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Abstract

Introduction

Congenital hyperinsulinism and hyperammonemia (CHH) is caused by gain of function of glutamate dehydrogenase (GDH). The genetic abnormalities are known to be located in three specific regions on the GDH protein. We describe here three different missense mutations identified in five new Japanese patients with CHH. And to study the genotype-phenotype correlations in patients with GLUD1 mutations, we analyzed previously reported Japanese cases.

Methods

An Epstein-Barr virus-transformed lymphoblastoid cell line was established from the 5 patients and control subjects, and was used for enzymatic and molecular analyses.

Results

All patients developed seizures with loss of consciousness associated with hypoglycemia and had persistent hyperammonemia. All patients had similar basal GDH activity of lymphoblasts and insensitivity to GTP inhibition. Genetic studies identified heterozygous I444M mutation in Patient 11, S217C mutation in Patient 1, and H262Y mutation in Patients 2, 3, and 4. Patients 3 and 4 were child and father, respectively. COS cell expression study confirmed that I444M and H262Y mutations were disease-causing genes.

Conclusions

We identified three mutations (I444M, H262Y, and S217C), and the former is a newly described mutation. A summary of 17 reported Japanese patients (10 boys and 7 girls) with GDH mutations showed 8 patients had mutation at the site of the GTP-binding region, 2 at the

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site of the antenna-like structure, and 7 at the site of the hinge region. Analysis of the reported cases showed no clear association between clinical phenotype and mutation sites. However, G446D mutation seems to be associated with serious abnormalities.

Key Words: Hyperinsulinism/hyperammonemia; Glutamate dehydrogenase; GLUD1; Mutation, Genotype-phenotype correlation

Introduction

Congenital hyperinsulinism and hyperammonemia (CHH) is a disease characterized by symptomatic hypoglycemia from the neonatal and infantile period with persistent hyperammonemia. In 1998, Stanley et al¹⁾ proposed that abnormalities of a single gene of the glutamate dehydrogenase (GDH) as the cause of CHH. GDH is a mitochondrial enzyme that catalyzes reversible oxidative deamination of L-glutamate to 2-oxoglutarate using cofactors nicotinamide adenine dinucleotide (NAD) and/or NAD phosphate (NADP). GDH is controlled by allosteric effectors of inhibition by GTP and NADH and activation by ADP, NAD⁺, and leucine. Patients with CHH show reduced sensitivity to inhibition by GTP for the altered form in the GTP binding site of GDH (in other words, "gain of function" of GDH causes CHH). It was proposed that this altered form of GDH leads to higher oxidation rates and ATP production. In pancreatic β -cells, the ATP/ADP ratio increases due to GDH dysregulation resulting in closure of ATP-sensitive potassium (KATP) channels and depolarization. Such depolarization causes calcium influx and excessive secretion of insulin. In addition to the KATP channels, non-selective cation channels are also reported to be involved in the depolarization²⁾. Unlike the genetic defects of sulfonylurea receptor (SUR1) and inward rectifier K⁺ channel (Kir 6.2) that cause hyperinsulinemic hypoglycemia from the neonatal period, diazoxide that acts on KATP channels effectively maintains blood glucose at normal levels^{3,4)}.

With regard to hyperammonemia, activated GDH in the liver and other tissues consumes massive amounts of glutamate and produces ammonia. In addition to this, the production of N-acetylglutamate, which is one of the positive effectors of carbamoyl phosphate synthetase, decreases due to low levels of glutamate in hepatocytes^{1,5)}. However, it is reported that administration of carbamylglutamate does not result in normalization of hyperammonemia⁶⁾.

Initial reports described mutations in the GDH protein located in the GTP allosteric sites of amino acid codons between 445 and 455¹⁾. Subsequent studies reported that the mutations at other GDH sites cause CHH¹⁰⁻²⁵⁾. The gene mutations currently detected are located at three sites on the GDH protein; the hinge region^{1,10-18)}, the GTP binding region^{13,14,18-23)} and the antenna-like structure^{16,24,25)}. We demonstrated that mutations at both the site of the hinge region and the antenna-like structure were associated with dysregulation of GDH activity through different mechanisms²⁵⁾.

In this report, we describe three different missense mutations identified in five new Japanese patients with CHH. We also analyzed the correlation between the clinical phenotypes and the genotypes in 17 Japanese patients with CHH.

Materials and methods

Patients

Patient 11, a 13-year-old female, developed the first afebrile seizure at 15 months of age.

Since then she was treated with antiepileptic agents, although she continued to have several episodes of seizure. She had a short stature (less than mean for age $-2SD$). During an acute seizure attack, blood glucose was 30 mg/dL with plasma ammonia concentration of $>100 \mu\text{mol/L}$. The electroencephalogram (EEG) showed generalized regular spike-and-wave discharge at 3 Hz. She was treated with diazoxide, which resulted in normalization of blood glucose level and disappearance of hypoglycemic episodes.

Patient 1 suffered a febrile convulsion at 10 months of age and had an afebrile convulsion at 3 years and 5 months of age. Blood glucose level was 20-30 mg/dL, and insulin level was 17.5 $\mu\text{g/dL}$. No remarkable abnormalities were detected on analysis of urinary organic acid, endocrinological tests, or abdominal CT imaging. The plasma ammonia level was 60-83 $\mu\text{mol/L}$. At 6 years of age, EEG abnormality was identified and antiepileptic medication was initiated.

Patient 2 was born at 41 weeks of gestation, weighting 2715 g, after an uneventful pregnancy and delivery. At 10 months of age, he developed the first afebrile seizure after a meal. During the attack, blood glucose level was 29 mg/dL, and plasma ammonia concentration was 142 $\mu\text{mol/L}$. Aminogram of blood and urine showed unremarkable changes. Analysis of urinary organic acid and acylcarnitine indicated no metabolic diseases. Endocrinological abnormalities such as growth hormone deficiency were not detected. Ultrasound and MRI revealed no abnormalities in the liver or pancreas. He was treated with low protein diet, but hypoglycemic episodes and hyperammonemia continued. Finally, he was treated with diazoxide, which resulted in improvement of blood glucose level.

Patient 3 was born after uneventful pregnancy and delivery. At 22 months of age, he had tonic seizures and was treated with sodium valproate. Blood analysis at 24 months of age showed blood glucose level of 28 mg/dL, insulin level 18.3 $\mu\text{g/dL}$, and plasma ammonia of 166 $\mu\text{mol/L}$. No remarkable abnormalities were detected in urinary organic acid analysis, endocrinological tests, or on ultrasound and MRI imaging. The first words were spoken at approximately 22 months.

Patient 4 is the father of Patient 3. He lost consciousness at nine months of age. He was diagnosed with leucine-sensitive hypoglycemia. Subtotal pancreatectomy was performed at 11 years of age.

Measurement of GDH activity

An Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line was established from the patients and control subjects, and used for enzymatic and molecular analyses. The lymphoblastoid cells of patients and controls who had no episodes of hypoglycemia ($n=5$) were homogenized in 10 mM Tris-acetate (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA) with a Potter-Elvehjem homogenizer on ice. The cell lysate was centrifuged, and the supernatant was used as the enzyme source. The protein concentration of the supernatant was measured using the Dc Protein Assay (Bio-Rad, Richmond, CA). GDH activity was measured by measuring the oxidation of NADH. The enzyme source was pre-incubated with 50 mM Tris-acetate (pH 8.0), 50 mM NH_4Cl , 0.1 mM NADH, and 2 mM EDTA, for 5 min at 25°C. GDH activity was measured after the addition of 5 mM alpha-ketoglutarate and was monitored by the reduction in UV absorption at 340 nm using a spectrophotometer DU-60 (Beckman, Fullerton, CA). Various concentrations of GTP were added to the reaction mixtures for the allosteric effect of GDH, as described previously²⁵.

Identification of human GDH mutations

Genomic DNA was isolated from lymphoblasts transformed with EBV. Each exon and its flanking intronic region were amplified with a pair of human GDH-specific oligonucleotide primers using polymerase chain reaction (PCR). The products were purified and sequenced by the dye terminator method using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT). Primers used for the PCR amplification in exons 6, 7, 10, 11, and 12 of the human GDH gene (GenBank accession numbers X66300-66312) were as follows: for exon 6; sense primer of 5'-ACTTTACTTAATGTCTATCAGT-3', antisense primer of 5'-ATTGGACTTGACATTTTCATTCT-3', and sequencing primer of 5'-ATTCTTTCAATTACCCCTC-3'. For exon 7; sense primer of 5'-GCTGTCTATAACCAGGACTTT-3', antisense primer of 5'-TGGACCCATGTTGCTGCCAT-3', and sequencing primer of 5'-CAATTAATAGCTGCACCAGAGT-3'. For exon 10; sense primer of 5'-GTGTGAAATTCCTGATACTGGCT-3', antisense primer of 5'-GTGAGTTTGGCGAACAAGATTAT-3', and sequencing primer of 5'-AGGTTTCATTTTCAGCTTTTAC-3'. For exon 11; sense primer of 5'-TGTAGTGTCTGTTCAAGAGAG-3', antisense primer of 5'-ACACACATGTCACGCACTTAC-3', and sequencing primer of 5'-CTATGCCGCAGATGAAATCC-3'. For exon 12; sense primer of 5'-ACAGGGACACAAAGCAGGTC-3', antisense primer of 5'-ACAGTCTGGCGGCTGAGATAG-3', and sequencing primer of 5'-ATGGTTGAGTTGCACTTCAT-3'.

Expression Analysis

Mutant human GDH cDNA was synthesized by specific base substitutions using site-directed mutagenesis into a eukaryotic expression vector (pCDNA3; Invitrogen, Carlsbad, CA) containing the full-length human GDH cDNA. Mutant or normal GDH cDNAs were introduced into COS cells by electroporation with a Gene Pulser (Bio-Rad) at 200 V with 960 mF capacitance. The cells were harvested after 72 h culture. GDH activity was determined in the homogenates of COS cells transfected with mutant and normal constructs as described above to ensure reproducibility.

Results

GDH activity

The activity and allosteric response of GDH in lymphoblasts from the patients and control subjects are shown in Figure 1. The basal GDH activities in Patients 1 to 4 and 11 were similar to that of the control, which were 14.1 nmol/mg of protein/min for Patient 11, 8.2 for Patient 1, 13.2 for Patient 2, 15.1 for Patient 3, 10.7 for Patient 4, and 12.7 for the control. The inhibitory effects of GTP were lower in Patients 11 and 1 to 4 than the control, while the half-maximal inhibitory concentrations of GTP in Patients 11 and 1 to 4 were increased to 180, 150, 120, 360, and 130 nmol/L, respectively (Fig. 1). The half-maximal inhibitory concentrations of GTP for controls were 60.6 ± 11.1 nmol/L.

Identification of GDH mutation

Sequence analysis was performed in all five patients to characterize GDH genes. The mutation in Patient 1 was heterozygous A-to-G transition at nucleotide position 1504 in exon 11, resulting in the replacement of isoleucine (ATA) by methionine (ATG) at codon 444 (I444M). Patients 2 to 4 had heterozygous C-to-T transition at nucleotide position 956 in exon 7, resulting in the replacement of histidine (CAC) by threonine (TAC) at codon 262 (H262Y). Patient 5 showed heterozygous C-to-G mutation at nucleotide position 822 in exon 6, resulting in the

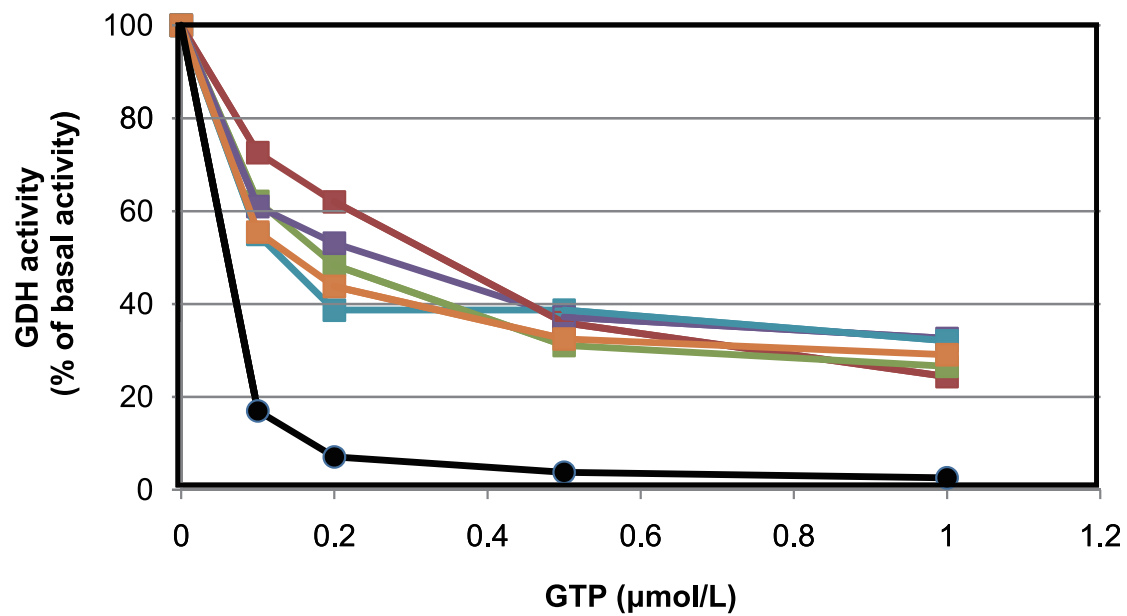


Figure 1. Effects of GTP on glutamate dehydrogenase (GDH) activity in lymphoblasts of Patients 11, 1-4, and control subjects. Orange line, Patient 11; red, Patient 1; green, Patient 2; purple, Patient 3; blue, Patient 4; and black, controls (n=5).

replacement of serine (TCT) by cysteine (TGT) at codon 217 (S217C).

Confirmation of mutation by expression analysis

To determine whether the I444M and H262Y substitutions were associated with GDH activities in lymphoblasts and to assess the potential GDH activities of both substitutions, we reconstructed each mutant in the expression vector pCDNA3 by site-directed mutagenesis. About the S217C mutation, McMullan showed the enzyme analysis²⁰. The GDH activities were established by correcting the transfection efficiency into COS cells. The allosteric responses of

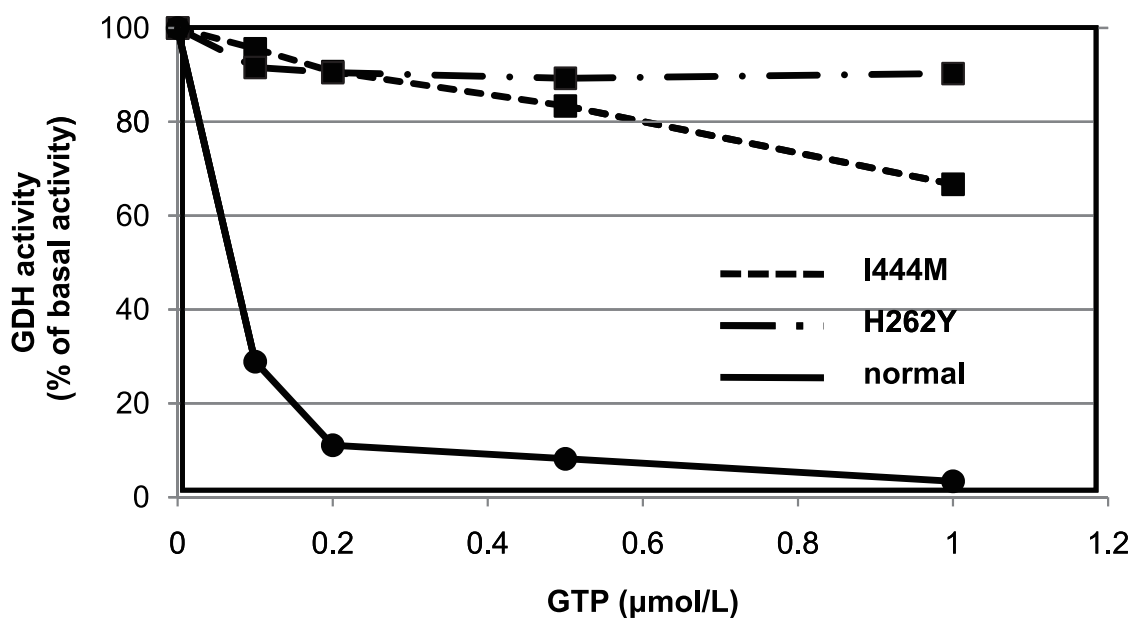


Figure 2. Effects of GTP on glutamate dehydrogenase (GDH) activity in COS cells transfected with normal or the indicated mutant cDNAs constructs by expression analysis.

GDH mutant and normal constructs are shown in Figure 2. The inhibitory effects of GTP were lower in COS cells transfected with the I444M and H262Y constructs than normal constructs. The basal GDH activities in COS cells transfected with the I444M, and H262Y constructs (73.2 and 61.0 nmol/mg of protein/min respectively) were similar to that of normal constructs (85.4 nmol/mg of protein/min).

Discussion

In the present study, we reported a mutation in exon 11 at amino acid codon 444 located in the hinge region (I444M), and mutations at codon 217 (S217C) and codon 262 (H262Y) located in the GTP binding site. I444M is a novel mutation, while H262Y was reported previously by Raizen et al¹⁴⁾ in a patient who developed myoclonus and lethargy in infancy and experienced improvement after the initiation of diazoxide treatment without abnormal EEG recordings. Mutation S217C was reported by MacMullen et al²⁰⁾ in a patient who did not show increased basal GDH activity but had significantly higher level of half-maximal inhibitory concentrations of GTP (110 nmol/L) than controls (48±17 nmol/L). The clinical phenotypes of our patients with H262Y and S217C were not different from those of the reported patients with the same mutations.

A total of 17 Japanese patients (10 boys and 7 girls) in 16 families including the patients described in this study had 11 mutations. Eight patients had mutation at the site of the GTP-binding region (Patient 1 to 8), two had mutation at the site of the antenna-like structure (Patient 9 and 10), and seven had mutation at the site of the hinge region (Patient 11 to 17). Most of the mutations have been reported also in European patients. A296E, R322H, and I444M mutations are reported only in Japanese. S445L was prevalent and was found in 4 patients.

In a previous study, we reported that the basal GDH activity of L413V mutation at the site of

Table 1. Summary of the reported Japanese patients with the hyperinsulinism-hyperammonemia syndrome

#	Sex	Age at onset	Amino acid change	Exon	Treatment	Blood glucose (mg/dL)	NH ₃ (μmol/L)	Mental retardation	Ref
1	M	10 months	S217C	6	Diazoxide	20-30	60-83	—	*
2	M	10 months	H262Y	7	diazoxide	26-40	77-142	—	*
3	M	22 months	H262Y	7	diazoxide	30-40	153-166	+	*
4	M	9 months	H262Y	7	pancreatectomy	unknown	unknown	—	*
5	M	6 days	R265K	7	pancreatectomy	N/A	N/A	+	11
6	F	7 months	Y266C	7	diazoxide	30	128	—	21
7	F	6 months	E296A	7	diazoxide	N/A	N/A	—	11
8	F	4 years	R322H	7	diazoxide	23	125	+	22
9	M	0 day	N410T	10	diazoxide	10-60	150-236	+	24
10	M	4 months	L413V	10	diazoxide	13	60-148	+	25
11	F	15 months	I444M	11	diazoxide	30	100	—	*
12	F	4 months	S445L	12	diazoxide	N/A	N/A	—	11
13	F	16 days	S445L	12	diazoxide	N/A	N/A	—	11
14	M	11 months	S445L	12	diazoxide	N/A	N/A	+	11
15	F	10 months	S445L	12	diet	40	178	—	17
16	M	5 days	G446D	12	pancreatectomy	8	148	+	25
17	M	1 day	G446D	12	pancreatectomy	10	160-230	+	12

N/A, not available; and *, the present study.

antenna-like structure was double that of the control subjects and patients with G446D mutation at the hinge region²⁵). Both L413V and G446D mutations showed similar insensitivity for GTP inhibition. The same assay system, the basal GDH activities in patients with the H262Y and S217C mutations in the GTP binding site were similar to those of the control and patients with mutations in the hinge regions. The inhibitory effects of GTP were lower in all patients compared with the controls. The expression analysis of COS cells transfected with mutants showed marked inhibitory regulation of GTP to GDH activity. In CHH, mutations have been detected at three sites of GDH structure. These different locations of mutations between the α -helix of antenna-like structure and the GTP binding/the hinge regions had different effects on GDH enzyme.

In patients with CHH, it is difficult to define the genotype-phenotype relation because hypoglycemic episodes have marked effects on development and neurological symptoms. 1) Studies to date have not reported clearly the genotype-phenotype correlation. 2) Ammonia levels correlate with dysregulation of GDH, and ammonia levels rather than hypoglycemia correlate with the severity of the disease²⁷). 3) Certain mutations at the GTP binding sites correlate with the development of epilepsy^{16,26}). In this study of 17 patients, hypoglycemic episodes occurred between day 0 of birth and 4 years of age. One patient with a mutation at the GTP binding site and at the antenna-like structure each and three patients with a mutation at the hinge region experienced the onset within 1 month of life. Glucose levels could be controlled by administration of diazoxide in most of the patients. Two patients with H262Y and R265K mutations at the GTP binding sites and two patients with the same mutation of G446D at the hinge region required subtotal pancreatectomy. Developmental delays were identified in three patients with a mutation at the GTP binding sites, one patient with a mutation at the antenna-like structure, and three patients with a mutation at the hinge region. In this study, patients with a mutation at the GTP binding site did not have neurological complications and comparison of ammonia levels did not show a definite correlation.

The two patients with G446D mutation (Patients 10 and 17, Table 2) developed hypoglycemic episodes (glucose level ≤ 10 mg/dL) within one week after birth and required subtotal pancreatectomy due to poorly controlled glucose levels. Moreover, developmental delays were found in the two patients. The ammonia level in Patient 17 tended to be slightly higher than that of Patient 10. The half-maximal inhibitory concentration of GTP in lymphoblasts of Patient 16 was higher ($0.86 \mu\text{mol/L}$)²⁵) than those of patients with L413V, I444M, H262Y, and S217C mutations that were analyzed by our group. Stanley et al¹⁰) also reported that the half-maximal inhibitory concentration of GTP in G446D mutation was double that of other mutations. Based on these results, this mutation seems to be associated with severe abnormalities. The relationship between genotype and phenotype in patients with CHH should be considered for each gene mutation rather than for the three mutation sites on the stereostructure. Accumulation of data of more patients with CHH is warranted.

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