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RESEARCH REPORT

Glycogen storage disease type IV: novel mutations and molecular characterization of a heterogeneous disorder

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Abstract Glycogen storage disease type IV (GSD IV; Andersen disease) is caused by a deficiency of glycogen branching enzyme (GBE), leading to excessive deposition of structurally abnormal, amylopectin-like glycogen in affected tissues. The accumulated glycogen lacks multiple branch points and thus has longer outer branches and poor solubility, causing irreversible tissue and organ damage. Although classic GSD IV presents with early onset of hepatosplenomegaly with progressive liver cirrhosis, GSD IV exhibits extensive clinical heterogeneity with respect to age at onset and variability in pattern and extent of organ and tissue involvement. With the advent of cloning and determination of the genomic structure of the human GBE gene (*GBE1*), molecular analysis and characterization of underlying disease-causing mutations is now possible. A variety of disease-causing mutations have been identified in the *GBE1* gene in GSD IV patients, many of whom presented with diverse clinical phenotypes. Detailed biochemical and genetic analyses of three unrelated patients suspected to have GSD IV are presented here. Two novel missense mutations (p.Met495Thr and p.Pro552Leu) and a novel 1-bp deletion mutation (c.1999delA) were identified. A variety of mutations in *GBE1* have been previously reported, including missense and nonsense mutations, nucleotide deletions and insertions, and donor and acceptor splice-site mutations. Mutation analysis is useful in confirming the diagnosis of GSD IV—especially when higher residual GBE enzyme activity levels are seen and enzyme analysis is not definitive—and allows for further determination of potential genotype/phenotype correlations in this disease.

Abbreviations

APBD	Adult polyglucosan body disease
FADS	Fetal akinesia deformation sequence
GBE1	Glycogen branching enzyme 1
GSD IV	Glycogen storage disease type IV (Andersen disease)

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Introduction

Glycogen storage disease type IV (GSD IV; Andersen disease; OMIM 232500) is a rare autosomal recessive disorder representing 0.3% of all glycogenoses (Moses and Parvari 2002). The disease is caused by deficient activity of the glycogen-branching enzyme (GBE, EC 2.4.1.18) (Brown and Brown 1983, 1989), which is encoded by the *GBE1* gene located on chromosome 3p14 (Thon et al. 1993). GBE [1, 4-α-D-glucan: 1, 4-α-D-glucan 6-α-D-(1, 4-α-glucano-transferase] participates with glycogen synthase in the synthesis of glycogen by transferring a chain containing a minimum of six α-1, 4-linked glycosyl units into an α-1, 6 position (Bannayan et al. 1976). GBE deficiency causes accumulation of an amylopectin-like structurally abnormal glycogen with fewer branch points in the affected tissue (Bannayan et al. 1976). The diagnosis of GSD IV is based on clinical symptoms combined with muscle or liver histology and electron microscopy findings and in vitro evidence of GBE deficiency in liver, muscle, or skin fibroblasts (Brown and Brown 1983, 1989; Barash et al. 1991). Prenatal diagnosis has also been performed by measurement of GBE activity in cultured amniocytes or chorionic villi cells. However, DNA mutation analysis can now complement enzyme activity studies, especially in prenatal fetal samples with equivocal results and in patients with higher levels of residual enzyme activity that overlap heterozygote levels (Shen et al. 1999; Akman et al. 2006).

GSD IV exhibits extensive clinical heterogeneity with respect to age of onset, as well as variability in pattern and extent of organ and tissue involvement. The most common form, the classic form, is characterized by hepatosplenomegaly with progression to lethal hepatic cirrhosis in the first few years of life, failure to thrive, and death by 5 years of age (Bao et al. 1996; Sahoo et al. 2002). Hepatic and cardiac variants have also been described, varying in severity and age at diagnosis. Additionally, the neuromuscular form of GSD IV is quite variable and may be subclassified into several different phenotypes. The most severe of these phenotypes presents perinatally as fetal akinesia deformation sequence (FADS), with arthrogryposis, hydrops, polyhydramnios, and pulmonary hypoplasia, and results in death at an early age, frequently due to cardiac or pulmonary compromise. Congenital and early-infantile phenotypes, presenting with hypotonia, skeletal muscle atrophy, and possibly cardiac and hepatic involvement, with varying degrees of severity and prognosis, have also been described. Later-onset (juvenile/adult) phenotypes are associated with myopathy or adult polyglucosan body disease (APBD) involving central and peripheral nervous system dysfunction (Moses and Parvari 2002; Bao et al. 1996; Tay et al. 2004; Shin 2006; Sindern et al. 2003; Burrow et al. 2006; Raju et al. 2008).

This study focuses on detailed biochemical and genetic evaluation of three unrelated GSD IV patients and compilation of GSD IV mutation data available to date in patients with variable presentations.

Materials and methods

GBE enzyme analysis

Three unrelated patients suspected to have infantile-onset GSD IV based on clinical presentation and histological evaluations were analyzed for residual GBE activity in snap-frozen liver biopsy tissue or cultured skin fibroblast cells employing standard spectrophotometric method and using phosphorylase as the indicating enzyme (Brown and Brown 1966, 1989). Enzyme activity was expressed as μmol/min/g tissue (liver) or nmol/min/mg protein (fibroblasts), as shown in Table 1.

Genomic DNA sequencing

Genomic DNA was extracted from peripheral blood leukocytes or cultured skin fibroblasts using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN, USA). All 16 coding exons of the *GBE1* gene were amplified by polymerase chain reaction (PCR) using flanking intronic primers designed by the Primer 3—PCR-primer selection program (http://www-genome.wi.

Table 1 Glycogen-branching enzyme gene (*GBE1*) mutation analysis and branching enzyme activity in three glycogen storage disease type IV (GSD IV) patients

Patient	Nucleotide change	Exon	Amino acid change	Branching enzyme activity in muscle or fibroblast
1	c.1604 A>G	12	p.Tyr535Cys	0 (L)
	c.1999delA	15	p.Thr667LeufsX40	434 (F)
2	c.1484 T>C	12	p.Met495Thr	254 (F)
	c.1543 C>T	12	p.Arg515Cys	
3	c.1655 C>T	13	p.Pro552Leu	180 (F)
Control	–	–	–	F=1300+/-390
				L=85+/-31

F skin fibroblasts (nmol/min/mg protein), *L* liver tissue (μmol/min/g)

mit.edu/cgibin/primer/primer3_www.cgi). Cycle sequencing was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem, Foster City, CA, USA) on the ABI 3700 DNA sequencer. Real-time quantitative PCR analysis of messenger RNA (mRNA) from patients' skin fibroblasts was also carried out using specific primers (cGBE-ex12-ATAAGTCGCTGG CATTTTGG/cGBE-ex16-GGATCTGCCGAATTGA) to rule out large exonic deletions. The *GBE1* gene was also sequenced from genomic DNA samples from 50 non-GSD IV controls (100 alleles) to rule out whether the identified variants were common polymorphisms.

Interspecies comparisons of GBE1 amino acid sequences

To determine whether the amino acid sequence changes identified altered conserved amino acids, GBE1 amino acid sequences were compared in multiple species by multiple alignment programs for amino acid sequences (MAFFT, version 6, http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Fig. 1), and the PolyPhen program was used to predict the pathogenicity of the change (Ramensky et al. 2002).

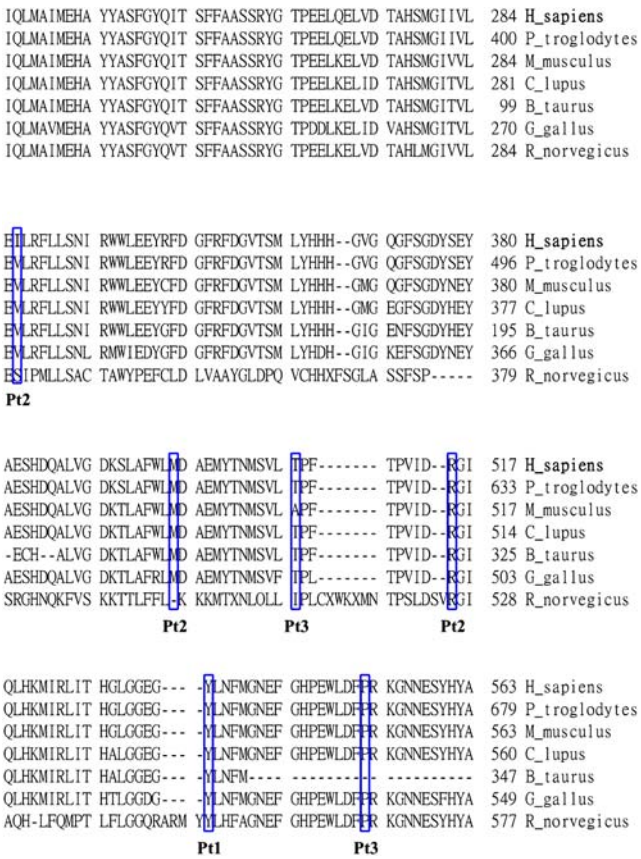

Fig. 1 Interspecies amino acid sequence alignment from glycogen-branching enzyme (GBE1). The *boxed* amino acids represent the amino acids that were altered in GBE1 in patients 1, 2, and 3, as compared with GBE1 sequences in other species

Mutational spectrum and genotype–phenotype correlation

GSD IV mutation literature data from the Human Genome Mutation Database (http://www.hgmd.cf.ac.uk) and other publications was reviewed, and mutations (n=34) and polymorphisms identified in the *GBE1* gene were compiled along with clinical phenotype (Table 2).

Results and discussion

Patient one is a French Canadian male who presented at 10 months of age with hypotonia, myopathy, and hepatomegaly. Liver histology revealed amylopectin-like accumulation of glycogen in the liver tissue, which is typical for the classic hepatic form of GSD IV. GBE activity was undetectable in his frozen liver tissue. However, some residual enzyme activity (approximately 30%) was seen in his cultured skin fibroblast cells (Table 1). Two mutations were identified by *GBE1* gene sequencing; c.1604A>G (p.Tyr535Cys) and c.1999delA (p.Thr667Leu; p.X703GluextX4) (Fig. 2a). Both parents were confirmed to be carriers: c.1604A>G in the father and c.1999delA in the mother. The c.1999delA mutation results in a frameshift beginning at amino acid position 667 and eliminating the original termination codon to produce a protein three amino acids longer than normal. The missense mutation identified in this patient (p.Tyr535Cys) has recently been reported in heterozygous form in an adult patient with APBD (Massa et al. 2008). Figure 1 shows that Tyr535 is highly conserved using interspecies alignment comparison. As the missense mutation found in patient 1 has only been seen once before as a single allele (Table 2), and it has not been reported in homozygous state, it is not possible to predict any genotype–phenotype correlation. Prenatal diagnosis performed on a subsequent pregnancy in this family revealed the fetus to be a carrier, with only one mutation (c.1999delA).

Patient two was a Hispanic female who presented with a severe infantile phenotype. She had extremely low GBE activity in her skin fibroblast cells (Table 1). Liver and muscle biopsies could not be obtained on this patient. Molecular analysis of the *GBE1* gene revealed three heterozygous missense variants. Two of these were in exon 12 and were predicted to be pathogenic mutations: a novel missense mutation, c.1484T>C (p.Met495Thr) and a previously reported missense change, c.1543C>T (p.Arg515Cys) (Table 1; Fig. 2b). The novel missense change (p.Met495Thr) alters a conserved amino acid (Fig. 1) and thus could have a deleterious effect on normal protein function. This amino acid change was predicted to be "probably damaging" by the PolyPhen program (Ramensky et al. 2002). The second missense mutation (p.Arg515Cys) has previously been reported in a patient with classic features of GSD IV and

Table 2 Summary of glycogen-branching enzyme (*GBE1*) gene mutations previously reported in glycogen storage disease (GSD) IV patients

Location	Nucleotide change	Amino acid change	Number of alleles	Phenotype	References
Exon 1	c.38insA	p.Asp13GlufsX12	1	Classic	Bruno et al. 2004
Intron 1	c.143+1g>a	Disrupts splicing	2 (homozygous)	FADS	Bruno et al. 2004
Exon 2	c.288delA	p.Gly97GlufsX46	2 (homozygous)	Congenital	Nambu et al. 2003
Exon 4	c.454G>T	p.Glu152X	2[a] (homozygous)	Congenital	Lamperti et al. 2009
Exons 4–6	c.430_782del	p.Val144_Ser261del	2 (heterozygous sibs)	Congenital	Bruno et al. 2004
Exon 4–7	Genomic 25.5 kb del; 9 bp ins	p.Val144_Ser331del	2 (homozygous; German)	Congenital	Nolte et al. 2008
Exon 5	c.671T>C	p.Leu224Pro	2 (heterozygous sibs)	Nonprogressive hepatic	Bao et al. 1996; Shen et al. 1999
Intron 5	c.691+2t>c	Disrupts splicing	Not reported	Nonprogressive hepatic	Shin 2006
Intron 5	c.691+5 g>c	Disrupts splicing	2 (homozygous)	Congenital	Assereto et al. 2007
Exon 6	c.708G>C	p.Gln236His	1	Congenital mild	Burrow et al. 2006
Exon 6	c.728A>G	p.His243Arg	1	Congenital	Bruno et al. 2004
Exon 6	c.771T>A	p.Phe257Leu	1	Classic	Bao et al. 1996
Intron 6	c.783–1g>a	p.Arg262_Ser331del	1 or 2 (could not be determined from molecular analysis)	Congenital	Bao et al. 1996
Exon 7	c.784C>T	p.Arg262Cys	1	Congenital mild	Burrow et al. 2006
Exon 7	c.895G>T	p.Gly299X	3 (heterozygous sibs)	FADS	L′Hermine-Coulomb et al. 2005
Exon 8	c.986A>C	p.Tyr329Ser	Common mutation for APBD in Ashkenazi Jews; 2 heterozygous sibs with nonprogressive hepatic form	APBD, nonprogressive hepatic	Bao et al. 1996; Lossos et al. 1998; Shen et al. 1999; Ubogu et al. 2005
Exons 8–12	c.993_1618del	p.Trp332_Met539del	2[a] (homozygous)	Congenital	Tay et al. 2004
Exon 9	c.1077insT	p.Thr360TyrfsX17	4[a] (2 homozygous sibs; Turkish)	Congenital	Nolte et al. 2008
Exon 10	c.1239delT	p.Asp413GlufsX23	2 (heterozygous sibs; Hispanic)	Congenital	Tay et al. 2004; Akman et al. 2006
Intron 10	c.1336–1g>a	Predicted to disrupt splicing	2[a] (homozygous; Greek)	Congenital	Akman et al. 2006; Konstantinidou et al. 2008
Exon 12	c.1468delC	p.Leu490TrpfsX5	1 (heterozygous sibs; Hispanic)	Congenital	Tay et al. 2004; Akman et al. 2006
Exon 12	c.1471G>C	p.Ala491Tyr	3 (heterozygous sibs)	FADS	L′Hermine-Coulomb et al. 2005
Exon 12	c.1484T>C	p.Met495Thr	1 (Hispanic)	Infantile	Patient 2
Exon 12	c.1543C>T	p.Arg515Cys	1; 1 (Hispanic)	Classic; Infantile	Bao et al. 1996; Patient 2
Exon 12	c.1544G>A	p.Arg515His	1 (German, non-Ashkenazi)	APBD	Ziemssen et al. 2000; Sindern et al. 2003
Exon 12	c.1558delC	p.His520IlefsX3	1	Congenital	Janecke et al. 2004; Konstantinidou et al. 2008
Exon 12	c.1570C>T	p.Arg524X	2	Classic	Bao et al. 1996; Bruno et al. 2004
Exon 12	c.1571G>A	p.Arg524Gln	2 (1 in childhood form; 1 in German, non-Ashkenazi APBD)	Classic, APBD	Bruno et al. 1999; Ziemssen et al. 2000; Sindern et al. 2003
Exon 12	c.1604A>G	p.Tyr535Cys	1 (Italian); 1 (French-Canadian)	Infantile; APBD	Massa et al. 2008; Patient 1
Exon 13	c.1634A>G	p.His545Arg	4[a] (homozygous sibs; Syrian)	FADS	Bruno et al. 2004
Exon 13	c.1643G>A	p.Trp548X	2 (homozygous)	Congenital	Assereto et al. 2007

Exon 13	c.1655C>T	p.Pro552Leu	1	Infantile	Patient 3
Exon 13	c.1774G>T	p.Glu592X	2 (heterozygous sibs)	Congenital	Bruno et al. 2004
Exon 14	c.1883A>G	p.His628Arg	1	Childhood	Bruno et al. 2004
Exon 14	c.1909C>T	p.Arg637X	2	Congenital	Bruno et al. 2004; Janecke et al. 2004; Konstantinidou et al. 2008
Exon 15	c.1999delA	p.Thr667LeufsX40	1 (French-Canadian)	Infantile	Patient 1
Exon 16	g.exon 16 del	p.Val685_Asn702del	2[a] (homozygous, Chinese)	Congenital	Raju et al. 2008

FADS fetal akinesia deformation sequence, *APBD* adult polyglucosan body disease

[a] Consanguinity has been reported

caused 80% reduction of GBE activity in transient expression studies in COS cells compared with wild-type controls (Bao et al. 1996) (Table 2). It is possible that compound heterozygosity for two missense mutations could result in the severe classic GSD IV phenotype observed in this patient. The third missense change (c.1000A>G, p.Iso334Val) was a known benign polymorphism based on information in the human Single Nucleotide Polymorphism database (dbSNP) (rs2172397) and amino acid alignment map.

Patient three was an infant presenting with severe hypotonia and hepatomegaly as well as reduced skin fibroblast GBE activity (Table 1). *GBE1* molecular analysis revealed two heterozygous variants, c.1655C>T (p.Pro552-Leu) and c.1519A>G (p.Thr507Ala). One variant, p.Pro552Leu, is a novel missense mutation that changes a conserved amino acid (Table 1, Fig. 2c, Fig. 1) and is predicted to be "probably damaging" by the PolyPhen program (Ramensky et al. 2002). The second variant, p.Thr507Ala, is a benign polymorphism reported in the dbSNP (rs2228389). No other pathogenic mutation could be found in this patient. It is possible that the second mutation is either a large deletion, which could not be detected by routine gene sequencing, or that it resides in a region of the gene that was not sequenced, such as intronic or promoter regions. Due to unavailability of parental DNA sample for testing, phase and biochemical consequence of these two missense changes could not be determined.

The majority of mutations found in this patient cohort are missense mutations (four out of five), and one mutation is a novel deletion mutation that results in a frameshift that alters the carboxy terminus of the protein. Two missense mutations are novel and were not observed in any of the 50 control DNA samples analyzed. The other two missense changes (p.Arg515Cys and p.Tyr535Cys) seen in our infantile-onset GSD IV patients (classic phenotype) have been reported before in a nonlethal infantile patient and an adult patient, respectively (Table 2).

Previously reported mutations in the *GBE1* gene

Thirty-four mutations in the *GBE1* gene have been published to date (Table 2) and cause a range of clinical phenotypes, from congenital to adult-onset GSD IV, representing extensive genetic heterogeneity. In addition, we identified three novel mutations in this study. Reported mutations include missense (n=13) and nonsense (n=6) mutations, intronic donor and acceptor splice-site mutations (n=5), small deletion frameshift mutations (n=4), small insertion frameshift mutations (n=2), and large deletions (n=4) (Table 2). The molecular basis underlying the pronounced range of severity and differences in organ involvement in patients with GSD IV is not well understood. Suggestion of the existence of tissue-specific

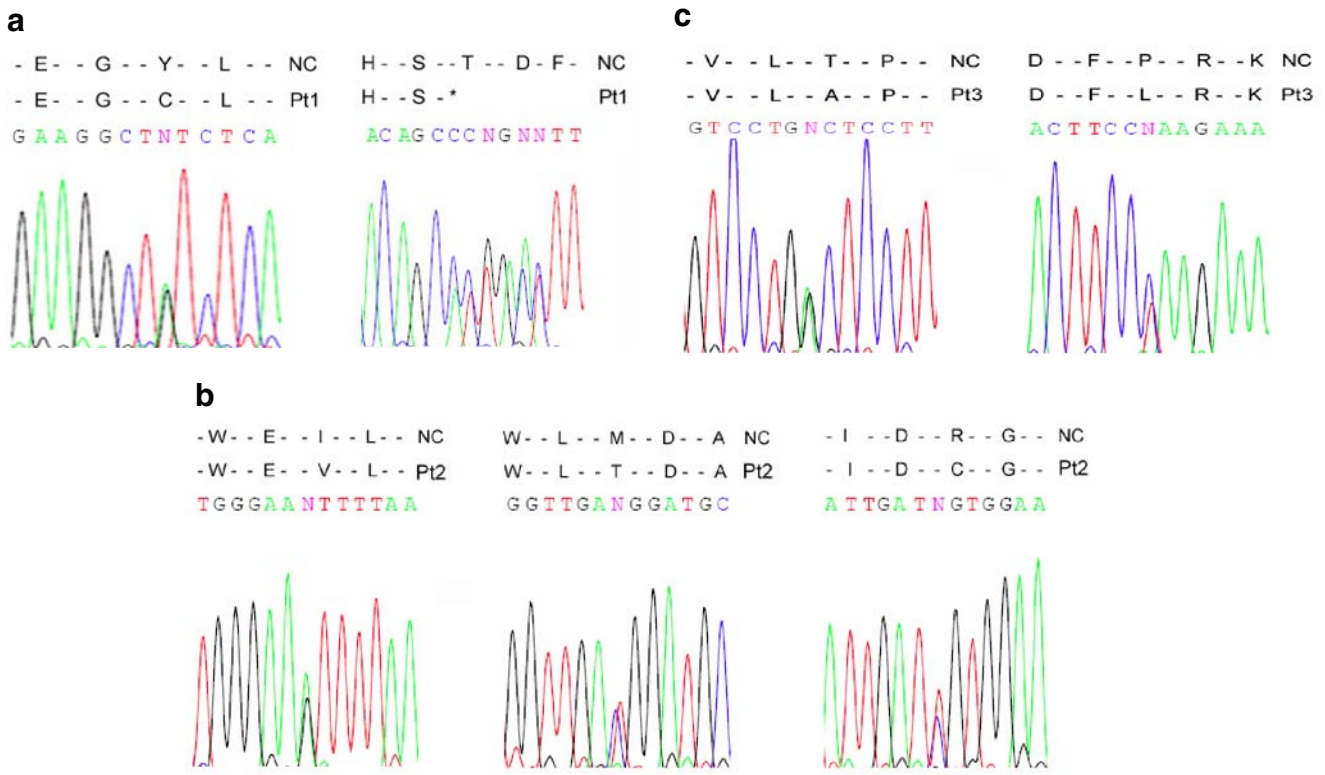

Fig. 2 The sequence analysis of glycogen-branching enzyme (*GBE1*) gene variation in patient 1(**a**), patient 2(**b**), and patient 3 (**c**)

isoforms of GBE1 as an explanation for clinical variability (Greene et al. 1987) has not been supported (Moses and Parvari 2002).

In general, patients with two missense mutations tend to have a milder form of the disease than those with two null mutations (Ziemssen et al. 2000; Bruno et al. 2004; Nolte et al. 2008). For example, to date, all mutations found in patients with APBD, at the milder end of the clinical spectrum, have been missense mutations (Ziemssen et al. 2000; Lossos et al. 1998; Massa et al. 2008). Most of these patients are of Ashkenazi Jewish descent who are homozygous for the p.Tyr329Ser mutation. Transient expression studies in COS-7 cells indicated that this mutation decreases GBE activity to about 50% of normal (Bao et al. 1996). The p.Tyr329Ser mutation has also been found in patients with the nonprogressive hepatic form of GSD IV, in compound heterozygosity with a second missense mutation, but not in patients with more severe forms of the disease (Bao et al. 1996). This suggests that the retained activity of the p.Tyr329Ser results in a less severe clinical phenotype. Similarly, the p.Arg525Gln missense mutation, which has been found only in APBD and the nonprogressive liver form of GSD IV, may also be a milder mutation with higher levels of retained GBE activity, but this has not been shown experimentally. The milder effect of a missense mutation, with retention of higher enzyme activity, is also suggested by the report of a child with congenital onset but mild clinical course of GSD IV who had two missense mutations (Burrow et al. 2006). In addition, two children with classic GSD IV have also been reported, each of whom had a known null mutation in combination with a missense mutation, with higher residual enzyme activity, as shown by expression studies (p. Arg515Cys with 20% normal activity and p.Phe257Leu with 27% normal activity) (Bao et al. 1996).

On the severe end of the GSD IV clinical spectrum, patients with congenital presentation tend to have at least one and often two protein-truncating mutations or large deletions (Bao et al. 1996; Bruno et al. 2004; Janecke et al. 2004; Nolte et al. 2008; Nambu et al. 2003; Assereto et al. 2007; Lamperti et al. 2009), which likely result in production of no functional GBE. Splicing mutations, on the other hand, can disrupt GBE1 protein deleteriously and result in nonprogressive hepatic or congenital phenotype (Akman et al. 2006; Shin 2006; Konstantinidou et al. 2008).

Whereas it is evident that different mutations will have different effects on the level of GBE activity, it is also possible that different mutations could lead to variable sensitivity of any mutant protein to proteases or post-translational modifications in different cell types, thus explaining differences in organ involvement. The expression of clinical disease may also be determined by other genetic

factors, such as alterations in other genes involved in glycogen metabolism, and environmental factors (Ziemssen et al. 2000; Ubogu et al. 2005).

Polymorphisms in the *GBE1* gene

Besides these pathogenic mutations, there were 17 new and reported polymorphisms detected in the *GBE1* gene (Table 2), according to the bioinformatics Pipeline for the Institute of Biomedical Sciences (IBMS) Genetic Research (http://genepipe.ngc.sinica.edu.tw/visualsnp) analysis. These SNPs include ten missense SNPs (rs2228389, rs36099971, rs2172397, rs28763902, rs17856389, rs2229519, rs1042498, rs28763904, rs35196441, rs62267114), four synonymous SNPs (rs2229520, rs17019144, rs13320194, rs28763906), and three frame-shift SNPs (rs35221448, rs71778833, rs34025837). The predicted risk level of SNPs rs2172397 (observed in patient 2) and rs2228389 (observed in patient 3) was in the medium range. The two novel mutations seen in our patients were not reported in the SNP database of the *GBE1* gene (Table 1), suggesting that they are pathogenic mutations causing GSD IV (Table 2). Additional sequence variants of unknown clinical significance have been found through sequencing clinical samples (Prevention Genetics, WI, USA; www.preventiongenetics.com), including c.-35dupC, c.143+10 g>t, c.292G>C (p.Val98Leu), c.607C>A (p.His203Asn), c.760A>G (p.Thr254Ala), and c.998A>T (p.Glu333Val). A benign amino acid change with normal activity in transient expression assays (p.Ala214Thr) has also been reported (Bao et al. 1996).

Conclusions

GBE enzyme activity levels were observed to be abnormal in all patients evaluated and in all tissues studied. However, the degree of residual enzyme activity was variable. There appears to be a reasonable correlation between clinical organ involvement and histological and biochemical abnormalities seen, although each patient had data available from only one or two of the three commonly examined tissues (skin fibroblasts, liver, and skeletal muscle). All patients were compound heterozygotes for mutations in the *GBE1* gene, which makes it difficult to predict genotype–phenotype correlations.

The diagnosis in our patients was clear after clinical, histological, and biochemical analyses. Mutation analysis served a confirmatory role. However, we recommend that genetic confirmation be obtained whenever possible in patients with suspected GSD IV to provide more data for genotype–phenotype correlations in this extremely rare disease. Three of the missense mutations in our patients are located in exon 12, which has previously been reported to be mutation hotspot (Moses and Parvari 2002). Based upon the prevalence of mutations in and around exon 12, we suggest that this exon should be sequenced first when performing mutation analysis for GSD IV. Also, in cases in which the biochemical analyses are equivocal or conflicting between tissues, mutation analysis can provide crucial diagnostic information.

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