Case Report

Analysis of GBE1 mutations via protein expression studies in glycogen storage disease type IV: A report on a non-progressive form with a literature review

Hiroyuki Iijimaa, Reiko Iwanoa, Yukichi Tanakab, Koji Muroyaa, Tokiko Fukudac, Hideo Sugied, Kenji Kurosawae, Masanori Adachia,⁎

a Department of Endocrinology and Metabolism, Kanagawa Children’s Medical Center, Mutsukawa 2-138-4, Minami-ku, Yokohama 232-8555, Japan
b Department of Pathology, Kanagawa Children’s Medical Center, Mutsukawa 2-138-4, Minami-ku, Yokohama 232-8555, Japan
c Department of Pediatrics, Hamamatsu University School of Medicine, Handayama, 1-20-1 Higashi-ku, Hamamatsu 431-3192, Japan
d Faculty of Health and Medical Sciences, Tokoha University, Sena, 1-22-1 Aoi-ku, Shizuoka 420-0911, Japan
e Division of Medical Genetics, Kanagawa Children’s Medical Center, Mutsukawa 2-138-4, Minami-ku, Yokohama 232-8555, Japan

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ABSTRACT

Background: Glycogen storage disease type IV (GSD IV), caused by GBE1 mutations, has a quite wide phenotypic variation. While the classic hepatic form and the perinatal/neonatal neuromuscular forms result in early mortality, milder manifestations include non-progressive form (NP-GSD IV) and adult polyglucosan body disease (APBD). Thus far, only one clinical case of a patient with compound heterozygous mutations has been reported for the molecular analysis of NP-GSD IV. This study aimed to elucidate the molecular basis in a NP-GSD IV patient via protein expression analysis and to obtain a clearer genotype-phenotype relationship in GSD IV.

Case presentation: A Japanese boy presented hepatosplenomegaly at 2 years of age. Developmental delay, neurological symptoms, and cardiac dysfunction were not apparent. Observation of hepatocytes with periodic acid-Schiff-positive materials resistant to diastase, coupled with resolution of hepatosplenomegaly at 8 years of age, yielded a diagnosis of NP-GSD IV. Glycogen branching enzyme activity was decreased in erythrocytes. At 13 years of age, he developed epilepsy, which was successfully controlled by carbamazepine.

Molecular analysis: In this study, we identified compound heterozygous GBE1 mutations (p.Gln46Pro and p.Glu609Lys). The branching activities of the mutant proteins expressed using E. coli were examined in a reaction with starch. The result showed that both mutants had approximately 50% activity of the wild type protein.

Conclusion: This is the second clinical report of a NP-GSD IV patient with a definite molecular elucidation. Based on the clinical and genotypic overlapping between NP-GSD IV and APBD, we suggest both are in a continuum.

1. Introduction

Glycogen storage disease type IV (GSD IV; Andersen disease [1]; OMIM #232500) is a rare autosomal recessive metabolic disorder caused by a deficiency of amylo-(1,4 to 1,6)-transglucosidase (EC 2.4.1.18, 1,4-alpha-glucan-branching enzyme, GBE). It is characterized by the accumulation of an amylpectin-like glycogen (polyglucosan) in multiple organs, such as the liver, muscle, heart, and the central and peripheral nervous systems [2]. Several phenotypic categories have been reported for GSD IV [3]. The classic hepatic form is the most common, wherein patients progress rapidly to cirrhosis and tend to die no later than 5 years of age, unless liver transplantation is attempted [1,4–8]. Meanwhile, patients with the non-progressive form (NP-GSD IV) display hepatosplenomegaly and elevated transaminase levels, which regress spontaneously without any features of cirrhotic, neurologic, muscular, or cardiac involvement [4,9]. In addition, a neuromuscular form has been reported, which is further sub-divided in accordance with the age at onset (perinatal, neonatal, juvenile, or adult). Patients with the perinatal form present in utero fetal akinesia deformation sequence, polyhydramnios, fetal hydrops, arthrogryposis,

Abbreviations: APBD, adult polyglucosan body disease; GBE, 1,4-alpha-glucan-branching enzyme; GSD IV, glycogen storage disease type IV; NP-GSD IV, non-progressive form of glycogen storage disease type IV; RT-PCR, reverse transcriptase-polymerase chain reaction; WT, wild type

⁎ Corresponding author.
E-mail address: madachi@mars.sannet.ne.jp (M. Adachi).

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and perinatal death [5,10–19]. Those with the neonatal form present hypotonia, muscular atrophy, and cardiomyopathy immediately postpartum, and usually die in the neonatal period [4–6,16,18,20–24]. The juvenile form is dominated by myopathy or cardiomyopathy with pubertal or young adult onset [5,25]. The adult form, also known as adult polyglucosan body disease (APBD), is characterized by adult-onset multisystem disorder including myopathy or neurological involvement such as neurogenic bladder, seizure, or spastic paraplegia with vibration loss and numbness [26–32].

The aforementioned various manifestations in GSD IV result from mutations in a single responsible gene, GBE1 (*607839). GBE1 is located on chromosome 3p14, consists of 16 exons, and encodes a protein of 702 amino acid residues [33]. Thus far, 52 different GBE1 mutations have been reported, including missense, nonsense, deleterious, insertion, and splice-site mutations (Fig. 1). To our knowledge, however, only two mutations (p.Leu224Pro and p.Tyr329Ser) in a single patient have been identified in NP-GSD IV [4]. Interestingly, one of these mutations (p.Tyr329Ser) is a founder mutation of APBD among individuals of Ashkenazi-Jewish descent [27,31]. In addition, functional protein expression analysis has been performed in only one study [4].

Herein, we describe a case of NP-GSD IV caused by novel missense GBE1 mutations and present a review of the literature to obtain a clearer genotype-phenotype relationship in GSD IV.

2. Case report

A Japanese boy was referred to our hospital at 2 years of age because of elevated serum transaminases. He was born after an uneventful pregnancy and had no significant family history. At the time of admission, his height and weight were 80.9 cm (−1.5 SD) and 10.54 kg (−0.9 SD), respectively. He had hepatosplenomegaly (liver 6 cm below the right costal margin and spleen 1.5 cm below the left costal margin). His developmental milestones were normal, and he did not present any neurologic symptoms. Laboratory data indicated an elevation of serum transaminases (AST 221 IU/l and ALT 124 IU/l), without any other

Fig. 1. Organization of the GBE1 gene, and disease-associated mutations hitherto reported. The number above each box indicates the exon number. References are denoted in parentheses. The mutations identified in our patient are indicated by an asterisk. Null mutations such as intragenic deleterious, nonsense, frameshift, and splice-site mutations are underlined. Herein, we gathered neonatal and perinatal forms in a mass because their diagnostic criteria are not strictly determined and clinical outcomes in these forms do not differ significantly. Null mutations, except for those located in exons 15 and 16, tend to associate with more severe forms of glycogen storage disease type IV (GSD IV), such as classic hepatic form or perinatal/neonatal neuromuscular forms. The same mutations are often reported in unrelated patients with milder forms, such as non-progressive-GSD IV (NP-GSD IV) and adult polyglucosan body disease (APBD).
metabolic derangements such as hypoglycemia, hyperlipidemia, hyperuricemia, or hyperlactacidemia. Viral hepatitis and autoimmune hepatitis were denied upon appropriate laboratory tests. In the oral glucose tolerance test (2.5 g/kg), an adequate increase in blood lactate levels was observed (fasting, 10.3 mg/dl; 60 min, 19.0 mg/dl, and 120 min, 16.7 mg/dl). Glucagon stimulation test (0.03 mg/kg) on fasting yielded a significant response regarding blood glucose, with 74 mg/dl before, and 113 mg/dl 30 min after stimulation. Upon liver biopsy, periodic acid-Schiff-positive cytoplasmic inclusions were reported with partial resistance to diastase digestion (Fig. 2), which yielded a diagnosis of GSD IV. There were, however, no histological changes or laboratory data indicating cirrhosis. Moreover, serum transaminase levels normalized at 5 years of age, followed by a disappearance of hepatosplenomegaly at 8 years of age. Cardiac function was adequate, as verified via ultrasonic cardiography, and he did not present any myopathic symptoms. However, he developed epilepsy at 13 years of age and was administered carbamazepine therapy. There were no abnormal findings in brain magnetic resonance image taken at 14 years of age. At present, he is 17 years old and has normal liver function, without discernible hepatosplenomegaly. Except for favorably controlled epilepsy, he has not shown any neurological symptoms, such as ataxia, muscle weakness, gait disturbance and urinary retention. He is now studying for college entrance examination.

3. Materials and methods

Written informed consent was obtained from the patient and his parents for all experiments described herein, per the tenets of the Declaration of Helsinki, in addition to consent for publication.

3.1. Analysis of GBE activity

Erythrocyte GBE activity was analyzed using a previously described method [34,35]. Phosphorylase b kinase enzyme activity in erythrocytes was measured to eliminate the possibility of sample inactivation.

3.2. GBE1 mutation analysis

GBE1 mutation analysis was performed in gDNA extracted from peripheral blood leukocytes of the patient and his parents. Direct Sanger sequencing of the GBE1 gene was performed after amplification of all 16 exons and intron/exon boundaries via PCR with an Applied Biosystems® Veriti Thermal Cycler (Thermo Fisher Scientific, MA, USA) in accordance with a previously described method [22]. Next-generation sequencing was performed using MiSeq® bench-top sequencer (Illumina, San Diego, CA, USA) with a TruSight® One Sequencing Panel (Illumina). Common genetic variations were identified using our in-house and public databases (dbSNP, 1000 Genomes Project, NHLBI Exome Sequencing Project, Human Genetic Variation Database (HGVD), NCBI ClinVar and Human Gene Mutation Database).

3.3. Reverse transcriptase-PCR (RT-PCR)

GBE1 sequence was obtained from GenBank (NG_011810), which was then applied to the human genome assembly (http://genome.ucsc.edu) using BLAT to identify exon/intron boundaries. Gene-specific PCR primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and NCBI primer BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Supplemental Table 1). Total RNA was extracted from leukocytes, using the ISOGEN II® reagent (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Total RNA was eluted in a final volume of 20 μl of RNase-free water and stored at −80 °C until use. RT-PCR was performed with the PrimeScript® One Step RT-PCR Kit (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer’s instructions.

3.4. In silico analysis

Phylogenetic information was obtained via Vertebrate Multiz Alignment & Conservation in UCSC Genome Browser (https://genome.ucsc.edu/). We used PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://asia.ensembl.org/index.html) predictive
algorithms to evaluate the pathogenicity of the identified sequence variants. PyMOL software (https://pymol.org/2/) was used to speculate potential conformational changes in mutant GBE1.

3.5. Functional analysis of mutant GBE proteins

To evaluate the pathogenicity of the sequence variations in GBE1 identified in the patient, functional analysis with in vitro expression experiments was conducted as follows. We used E. coli BL21 (DE3) cells (Merck, Darmstadt, Germany) to express wild type (WT) and mutant GBE proteins. Branching activity of each protein was then measured by the previously described method that utilizes amylose-iodine absorbance spectrum [37]. Because amylose, which constitutes starch with amylopectin, has a linear structure, GBE can act on amylose to form branching points even in the absence of other enzymes required for glucose-chain elongation. Once branched, amylose will fail to develop amylose-iodine specific absorbance spectrum at 660 nm (A660). Accordingly, by measuring the reduction of A660 by spectrometry, GBE activity can be estimated.

3.5.1. Construction of WT and mutant GBE1 cDNAs

To construct cDNA sequences containing either p.Gln46Pro or p.Glu609Lys mutations, site-directed mutagenesis was performed using pET-21b vector Novagen (Merck). Each cDNA, including WT, was artificially synthesized and then inserted into the Ndel/XhoI sites of the vector. Stop codons were eliminated and polyhistidine-tags were inserted at the c-termini.

3.5.2. Protein expression, purification, and dialysis

WT and mutant cDNA in vector pET-21b were transfected into BL21 (DE3) cells (Merck). After incubation at 37 °C overnight, each colony was inoculated into Luria-Bertani (LB) liquid medium (supplemented with 100 µg/ml Ampicillin) and incubated at 37 °C with agitation (220 rpm) until the OD600 approached 0.6–1.0. Isopropyl-β-D-1-thio-galactopyranoside was added into the culture to induce target protein expression. After cell lysis by ultrasonication and centrifugation at 12,000 rpm for 15 min (low temperature), the supernatant was loaded onto a Ni NTA affinity column (Thermo Fisher Scientific) pre-equilibrated with lysis buffer (50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole). The column was then washed with wash Buffer (50 mM NaH2PO4, 300 mM NaCl, and 50–100 mM imidazole) to elute the irrelevant proteins, until the OD280 of the eluent approached baseline values. The eluent containing 100 mM imidazole was dialyzed with PBS buffer and analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12% resolving gel).

3.5.3. Spectrophotometric analysis of enzyme activity

The present assay employed starch as the substrate, which would be branched by GBE. After a certain incubation period, amylose content can be determined by staining with iodine. GBE activity was determined when the initial reaction velocity appeared linear.

The reaction mixture contained 6 mg/ml starch in 10 mM sodium phosphate buffer (pH 7.4), containing 100 mM sodium chloride. The reaction mixture (0.05 ml) was mixed with 1.0 ml iodine reagent prior to spectrophotometric measurement of A660. Each enzyme assay was performed in duplicate. One unit of GBE activity was defined as the amount of the decrease of 1 mg starch per minute at 37 °C during 30 min. In other words, Activity (U/mg) = ΔA660/S/T/C/V

S: slope of the standard curve, T: reaction time (30 min), C: concentration of the GBE protein, V: volume of the GBE protein (0.001 ml).

4. Results

4.1. Analysis of GBE activity

Erythrocyte GBE activity in the patient was lower (0.3 µmol Pi/min/g Hb) than that of the three control samples (2.4, 2.5, and 3.2 µmol Pi/min/g Hb). Positive disease control samples displayed a GBE activity of 0.2 µmol Pi/min/g Hb. Erythrocyte phosphorylase b kinase activity of the patient was normal.

4.2. GBE1 mutation analysis

Sanger sequencing of GBE1 revealed heterozygous missense mutations (c.137A > C [p.Gln46Pro, located in exon 1] and c.1825G > A [p.Glu609Lys, located in exon 14]) in the patient (Fig. 3A). The former mutation was detected in his father, whereas the latter mutation was detected in his mother, both being heterozygous mutations. A homozygous c.568A > G substitution was detected in the patient and in his father and was reported in 19% of 1207 healthy controls from the HGVd. This substitution was listed in the dbSNP database (rs2229519). Next-generation sequencing revealed the same mutations (p.Gln46Pro and p.Glu609Lys) in GBE1. According to HGVD, p.Gln46Pro was not listed, whereas p.Glu609Lys was listed with a frequency of 0.00206782. In addition, p.Glu609Lys was listed as rs772802187 in dbSNP.

4.3. RT-PCR

No aberrant splice variant was identified (data not shown). Direct sequencing of RT-PCR products revealed the same mutations (p.Gln46Pro and p.Glu609Lys).

4.4. In silico analysis

Both Gln46 and Glu609 are conserved throughout species (Fig. 3B). Upon PolyPhen-2 and SIFT analysis, p.Gln46Pro was predicted as “not damaging”. However, PolyPhen-2 and SIFT analyses predicted p.Glu609Lys to be “possibly damaging” and “damaging,” respectively (Fig. 3C). The modeled structure of Gln46Pro-GBE and Glu609Lys-GBE are shown in Fig. 3D. In both GBE proteins, the mutation was speculated to disrupt hydrogen bonds on the molecular circumference and to cause steric hindrance with Asp44 and Asn456, respectively (Fig. 3D).

4.5. Functional analysis of mutant GBE proteins

The enzyme activity of Gln46Pro-GBE and Glu609Lys-GBE was low (1.21 and 1.15 U/mg, respectively) compared with that of WT-GBE (2.18 U/mg), corresponding to 56% and 53% of WT-GBE activity, respectively (Table 1).

5. Discussion

Two novel missense mutations, p.Gln46Pro and p.Glu609Lys, were identified in a compound heterozygous state, in a potentially recessive pattern of inheritance. To our knowledge, this is the second report wherein enzymatic activity of mutant GBE proteins was verified on the basis of molecular expression analyses in any form of GSD IV. The present results indicate that Gln46Pro-GBE retained 56% and
**Fig. 3.** GBE1 mutation analysis.

Electropherogram of GBE1 gDNA of our patient, showing heterozygous missense mutations (arrow). (B) Glutamine at position 46 (Gln46) and glutamate at position 609 (Glu609) in *Homo sapiens* GBE1 are conserved. (C) PolyPhen-2 and SIFT analyses of the p.Gln46Pro and p.Glu609Lys mutations. (D) A structural model of GBE protein generated via PyMOL. Hydrogen bonding is indicated with dashed lines and steric hindrance is indicated with discs. (D-1, D-3) The native GBE structure shows that the Gln46 and Glu609 interact with Asp44 and Asn456, respectively. (D-2, D-4) The modeled structure of the mutant proteins (Gln46Pro-GBE and Glu609Lys-GBE) is speculated to have structural changes caused by steric hindrance with Asp44 and Asn456, respectively.
Glut609Lys-GBE 53% residual activity compared to WT-GBE, which may argue against the pathogenicity of the mutations. However, the only previous study based on molecular expression analysis reported that the activity of Tyr329Ser-GBE and Leu224Pro-GBE, responsible for NP-GSD IV, were 54% and 8.7% of WT-GBE, respectively [4]. In addition, most APBD patients of Ashkenazi-Jewish descent have a homozygous p.Tyr329Ser mutation [27,31]. Therefore, we assume that approxi-
mate physiological predictions failed to reveal the damaging nature of
p.Gln46Pro and p.Glu609Lys mutations are truly pathogenic.

A previous study reported a suitable genotypy-phenotype relationship in GSD-IV (Fig. 1). All null mutations, underlined in Fig. 1, have
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