neurogenic differentiation 4 (NEUROD4) in Japanese patients with MODY. Diabetes 2000: 49: 1955–1957.
20. Hinokio Y, Horikawa Y, Furuta H et al. Beta-cell transcription factors and diabetes: no evidence for diabetes-associated mutations in the hepatocyte nuclear factor-3beta gene (HNF3B) in Japanese patients with maturity-onset diabetes of the young. Diabetes 2000: 49: 302–305.
21. Hara M, Wang X, Paz VP et al. Identification of three missense mutations in the peroxisome proliferator-activated receptor alpha gene in Japanese subjects with maturity-onset diabetes of the young. J Hum Genet 2001: 46: 285–288.
22. Furuta H, Horikawa Y, Iwasaki N et al. Beta-cell transcription factors and diabetes: mutations in the coding region of the BETA2/NeuroD1 (NEUROD1) and Nkx2.2 (NKX2B) genes are not associated with maturity-onset diabetes of the young in Japanese. Diabetes 1998: 47: 1356–1358.
23. Hara M, Wang X, Paz VP et al. No diabetes-associated mutations in the coding region of the hepatocyte nuclear factor-4gamma gene (HNF4G) in Japanese patients with MODY. Diabetologia 2000: 43: 1064–1069.
24. Otabe S, Nakayama H, Fukutani T et al. Clinical and genetic features of childhood-onset type 2 diabetes in Japan. Acta Diabetol 2007: 44: 181–185.
25. Sone H, Ito H, Ohashi Y, Akanuma Y, Yamada N. Japan Diabetes Complication Study Group. Obesity and type 2 diabetes in Japanese patients. Lancet 2003: 361: 85.
26. Flanagan SE, Edghill EL, Gloyn AL, Ellard S, Hattersley AT. Mutations in KCNJ11, which encodes Kir6.2, are a common cause of diabetes diagnosed in the first 6 months of life, with the phenotype determined by genotype. Diabetologia 2006: 49: 1190–1197.
27. Yorifuji T, Kawai M, Momoi T et al. Nephropathy and growth hormone deficiency in a patient with mitochondrial tRNA (Leu(UUR)) mutation. J Med Genet 1996: 33: 621–622.
28. Drewes T, Senkel S, Holewa B, Ryffel GU. Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. Mol Cell Biol 1996: 16: 925–931.
29. Magnuson MA, Shelton KD. An alternate promoter in the glucokinase gene is active in the pancreatic ? cell. J Biol Chem 1989: 264: 15936–15942.
30. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985: 28: 412–419.
31. Urakami T, Kubota S, Nitadori Y, Harada K, Owada M, Kitagawa T. Annual incidence and clinical characteristics of type 2 diabetes in children as detected by urine glucose screening in the Tokyo metropolitan area. Diabetes Care 2005: 28: 1876–1881.
32. Domínguez-López A, Miliar-García Á, Segura-Kato Y et al. Mutations in MODY genes are not common cause of early-onset type 2 diabetes in Mexican families. J Pancreas (Online) 2005: 6: 238–245.
33. Ulinski T, Lescure S, Beaufils S et al. Renal phenotypes related to hepatocyte nuclear factor-1beta (TCF2) mutations in a pediatric cohort. J Am Soc Nephrol 2006: 17: 497–503.
34. Raile K, Klopocki E, Holder M, et al. Expanded clinical spectrum in hepatocyte nuclear factor 1b-maturity-onset diabetes of the young. J Clin Endocrinol Metab 2009: 94: 2658–2664.
35. Bellanné-Chantelot C, Clauin S, Chauveau D et al. Large genomic rearrangements in the hepatocyte nuclear factor-1beta (TCF2) gene are the most frequent cause of maturity-onset diabetes of the young type 5. Diabetes 2005: 54: 3126–3132.

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Short Report

Maternal uniparental isodisomy and heterodisomy on chromosome 6 encompassing a *CUL7* gene mutation causing 3M syndrome

Sasaki K, Okamoto N, Kosaki K, Yorifuji T, Shimokawa O, Mishima H, Yoshiura K-i, Harada N. Maternal uniparental isodisomy and heterodisomy on chromosome 6 encompassing a *CUL7* gene mutation causing 3M syndrome.
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We report a case of segmental uniparental maternal hetero- and isodisomy involving the whole of chromosome 6 (mat-hUPD6 and mat-iUPD6) and a cullin 7 (*CUL7*) gene mutation in a Japanese patient with 3M syndrome. 3M syndrome is a rare autosomal recessive disorder characterized by severe pre- and postnatal growth retardation that was recently reported to involve mutations in the *CUL7* or obscurin-like 1 (*OBSL1*) genes. We encountered a patient with severe growth retardation, an inverted triangular gloomy face, an inverted triangle-shaped head, slender long bones, inguinal hernia, hydrocele testis, mild ventricular enlargement, and mild mental retardation. Sequence analysis of the *CUL7* gene of the patient revealed a homozygous missense mutation, c.2975G>C. Genotype analysis using a single nucleotide polymorphism array revealed two mat-hUPD and two mat-iUPD regions involving the whole of chromosome 6 and encompassing *CUL7*. 3M syndrome caused by complete paternal iUPD of chromosome 6 involving a *CUL7* mutation has been reported, but there have been no reports describing 3M syndrome with maternal UPD of chromosome 6. Our results represent a combination of iUPDs and hUPDs from maternal chromosome 6 involving a *CUL7* mutation causing 3M syndrome.

Conflict of interest

None of the authors of this paper declares a conflict of interest.

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Key words: 3M syndrome – cullin 7 (*CUL7*) – Genome-Wide Human SNP Array 6.0 (SNP6.0) – maternal uniparental disomy of chromosome 6 (matUPD6)

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3M syndrome is a rare inherited autosomal recessive disorder characterized by pre- and postnatal growth retardation, characteristic facial features, and skeletal anomalies. Clinical features of 3M syndrome include large head circumference, broad forehead, a triangular facial outline, dolichocephaly, long philtrum, short stature, short thorax and neck, tall vertebral bodies, and slender

long bones and ribs. Males with 3M syndrome occasionally have hypogonadism and hypospadias (1–9). However, intelligence is unaffected and karyotype is normal on conventional chromosome analysis.

In patients with 3M syndrome, disease-causing mutations have been identified in the cullin 7 (*CUL7*, MIM *609577) and obscurin-like 1 (*OBSL1*, MIM *610991) genes (7–9). Mutations of *CUL7* are the major cause of 3M syndrome, accounting for 80% of previously reported cases, whereas *OBSL1* accounts for 20% of cases (8, 10).

Uniparental disomy (UPD) is the transmission pattern of either two copies of the identical chromosome (uniparental isodisomy; iUPD) or of both homologous chromosomes (uniparental heterodisomy; hUPD) from one parent with no contribution from the other parent (11). Phenotypes that are clinically associated with paternal UPD of chromosome 6 (patUPD6) and genomic imprinting have been established, but because of the rarity of maternal UPD of chromosome 6 (matUPD6), clinical features have not yet been established. Here, we report a patient with a homozygous mutation in *CUL7* due to a maternal iUPD of chromosome 6 (mat-iUPD6).

Materials and methods

Clinical report

A Japanese male patient with 3M syndrome was examined in this study. The patient was delivered by caesarean section at 36 weeks of gestation without a family history of 3M syndrome (Fig. 1a). His birth weight was 1000 g (−4.8 SD), length 33.0 cm (−6.8 SD), head circumference 30.2 cm (−1.5 SD), and Apgar score 7/9. Feeding difficulty was noted during the neonatal period. He remained in a neonatal intensive care unit for 2 months and was referred to our group because of developmental delay and muscle hypotonia at 4 months. The patient displayed anomalies including hypospadias, inguinal hernia, hydrocele testis, inverted triangular gloomy face, malar hypoplasia, long eyelashes, epicanthal folds, short nose, anteverted nares, full lips, long philtrum, pointed chin, short chest, grooved lower anterior thorax, hypermobility of joints, and slender long bones (Fig. 1a,b). Mild ventricular enlargement was observed by neuroradiological studies. His growth was severely retarded.

At 2 years 9 months, his height, weight, and head circumference were 69.3 cm (−4.6 SD), 6.8 kg (−6.7 SD), and 48 cm (−1.2 SD), respectively. His head size was disproportionately large compared to his height. Thus the patient was diagnosed as suffering from 3M syndrome. He could understand simple sentences, but could not speak nor sit alone. Partial growth hormone (GH) deficiency was noted. GH replacement therapy was started from 2 years. GH was effective without side effects. At 5 years, his height and weight were 84.8 cm (−5.9 SD) and 10 kg (−3 SD), respectively. He was moderately mentally retarded.

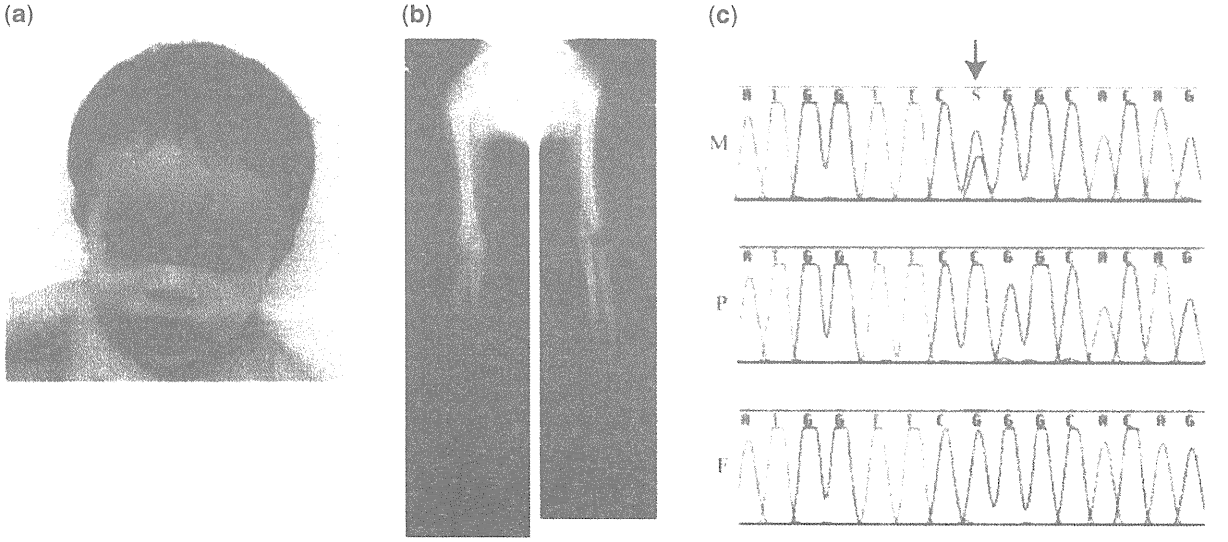

Fig. 1. Facial and skeletal features of the patient at 2 years 7 months of age. (**a**) Note the inverted triangular gloomy face, short nose, full lips, and long philtrum. (**b**) Note the slender long bones. (**c**) Electropherograms of the patient and parents. DNA sequence showing a single base change substituting cytosine for guanine, which results in p.R992P, in the patient. M, mother; P, patient; and F, father.

Conventional cytogenetic studies and FISH analysis

We obtained blood samples under written informed consent for participation in this study. Conventional cytogenetic examination of G-banded chromosomes from peripheral blood lymphocytes was performed. We also performed fluorescence *in situ* hybridization (FISH) analysis on lymphocyte metaphase spreads from the patient using two Bacterial Artificial Chromosome (BAC) clones containing *CUL7*, RP11-628J2 and RP11-653G5, as probes.

Genomic sequencing

Genomic DNA was extracted from peripheral blood following standard protocols. For mutation analysis, we designed primers to amplify all the coding exons of *CUL7* and the flanking intron sequences. Direct sequencing was carried out using a BigDye Terminator v3.1 Cycle sequencing Kit™ and separated on a Genetic Analyzer 3130*xl* (Applied Biosystems Inc., Foster City, CA). Sequence electropherograms were aligned with SEQUENCHER™ software (Gendcodes, Ann Arbor, MI) to visually inspect base alterations.

Microarray analysis

We performed genome-wide single nucleotide polymorphism (SNP) genotyping using Genome-Wide Human SNP Array 6.0 (SNP6.0) following the manufacturer's instructions (Affymetrix, Santa Clara, CA, http://www.affymetrix.com/index.affx). The data generated from Genotyping Console (GTC) 4.0 were loaded into CHROMOSOME ANALYSIS SUITE (CHAS) 1.0.1 software to display the results. We carried out UPD analyses of the patient using genotype data in trio. Genomic positions of SNPs corresponded to the March 2006 human genome (hg18).

Results

Genomic sequencing

We sequenced all 26 coding exons and flanking intronic regions of the *CUL7* gene, which spans a genomic region of approximately 16.3 kb, in the family. In the patient, we detected a homozygous missense mutation (c.2975G>C) in exon 15, which resulted in the substitution of proline for arginine at amino acid residue 992 (p.R992P) (Fig. 1c). The mother was a heterozygous carrier of the mutation, whereas the father was homozygous for the wild-type allele (Fig. 1c). The p.R992P mutation was not detected in 100 unrelated control individuals.

Conventional and molecular cytogenetic analyses

G-banding and FISH analysis at the *CUL7* locus showed a normal karyotype in the patient and the parents with no microdeletion at *CUL7* locus in the patient (data not shown).

Microarray analysis

To confirm paternity, and to find a small size deletion, we performed SNP6.0 analysis. However, no copy number variations (CNVs) were identified in the region containing both *CUL7* and *OBSL1* genes (Fig. 2a). The other variants overlap with reported regions of CNVs in the Database of Genomic Variants (http://projects.tcag.ca/variation) or were transmitted from the parents (data not shown).

To confirm matUPD6 in the patient, we examined the genotypes of the patient/father/mother trio. The results using informative markers indicated that there were two maternal heterodisomic regions (hUPD6-1 and hUPD6-2) and two maternal isodisomic regions (iUDP6-1 and iUPD6-2) in chromosome 6, respectively (Fig. 2 and Table 1). The results indicated that the patient had inherited two alleles from his mother, but none from his father, in chromosome 6. The final karyotype of this patient was 46,XY,upd(6)mat and arr 6p25.3p22.3(110,391–16,287,166)×2 htz mat,6p 22.3q12(16,290,223–65,796,893)×2 hmz mat,6q 12q25.1(65,799,990–150,517,779)×2 htz mat,6q 25.1q27(150,518,012–170,759,956)×2 hmz mat.

Discussion

We identified a causative homozygous mutation in *CUL7* in a patient with 3M syndrome. The results clearly indicate that mat-iUPD6 involving a mutant allele of the *CUL7* gene caused 3M syndrome in the patient.

matUPD6 is relatively rare and seven cases have been reported. The first case was a renal transplant patient who showed growth retardation at birth and mat-iUPD6 (12). The second case was a patient with congenital adrenal hyperplasia (CAH) resulting from a homozygous mutation in the 21-hydroxylase gene (*CYP21*), and had intrauterine growth retardation (IUGR) and mat-iUPD6 (13). The third case was a macerated male fetus from a pregnancy terminated at 23 weeks of gestation because of intrauterine death. The patient showed a mosaic trisomy 6 (14). The fourth case was a male patient with two clinical phenotypes, Klinefelter's syndrome and CAH. His karyotype was mosaic 48,XXY, +mar[30]/47,XXY[20] and

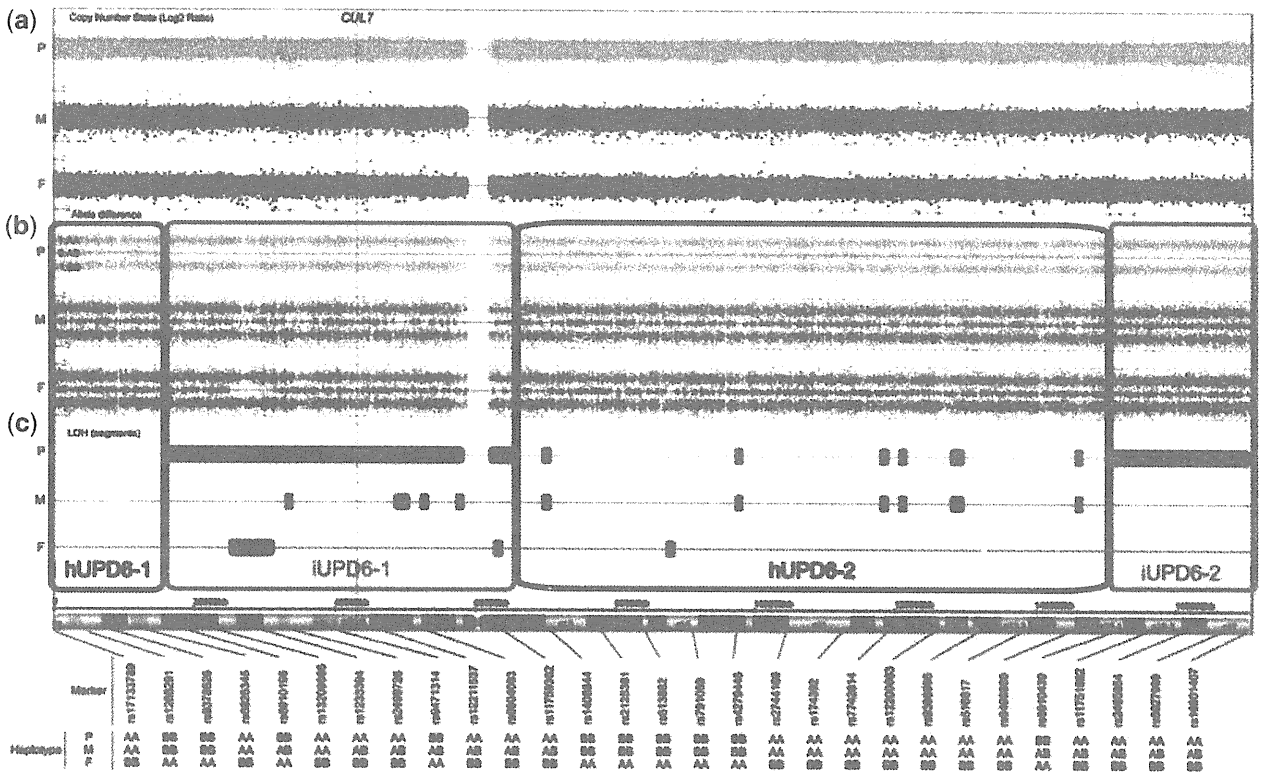

Fig. 2. SNP6.0 data. (**a**) Plots of the SNP6.0 data displayed in ChAS 1.0.1 showing the log2 ratio plot of copy number state, allele difference plot, and loss of heterozygosity (LOH) segment (purple box) (P, patient; M, mother; and F, father). (**b**) The allele difference graph represents the genotypes for each individual single nucleotide polymorphism (SNP). Dots with a value of 1, −1, and 0 represent SNPs with AA, BB, and AB genotypes, respectively. A vertical dashed line indicates the *CUL7* locus. (**c**) The LOH segment plot indicates nine LOH regions on chromosome 6. iUPD6-1 and iUPD6-2 denote the regions of uniparental isodisomy (red box). hUPD6-1 and hUPD6-2 denote the regions of uniparental heterodisomy (blue box). The genotypes on chromosome 6 indicate maternal heterodisomy or isodisomy in the affected offspring [only the uniparental disomy (UPD) markers are displayed].

Table 1. Examination of SNPs from a patient/father/mother trio[a]

			hUPD6-1	iUPD6-1	hUPD6-2	iUPD6-2
Genotype of trio (patient/father/mother)	iUPD	AA/BB/AB	0	534	0	318
		BB/AA/AB	0	576	3	304
	iUPD or hUPD	AA/BB/AA	178	543	605	272
		BB/AA/BB	196	506	563	262
Share genotype (patient/mother)	iUPD or hUPD	AA/AA	2,812	5,897	9,716	3,009
		BB/BB	2,799	5,785	9,557	2,919
	hUPD	AB/AB	1,699	19	6,384	12
		Total of share genotype	7,310	11,701	25,657	5,940
		Share genotype rate (%)	99.82	78.20	99.89	73.31
		Total SNP probe	7,323	14,963	25,684	8,103
		Start SNP	rs4959515	rs9370869	rs9354209	rs9384189
		Start position	110,391	16,290,223	65,799,990	150,518,012
		End SNP	rs9477050	rs9453156	rs7765984	rs6931065
		End position	16,287,166	65,796,893	150,517,779	170,759,956
		Size (bp)	16,176,776	49,506,671	84,717,790	20,241,945
		Cytoband	p25.3-p22.3	p22.3-q12	q12-q25.1	q25.1-q27

hUPD, uniparental heterodisomy; iUPD, uniparental isodisomy; iUPD or hUPD, UPD could not be defined as isodisomy or heterodisomy; SNP, single nucleotide polymorphism.

[a]Each row contains information on each matUPD6 inheritance block identified by trio haplotype analysis.

both the X chromosome and chromosome 6 showed maternal iUPD. This case also was notable for IUGR and growth retardation at 8 months of age (15). The fifth case was a fetus with IUGR at 29 weeks of pregnancy from a 45-year-old patient. The case was ascertained as trisomy 6 mosaicism in cultured chorionic villi but disomy in amniocytes; analysis of DNA markers in amniocytes and parental samples revealed mat-iUPD6 in disomy cells (16). The sixth case was a male infant with molybdenum cofactor deficiency who showed developmental delay. SNP analysis with the trio revealed that at least 6p21.1-6p24.3 were mat-iUPD6, but not another region were remain unclear (17). The seventh case was a patient with cleft lip and palate, and showed a complete maternal hUPD on chromosome 6 (mat-hUPD6). This case had no notable IUGR in the serial ultrasound examination (18). Taken together, IUGR and growth retardation were found in the cases with mat-iUPD6 (12, 13, 15–17), while these were not found in cases with mat-hUPD6 (14, 18). The IUGR and growth retardation in cases of mat-iUPD6 may be the result of homozygosity of chromosome 6. On the basis of these reports, no clear maternal imprinting effect of chromosome 6 can be established; however, recently, a complete gain of methylation phenotype at insulin-like growth factor 2 receptor was shown in patients with growth restriction (19).

The patient with homozygous mutation in *CUL7* and matUPD6 had clinical features compatible with 3M syndrome. However, the patient displayed certain features that have not been previously reported among patients with *CUL7* mutations such as mild mental retardation, inguinal hernia, hydrocele testis, and mild ventricular enlargement (7, 8, 20). Mild mental retardation is an especially characteristic phenotype in our case because most patients with 3M syndrome have normal intelligence. It is difficult to determine whether matUPD6 had a significant role in the development of certain feature in our case.

Here we report a case of 3M syndrome with a homozygous mutation that resulted from maternal iUPD, including the *CUL7* gene. Although complete paternal or maternal UPD for chromosome 6 has previously been reported, this is the first report of a patient with 3M syndrome who has a mixture of mat-hUPD6 and mat-iUPD6 regions. Our results emphasize that UPD should be considered possible mechanism for developing the autosomal recessive disorders including 3M syndrome.

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References

1. Winter RM, Baraitser M, Grant DB, Preece MA, Hall CM. The 3-M syndrome. J Med Genet 1984: 21: 124–128.
2. Feldmann M, Gilgenkrantz S, Parisot S, Zarini G, Marchal C. 3M dwarfism: a study of two further sibs. J Med Genet 1989: 26 (9): 583–585.
3. García-Cruz D, Cantú JM. Heterozygous expression in 3-M slender-boned nanism. Hum Genet 1979: 52: 221–226.
4. Mueller RF, Buckler J, Arthur R et al. The 3-M syndrome: risk of intracerebral aneurysm? J Med Genet 1992: 29: 425–427.
5. Le Merrer M, Brauner R, Maroteaux P. Dwarfism with gloomy face: a new syndrome with features of 3-M syndrome. J Med Genet 1991: 28: 186–191.
6. Spranger J, Opitz JM, Nourmand A. A new familial intrauterine growth retardation syndrome the "3-M syndrome". Eur J Pediatr 1976: 123: 115–124.
7. Huber C, Dias-Santagata D, Glaser A et al. Identification of mutations in *CUL7* in 3-M syndrome. Nat Genet 2005: 37: 1119–1124.
8. Huber C, Delezoide AL, Guimiot F et al. A large-scale mutation search reveals genetic heterogeneity in 3M syndrome. Eur J Hum Genet 2009: 17: 395–400.
9. Hanson D, Murray PG, Sud A et al. The primordial growth disorder 3-M syndrome connects ubiquitination to the cytoskeletal adaptor *OBSL1*. Am J Hum Genet 2009: 84: 801–806.
10. Huber C, Fradin M, Edouard T et al. OBSL1 mutations in 3-M syndrome are associated with a modulation of IGFBP2 and IGFBP5 expression levels. Hum Mutat 2010: 31: 20–26.
11. Engel E. A new genetic concept: uniparental disomy and its potential effect, isodisomy. Am J Med Genet 1980: 6: 137–143.
12. van den Berg-Loonen EM, Savelkoul P, van Hooff H, van Eede P, Riesewijk A, Geraedts J. Uniparental maternal disomy 6 in a renal transplant patient. Hum Immunol 1996: 45: 46–51.
13. Spiro RP, Christian SL, Ledbetter DH et al. Intrauterine growth retardation associated with maternal uniparental disomy for chromosome 6 unmasked by congenital adrenal hyperplasia. Pediatr Res 1999: 46: 510–513.
14. Cockwell AE, Baker SJ, Connarty M, Moore IE, Crolla JA. Mosaic trisomy 6 and maternal uniparental disomy 6 in a 23-week gestation fetus with atrioventricular septal defect. Am J Med Genet A 2006: 140: 624–627.
15. Parker EA, Hovanes K, Germak J, Porter F, Merke DP. Maternal 21-hydroxylase deficiency and uniparental isodisomy of chromosome 6 and X results in a child with 21-hydroxylase deficiency and Klinefelter syndrome. Am J Med Genet A 2006: 140: 2236–2240.
16. Haag M, Beischel L, Rokeach J et al. First prenatal detection of maternal uniparental disomy (UPD) of chromosome 6 and 'rescue' of trisomy 6 [abstract]. *Abstracts of the 57th Annual Meeting of the ASHG 2007*; Abstract no 2428.
17. Gümüş H, Ghesquiere S, Per H et al. Maternal uniparental isodisomy is responsible for serious molybdenum cofactor deficiency. Dev Med Child Neurol 2010: 52 (9): 868–872.

18. Salahshourifar I, Halim AS, Sulaiman WA, Zilfalil BA. Maternal uniparental heterodisomy of chromosome 6 in a boy with an isolated cleft lip and palate. Am J Med Genet A 2010: 152A (7): 1818–1821.
19. Turner CL, Mackay DM, Callaway JL et al. Methylation analysis of 79 patients with growth restriction reveals novelpatterns of methylation change at imprinted loci. Eur J Hum Genet 2010: 18: 648–655.
20. Maksimova N, Hara K, Miyashia A et al. Clinical, molecular and histopathological features of short stature syndrome with novel *CUL7* mutation in Yakuts: new population isolate in Asia. J Med Genet 2007: 44: 772–778.

Molecular and Clinical Analysis of Japanese Patients with Persistent Congenital Hyperinsulinism: Predominance of Paternally Inherited Monoallelic Mutations in the K_{ATP} Channel Genes

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Background: Preoperative identification of the focal form of congenital hyperinsulinism is important for avoiding unnecessary subtotal pancreatectomy. However, neither the incidence nor the histological spectrum of the disease is known for Japanese patients.

Aims: The aim of the study was to elucidate the molecular and histological spectrum of congenital hyperinsulinism in Japan.

Subjects: Thirty-six Japanese infants with persistent congenital hyperinsulinism were included in the study.

Methods: All exons of the ATP-sensitive potassium channel (K_{ATP} channel) genes (*KCNJ11* and *ABCC8*), the *GCK* gene, and exons 6 and 7 and 10–12 of the *GLUD1* gene were amplified from genomic DNA and directly sequenced. In patients with K_{ATP} channel mutations, the parental origin of each mutation was determined, and the results were compared with the histological findings of surgically treated patients. In one of the patients with scattered lesions, islets were sampled by laser capture microdissection for mutational analysis.

Results: Mutations were identified in 24 patients (66.7%): five in *GLUD1* and 19 in the K_{ATP} channel genes. Sixteen had a paternally derived, monoallelic K_{ATP} channel mutation predictive of the focal form. In 10 patients who underwent pancreatectomy, the molecular diagnosis correctly predicted the histology, more accurately than [18F]-3,4-dihydroxyphenylalanine positron emission tomography scans. Three patients showed focal lesions that occupied larger areas of the pancreas. Preferential loss of the maternal allele was observed in these islets.

Conclusion: The majority of the Japanese patients with K_{ATP} channel hyperinsulinism (84.2%) demonstrated paternally inherited monoallelic mutations that accurately predicted the presence of the focal form. (*J Clin Endocrinol Metab* 96: E141–E145, 2011)

Persistent congenital hyperinsulinism is the main cause of prolonged hypoglycemia in infancy. The most common etiology is an inactivating mutation in one of two genes, *ABCC8* or *KCNJ11*, which code for the two subunits of the pancreatic ATP-sensitive potassium (K_{ATP}) channel. The second most common is an activating mu-

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Abbreviations: DOPA, 3,4-Dihydroxyphenylalanine; GCK, glucokinase; GLUD1, glutamate dehydrogenase; K_{ATP}, ATP-sensitive potassium channel; MLPA, multiple ligation-dependent probe amplification; PET, positron emission tomography.

tation in the glutamate dehydrogenase (*GLUD1*) gene, which is found in cases of hyperinsulinemia-hyperammonemia syndrome followed by an activating mutation in the glucokinase (*GCK*) gene with a much rare incidence (1).

Because severely affected infants often experience profound neurological sequelae (2, 3), appropriate management of hypoglycemia is critically important. Infants resistant to medical treatment usually undergo subtotal pancreatectomy. Although the procedure is often effective at controlling hypoglycemia, residual hypoglycemia is not uncommon, and many of the infants develop insulin-dependent diabetes mellitus postoperatively (1, 4).

Notably, the recognition of the focal form of persistent congenital hyperinsulinism has changed clinical practice because precise pre- and intraoperative identification of focal lesions allows us to perform a partial resection of the pancreas, leading to a complication-free cure (1, 5, 6).

Focal lesions are found in individuals with a paternally inherited, monoallelic K_{ATP} channel mutation (5–7). Subsequent somatic loss of the maternal allele (most likely caused by paternal isodisomy) leads to a loss of the activities of the K_{ATP} channel and the adjacent tumor suppressors (*H19* and *CDKN1C*) normally expressed by the maternal allele. These cells gain a growth advantage eventually forming a focal lesion of insulin-overproducing β-cells (8).

It has been reported that approximately 40% of patients with K_{ATP} channel hyperinsulinism have monoallelic mutations (9, 10) and that up to 40–60% of surgically treated patients have the focal form (1, 6, 7). However, to date, neither the incidence of focal lesions nor the clinical spectrum of persistent congenital hyperinsulinism has been reported for Asians.

In this study, we performed a comprehensive mutational analysis of Japanese patients with this disorder and correlated the results with the histology of surgically treated patients.

Subjects and Methods

Subjects

The study subjects were 36 Japanese infants with persistent congenital hyperinsulinism. The inclusion criteria were as follows: 1) a plasma insulin level of greater than 3 μU/ml in the presence of hypoglycemia [plasma glucose < 45 mg/dl (2.5 mmol/liter)], 2) hypoglycemia lasting beyond 3 months of age, and 3) the absence of insulinoma. The patients were born in 2005–2010 except for those with hyperinsulinemia-hyperammonemia syndrome who were recruited over a longer period (born in 1999–2009). For mutational analysis, written informed consent was obtained, and the study protocol was approved by the institutional review board.

Mutational analysis

Genomic DNA was extracted from peripheral blood leukocytes using a QIAmp DNA blood kit (QIAGEN, Hilden, Germany) as recommended by the supplier. Then all exons and the exon-intron boundaries of the *KCNJ11*, *ABCC8*, and *GCK* genes were amplified from genomic DNA. For the *GLUD1* gene, only exons 6 and 7 (the antenna domain) and exons 10–12 (the GTP binding domain) were amplified because previously reported mutations were exclusively found in these regions. The amplification conditions and the sequences of the primers are available as supplemental data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. The amplified products were purified using the Wizard PCR Preps DNA purification system (Promega, Fitchburg, WI) and directly sequenced using the BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems, Foster City, CA).

Deletion mutations that might not have been detected by the PCR-sequencing strategy described above were analyzed by multiple ligation-dependent probe amplification (MLPA) of all 39 exons of the *ABCC8* gene. The analyses were performed using SALSA MLPA kit P117 (MRC Holland, Amsterdam, The Netherlands) as recommended by the manufacturer.

[18F]-3,4-dihydroxyphenylalanine (DOPA) positron emission tomography (PET)

[18F]-DOPA PET studies were performed at the PET facility of Kizawa Memorial Hospital basically, as described by Ribeiro *et al.* (11). The scan results were fused with those of a computed tomography scan taken at the same time to localize the focal lesion more accurately.

Laser capture microdissection (LCM)

The scattered islets of patient 10 were sampled by LCM using the PixCell IIe LCM system (Arcturus, Mountain View, CA). DNA was extracted from the pooled islets using a FASTPURE DNA kit (Takara-bio, Ohtsu, Japan). DNA extracted from a normal pancreatic area on the same slide was used as the control.

Results

Patient profiles and mutations

The profiles of the patients and the results of the mutational analyses are listed in Table 1. In patients with elevated ammonia at the initial presentation, only patients 1–5 showed persistent hyperammonemia. Those five had mutations in *GLUD1*. Of the remaining 31 patients, mutations were identified in 19 (61.3%): 18 in *ABCC8*, one in *KCNJ11*, and none in *GCK*. No exonic deletions were identified by MLPA, and the four novel missense mutations were not found in 100 normal controls. p.R836X and p.R998X in *ABCC8* were identified in five and three unrelated patients, respectively, possibly representing relatively common mutations in Japanese.

Interestingly, of these patients with K_{ATP} channel mutations, only two had biallelic mutations, whereas the

TABLE 1. Profiles of the patients with mutations

Patient no.	Gender	Onset	Glucose (mg/dl) [mmol/liter]	Insulin (μU/ml) [pmol/liter]	Ammonia (μg/dl) [μmol/liter]	Mutation					Medical treatment
						Gene	cDNA	Protein	Previously reported?	Parental origin	
1	F	9 months	38 [2.1]	4.8 [33]	83 [49]	*GLUD1*	c.661C>T	p.R221C	yes	ND	F, D
2	M	7 months	30 [1.7]	3 [21]	132 [77]	*GLUD1*	c.797A>G	p.Y266C	yes	ND	F, D
3	F	3 months	29 [1.6]	4 [28]	246 [144]	*GLUD1*	c.1336G>A	p.G446S	Yes	ND	F, D
4	M	10 months	<45 [2.5]	7.7 [53]	154 [90]	*GLUD1*	c.1229A>G	p.N410S	No	ND	F, D
5	M	0 d	10 [0.6]	10 [69]	250 [147]	*GLUD1*	c.1229A>C	p.N410T	Yes	ND	F, D
6[a]	F	2 d	31 [1.7]	30.2 [210]	78 [46]	*ABCC8*	c.382G>A c.3748C>T	p.E128K p.R1250X	Yes, Yes	Biparental	
7	M	2 d	5 [0.3]	7.5 [52]	131 [77]	*ABCC8*	c.2506C>T c.4575_4587del13	p.R836X p.M1524Mfs1539X	Yes, No	Biparental	F, O
8	M	0 d	<45 [2.5]	11 [76]	58 [34]	*ABCC8*	c.4516G>A	p.E1506K	Yes	Mat	F, D
9[a]	F	1 month	<20 [1.1]	42.4 [294]	NA	*ABCC8*	c.2506C>T	p.R836X	Yes	Pat	
10[a]	M	2 d	10 [0.56]	23.5 [163]	NA	*ABCC8*	c.4412-13G>A	—	Yes	Pat	
11[a]	F	0 d	33 [1.8]	46.6 [324]	79 [46]	*ABCC8*	c.3745G>T	p.V1249F	No	Pat	
12[a]	F	3 months	20 [1.1]	5.16 [36]	78 [46]	*ABCC8*	c.2992C>T	p.R998X	Yes	Pat	
13[a]	F	0 d	23 [1.3]	101 [701]	45 [24]	*ABCC8*	c.4608 + 1G>A	—	No	Pat	
14[a]	M	0 d	22 [1.2]	22.7 [158]	75 [44]	*ABCC8*	c.2992C>T	p.R998X	Yes	Pat	
15[a]	M	5 months	33 [1.8]	5.42 [38]	NA	*ABCC8*	c.2992C>T	p.R998X	Yes	Pat	
16[a]	M	0 d	28 [1.6]	38.7 [269]	66 [39]	*ABCC8*	c.331G>A	p.G111R	Yes	Pat	
17	F	2 months	15 [0.8]	9.9 [69]	90 [53]	*ABCC8*	c.61_62insG	p.V21Gfs88X	No	Pat	F, O
18	M	0 d	19.6 [1.1]	44 [306]	79 [46]	*ABCC8*	c.2506C>T	p.R836X	Yes	Pat	F, O
19	F	7 months	35 [1.9]	11.2 [78]	97 [57]	*ABCC8*	c.2506C>T	p.R836X	Yes	Pat	F, O
20	M	4 months	<45 [2.5]	7.5 [52]	84 [49]	*ABCC8*	c.3928_3929insG	p.A1310Gfs1405X	No	Pat	F, O
21	M	2 d	38 [2.1]	3.4 [24]	91 [53]	*ABCC8*	c.4186G>T	p.D1396Y	No	Pat	F
22	F	0 d	9 [0.5]	22 [153]	NA	*ABCC8*	c.2506C>T	p.R836X	Yes	Pat	F, O
23	M	2 d	0 [0]	17.3 [120]	317 [186]	*ABCC8*	c.4412-13G>A	—	Yes	Pat	F, D
24[a]	M	0 d	33 [1.8]	21.9 [152]	75 [44]	*KCNJ11*	c.637G>A	p.A213T	No	Pat	

The clinical data are those at the initial presentation. Of the medically treated patients with monoallelic, paternally inherited K_{ATP} channel mutations (patients 17–23), none reported a family history of hypoglycemia. F, Frequent feeding; D, diazoxide; O, continuous sc injection of octreotide; M, male; F, female; Pat, paternal; Mat, maternal; NA, not available; ND, not determined.

[a] Patients who underwent surgery.

other 17 had monoallelic mutations. Furthermore, 16 of 17 of the mutations were of paternal origin. The single maternally inherited mutation was identical to a mutation previously reported by Huopio *et al.* (12) as a mutation causing hyperinsulinism in infancy and diabetes mellitus in adulthood. In fact, the mother of the patient developed diabetes at the age of 13 yr, and the maternal grandmother developed a mild form of diabetes during adulthood. Therefore, from the results of the mutational analyses, the incidence of a paternally inherited monoallelic mutation suggesting the presence of a focal lesion appears to be much higher in Japanese (84.2% of K_{ATP} channel hyperinsulinism cases).

Clinical studies and LCM studies

None of the patients with paternally inherited K_{ATP} channel mutations responded to diazoxide except for patient 23 who partially responded at the maximal dose of 25 mg/kg · d. Pancreatectomy was performed on 10 patients who were resistant to medical therapy, one with a biallelic *ABCC8* mutation (patient 6) and nine with monoallelic paternally inherited mutations, eight in *ABCC8* (patients 9–16), and one in *KCNJ11* (patient 24). [18F]-DOPA PET scans were performed in all patients preoperatively. The patient with the biallelic mutation (patient 6) showed typical diffuse uptake. Of the nine patients with monoallelic mutations, four showed a single focal uptake pattern (patients 9, 12, 15, and 16); two (patients 14 and 24) showed multifocal uptake; and the other three (patients 10, 11, and 13) showed irregular uptake throughout the pancreas, which was difficult to distinguish from that o diffuse lesions. The six patients with focal or multifocal uptake underwent partial resection of the pancreas. Histological examination revealed a single focal lesion in these patients. Five were almost completely cured, and one showed residual but milder hypoglycemia. Of the three patients who demonstrated irregular uptake during the PET study, two underwent subtotal pancreatectomy because their intraoperative findings did not rule out the presence of diffuse lesions. In one of these two patients (patient 13), postoperative histology revealed a large focal lesion in the tail and the body of the pancreas. In the other patient (patient 11), abnormal islets were found throughout the pancreas. The presence of normal islets in part of the pancreas suggested the diagnosis of a giant focal lesion. In the third patient (patient 10) with irregular [18F]-DOPA uptake (Fig. 1B), an arterial stimulation venous sampling study suggested the presence of a lesion in the body or the tail of the pancreas (Fig. 1A). Intraoperatively, no focal lesion could be identified by inspection or palpation. Although the margins of the lesion could not be clearly determined, partial resection was performed at 2.5 cm from the tail. This patient was also clinically cured after surgery. Postoperative histology revealed scattered, relatively large islets with a diameter of up to 700 μm clustered within the tail and the body. Each islet appeared to be separated by normal acinar cells, and no