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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

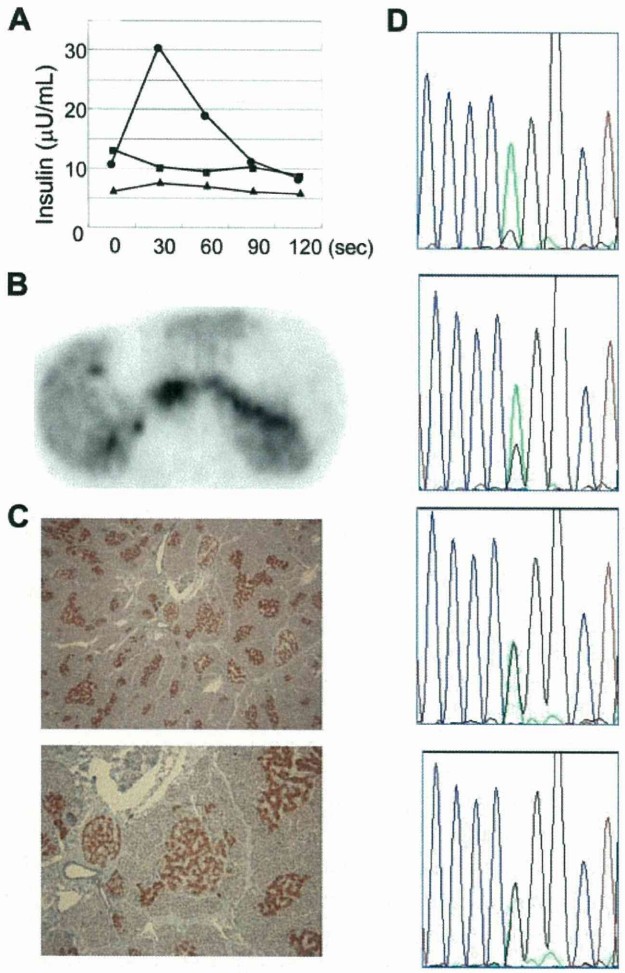

FIG. 1. Results of different diagnostic modalities in patient 10. A, Results of arterial stimulation venous sampling studies. The insulin concentration of the right hepatic vein was measured after the injection of calcium into the splenic (*filled circles*), gastroduodenal (*filled rectangles*), and superior mesenteric (*filled triangles*) arteries. An insulin response was observed only after stimulation of the splenic artery. B, A curved planar reconstruction of a [18F]-DOPA PET scan. The uptake in the head probably reflects an artifact. C, Chromogranin A staining of the resected pancreas showing the area in which abnormal islets were most densely distributed. Magnification, ×40 (*upper panel*), ×80 (*lower panel*). D, Mutational analysis of abnormal islet samples. The *upper two panels* show the results of two separate analyses of 30 (*upper panel*) and 40 (*lower panel*) islet samples. The *lower two panels* show the results of a similar analysis of an adjacent normal pancreatic area. The paternally inherited A allele (*green*) predominates in the abnormal islets, whereas the A and the wild-type G alleles (*black*) have similar intensities in the normal area of the pancreas.

single lesion composed of a solid β-cell cluster was identified by serial sections of the specimen (Fig. 1C). LCM was performed twice to collect samples from 30 and 40 of these islet clusters. Mutational analysis of the pooled DNA collected from these LCM samples revealed the predominance of the paternally inherited mutant allele within these scattered large islets compared with the surrounding normal pancreatic tissue (Fig. 1D).

Discussion

The most important finding of this study is the higher incidence of paternally inherited, monoallelic K_{ATP} channel mutations in Japanese patients with congenital hyperinsulinism ($P < 0.005$ by the sign test), which suggests that the majority of Japanese patients have the focal form. Although the number of patients is small, we believe our results represent the situation of the whole country for several reasons. First, a national survey in 2008–2009 conducted by the Ministry of Health, Labor, and Welfare of Japan estimated the incidence of persistent congenital hyperinsulinism as 1:35,400 births. Our study captured 23% of all cases during that period. Second, the patients were referred without geographical biases because ours is the only laboratory currently offering a comprehensive molecular diagnosis in Japan. Third, a previous report by Ohkubo *et al.* (13) also reported a high frequency (seven of 10) of monoallelic mutations in Japan. In contrast, patients with hyperinsulinism-hyperammonemia syndrome were collected somewhat arbitrarily over a longer period; therefore, the apparent higher incidence might not represent the actual incidence in Japan.

Conflicting results have been reported for the diabetogenesity of p.E1506K in *ABCC8* (12, 14, 15). The association might be a chance observation or might reflect a difference in the genetic background. If the association does exist, that might be due to the specific nature of the mutation, which confers the instability of the β-cells such as altered membrane potential of the cells.

Molecular diagnosis correctly predicted the histology in all patients who underwent pancreatectomy. On the contrary, the ability of [18F]-DOPA PET scans to identify focal lesions was inferior compared with the results of previous reports for other populations (16, 17). Histologically, at least two patients with ambiguous PET results had large focal lesions. The third patient (patient 10) appeared to have unusually scattered islets for a focal lesion. However, there remains the possibility that these islets are actually interconnected and represents a focal lesion with greater admixture of exocrine tissues. Although the number of patients was too small to draw a definite conclusion, larger lesions might be more common in the Japanese.

The reason that the incidence of the focal form of the disease is higher in Japanese is unclear. One possibility is that Japanese have a higher incidence of somatic isodisomy. If this occurred during the earlier stages of development, it would lead to the development of Beckwith-Wiedemann syndrome. However, the incidence of this syndrome caused by paternal isodisomy is not particularly higher in Japanese (18). Alternatively, cells with mutations common in Japanese might be more prone to develop into

a focal lesion, by either promoting a second hit of isodisomy or conferring a growth advantage after the disomic event. Further studies are necessary to address this question.

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ORIGINAL ARTICLE

Diagnostic accuracy of [^{18}F]-fluoro-L-dihydroxyphenylalanine positron emission tomography scan for persistent congenital hyperinsulinism in Japan

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Summary

Objective We aimed to elucidate the accuracy and limitations of [^{18}F]-fluoro-L-dihydroxyphenylalanine ([^{18}F]DOPA) positron emission tomography (PET) for Japanese patients with congenital hyperinsulinism. Although [^{18}F]DOPA PET is reported to be useful for precisely localizing the focal form of congenital hyperinsulinism, previous reports are mostly from European and North American centres.

Patients Seventeen Japanese infants with congenital hyperinsulinism.

Measurements [^{18}F]DOPA PET studies were carried out, and the results were assessed by simple inspection or by a quantitative measurement termed the 'Pancreas Percentage', which expresses the uptake of the head, body or tail of the pancreas as a percentage of the total maximum standardized uptake value of the whole pancreas. The results were compared with those of other studies, including genetic analysis and histology.

Results By simple inspection, when a single focal uptake was obtained, the localization and histology were correct in all cases that underwent pancreatectomy. However, the overall results were consistent with the molecular diagnosis and histology in only 7/17 and 6/12 patients, respectively. The inaccuracy of PET studies by inspection was because of elevated background uptake that mimicked a diffuse or multifocal appearance. The accuracy improved substantially using the Pancreas Percentage; it was consistent with the molecular diagnosis and histology in 10/17 and 9/12 patients, respectively.

Conclusions In contrast to the results of previous reports, [^{18}F]DOPA PET appears to be less efficient for diagnosing Japanese patients with congenital hyperinsulinism. However, the diagnostic accuracy is substantially improved when this technique is combined with the Pancreas Percentage.

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Introduction

Congenital hyperinsulinism in infancy is characterized by prolonged hyperinsulinism and severe hypoglycaemia. This disorder is most often associated with inactivating mutations in one of two genes, *ABCC8* and *KCNJ11*, which encode the two subunits of the pancreatic ATP-sensitive potassium (K_{ATP}) channel.[1,2]

There are two main histopathological forms of K_{ATP} channel hyperinsulinism: diffuse and focal.[1,3] In the diffuse form, mutations are present in both alleles of the K_{ATP} channel genes; this form is associated with insulin oversecretion from all β cells in the pancreas. In the focal form, a mutation is present in the paternal allele.[4,5] The subsequent somatic loss of the maternal allele containing the K_{ATP} channel genes and the adjacent paternally imprinted tumour-suppressor genes lead to a growth advantage for insulin-overproducing β cells, which eventually form a focal lesion in the pancreas.

As uncontrolled severe hypoglycaemia causes neurological complications, surgical treatment is required if medical treatment is not effective. The diffuse form is usually treated by subtotal pancreatectomy. Although this surgery is effective for controlling hypoglycaemia, many patients develop insulin-dependent diabetes mellitus postoperatively. In contrast, the focal form can be cured by a partial pancreatectomy without complications as long as the localization of the lesion is known pre- or intra-operatively.

In 2003, Otonkoski *et al.*[6] reported that positron emission tomography (PET) using [^{18}F]-fluoro-L-dihydroxyphenylalanine ([^{18}F]DOPA) effectively localizes focal lesions. Since then, several studies have reported the efficacy of [^{18}F]DOPA PET scanning for

detecting the localization of focal lesions.[2,7–11] However, previous studies mainly focus on Caucasian subjects, there are no comprehensive [^{18}F]DOPA PET studies on Asian subjects.

In a previous study on the molecular analysis of Japanese patients,[7] we briefly reported the PET appearance of 10 surgically treated patients. In the present study, we extended the analysis to include more patients who were treated with or without surgery. We unexpectedly encountered difficulties in diagnosing Asian subjects, which was in contrast with previous studies with subjects of other ethnicities. In an attempt to overcome the ambiguity of the interpretation of PET results, we employed a quantitative method for the evaluation of pancreatic [^{18}F]DOPA uptake.

Subjects and methods

Subjects

The study population included 17 Japanese infants (nine boys and eight girls; age range, 2–37 months) with persistent congenital hyperinsulinism who were referred to Kizawa Memorial Hospital, Japan, between July 2005 and June 2010. Diagnoses were based on the following criteria: (i) plasma insulin level >3 μU/ml in the presence of hypoglycaemia [plasma glucose <45 mg/dl (2·5 mM)]; (ii) hypoglycaemia lasting beyond 3 months of age and (iii) the absence of an insulinoma. The demographic features of the patients are shown in Table 1. Written informed consent was obtained from their guardians, and the study protocol was approved by the Institutional Review Board. In this study, we also included patients with biallelic mutations in the K_{ATP} channel genes, in whom diffuse lesions were expected. The guardians were told that the PET study would only serve to confirm the diffuse nature of the disease and that part of the study's purpose was for this population to serve as a control for future patients.

[^{18}F]DOPA PET analysis

[^{18}F]DOPA PET studies were performed using an ADVANCE NXi scanner (GE, Milwaukee, WI, USA) at Chubu Medical Center for Prolonged Traumatic Brain Dysfunction, as described by Ribeiro *et al.*[8]

If the patient was on diazoxide or glucagon, these were discontinued for at least 2 days before the study, whereas octreotide treatment was continued. Prior to the study, patients fasted for 6 h and normoglycaemia was maintained by intravenous glucose infusion. PET acquisition was performed under light sedation with thiamylal sodium.

[^{18}F]DOPA was manufactured on the day of the study using CYPRIS-HM18 cyclotron (Sumitomo, Tokyo, Japan). After 3 min of transmission scanning, [^{18}F]DOPA (5 MBq/kg) was infused intravenously over 1 min. Five-minute acquisitions of PET scanning (in the two-dimensional acquisition mode) were consecutively performed for up to 60 min. The PET scan results were incorporated with those of a CT scan taken simultaneously to localize the focal lesion more accurately.

Table 1. [^{18}F]DOPA PET analysis of 17 patients with congenital hyperinsulinism, and comparison with genetic, ASVS and histology results

Patient/sex	Origin/gene	Diagnosis by histology	Age at PET (months)	Diagnosis by inspection	Standardized uptake value			Pancreas %			Diagnosis by pancreas %	Diagnosis by ASVS
					Head	Body	Tail	Head	Body	Tail		
Surgery												
1/F	Bip/ABCC8	Diffuse	4	Diffuse	6·3	5·8	4·6	100	92	73	Diffuse	No
2/M	Bip/ABCC8	Diffuse	2	Diffuse	5·1	3·8	4·0	100	75	78	Diffuse	No
3/F	Bip/ABCC8	Diffuse	37	Diffuse	9·0	7·7	6·7	100	86	74	Diffuse	Focal (H)
4/F	Pat/ABCC8	Focal (H)	13	Focal (H)	6·9	3·6	2·8	100	52	41	Focal (H)	Focal (H)
5/F	Pat/ABCC8	Focal (H)	7	Focal (H)	5·7	3·8	3·0	100	67	53	Focal (H)	No
6/M	Pat/ABCC8	Focal (H)	2	Focal (H)	4·5	2·9	2·8	100	64	62	Focal (H)	No
7/M	Pat/KCNJ11	Focal (H)	2	Irregular (H, B)	3·8	2·5	2·5	100	66	66	Focal (H)	No
8/M	Pat/ABCC8	Focal (B)	2	Irregular (H, B)	4·4	7·1	2·4	62	100	34	Focal (B)	Focal (B)
9/M	Pat/ABCC8	Focal (H)	8	Not detected	1·8	2·1	1·6	86	100	73	Not detected	Focal (H)
10/F	Pat/ABCC8	Large focal (B, T)	5	Irregular (H, B)	2·4	3·7	1·8	65	100	49	Focal (B)	No
11/M	Pat/ABCC8	Large focal (B, T)	17	Irregular	6·8	5·3	5·0	100	78	74	Diffuse	Focal (B, T)
12/F	Pat/ABCC8	Large focal (H, B, T)	4	Irregular	4·0	4·1	4·1	98	100	100	Diffuse	No
No surgery												
13/M	Pat/ABCC8	(complete remission)	9	Focal (T)	2·4	2·9	5·0	48	58	100	Focal (T)	Normal
14/M	Pat/ABCC8	(partial remission)	5	Irregular	5·1	4·7	7·0	73	67	100	Diffuse (H, T)	No
15/M	Pat/ABCC8	(partial remission)	23	Irregular	4·5	3·7	3·4	100	82	76	Diffuse	No
16/F	Pat/ABCC8		16	Irregular	3·8	3·8	2·9	100	100	76	Diffuse	Diffuse
17/F	Pat/ABCC8		26	Diffuse	6·0	5·4	6·3	95	86	100	Diffuse	No

M, male; F, female; Bip, biparental mutation; Pat, paternal mutation; Diffuse, diffuse lesion/uptake; Focal, focal lesion/uptake; Irregular, irregular uptake; H, head of the pancreas; B, body of the pancreas; T, tail of the pancreas; complete remission, without any treatment; partial remission, frequent feeding alone; ASVS, arterial stimulation venous sampling analysis; [^{18}F]DOPA PET, [^{18}F]-fluoro-L-dihydroxyphenylalanine positron emission tomography.

Image interpretation

PET images were interpreted by one of the authors, H.N., who is a board-certified radiologist and has experience of over 6870 PET studies. By inspection, focal lesions were identified when the local uptake of [^{18}F]DOPA was obviously higher than that of the remaining pancreatic tissue. Conversely, when the uptake was nearly uniform, the result was considered to represent a diffuse lesion.

In addition to the simple visual inspection, we also employed an objective index of [^{18}F]DOPA uptake termed the 'Pancreas Percentage'. The whole pancreas was first divided into three regions of interest: the head, body and tail. The standardized uptake value (SUV) of each region was then measured 30 min after [^{18}F]DOPA injection. The SUV of the region with the greatest uptake was defined as 100%. The values for the remaining regions were then expressed as percentages compared to that of the region with the greatest uptake. When the Pancreas Percentage of any region was >70% and the SUV was >2·5, the region was considered to be a lesion. For example, when all regions met the criteria, the PET scan was assumed to show diffuse uptake. Similarly, when a single region met the criteria, it was considered to represent a single focal region.

Molecular diagnosis

Prior to the PET studies, all patients received a molecular diagnosis of K_{ATP} channel hyperinsulinism. The methodological details and part of the results of the mutational analyses have been described previously.[7] Briefly, all exons, exon–intron boundaries, and the promoter region of the K_{ATP} channel genes, *ABCC8* and *KCNJ11*, were amplified from genomic DNA and directly sequenced. The parental origin of each mutation was determined by an analysis of the parents. Molecular diagnoses of diffuse and possible focal forms were made on the basis of biallelic and paternally inherited monoallelic K_{ATP} channel mutations, respectively.[7]

Arterial stimulation venous sampling analysis

Arterial stimulation venous sampling (ASVS) analysis was performed under general anaesthesia. All medical treatments were halted 2 days before examination. During the study, normoglycaemia was maintained by glucose infusion. A catheter was placed in the right hepatic vein for sampling. After selective catheterization of the superior mesenteric, gastroduodenal, and splenic arteries, calcium gluconate (0·0125 mmol/kg) was rapidly injected through the catheter in each selectively catheterized artery. Hepatic venous blood was obtained before and 30, 60, 90, 120 and 150 s after calcium injection. A positive finding was defined as a twofold increase in insulin levels in the 30- or 60-s samples (or both) obtained from the hepatic vein.

Results

The patient profiles and results of the [^{18}F]DOPA PET analyses, molecular analyses, ASVS and histological findings are summarized in Table 1.

By inspection, four patients were found to have single focal uptake (patients 4, 5, 6 and 13) and four others were found to have diffuse uptake (patients 1, 2, 3 and 17). We unexpectedly encountered difficulties in assigning the other patients as having diffuse or focal forms. One patient (patient 9) appeared to have no uptake, while others showed irregular uptakes with a variety of background uptakes resembling double focal (patients 7, 8 and 10) or irregular diffuse uptake (patients 11, 12, 14, 15 and 16). Overall, by inspection, the PET diagnosis was consistent with the molecular diagnosis in only 7 of 17 patients. Representative results of these PET scans are shown in Fig. 1.

As it appears that higher background uptake, especially in the head, makes the interpretation of PET scans more difficult, we employed an objective index—the Pancreas Percentage—and reanalysed the PET results. The idea was to smooth out the irregular background uptake and identify the focal lesion with the highest uptake, assuming that multiple focal lesions are extremely rare. Cut-off values were set by taking into account the PET images of patients with known diffuse lesions. When using the Pancreas Percentage, the PET diagnosis was more strongly correlated with the molecular diagnosis; it was consistent in 10 of 17 patients.

Pancreatectomy was performed in 12 patients who exhibited resistance to medical treatment. For patients with paternal mutations, partial resection of the pancreas was attempted using extensive intra-operative biopsies guided by the PET results. In patient 9, who showed no uptake by PET, extreme intensification of PET signals revealed faint uptake in the head (Fig. 1, f2). During surgery, the corresponding area of the pancreas was repeatedly biopsied, which lead to the identification and resection of a thin lesion that was 6 mm in diameter.

Postoperative histology revealed that the molecular diagnosis correctly predicted the histology in all cases. PET diagnosis by inspection was correct in only 6 of 12 cases, whereas the diagnosis was correct in 9 of 12 cases when the Pancreas Percentage was used. Excluding patient 9 whose lesion was initially not visible, two patients (patients 11 and 12) were incorrectly diagnosed using the Pancreas Percentage; histology showed that these two patients had large and somewhat scattered lesions. The diagnosis of the focal form was made through the identification of a region of the pancreas with normal islets. Patient 11 exhibited loss of heterozygosity according to molecular analysis.[7]

Discussion

The present study is the first to report the diagnostic accuracy of [^{18}F]DOPA PET for Asian patients with congenital K_{ATP} channel hyperinsulinism. Although the number of patients is relatively small, two important findings were obtained through this study. First, [^{18}F]DOPA PET appears to be less effective for localizing lesions in Asians than that reported by previous studies mainly from European and North American centres.[2,8–11] Second, the diagnostic accuracy improved substantially (reaching 75%, 9/12) when a quantitative index, the Pancreas Percentage, was employed to analyse the PET results. Three patients were erroneously diagnosed using the Pancreas Percentage; one presented with a lesion

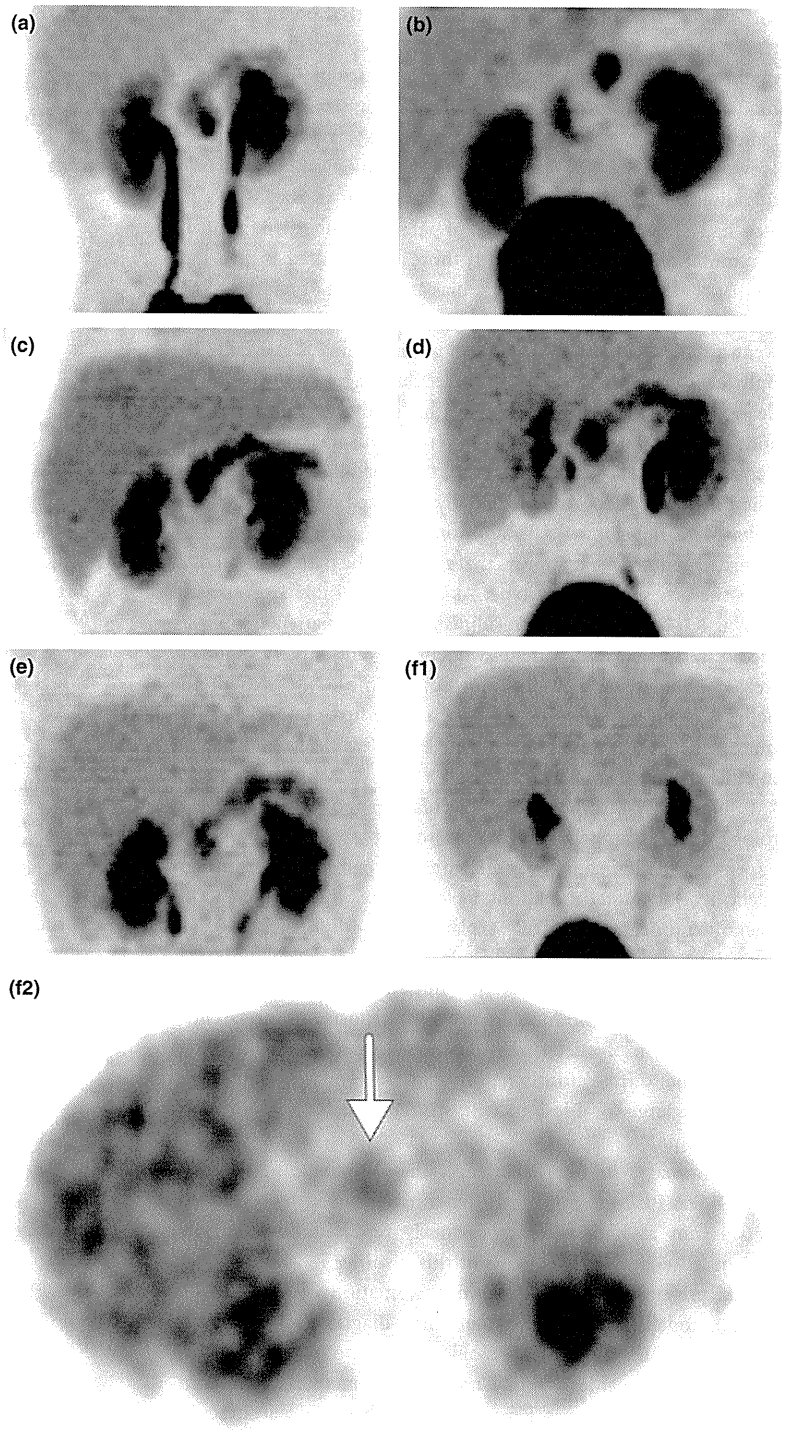

Fig. 1 Representative patterns of [^{18}F]DOPA uptake. Maximum intensity projection (a–f1) and axial image (F2) obtained 30 min after injection. (a, Patient 4): a single focal uptake in the pancreatic head with paternal mutation; histologically, it was a pancreatic head lesion; (b, patient 8): irregular uptake in the pancreatic head and body with a paternal mutation; histologically, it was a pancreatic body lesion (the uptake in the head was false positive); (c, patient 1): typically diffuse uptake with biparental mutations; histologically, it was a diffuse lesion; (d, patient 11): irregular diffuse uptake with a paternal mutation; histologically, it was a large focal lesion (the uptake in the pancreatic head was false positive); (e, patient 12): irregular diffuse uptake with a paternal mutation; histologically, it was a large focal lesion; (f1, patient 9): no uptake with a paternal mutation; histologically, it was a thin lesion in the pancreatic head; (f2, patient 9): retrospective axial imaging revealed weak uptake in the pancreatic head (white arrow). DOPA, dihydroxyphenylalanine.

that was probably smaller and thinner than the detection limits of this methodology. Otonkoski *et al.*[9] report that the smallest focal lesion detected using [^{18}F]DOPA PET is 4 × 5 mm. Likewise, Hardy *et al.*[10] report that the minimum size of focal adenomatosis that can be recognized by [^{18}F]DOPA PET scans is 6 ± 2 mm. In the present study, the smallest lesion detected was 8 mm (patient 4). The other two patients who were erroneously diagnosed as having the diffuse form actually had large focal lesions, which are inherently difficult to differentiate from the diffuse form.

The reason why we observed an increased incidence of irregular appearances on PET scans remains unclear; one possibility is simply a lack of experience. The first [^{18}F]DOPA PET scan for congenital hyperinsulinism in Japan was performed in 2005. Since then, all scans in this country have been performed by our group. In spite of

this, our experience is limited as compared with those of large centres in the US and in Europe. Furthermore, correct interpretation might require more experience in this particular imaging technique, even for experienced nuclear radiologists. The use of the Pancreas Percentage might counteract this lack of experience. However, another possibility is the actual biological differences in this disease in Japanese patients. For unknown reasons, our subjects had a relatively large amount of paternally inherited monoallelic mutations as compared with those documented in previous reports.[2,8] This is not caused by a bias at the level of referral to the PET studies. Prior to the PET studies, 16 of the 17 patients underwent a molecular testing in our laboratory which is currently the only facility routinely offering a molecular diagnosis of this disorder in Japan. During the period of this study, we diagnosed five additional cases of K_{ATP} channel hyperinsulinism who did not participate in the PET study; only one of them had biallelic mutations, whereas the other four had paternally inherited monoallelic mutations (data not shown). In addition to the excess of paternally inherited mutations, it appears that many of the patients presented with the less severe phenotype. As shown in Table 1, even with known K_{ATP} channel hyperinsulinism, 5 of 17 patients could be medically treated and patients who underwent surgery could be treated by octreotide at least for some time. Only two of the patients received pancreatectomy within the first 4 months. These milder phenotypes might be correlated with less clear uptake, making PET diagnosis more difficult.

Interestingly, in our series, molecular diagnosis predicted histology most accurately. In contrast to our experience, previous papers report that some patients with paternally inherited monoallelic K_{ATP} channel mutations have a diffuse histology.[2,12] This phenomenon can be partially explained by additional undetected mutations in the maternal allele.[12] However, this speculation does not explain the excess of paternal mutations exhibited by these patients, which is common in previous reports. As preferential underdetection of maternally inherited mutations is unlikely, some of the 'diffuse' forms reported in these publications might actually be large focal lesions with scattered islets, as reported by Yorifuji *et al.*[7]

In summary, in our experience, the diagnostic accuracy of [^{18}F]DOPA PET for congenital hyperinsulinism in Japanese subjects is not as high as that previously reported for Caucasian patients. Preoperative diagnosis of this disorder in Asian subjects should preferably be performed in combination with other diagnostic modalities, especially molecular diagnosis. Nonetheless, [^{18}F]DOPA PET is an indispensable methodology that can accurately localize focal lesions in a noninvasive manner. When combined with the Pancreas Percentage, the diagnostic accuracy of [^{18}F]-fluoro-L-dihydroxyphenylalanine positron emission tomography is augmented, even in Japanese subjects.

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Conflicting interests and financial disclosure

Nothing to declare.

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Novel Insights from Clinical Practice

HORMONE RESEARCH IN PÆDIATRICS

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Lasting ^{18}F-DOPA PET Uptake after Clinical Remission of the Focal Form of Congenital Hyperinsulinism

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Established Facts

- ^{18}F-DOPA positron emission tomography (PET) is a useful tool for detecting the localization of the focal form of K_{ATP} channel hyperinsulinism.
- K_{ATP} channel hyperinsulinism occasionally resolves itself spontaneously, which has been attributed to the apoptotic death of abnormal β-cells in previous studies.

Novel Insights

- Uptake of ^{18}F-DOPA does not always correlate with the insulin-secreting capacity of β-cells.
- Spontaneous resolution could be a functional process rather than the result of the apoptotic death of abnormal β-cells.

Key Words
Congenital hyperinsulinism · Hypoglycemia · Glucose · ^{18}F-DOPA

Abstract

Background: Positron emission tomography (PET) using ^{18}F-DOPA is a useful tool for detecting the focal forms of congenital hyperinsulinism. ^{18}F-DOPA is taken up by aromatic L-amino acid decarboxylase in pancreatic β-cells. However, the role of this enzyme in insulin secretion is unknown. ***Subjects and Methods:*** A Japanese boy who presented with symptomatic hyperinsulinemic hypoglycemia at the age of 2 days and spontaneous resolution at 1 year and 10 months was subjected to mutational analysis and repeated ^{18}F-DOPA PET scans. ***Results:*** Mutational analysis revealed a paternally inherited monoallelic mutation, c.4186G>T (p.D1396Y), in the *ABCC8* gene, and an ^{18}F-DOPA PET scan revealed focal uptake in the body of the pancreas. The patient was successfully treated with frequent feeding. A follow-up PET scan revealed virtually identical uptake to that observed previously. However, in the arterial stimulation-venous sampling proce-

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dure, no significant insulin release was observed. He was placed on a normal diet, and no hypoglycemia recurrence was observed. ***Conclusion:*** This case demonstrates two important findings. Firstly, the uptake of ^{18}F-DOPA does not correlate with the insulin-secreting capacity of the lesion. Secondly, clinical remission could be a functional process not necessarily accompanied by the apoptotic death of abnormal β-cells.

Table 1. Results of the ASVS procedure

Time	Insulin, μU/ml		
	splenic	gastroduodenal	superior mesenteric
0 s	6.2	5.0	4.7
30 s	5.2	5.0	8.2
60 s	6.9	8.2	5.8
90 s	7.6	9.9	7.7
120 s	5.2	7.7	3.7

Introduction

Congenital hyperinsulinism is the most common cause of persistent hypoglycemia in the neonatal/infantile period. Since severely affected patients often develop profound neurological sequelae [1, 2], surgical resection of the pancreas is mandated when medical treatment is not effective. Regarding surgical treatment, it is critically important to differentiate between the two histological forms of the disorder, the diffuse and focal forms. To surgically treat the diffuse form, subtotal pancreatectomy is indicated. However, the result of this procedure is often not satisfactory, and many patients suffer residual hypoglycemia or develop diabetes mellitus postoperatively [3]. On the contrary, patients with the focal form can be cured by partial resection of the pancreas without complications [4, 5].

The focal form is known to occur in individuals with a monoallelic, paternally inherited mutation in the *ABCC8* or *KCNJ11* gene, which code for the two subunits of the ATP-sensitive potassium channel (K_{ATP} channel) [4]. Several diagnostic modalities have been developed to identify focal lesions preoperatively including mutational analysis, the arterial stimulation-venous sampling (ASVS) procedure, pancreatic venous blood sampling, and positron emission tomography (PET) using [^{18}F]-fluoro-L-DOPA (^{18}F-DOPA PET). Currently, ^{18}F-DOPA PET is becoming the modality of choice due to its noninvasiveness and accuracy [6–11]. However, in this report, we present a patient with the focal form of congenital hyperinsulinism, in whom disease activity did not correlate with ^{18}F-DOPA uptake.

Case Report

The patient was a Japanese boy born after 39 weeks of uneventful pregnancy with a birth weight of 3,630 g. There was no family history of hypoglycemia or diabetes mellitus. On day 2, he presented with generalized convulsions accompanied by hypoglycemia (14 mg/dl, 0.78 mmol/l). A subsequent laboratory examination revealed hypoglycemia (39 mg/dl, 2.15 mmol/l) in the presence of an elevated serum insulin level (29.1 μU/ml, 202 pmol/l), and the peak glucose infusion rate to maintain normoglycemia was 10 mg/kg/min. Otherwise, endocrinological and metabolic screening did not reveal any abnormalities. A diagnosis of congenital hyperinsulinism was made, and mutational analysis using DNA extracted from peripheral blood leukocytes revealed a paternally inherited heterozygous mutation, c.4186G>T (p.D1396Y), in the *ABCC8* gene. Although functional studies were lacking, this mutation appeared to be pathogenic since it was not listed in the dbSNP (build 13.2, http://www.ncbi.nlm.nih.gov/snp/) and the Japanese SNP (http://snp.ims.u-tokyo.ac.jp/) databases, and both the SIFT (http://sift.bii.a-star.edu.sg/) and the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph/index.html) programs predicted a detrimental effect of this mutation. In addition, the mutation was not found in 106 Japanese control subjects (data not shown). No other mutations were identified in the *KCNJ11*, *GCK*, or *GLUD1* genes. ^{18}F-DOPA PET revealed strong focal uptake in the body of the pancreas, leading to a diagnosis of the focal form of K_{ATP} channel hyperinsulinism (fig. 1, left panels).

Treatment with 15 mg/kg diazoxide was not effective at preventing hypoglycemia. Since the patient remained free of symptoms due to frequent feeding (10–12 times a day), despite occasional hypoglycemia (<40 mg/dl, 2.22 mmol/l), he was treated conservatively under frequent self-monitoring of blood glucose.

As the patient remained symptom-free, and self-monitored blood glucose had gradually progressed towards normoglycemia, he underwent a follow-up PET scan at the age of 1 year and 10 months. The uptake in the body of the pancreas was virtually identical to that observed in the previous PET scan (fig. 1, right panels). To investigate the discrepancy between the PET results and clinical improvement, an ASVS study was performed, which revealed low basal and stimulated insulin secretion following calcium infusion into the splenic, gastroduodenal, or superior mesenteric arteries (table 1). Due to these results, he was placed on a normal diet involving three meals a day, and no further hypoglycemia was observed on frequent blood glucose monitoring. Blood glucose after a 12-hour fast constantly remained within a range of 76–98 mg/dl (4.21–5.44 mmol/l).

Mutational Analysis

Mutational analysis was performed as described previously [12]. Briefly, all exons and the exon-intron boundaries of the

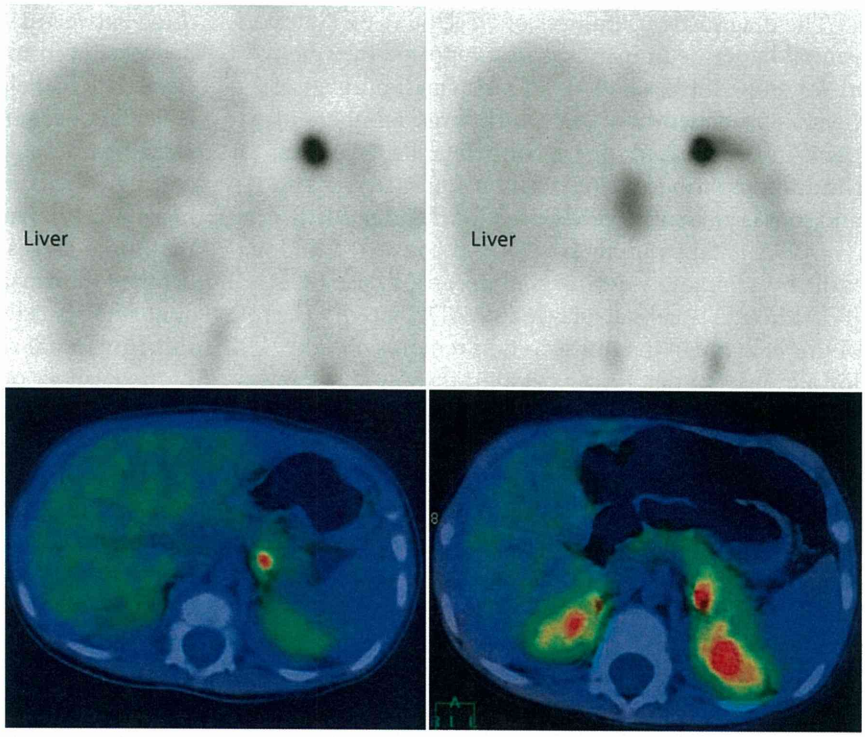

Fig. 1. ^{18}F-DOPA PET scans taken at age 8 months (left) and again at 1 year and 10 months (right). Upper panels show coronal images of abdominal PET scans and lower panels show fused axial PET/CT images. The maximal standardized uptake values for these lesions were 5.0 (left) and 6.8 (right), respectively.

KCNJ11, *ABCC8*, and *GCK* genes were amplified from leukocyte genomic DNA. Then, the amplified products were purified using the Wizard PCR Preps DNA purification system (Promega, Wisc., USA) and directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Calif., USA). For the *GLUD1* gene, only exons 6–7 (the antenna domain) and 10–12 (the GTP binding domain) were sequenced since the previously reported mutations were exclusively found in these regions. In addition, 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 using the SALSA MLPA kit P117 (MRC-Holland, Amsterdam, The Netherlands).

ASVS Procedure

The ASVS study was performed as described by Abernethy et al. [13]. Briefly, a sampling catheter was inserted from the femoral vein of the infant and placed into the right hepatic vein. Then, another catheter was inserted from the femoral artery and sequentially placed into the splenic, gastroduodenal, and the superior mesenteric arteries, and in each artery, calcium gluconate (0.0125 mmol of calcium per kilogram body weight) diluted in 2 ml of saline were infused over 10 s. Blood samples were collected at 30-second intervals for 150 s, and the concentrations of calcium, insulin, and glucose were determined for each sample.

^{18}F-DOPA PET

^{18}F-DOPA PET studies were performed at the PET facility of Kizawa Memorial Hospital, as described by Ribeiro et al. [10]. The scan results were fused with those of a CT scan taken at the same time in order to localize the focal lesion more accurately. Currently, Kizawa Memorial Hospital is the only facility in Japan performing ^{18}F-DOPA PET for congenital hyperinsulinism. All PET results were interpreted by one of the authors, H.N., who is a board-certified radiologist with a personal experience of over 7,000 PET studies including 28 with ^{18}F-DOPA.

Discussion

In this paper, we report on a patient with the focal form of congenital hyperinsulinism whose focal uptake on ^{18}F-DOPA PET remained virtually unchanged despite clinical remission of hypoglycemia. As the routine clinical practice, the lesion should have been resected immediately following the first PET scan to avoid the risk of possible brain damage. For this particular patient, however, we continued the diet therapy until clinical remission. As a result, this case highlighted two important findings. Firstly, the uptake of ^{18}F-DOPA does not correlate with the insulin-secreting capacity of the lesion, and secondly, clinical remission is probably not caused by the apoptotic death of insulin-producing cells.

The diagnostic usefulness of ^{18}F-DOPA PET for congenital hyperinsulinism was first reported by Ribeiro et al. [6] and Otonkoski et al. [7] for a small number of patients. Subsequently, studies with large numbers of patients in Europe and the US confirmed its usefulness for the differentiation and localization of focal lesions [8–11], and so it is currently widely used for this disorder.

DOPA is taken up by β-cells and converted to dopamine by aromatic L-amino acid decarboxylase (AADC) [8]. Although β-cells display high AADC activity, the role of this enzyme in insulin secretion remains obscure. Using a mouse model, Ericson et al. [14] showed that intravenously injected L-DOPA accumulates within β-cell secretory granules and inhibits insulin secretion. Also in a mouse model, Lundquist et al. [15] demonstrated that intravenously administered L-DOPA is rapidly converted to dopamine and inhibited glucose-induced insulin secretion from β-cells. The inhibition was reversed with an AADC inhibitor, suggesting that dopamine and not DOPA is responsible for this inhibition [15]. In contrast, de Lonlay et al. [8] demonstrated that insulin secretion in a patient with congenital hyperinsulinism was not affected by the administration of an AADC inhibitor. An in vitro study using rat INS cells also suggested that insulin secretion is not affected by AADC inhibitor treatment [8]. In addition, in adult patients with insulinoma, ^{18}F-DOPA PET could not sensitively detect tumors, suggesting that DOPA uptake is not directly related to the insulin-secreting capacity of the cells [16]. The fact that DOPA uptake by the focal lesion remained virtually identical despite clinical resolution also suggests that the role of AADC in insulin secretion, if any, is relatively small.

It is known that congenital hyperinsulinism is often resolved spontaneously as the patient grows older. The time to resolution differs from case to case; it occurs most often between 1–5 years of age but can happen as early as 8 weeks, even in cases of diffuse form hyperinsulinism caused by biallelic mutations in the K_{ATP} channel genes [17]. The mechanism leading to spontaneous resolution remains unclear. The apoptotic death of insulin-oversecreting β-cells has been proposed as a possible mechanism [18, 19]. However, our case showed that, at least in the initial stages, clinical resolution occurs without the death of the causative β-cells. Since our case suggests that abnormal β-cells lose their responsiveness to calcium, this functional shutdown could be caused by a decrease in the number of functional K_{ATP} channels, as is seen in MIN6 cells that have been chronically treated with sulfonylurea [20]. Physiologically, spontaneous resolution of K_{ATP}-channel hyperinsulinism resembles the phenomenon of β-cell failure following prolonged sulfonylurea treatment of patients with diabetes mellitus. Interestingly, it has been reported that the loss of insulin secretory capacity following prolonged glibenclamide treatment is initially functional and reversible, although the eventual result is known to be the absolute loss of β-cell mass [21]. Similar sequence of events might be operating behind the spontaneous resolution of congenital hyperinsulinism. Understanding the mechanism of the spontaneous resolution of this condition might lead to an efficient medical therapy if we could manipulate the process.

Acknowledgements

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CD36 deficiency predisposing young children to fasting hypoglycemia

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Abstract

Fatty acid (FA) β-oxidation defects cause hypoglycemia. Our aim was to determine if CD36—a membrane transporter for long-chain FAs—deficiency predisposes children to hypoglycemia. After overnight fasting, we measured parameters for carbohydrate and FA metabolisms at 12-, 14-, and 16-hour fasting points in 51 preschool children with histories of episodic hypoglycemia and 49 age-matched healthy controls. Simultaneously, the expressions of CD36 on platelets and monocytes were examined to determine the phenotypes. Six of the 51 hypoglycemic children and none of the 49 control children were diagnosed as having type I CD36 deficiency. Four and 3 children were diagnosed as having type II CD36 deficiency, respectively. Hypoglycemia was often recurrent in the type I CD36 group. At any fasting point, the type I CD36 group showed significantly lower blood glucose and insulin concentrations than the other groups: glucose, $P < .001$ vs control group and $P < .01$ or $P < .001$ vs type II/wild-type CD36 hypoglycemic groups; insulin, $P < .001$ vs control group and $P < .01$ vs type II/wild-type CD36 hypoglycemic groups. Free FA concentration in the type I group was always 1.5- to 2.0-fold higher than that in the other groups, whereas the total ketone body concentration was consistently about two thirds of that in the other groups. Among the type II, wild-type, and control groups, there were no significant differences in the parameters except that the wild-type group showed significantly lower FFA concentration ($P < .05$). These results suggested that type I CD36 deficiency but not type II CD36 deficiency predisposes preschool children to hypoglycemia.

1. Introduction

CD36 is a multifunctional membrane-associated glycoprotein with a molecular weight of 88 kd [1-5]. CD36 is a receptor for collagen and thrombospondin on platelets and oxidized low-density lipoproteins on macrophages [1-4]. CD36 also plays an important role in the uptake of long-chain fatty acids (LCFAs) in the heart, skeletal muscle, adipose tissue, and small intestine [5].

Based on its expression patterns on platelets and monocytes, CD36 deficiency is classified into 2 subgroups: type I and II [6]. Type I CD36 deficiency lacks CD36 expression on

Authors' contributions: This study was conducted through the leadership of Dr Takashi Miida. Hironori Nagasaka, Takashi Miida, Ken-ichi Hirano, and Hitoshi Chiba made a design for this study. Hironori Nagasaka, Tohru Yorifuji, Tomozumi Takatani, Yoshiyuki Okano, Hirokazu Tsukahara, and Tetsuya Ito collected blood samples from the enrolled children after the informed consents from the children's parents. Hidekatsu Yanai, Satoshi Hirayama, and Ken-ichi Hirano performed statistical analyses together with the determinations of CD36 phenotypes. Tomozumi Takatani and Tohru Yorifuji performed gene analyses. Takashi Miida, Hidekatsu Yanai, Shu-Ping Hui, and Satoshi Hirayama interpreted the data and described the figures. Satoshi Hirayama, Hironori Nagasaka, and Takashi Miida described this manuscript.

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both cell types, whereas type II CD36 deficiency lacks expression only on platelets. Most type I cases are either homozygous or compound heterozygous for CD36 gene mutations, whereas type II cases are often free of CD36 gene mutations [7-9]. CD36 deficiency is one of the common genetic disorders in Japan [9-10]. As the metabolic manifestations of CD36-deficient adult subjects, decreased insulin sensitivity and postprandial hypertriglyceridemia have been reported [10-13]. However, for children, its clinical manifestations and the prevalence have been scarcely studied [14,15].

Hypoglycemia is highly prevalent in children, but its underlying disease or condition cannot be identified in most cases [16-18]. Children with fatty acid (FA) β-oxidation defects often show profound hypoglycemia [19].

The present study aimed to elucidate whether CD36 deficiency is attributable to the development of hypoglycemia in young children as other FA β-oxidation defects. We examined the prevalence of CD36 deficiency among preschool children with histories of hypoglycemia and examined the glucose and FA metabolism in them with special reference to CD36 phenotype.

2. Subjects and methods

2.1. Subjects

From 2004 to 2008, we prospectively screened 198 consecutive preschool children brought to our local affiliated hospitals for unconsciousness and/or seizures in the morning (Fig. 1).

Fifty-three children (23 girls and 30 boys, aged 1.8-5.2 years) were found to have hypoglycemia. Their blood glucose (BG) concentrations were only 25 to 42 mg/dL. Blood gas analyses revealed that their base excess ranged from −2.7 to −6.1 mEq/L. All these children had bradycardia or tachycardia with excessive sweating, suggesting hypoglycemia-induced autonomic responses. Their symptoms disappeared immediately after intravenous glucose infusions.

The 53 children were referred to our institutions for further examinations at 1 to 3 months after their episodes of hypoglycemia. At the time of admission for an extended fasting test, their ages were 2.1 to 5.5 years; and they were free of symptoms suggestive of any disorders. As age-matched healthy controls, we enrolled 49 children (22 girls and 27 boys) aged 2.1 to 4.6 years.

Both hypoglycemic and control children had no medical problems during their newborn and infancy periods and had grown completely healthy. There were no significant differences in birth weight and gestational age between both children: hypoglycemic children—2673 to 3462 g and 37 to 41 weeks; control children—2790 to 3369 g and 38 to 40 weeks.

Informed consent was obtained from the parents before enrolling these children in this study. The protocol was approved by the medical ethics committees of the participating institutions.

2.2. Study design

Firstly, we excluded children with metabolic or hormonal diseases that cause hypoglycemia. To diagnose hyperinsulinemia; hyperthyroidism; growth hormone deficiency; FA β-oxidation disorders; organic acidemia; fructose-1,6-diphosphatase deficiency; and glycogen storage disease, we specifically examined the profiles of blood amino acids and acylcarnitine, and blood concentrations of ammonia, lactate, insulin, growth hormone, insulin-like growth factor–1, free thyroxine, free thyronine, thyroid-stimulating hormone, and cortisol. We also examined profiles of urinary organic acids.

Secondly, the children with histories of hypoglycemia were divided into 3 subgroups according to the CD36 expression patterns on the platelets and monocytes by flow cytometry: Type I CD36, type II CD36, and wild-type hypoglycemic groups. The CD36 expression patterns in the control children were also examined.

For the 3 hypoglycemic groups and the control group, extended fasting tests were performed. At 7:00 to 7:30 PM on the day before blood sampling, we provided the children with suppers containing one third of the daily required calories for children of these ages. Fasting blood samples were collected from cubital veins for biochemical assays 3 times in the morning (12, 14, and 16 hours after supper). Body weight and height SD scores were also recorded for all enrolled children.

2.3. Biochemical assays

Fasting BG and insulin concentrations were determined by an enzymatic method and an enzyme immunoassay using a commercial kit (TOSOH-II; Tosoh, Tokyo, Japan), respectively. Serum total cholesterol and triglycerides were measured enzymatically using an automated analyzer. Low-density lipoprotein cholesterol and high-density lipoprotein cholesterol were determined by homogenous assays. Serum concentrations of free FA (FFA) and total ketone bodies (TKB) were measured by enzymatic methods using commercial kits (NEFA-SS kit EIKEN; Eiken Chemicals, Tokyo, Japan, and Total-ketone body kit; Kainos Laboratories, Tokyo, Japan, respectively). Acylcarnitine profiles were examined by tandem mass spectrometry as described previously [20].

2.4. CD36 phenotyping

Phenotypes of CD36 were determined by flow cytometry using platelets and monocytes as described previously [6]. Fasting venous blood was drawn into a tube containing EDTA-K_2 to prepare platelet-rich plasma (PRP). In monocyte assays, PRP was processed in a Multi-Q-Prep (Coulter, Miami, FL) for hemolysis and fixation. The prepared PRP was then mixed with a fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody (Mab) (FA6-152; Immunotech, Miami, FL) [6]. To detect CD36 expressions on platelets or monocytes, the CD36 signal was gated with either a phycoerythrin-conjugated anti-CD42b Mab (AN51; Dako, Copenhagen, Denmark) using an EPICS Profile II flow cytometer (Coulter, Miami, FL) or an

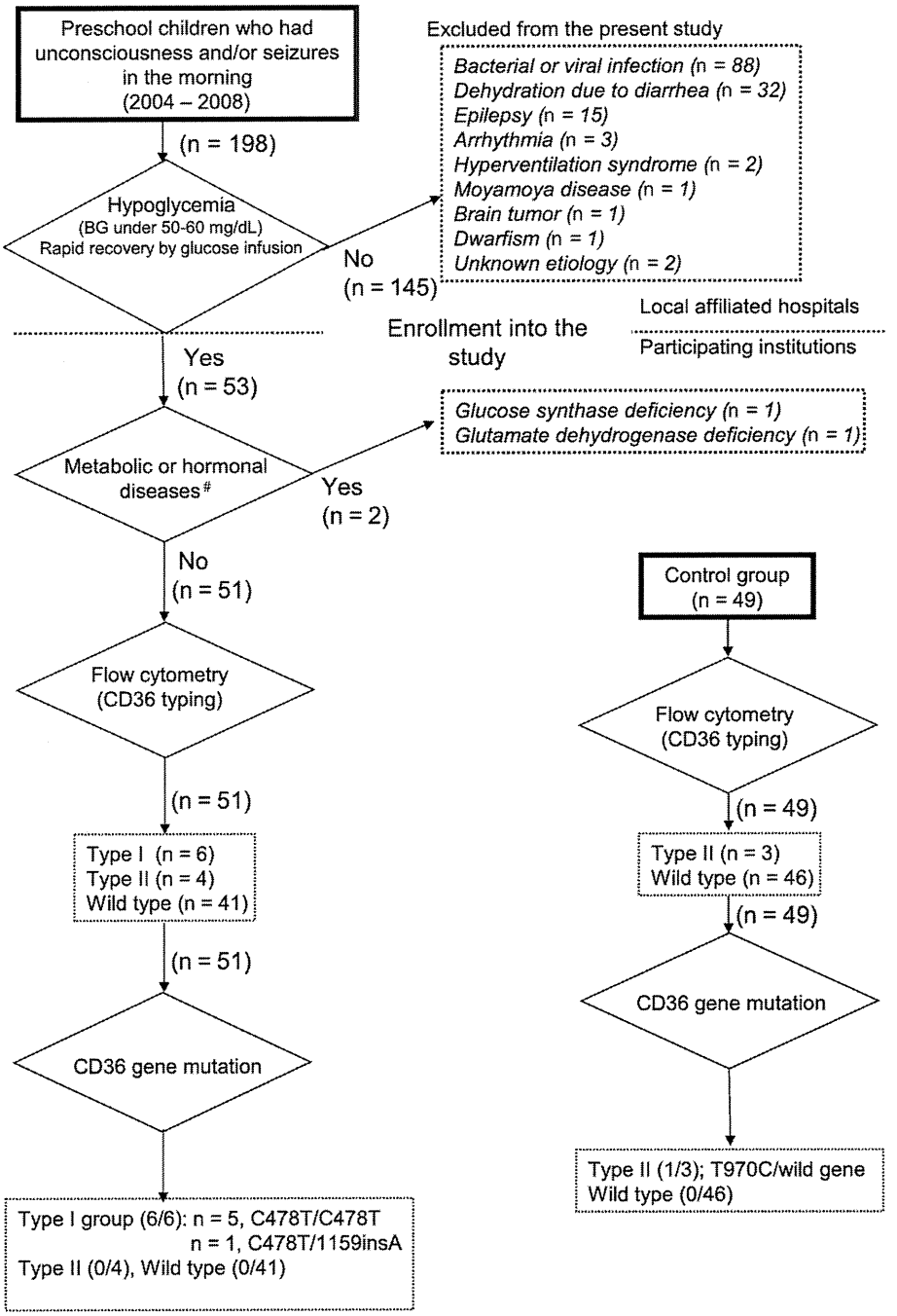

Fig. 1. Flowchart for classifying hypoglycemic children. #Profiles of blood amino acids and acylcarnitine, plasma ammonia, and lactate levels were measured. Insulin, growth hormone, insulin-like growth factor–1, free thyroxine, free thyronine, thyroid-stimulating hormone, and cortisol were also examined to detect endocrinologic disorders leading to hypoglycemia.

FITC-conjugated anti-CD14 Mab (MY4-FITC, Coulter) using an XL-MCL flow cytometer (Coulter).

2.5. CD36 gene analysis

In children with types I or II CD36 deficiencies, 3 common mutations of the CD36 gene, irrespective of histories with hypoglycemic episodes, were determined: (*a*) a substitution of T for C at nt 478 in exon 4 (C478T), (*b*) an AC deletion at nt 539 in exon 5 (539delAC), and (*c*) an A insertion at nt 1159 in exon 10 (1159insA) [7-9,14]. A previous study showed that 478T mutation impairs the maturation of the CD36 precursor, leading to CD36 defects on both platelets and macrophages [7]. Both

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).