RESEARCH ARTICLE

Molecular analysis of glycogen storage disease type Ia in Iranian Azeri Turks: identification of a novel mutation

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Abstract

Glycogen storage diseases (GSDs) are caused by abnormalities in enzymes that are involved in the regulation of gluconeogenesis and glycogenolysis. GSD I, an autosomal recessive metabolic disorder, is the most common GSD and has four subtypes. Here, we examined GSD Ia caused by the defective glucose-6-phosphatase catalytic (*G6PC*) gene. We investigated the frequency of GSD Ia and clarified its molecular aspect in patients with the main clinical and biochemical characteristics of GSD, including 37 unrelated patients with a mean age of three years at the time of diagnosis. All patients belonged to the Azeri Turkish population. Hypoglycaemia and hypertriglyceridaemia were the most frequent laboratory findings. Mutations were detected by performing direct sequencing. Mutation analysis of the *G6PC* gene revealed that GSD Ia accounted for 11% in GSD patients with involvement of liver. Three patients were homozygous for R83C mutation. In addition, a novel stop mutation, Y85X, was identified in a patient with the typical features of GSD Ia.

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Introduction

Glycogen storage diseases (GSDs) are a heterogeneous group of inherited disorders caused by defects in glycogen degradation and synthesis. GSDs are mainly observed in children and it mostly affects the liver, muscle and heart (Koshy *et al.* 2006). GSDs can be differentiated based on clinical, biochemical and enzymatic examination of the liver tissue. Based on enzyme deficiency, they are classified into 10 major types (Mundy and Leeb 2004), and GSD I, GSD III, GSD VI and GSD IX usually affect the liver severely (Kido *et al.* 2013). GSD I, which was first described by von Gierke, is the most common form of GSD (Özen and Bayraktar 2007). GSD I has four subtypes, namely GSD Ia, characterized by defects in glucose-6-phosphatase catalytic subunit (G6 Pase); GSD Ib, presented by deficiency in glucose-6-phosphate translocase; GSD Ic, described by defects in a putative phosphate transporter; and GSD Id, caused by defects in a putative glucose transporter (Chou and Mansfield 2008; Kasapkara *et al.* 2012).

GSD Ia is a recessively inherited metabolic disorder prevalence of approximately about one in 100,000 live births (Mundy and Leeb 2004; Zheng *et al.* 2015). It is caused by a defect in the glucose-6-phosphatase catalytic (*G6PC*) system, which catalyzes the hydrolysis of G6P to phosphate and glucose. *G6PC* is a single-copy gene and is located on chromosome 17q21 (Lei *et al.* 1994a, b). It consists of five exons spanning approximately 12.5-kb DNA and has tissue-restricted expression, predominantly in liver, kidney and intestine (Lei *et al.* 1993, 1994a, b). The gene encodes a hydrophobic protein containing 357 amino acids and nine transmembrane domains (Pan *et al*. 1998). Analysis of mutations in the *G6PC* gene can be performed to obtain more conclusive results (Mundy and Leeb 2004; Liang *et al.* 2013).

Defects in the *G6PC* system increase glucose-6-phosphate (G6P) levels in the cytoplasm. This triggers alternative metabolic pathways that lead to the accumulation of glycogen in liver and kidneys, thus contributing to progressive hepatomegaly and nephromegaly. Elevated cytoplasmic G6P levels result in metabolic consequences, such as hypercholesterolamia, hypertriglyceridamia, hyperuricaemia and lactic acidamia (Janecke *et al.* 2001; Eminoğlu *et al.* 2013). To date, various mutations have been reported in the *G6PC* gene, including missense (the most prevalent form), nonsense, insertion/deletion and splicing (Rake *et al.* 2000; Chou and Mansfield 2008). Here, we present molecular analysis

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of suspected GSD Ia patients to provide a DNA-based method for carrier and prenatal diagnosis in Iranian Azeri Turkish patients.

Materials and methods

Study subjects

In the present study, we included 37 unrelated patients with a mean age of 3 years (age range between 5 months and 13 years) who demonstrated clinical features of GSD (Froissart *et al.* 2011; Kishnani and Chen 2015). The patients were screened by their physicians at a children's hospital in Tabriz between February 2011 and July 2014, and were referred to our laboratory for molecular investigation (table 1).

Of the 37 patients, 34 were from the northwestern region of Iran and three were from the Republic of Azerbaijan (located to the northwest of Iran). All patients had the same ethnic background (Azeri Turkish population).

The study was approved by the institutional review board and ethical committee of the Tabriz University of Medical Sciences, Tabriz, Iran. Informed consent was obtained from the families of the patients. Diagnostic criteria were based on the clinical and biochemical features of GSD; however, liver biopsy, enzyme analysis and mutation analysis were not performed (Froissart *et al.* 2011; Kishnani and Chen 2015).

We first measured the levels of serum creatine kinase (CK) and creatine phospho kinase (CPK), and excluded three patients with high CK and CPK levels from molecular analysis due to suspected GSD-III (Kishnani *et al.* 2010; Mogahed *et al.* 2015; Zheng *et al.* 2015).

Preparation of genomic DNA

Approximately 5 ml of intravenous blood samples were collected from all the patients into EDTA vacutainers and used for DNA extraction. Genomic DNA was extracted from leucocytes present in the peripheral blood by using the salting-out method.

Table 1. Clinical, biochemical and molecular genetic characteristics of 37 GSD Ia patients.

ID	Age (year)	Sex	FBS (mg/dL)	TG (mg/dL)	SGPT (U/L)	CPK (U/L)	Mutation
1	6	M	50	166	318	3396	EX
2	NA	M	43	220	200	220	–
3	2	F	45	190	NA	89	–
4	5	M	65	159	502	108	–
5	6	F	49	187	176	189	–
6	1	M	44	240	200	200	R83C
7	2.5	M	81	302	62	118	–
8	3	F	47	280	77	68	–
9	2	M	51	169	440	49	c.84C>T (rs758804611)
10	4	F	50	140	69	114	–
11	2	F	48	1252	30	99	R83C
12	1.5	M	25	540	89	79	R83C
13	5	F	37	300	59	217	–
14	3	M	32	560	143	135	–
15	0.4	M	43	430	206	120	Y85X
16	0.5	F	44	235	68	68	–
17	1.5	F	47	306	199	NA	–
18	1	M	57	340	301	87	c.340 + 10C>A
19	2	M	44	440	405	99	–
20	NA	F	69	112	36	130	–
21	2	M	39	340	94	59	–
22	4	M	49	280	76	NA	–
23	1.8	F	100	134	146	279	–
24	1	F	45	570	132	110	–
25	2	F	62	355	76	99	–
26	NA	F	83	135	55	69	c.*23T>C (rs2229611)
27	2	M	48	413	92	112	–
28	15	M	46	221	99	205	–
29	5	M	85	251	45	2922	EX
30	2	M	42	400	49	123	–
31	NA	F	38	198	68	160	–
32	3	M	71	304	58	130	–
33	3	F	51	112	49	NA	c.18T>C (rs144652516)
34	5	F	44	169	157	125	–
35	NA	F	46	186	170	560	EX
36	3	M	48	420	45	140	–
37	4	F	49	230	63	100	–

M, male; F, female; FBS, fasting blood sugar; TG, serum triglycerides; NA, not available; SGPT, serum glutamate pyruvate transaminase; CPK, serum total creatine phosphokinase; EX, excluded from sequencing analysis.

PCR amplification and sequencing

Complete *G6PC* exons 1, 2, 3, 4 and 5 and intron/exon borders were amplified by performing PCR. Amplification was performed using a 25-μL reaction mixture containing 50 ng of genomic DNA, 200 μmol/L of dNTPs, 10 pmol of each primer, 1.5 U of *Taq* DNA polymerase, 1× PCR buffer (10 mmol/L of KCl, 10 mmol/L of Tris-HCl (pH 8.3), 1.5 mmol/L of $MgCl_2$) and 6 mmol/L of $MgCl_2$ (Cinnagene, Tehran, Iran). PCR was performed using the following protocol: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 s; annealing for 40 s; extension at 72°C for 40 s; and final extension at 72°C for 5 min (Gene Amp@ PCR System, Bio-Rad, USA). PCR-amplified fragments were purified, and mutation analysis was performed by direct DNA sequencing using reverse and forward primers (Macrogen, Seoul, South Korea).

Results

In total, 70% patients had an affected family member. Consanguineous marriage was observed in the families of 74% patients. Major findings of biochemical and physical examinations at the time of diagnosis were hypoglycaemia (blood sugar, <50 mg/dL) in 27 patients and increased serum triglycerides (>200 mg/dL) in 24 patients (table 1). Hepatomegaly was observed in all the patients, with most patients demonstrating a doll-like face. DNA sequencing demonstrated that three patients were homozygous for a substitution mutation 326C>T (R83C). Mutation analysis of *G6PC* identified a novel mutation, Y85X, in homozygous form in one patient (figure 1).

The 233C>A (Y85X) mutation leads to the substitution of tyrosine by a stop codon. This substitution results in the premature stop codon producing a truncated protein, which is only 24% of the original full-length protein and is predicted to have no catalytic activity. The newly identified mutation was present in an affected child born to consanguineous healthy, but carrier parents. The affected patients with the detected mutations belonged to four unrelated Iranian Azeri families. Mutation analysis of DNA obtained from their parents demonstrated that they carried the same mutation in the heterozygous form (figure 2). Other patients did not have mutations in *G6PC*. Meanwhile, three SNPs (rs758804611, rs222961 and rs144652516) were found in heterozygote status in three different patients other than the mutants (table 1).

Discussion

Characterization of *G6PC* and identification of mutations in this gene provide a DNA-based tool to diagnose patients clinically suspected of having GSD Ia. Moreover, mutation analysis of a family at risk of conceiving offspring with GSD Ia offers a DNA-based tool for prenatal diagnosis. Early diagnosis allows the employment of adequate

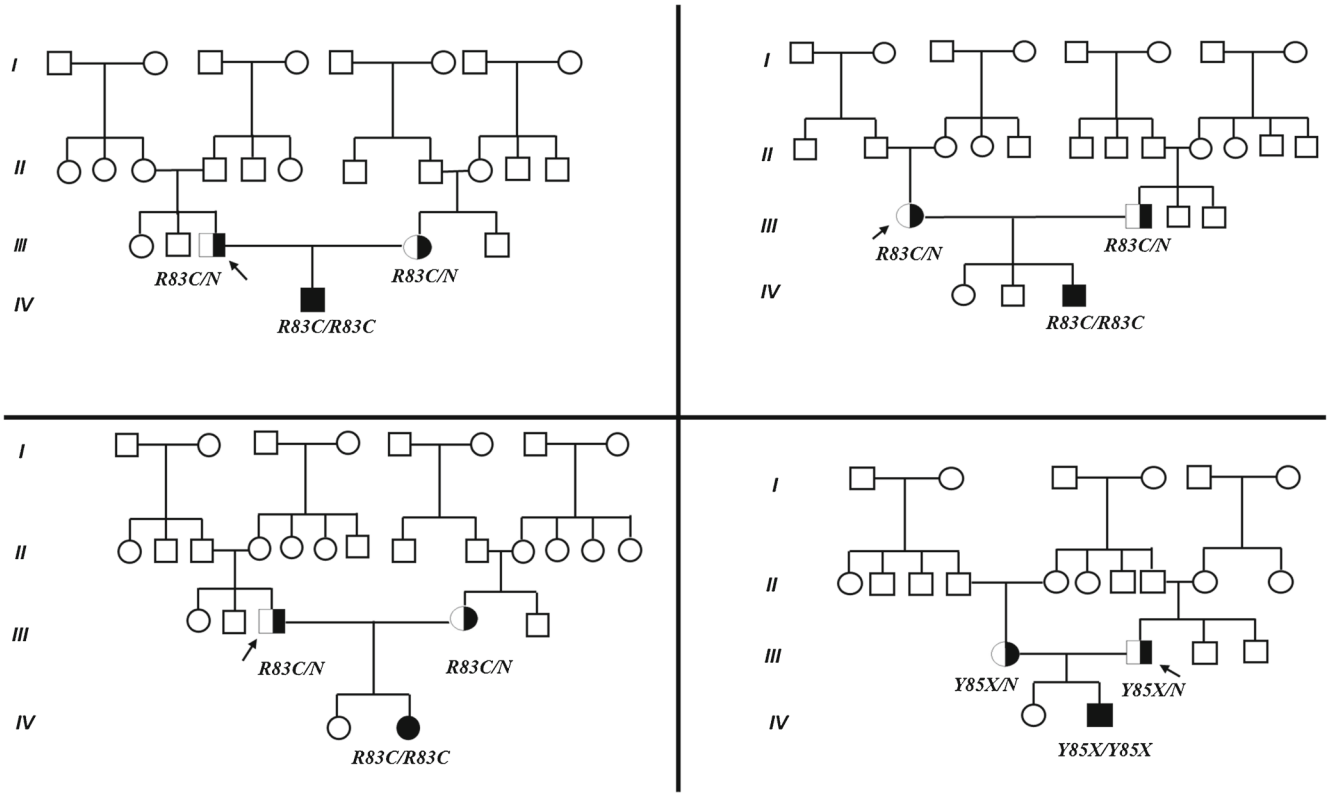

Figure 1. Pedigree patterns in four families with GSD Ia.

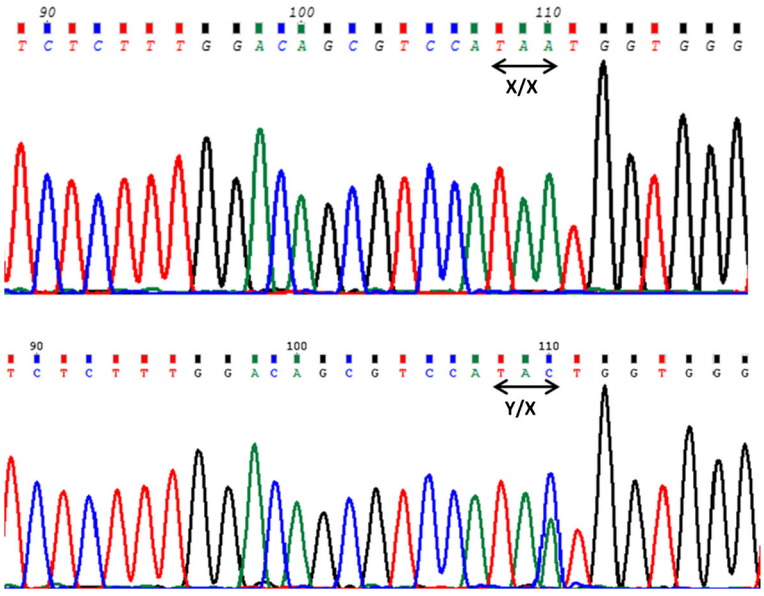

Figure 2. Sequencing analysis of fragment including Y85X mutation in homozygous and heterozygous status on exon 2.

metabolic control strategies and treatments to prevent complications and thus increases the quality of life (Mogahed *et al.* 2015). The rates of consanguineous marriages are high in Iran, therefore, a high prevalence of autosomal recessive metabolic diseases, such as hepatic glycogenoses is predictable (Hasanzadeh-Nazarabadi *et al.* 2006; Gialluisi *et al.* 2012). The prevalence of hepatic glycogenoses in Iran is unknown, and no case studies or detailed mutation studies on GSD have been reported to date (Motamed *et al.* 2011). Clinical diagnosis of GSD in patients included in the present study was not previously confirmed by performing enzyme assay of biopsied liver tissue or mutation analysis.

Mutation analysis of *G6PC* was performed for 34 patients with GSD who belonged to 34 unrelated families, and who had clinical and biochemical characteristics resembling those of GSD Ia. Direct DNA sequencing was performed to screen mutations in the coding region, intron/exon junctions and 5′ UTRs and 3′ UTRs of the *G6PC* gene. Upon molecular investigation, four of 34 GSDs (12%) with liver involvement were verified as being affected by GSD Ia. Molecular analysis of parents of these patients with GSD Ia, identified mutant alleles of *G6PC* in both parents. The R83C mutation in *G6PC* occurs at a CpG dinucleotide rich and is a C>T transition at nucleotide 326. The R83C mutation is common in Czech and Slovak (40%) (Kozák *et al.* 2000), Italian (46.2%) (Stroppiano *et al.* 1999) and Turkish (68.5%) patients with GSD Ia (Eminoğlu *et al.* 2012), and accounts for six of eight mutant alleles of *G6PC* in the present study. The active site mutation, R83C is associated with undetectable phosphohydrolase activity of G6 Pase in liver biopsy of patients (Lei *et al.* 1994a, b). This is consistent with the results of *in vitro* investigations that Arg to Cys substitution at codon 83 abolishes the phosphohydrolase activity of G6 Pase (Lei *et al.* 1994a, b). The homozygous Y85X nonsense mutation, producing a truncated protein, is predicted to be associated with no residual activity.

This evidence eases genotype–phenotype description, at least for patients homozygous for these two mutations. All patients affected by GSD Ia, either with R83C or with the novel mutation (Y85X), are associated with younger age at the time of diagnosis (<2 year) and with clinical findings including hepatomegaly, growth retardation and doll face. Triglyceride serum uric acid values were higher in all the four patients, and they also suffered from severe hypoglycaemia (table 1). Because of insufficient sample size and lack of allelic heterogeneity, statistical analysis and comparison between biochemical parameters associated with different mutations could not be performed. As reported previously, GSD Ia patients exhibit phenotypic heterogeneity; moreover, there is no clear evidence for a firm genotype–phenotype relationship for each GSD Ia gene mutation (Matern *et al.* 2002; Chou and Mansfield 2008). The GSD types with liver involvement are best distinguished through enzyme studies of liver tissue obtained by wedge biopsy. However, clinical, biochemical and histological features may suggest the type of GSD (Kishnani and Chen 2015). Type I, III and IX constitute 80% of hepatic GSDs (Chen 2001). In this study, around 12% (four of 37) of patients suffered from GSD Ia and the remaining patients might be affected by other subtypes of GSD which are not analysed. On the other hand, the primers that we designed and used allowed only the detection of mutations located in the coding region and intron/exon junctions and 5′ UTRs and 3′ UTRs of the gene. Therefore, mutations in the intron regions of *G6PC* gene, which may affect the rate of gene transcription or stability of the *G6PC* mRNA were not sequenced and eluded detection.

Finally, these results emphasize that GSDs are multisystem diseases that are particularly hard for clinicians to

diagnose, and suggest that molecular diagnosis of GSDs is helpful to verify disease type and to offer appropriate clinical approach. Moreover, knowledge of the family's pathogenic mutations facilitates to predict the risk of recurrence for future children; thus, it assists in counselling, carrier testing and prenatal diagnosis.

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