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Glycogen storage disease type Ia: molecular study in Brazilian patients

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Abstract Mutations in the glucose-6-phosphatase (*G6Pase*) gene are responsible for glycogen storage disease type Ia (GSDIa). This disease is characterized by growth retardation, hepatomegaly, hypoglycemia, hyperlipidemia, and lactic acidosis. In this study, we report mutations in the *G6Pase* gene in 8 of 25 Brazilian patients with clinical symptoms of GSDIa. Five previously described mutations (R83C, Q347X, V338F, D38V, and G68R) were detected. The two most common mutations identified were R83C and Q347X, accounting for 8 of 14 (57.14%) mutant alleles. A 1176 single-nucleotide polymorphism and two intronic mutations (IVS3-58T>A and IVS4+10G>A) were also analyzed. We used the minigene strategy in order to verify the effect of these intronic mutations on the splicing mechanism. This study emphasizes that molecular genetic analysis is a reliable and convenient alternative to the assay of enzyme activity in a fresh liver biopsy specimen for diagnosing GSDIa.

Key words Glycogen storage disease type Ia · Glucose-6-phosphatase · Mutation · DNA-based diagnosis · Brazilian

Introduction

Type Ia glycogen storage disease (GSDIa; von Gierke disease; MIM no. 232200), is caused by a deficiency of glucose-6-phosphatase (*G6Pase*) activity in the liver, kidney, and

intestinal mucosa, with the excessive accumulation of glycogen in these organs. The *G6Pase* enzyme catalyses the terminal steps in gluconeogenesis and glycogenolysis and the conversion of glucose-6-phosphate (G6P) to glucose and phosphate. It is considered to be the key enzyme in glucose homeostasis. GSDIa is clinically characterized by short stature, doll-like facies, growth retardation, hepatomegaly, lactic acidemia, hyperuricemia, and hyperlipidemia (Chen and Burchell 1995). Current treatments of GSDIa are directed toward controlling symptomatic hypoglycemia. Patients receive continuous nasogastric infusion of glucose or frequent oral administration of uncooked cornstarch. Both treatments significantly reduce the metabolic abnormalities of GSDIa and greatly improve the prognosis, but the underlying pathological process remains untreated.

Somatic gene therapy is one attractive alternative to dietary therapy for GSDIa, whereby a functional recombinant *G6Pase* gene is targeted to the affected organ in vivo (Zingone et al. 2000). GSDIa, the most prevalent form of glycogen storage disease, is transmitted as an autosomal recessive trait, with an estimated overall frequency of 1:100,000. While a tentative diagnosis of GSDIa can be made from the characteristic clinical picture and various loading tests, an assay of *G6Pase* activity in liver biopsy specimens is essential to distinguish the disease from glycogen storage disease type Ib. Glycogen storage disease type Ib is associated with neutropenia and neutrophil dysfunction, which indicates that the putative *G6P* translocase gene (*G6PT1*), recently cloned by Gerin et al. (1997), is likely to have an important function in leukocytes. Investigation of patients diagnosed with GSDI non-a, (GSD Ib, Ic, or Id) showed mutation in the *G6PT1* gene, therefore indicating that they were GSDIb patients. At present, available evidence indicates that there are only two types of GSDI: GSDIa and GSDI non-a. Diagnosis can be done by enzymological tests and confirmed by mutation analysis of the respective genes (Veiga-da-Cunha et al. 2000). Characterization of the *G6Pase* gene by Lei et al. (1993) enabled the identification of the mutations causing GSDIa. This provides an option to apply DNA-based diagnosis for carrier detection and prenatal diagnosis. Identification of the

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mutations causing GSDIa provides an insight into the structure-function relation of G6Pase catalysis by revealing the structural roles of specific amino acid residues (Parvari et al. 1999). The *G6Pase* human gene is located on chromosome 17q, contains five exons, spans 12.5kb, and encodes endoplasmic reticulum membrane-associated protein containing 357 amino acid residues (Lei et al. 1993; Chou and Mansfield 1999). So far, more than 50 different mutations in the *G6Pase* gene have been identified.

In the present study, we describe the results of mutation analysis of the *G6Pase* gene in 24 unrelated Brazilian GSDIa families, and the construction of minigenes in order to analyze whether two intronic mutations, one located in intron 3 and the other in intron 4, could be responsible for an aberrant mRNA splicing. Exon skipping is a common mutational mechanism, usually caused by changes in the consensus sequences at splice sites or lariat branch-point regions. Sequences outside these regions can also affect the inclusion or exclusion of exons. Such splicing enhancers may be located in introns or exons, and alternative splicing patterns could be determined by the visualization of a sized transcription product. In our study, we used the minigene strategy to determine the pathological nature of these intronic mutations.

Subjects and methods

Subjects

Twenty-five GSDIa patients from 24 unrelated families were investigated. Fourteen families came from the south-east and the other 10 came from the southern part of Brazil. The diagnosis of GSDIa was based on the typical clinical and laboratory findings, such as hepatomegaly, recurrent hypoglycemia, hyperuricemia, and doll-like facies. The diagnosis was confirmed in only 4 patients by the measurement of enzyme activity in fresh liver biopsy specimens. Twenty-one patients had not undergone a liver biopsy prior to our molecular genetic analyses, but displayed the characteristic clinical and laboratory findings of GSDIa. All patients gave their informed consent prior to their inclusion in this study.

Methods

Identification of mutations in GSDIa patients. Genomic DNA was extracted from peripheral leukocytes, using standard procedures. The five exons of the *G6Pase* gene and their flanking intron-exon junctions were polymerase chain reaction (PCR) amplified, using previously described primers (Lei et al. 1993). Sequence analysis was performed by the cycle-sequencing method, using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, São Paulo, SP, Brazil) and a Pharmacia (São Paulo, SP, Brazil) $\alpha^{33}\text{P}$ -labeled terminator pack. The sequencing reaction was performed according to the manufacturer's protocol.

Polymorphism analysis. A single-nucleotide polymorphism occurs at position 1176, where the presence of T instead of C generates a *DraI* restriction site. The PCR product of exon 5 is 647bp in size. In the presence of a T nucleotide at position 1176, *DraI* restriction enzyme cuts the PCR product into a 572-bp and a 75-bp fragment. Enzymatic digestion of PCR products was performed as described elsewhere (Lam et al. 1998).

Construction of minigenes. The effects of the IVS3-58T>A and IVS4+10G>A mutations, located in introns, 3 and 4, respectively, were investigated using a minigene strategy. The constructions exon 3-intron 3-exon 4 (minigene 1) and exon 4-intron 4-exon 5 (minigene 2), were PCR amplified from the patients, and from control genomic DNA using the following set of primers (minigene 1): 5'-CCATGGGCAC AGCAGGTCTAGACTAC-3' (sense); 5'-CTGACGGAT CCAGCAACAACCTTGATG-3' (antisense); (minigene 2): 5'-GGGATTCTAGACTGTGCAGCTGAATGTCTG-3' (sense) (Kajihara et al. 1995); 5'-CTGGGTTTCTCCAGG ATCCACAGGAGGTC-3' (antisense) (Kajihara et al. 1995). The fragments were digested with *XbaI* (within sense primer) and *BamHI* (within antisense primer), purified with a Concert Rapid Gel Extraction System (GIBCO BRL, Grand Island, NY, USA), and then ligated to the expression vector pSVL (Pharmacia LKB, São Paulo, SP, Brazil) to create minigene constructs that included the *G6Pase* sequence containing the intronic mutation.

In-vitro transient expression. COS-7 cells were grown in Dulbecco's modified Eagle's medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% (vol/vol) fetal bovine serum (Cultilab). The wild-type and the mutant pSVL vector were transfected into COS-7 cells by the calcium-phosphate method, using a calcium phosphate transfection system (GIBCO BRL), to transient expression. After incubation at 37°C for 72 h, the transfected cells were suspended in a small volume of isotonic buffer. Total mRNA was extracted with a QuickPrep Micro mRNA Purification Kit (Amersham); it was reverse-transcribed, and then PCR amplified, using a ThermoScript reverse transcription (RT)-PCR System (GIBCO BRL). The specific primers for the cDNA amplification were (minigene 1): 5'-ACTACG TGATGGTCACATCT-3' (sense); 5'-CAGCAACA ACTTGATGAGGA-3' (antisense); (minigene 2): 5'-GTCGTGCAGCTGAATGTCTG-3' (sense) (Kajihara et al. 1995); 5'-TCCAGAGTCCACAGGAGGTC-3' (antisense) (Kajihara et al. 1995). Ten microliters of the PCR products were electrophoresed in 2% agarose gel.

Results

Mutation analysis of the *G6Pase* gene was performed by direct sequencing. We identified mutations on both alleles in eight patients studied. The mutations identified are listed in Table 1. A total of five different mutations was detected; four missense mutations (G68R, V338F, D38V, and R83C),

Table 1. Distribution and frequency of mutations in 14 *G6Pase* alleles of GSDIa patients

Mutation name	Nucleotide change	Exon	No. of alleles	Frequency (%)
R83C	326C → T	2	4	28.57
Q347X	1118C → T	5	4	28.57
G68R	281G → A	1	2	14.28
V338F	1012G → T	5	2	14.28
D38V	192A → T	1	2	14.28
Total			14	100

GSDIa, Type Ia glycogen storage disease

and one nonsense mutation (Q347X). R83C and Q347X were the most common mutations in the patients, accounting for 8 of 14 (57.14%) mutant alleles. Two patients were homozygous for the R83C mutation and two were homozygous for the Q347X mutation. One patient was homozygous for the G68R mutation, which was first described in this country (Sartorato et al. 1998), two brothers were homozygous for V338F (2/14), and one patient was homozygous for D38V.

We also studied a known polymorphism, 1176T/C, described by Lam et al. (1998). The polymorphism (nucleotide C) was present in 70% of the GSDIa alleles and in 60% of the alleles from 50 healthy controls. According to Wong et al. (1998), the nucleotide 1176C is in linkage disequilibrium with the R83C mutation, and this was confirmed in our patients. The 1176 nucleotide polymorphic marker can be used in the carrier and prenatal diagnosis of GSDIa families that have unidentified mutations and are informative for this marker.

The patient with the G68R mutation in exon 1 also presented one intronic mutation (IVS3-58T>A) in a homozygous condition. This mutation was investigated by the minigene technique, and the construction, exon 3-intron 3-exon 4, cloned into the pSVL vector, had a size of 1.6kb. After the transfection, cDNA was amplified by PCR and the amplification product had an expected size of 171pb for the mutant and the control construction. No differences between the normal and mutant sequence were detected, showing that the IVS3-58T>A mutation did not cause an alternative splicing mechanism.

The IV4S+10G mutation was found in a heterozygous condition in a patient who did not have any other mutations in the gene coding region. The intron-exon junctions from the *G6Pase* gene of this patient were amplified and direct sequenced, and no other mutations were found. The minigenes containing exon 4-intron 4-exon 5 cloned into the pSVL vector had a size of 1.64kb. After the transfection, cDNA was amplified by PCR and the amplification product had an expected size of 236pb for the mutant and the control construction. This demonstrates that there were no differences between the normal and mutant transcripts, and therefore the IV4S+10G mutation is not responsible for an aberrant splicing in the gene.

Discussion

In terms of phenotype-genotype correlation, patients with the R83C mutation showed clinical and biochemical manifestations that were similar to the known pattern of the disease. This mutation has already been examined for G6Pase activity in transient expression assays (Lei et al. 1993) and the mutant had no detectable phosphohydrolase activity. It alters Arg⁸³, an amino acid residue that contributes to the active center of the enzyme. The stringent structural requirement of codon 83 suggests that this amino acid residue in G6Pase may also be involved in positioning the phosphate (Lei et al. 1995). The mutation Q347X, which converts a glutamine at position 347 to a stop codon, inactivates G6Pase activity, because it causes truncation of the carboxy terminal 11 amino acid residues in human G6Pase (Lei et al. 1994). A similar mutation frequency in the *G6Pase* gene was observed in Caucasian patients from the United States (Lei et al. 1995) and northwest Europe (Chevalier-Porst et al. 1996; Rake et al. 1999), where R83C and Q347X account for approximately 50% of all mutant alleles. The ethnic origins of the Brazilian population are highly heterogeneous, with the population being composed of immigrants from Europe, Africa, and Asia, as well as Amerindian groups, resulting in a complex race admixture. The Portuguese contribution is one of the most ancient and predominant ones, beginning in the sixteenth century with the colonization of Brazil. These observations indicate that the R83C and Q347X mutations may be relatively common in Caucasians and could have spread through European migration mainly in recent centuries.

The G68R mutation is situated in the transmembrane helices of the G6Pase enzyme, and, although no enzymatic assay has been done, an alteration in this region may influence the stability of the enzyme structure and, consequently, its activity.

The V338F mutation was recently described in a compound heterozygous Dutch GSDIa patient (Rake et al. 1999). Although it remains to be demonstrated by expression analysis whether this mutation leads to G6Pase deficiency, V338F is a true mutation, considering that this mutation is recurrent in homozygous form in GSDIa patients. According to Rake et al. (1999), the substitution of valine by phenylalanine at codon 338 could affect the activity or stability of G6Pase, because phenylalanine contains an aromatic ring instead of the aliphatic nonpolar side chain contained in valine.

In the *G6Pase* gene, we identified another missense mutation (D38V) in exon 1 in a patient whose GSDIa diagnosis was confirmed by the lack of G6Pase activity shown in the biochemical analysis of a liver biopsy sample. This mutation, which was previously described only in the heterozygous condition (Chevalier-Porst et al. 1996), changes an acidic amino acid residue (aspartic acid) to a nonpolar hydrophobic one (valine) in the middle of the first predicted transmembrane spanning domain. Analysis of this mutation by site-directed mutagenesis, followed by transient expres-

sion, demonstrated that the D38V mutation abolishes G6Pase activity.

In 17 patients with clinical GSDIa symptoms, we did not find mutations in the coding region that could be responsible for the disease. Although they had the clinical characteristics of GSDIa, these patients had not undergone a liver biopsy for the biochemical analysis of G6Pase activity, and we can presume that the alteration in the *G6PC* gene, responsible for the patient's phenotype, could be located in the noncoding region, or that they are GSDI non-a patients.

In GSDIa, the apparent absence of phenotypical heterogeneity could be related to our inability or failure to recognize phenotypes, which was demonstrated by the study of one of our patients who had the D38V mutation. She was 39 years old at the time of biochemical investigation, and the typical symptoms of GSDIa had been absent during infancy. The biochemical investigation was performed mainly because of the existence of GSDIa family history. So we could speculate that GSDIa could, in fact, be a disease with a large phenotypic variability. It is possible that mild cases may remain misdiagnosed, as the enzyme assay is performed only in severe cases when a liver biopsy is required considering hepatomegaly, and occasionally, liver biopsies may be performed without enzymatic analysis.

In conclusion, the molecular genetic analysis presented in this study is a noninvasive, highly efficient, and reliable method for the diagnosis of GSDIa. It is a convenient alternative to enzyme studies in liver tissue obtained by biopsy.

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References

Chen YT, Burchell A (1995) Glycogen storage disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The metabolic and molecular bases of inherited disease*. McGraw-Hill, New York, pp 935–965

Chevalier-Porst F, Bozon D, Bonardot A, Bruni N, Mithieux G, Mathieu M, Maire I (1996) Mutation analysis in 24 French patients with glycogen storage disease type 1a. *J Med Genet* 33:358–360

Chou JY, Mansfield BC (1999) Molecular genetics of type 1 glycogen storage diseases. *TEM* 10:104–113

Gerin I, Veiga-da-Cunha M, Achouri Y, Collet J, Schaftingen EV (1997) Sequence of a putative glucose 6-phosphate translocase, mutated in glycogen storage disease type 1b. *FEBS Lett* 419:235–238

Kajihara S, Matsuhashi S, Yamamoto K, Kido KM, Tsuji K, Tanae A, Fujiyama S, Itoh T, Tanigawa K, Uchida M, Setoguchi Y, Motomura M, Mizuta T, Sakai T (1995) Exon redefinition by a point mutation within exon 5 of the glucose-6-phosphatase gene is the major cause of glycogen storage disease type 1a in Japan. *Am J Hum Genet* 57:549–555

Lam CW, Liang MH, Pang CP, Tong SF, Wong LJC (1998) A novel *DraI* polymorphism in the 3' untranslated region of human glucose-6-phosphatase gene: useful for carrier detection and prenatal diagnosis of glycogen storage disease type 1a. *Clin Genet* 53:502–503

Lei KJ, Shelly LL, Pan CJ, Sidbury JB, Chou JY (1993) Mutations in the glucose 6-phosphatase gene that cause glycogen storage disease type 1a. *Science* 262:580–583

Lei KJ, Pan CJ, Shelly LL, Liu JL, Chou JY (1994) Identification of mutations in the gene for glucose-6-phosphatase, the enzyme deficient in glycogen storage disease type 1A. *J Clin Invest* 93:1994–1999

Lei KJ, Shelly LL, Baochuan L, Sidbury JB, Chen Y, Nordlie RC (1995) Mutations in the glucose 6-phosphatase gene are associated with glycogen storage disease types 1a and 1aSP but not 1b and 1c. *J Clin Invest* 95:234–240

Parvari R, Isam J, Moses SW (1999) Glycogen storage disease type 1A in three siblings with the G270V mutation. *J Inher Metab Dis* 22:149–154

Rake JP, ten Berg AM, Verlind E, Visser G, Niezen-Koning KE, Buys CHCM, Smit GPA, Scheffer H (1999) Glycogen storage disease type 1a: four novel mutations (175delGG, R170X, G266V and V338F) identified. *Hum Mut* 13:173–176

Sartorato EL, Reis FC, Norato DYJ, Hackel C (1998) A novel mutation in a Brazilian patient with glycogen storage disease type 1a. *J Inher Metab Dis* 21:447

Veiga-da-Cunha M, Gerin I, Van Schaftingen E (2000) How many forms of glycogen storage disease type I? *Eur J Pediatr* 159:314–318

Wong LJ, Liang MH, Hwu WL, Lam CW (1998) Linkage disequilibrium and linkage analysis of the glucose-6-phosphatase gene. *Hum Genet* 103:199–203

Zingone A, Hiraiwa H, Pan CJ, Lin B, Chen H, Ward JM, Chou JY (2000) Correction of glycogen storage disease type 1a in a mouse model by gene therapy. *J Biol Chem* 275:828–832