Mutations in the glucose-6-phosphate transporter (G6PT) gene in patients with glycogen storage diseases type 1b and 1c

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Abstract **Glycogen storage diseases type 1 (GSD 1) are a group of autosomal recessive disorders characterized by impairment of terminal steps of glycogenolysis and gluconeogenesis. Mutations of the glucose-6-phosphatase gene are responsible for the most frequent form of GSD 1, the subtype 1a, while mutations of the glucose-6-phosphate transporter gene (G6PT) have recently been shown to cause the non 1a forms of GSD, namely the 1b and 1c subtypes. Here, we report on the analysis by single-stranded conformation polymorphism (SSCP) and/or DNA sequencing of the exons of the G6PT in 14 patients diagnosed either as affected by the GSD 1b or 1c subtypes. Mutations in the G6PT gene were found in all patients. Four of the detected mutations were novel mutations, while the others were previously described. Our results confirm that the GSD 1b and 1c forms are due to mutations in the same gene, i.e. the G6PT gene. We also show that the same kind of mutation can be associated or not with evident clinical complications such as neutrophil impairment. Since no correlation between the type and position of the mutation and the severity of the disease was found, other unknown factors may cause the expression of symptoms, such as neutropenia, which dramatically influence the severity of the disease.**

Key words: Glycogen storage disease; Glucose-6-phosphate; Mutation

1. Introduction

Glycogen storage disease type 1 (GSD 1 or von Gierke disease) includes a clinically, biochemically and genetically heterogeneous group of autosomal recessive disorders [1]. The basic defects reside in the impairment of the terminal steps of glycogenolysis and gluconeogenesis, at different levels.

Mutations of the glucose-6-phosphatase gene (G6Pase), which lead to the enzyme deficiency, are responsible for the most frequent form of GSD 1, the subtype 1a [1]. This gene is not mutated in patients in which there are biochemical evidences of defects in the glucose-6-phosphate transport system [2]. GSD 1 patients diagnosed as non 1a have been subdivided at least in 1b and 1c subtypes on the basis of clinical and biochemical parameters [1]. The recent cloning of the cDNA for a putative endoplasmic reticulum glucose-6-phosphate transporter (G6PT) [3] has enabled the search for mutations in this gene in GSD 1 non a patients [4–9]. Recent data from others [5–9] indicate that mutations in the G6PT gene are present in both 1b and 1c patients so far investigated.

We report on the genetic analysis of 14 additional patients affected by GSD 1 non a. Six of them were clinically and biochemically diagnosed as 1b subtypes, five as 1c subtypes and three simply as 1 non a forms. Mutations of the G6PT gene were detected in all cases. Four of the mutations detected were novel, while the others were previously described. Although the mutations appear to be scattered throughout the entire G6PT gene, there seems to be a proportionally higher number of mutations in exons 3 and 8. Present and previously reported data [5–9] strongly suggest that the G6PT gene is involved in most, if not all, of the GSD 1 non a cases.

2. Materials and methods

2.1. Patients

DNAs from 14 non 1a GSD patients and from their parents, when available, were collected. Two patients (no. 38 and 39) were siblings. The Caucasian ethnic background is common for all the patients studied. In 11 patients (no. 22, 25, 28, 32, 35, 37–41 and 49), the diagnosis for GSD 1b or 1c was based on clinical features (hepatomegaly, hypoglycemia and lactic acidosis) and on kinetic analysis of G6Pase system activity in liver biopsy samples [1]. Patients 42, 43 and 44 were simply diagnosed as type 1 non a, since (in addition to the clinical symptoms as above) no mutations were found in the glucose-6-phosphatase enzyme gene. Neutropenia and impaired neutrophil function were clinically evident in seven out of the 14 patients investigated.

2.2. Mutational analysis

Peripheral blood lymphocytes isolated from all the individuals were used for extraction of genomic DNA using a standard protocol [4].

All the nine exons of the G6PT gene were amplified by the polymerase chain reaction (PCR), using the intronic primers described elsewhere [4] and subsequently screened by single-strand conformation polymorphism (SSCP) analysis. PCR reactions were performed in 25 μl containing 100 ng of genomic DNA, 1×PCR buffer (Perkin-Elmer), 0.5 pmol/l of each primer, 180 μM of each dNTP and 1 U *Taq*

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DNA polymerase (Perkin-Elmer). The PCR conditions were 95°C for 5 min and then 30 cycles at 95°C for 40 s, 60°C for 30 s and 72°C for 30 s and the final extension was 72°C for 4 min. The amplified samples were diluted 1:1 in formamide buffer (95% formamide, 10 mM NaOH, 0.025% bromophenol blue and 0.025% xylene cyanol), denatured at 95°C for 5 min and cooled on ice. Four–8 μl of PCR products was loaded on a non-denaturing polyacrylamide gel containing 6% acrylamide, prepared with a 99:1 ratio between acrylamide and bis-acrylamide. Gels were run in a cold room for 1–4 h at 30 W. DNA bands were visualized by silver staining. PCR products that revealed an aberrant conformer were first re-confirmed by an independent PCR and than purified using Qiagen purification columns according to the manufacturer's instructions and both strands were sequenced using the Sequenase 2.0 kit (USB). In the cases in which the SSCP method did not allow us to detect conformational variants (four mutations in four patients), direct sequencing of all the exons was performed.

3. Results

SSCP analysis and DNA sequencing of the coding region and exon-intron boundaries of the G6PT gene allowed us to identify causative mutations in all of the 14 patients investigated, who have been diagnosed as GSD type 1b, 1c or 1 non a (Table 1). Collectively, 13 different mutations were detected (Table 1) and four novel mutations were identified. A dinucleotide (GG) insertion after nucleotide 514 in exon 2 was found in both alleles of patient 22, which results in a change in the reading frame after Gly-115 and premature termination of translation after amino acid 128 (reading frame shift (RFS) after Gly-115, Stop at 128). Patients 25 and 35 were found to carry a 693–696delTGTG mutation in exon 3, inherited from the mother in both cases, causing a change in the reading frame after Ala-174 and termination of translation after amino acid 210 (RFS after Ala-174, Stop at 210). Patient 25 was found to carry also the missense mutation previously reported as C251T, changing the Arg-28 to Cys [5]. This mutation of paternal origin was detected through direct sequencing, while no other mutation was found in patient 35 in the remaining allele, although all the exons and exon-intron junctions were sequenced. Patient 42 showed a G to A transition in position 1068, leading to the Arg-300-His substitution in exon 6 previously described [4]. In this patient, we detected, after direct sequencing, another novel mutation (G580A) leading to a Trp-137-Stop in exon 3. An additional novel point mutation (G1268A) in exon 8 which leads to a Ala-367-Thr substitution was found in patient 44, together with a nonsense mutation (C911T) previously reported [5], which causes the substitution of the codon for Gln in position 248 with a stop codon. All the other patients carry mutations previously described. Patient 28 carried a G371A mutation (detected through direct sequencing), which leads to a Gly-68-Arg change in exon 2 [5] and a silent mutation in exon 9 (G1393A, codon 408) already described [5]. No other pathogenic mutation was found in the other allele. Patient 32 was found to be homozygous for the frequently detected 2 bp deletion (1211–1212 CT) in exon 8 [5].

The C911T mutation was detected also in patient 37, who, in addition, carried the 1211–1212 CT deletion. Patient 43 carried the 1211–1212 CT deletion and the T716C mutation in exon 3, changing Cys-183 to Arg in the other allele [8].

Among the GSD 1c patients, two brothers (patients 38 and 39) were homozygous for a G228A nucleotide substitution which causes a Gly-20-Asp substitution in exon 1 [5]. Patient 40 was found to be homozygous for a Gly-149-Glu in exon 3 (G615A) [8]. Patient 41 was found to be homozygous for a 7 bp deletion (338–344 TCGGCAG del) in exon 2, which results in a frameshift after Gln-56 and premature termination of translation (RFS after Gln-56, Stop at 72) [8]. Patient 49

Table 1
Mutations identified in 14 GSD 1b/1c patients

Patient	Diagnosis	Neutropenia	Mutation	Effect on amino acid sequence
22	1b	−	514insGG (exon 2)*	RFS after Gly-115. Stop at 128
			514insGG (exon 2)	RFS after Gly-115. Stop at 128
25	1b	+	693–696delTGTG (exon 3)*	RFS after Ala-174. Stop at 210
			C251T (exon 1)	Arg-28-Cys
28	1b	+	G371A (exon 2)	Gly-68-Arg
			G1393A (exon 9)	Silent
32	1b	+	1211–1212delCT (exon 8)	RFS after Ala-347. Stop at 400
			1211–1212delCT (exon 8)	RFS after Ala-347. Stop at 400
35	1b	+	693–696delTGTG (exon 3)	RFS after Ala-174. Stop at 210
37	1b	+	C911T (exon 4)	Gln-248-Stop
			1211–1212delCT (exon 8)	RFS after Ala-347. Stop at 400
38	1c	−	G228A (exon 1)	Gly-20-Asp
			G228A (exon 1)	Gly-20-Asp
39	1c	−	G228A (exon 1)	Gly-20-Asp
			G228A (exon 1)	Gly-20-Asp
40	1c	−	G615A (exon 3)	Gly-149-Glu
			G615A (exon 3)	Gly-149-Glu
41	1c	−	338–344delTCGGCAG (exon 2)	RFS after Gln-56. Stop at 72
			338–344delTCGGCAG (exon 2)	RFS after Gln-56. Stop at 72
42	1 non a	+	G1068A (exon 6)	Arg-300-His
			G580A (exon 3)*	Trp-137-Stop
43	1 non a	+	T716C (exon 3)	Cys-183-Arg
			1211–1212delCT (exon 8)	RFS after Ala-347. Stop at 400
44	1 non a	−	C911T (exon 4)	Gln-248-Stop
			G1268A (exon 8)*	Ala-367-Thr
49	1c	−	1211–1212delCT (exon 8)	RFS after Ala-347. Stop at 400
			1211–1212delCT (exon 8)	RFS after Ala-347. Stop at 400

RFS, reading frame shift. The position of mutations is given according to the numbering of the previously published cDNA sequence [3]. An asterisk (*) indicates a novel mutation.

Table 2

Nucleotide change	Exon	Effect on amino acid sequence	Frequency %	References
Missense mutations				
G1184T	8	Gly-339-Cys (TR 8)	8.7	[3,5]
G228A	1	Gly-20-Asp (TR 1)	4.8	[5]
T521C	2	Trp-118-Arg (CL 1)	4.0	[6,10]
G252A	1	Arg-28-His (IL 1)	3.2	[8]
G615A	3	Gly-149-Glu (TR 3)	3.2	[8]
C251T	1	Arg-28-Cys (IL 1)	1.6	[5]
G371A	2	Gly-68-Arg (IL 1)	1.6	[5]
G431A	2	Gly-88-Asp (TR 2)	1.6	[5]
T716C	3	Cys-183-Arg (TR 4)	1.6	[8]
G1068A	6	Arg-300-His (CL 3)	1.6	[4]
A332C	2	Ser-55-Arg (IL 1)	0.8	[5]
G617A	3	Gly-150-Arg (TR 3)	0.8	[5]
G1268A	8	Ala-367-Thr (TR 9)	0.8	Novel
Nonsense mutations				
C911T	4	Gln-248-Stop	4.8	[5,8]
G1348A	9	Trp-393-Stop	1.6	[8]
G1232T	8	Glu-355-Stop	0.8	[3,5]
G456A	2	Trp-96-Stop	0.8	[5]
G580A	3	Trp-137-Stop	0.8	Novel
C821T	4	Gln-218-Stop	0.8	[5]
Deletions/insertions				
1211–1212delCT	8	ΔRF after Ala-347, Stop at 400	20.6	[4,5,8,9]
338–344delTCGGCAG	2	ΔRF after Gln-56, Stop at 72	3.2	[8]
350delG	2	ΔRF after Tyr-60, Stop at 94	1.6	[4]
514insGG	2	ΔRF after Gly-115, Stop at 128	1.6	Novel
629delA	3	ΔRF after Pro-153, Stop at 211	1.6	[5]
693–696delTGTG	3	ΔRF after Ala-174, Stop at 210	1.6	Novel
1094delGCTG/insTC	6	ΔRF after Met-308, Stop at 323	1.6	[6]
1103ins12	6	Four amino acid repeat after Met-311	1.6	[5]
1105insA	6	ΔRF after Thr-312, Stop at 325	1.6	[5]
1094delG	6	ΔRF after Met-308, Stop at 311	0.8	[4]
1205insC	8	ΔRF after Pro-345, Stop at 401	0.8	[5]
Splice site mutations				
1292+3del**aagt** (TGgt**aagt**gtt → TGgtgtt)	E. 8/ I. 8	Truncated protein?	6.3	[5]
317+1 G → T (GG**g**t → GG**t**t)	E. 1/I. 1	Truncated protein?	1.6	[9]
1293-2del**ag** (c**ag**TG → cTG)	I. 8/E. 9	Truncated protein?	1.6	[5]
317+1 G → A (GG**g**t → GG**a**t)	E. 1/I. 1	Truncated protein?	0.8	[6]
318-2 A → C (**a**gGG → **c**gGG)	I. 1/E. 2	Truncated protein?	0.8	[10]
550+1 G → T (AG**g**t → Ag**t**t)	E. 2/I. 2)	Truncated protein?	0.8	[8]
1041ins9 (agGCGG → ag**GCTCCATAGG**CGG)	I. 5/E. 6	Gly-292-Pro Leu-293-Stop	0.8	[5]

CL, cytosolic loop; IL, intraluminal loop and TR, transmembrane region according to the model proposed by Pan et al. [11].

was found to be homozygous for the 2 bp deletion (1211–1212 CT) in exon 8.

4. Discussion

Identification of the G6PT cDNA [3] and characterization of the structure of the gene [4–7] has allowed us to carry out mutational analysis of the G6PT gene in 14 patients who have been diagnosed as GSD type 1b, 1c and 1 non a (Table 1). Present results are in agreement with other recent reports [5–9], which indicate that this gene is the cause of the majority of, if not all, GSD 1b and 1c. Seven patients (including two siblings) were homozygous for the identified mutation in the G6PT gene (Table 1). The other seven patients resulted to be heterozygous: five of them were characterized in both the alleles, whereas in the other two patients, SSCP analysis and complete sequencing revealed mutations in only one allele (Table 1, patients 28 and 35). The incompleteness in revealing all mutations might be ascribed to mutations in the 5′ and 3′ untranslated regions, since genetic heterogeneity has not been reported to date.

Overall, nine different single nucleotide substitutions, three deletions and one insertion account for 26 mutations found out of 28 chromosomes: no major rearrangements were observed (Table 1). We have identified four novel mutations in the G6PT gene: a dinucleotide insertion (514insGG), a four base deletion (693–696delTGTG), a nonsense mutation (G580A) and a missense mutation (G1268A). Including these, the known mutations of the G6PT rise to 37 as summarized in Table 2.

An important observation arising from the presently available data (Table 2) is that the same mutation has been found either in GSD 1b or GSD 1c diagnosed patients. In the present study, the Gly-20-Asp and the Gly-149-Glu, previously described in GSD 1b patients [5,8], were respectively found in three GSD 1c patients (siblings 38 and 39 and patient 40). Moreover, the 338–344delTCGGCAG mutation causing a RFS after Gln-56 and the production of a truncated protein was first described in a 1b patient [8] and in the present work, we found it in a 1c patient (patient 41). Additional examples confirm that the G6PT gene is involved in one non a subtype of GSD. The four amino acid repeat after Met-311 and the splice site mutation between exon 8 and intron 8 (1292+3delaagt) have both been described either in 1b or 1c patients [5]. It may be noteworthy to highlight that also the most frequent mutation of the G6PT gene (1211–1212del CT)

has already been described either in GSD 1b or 1c patients [4,5,8,9]. In the present set of patients, the 1211–1212del CT was found in four patients: in the homozygous state in patients 32 and 49 (1b and 1c, respectively) and in the heterozygous state in patients 37 and 43 (1b and non 1a, respectively). It can be concluded, therefore, that not only the same gene is involved in both GSD 1b and 1c subtypes, but that no specific mutation is associated to each of the two subtypes.

Interestingly, the same mutation in the G6PT gene is not necessarily associated with the appearance of a clinically evident neutrophil impairment. Patient 32 was diagnosed as GSD 1b patient, suffers from neutrophil dysfunction and has the same kind of mutation in the homozygous state (1211–1212delCT), characterizing patient 49, who was diagnosed as GSD 1c and does not show symptoms of neutrophil impairment. In addition, a previously reported GSD 1 non a patient affected by severe neutropenia was found to be homozygous for Gly-149-Glu mutation [8], whilst patient 40, who is also homozygous for Gly-149-Glu mutation, has no evident neutrophil dysfunction. These observations indicate that neutrophil impairment, which often has been assumed to be a distinctive feature of GSD 1b, cannot be considered so anymore.

Although the mutations appear to be scattered throughout the entire G6PT gene, there seems to be a proportionally higher number of mutations in exons 3 and 8. According to the model proposed by Pan [11] for the transmembrane topology of G6PT, we conclude that the mutations we detected are equally distributed in the transmembrane region, in the luminal side and in the cytoplasmic side. This is also true if we take into consideration all the known mutations of the G6PT gene. All the known missense mutations causing amino acid substitutions in the transmembrane region of the protein are responsible for the conversion of a hydrophobic amino acid to a hydrophilic amino acid, Gly-339-Cys. This can possibly cause an abnormal folding of the transporter in the ER membrane and, consequently, a functional anomaly. On the other hand, nonsense mutations and insertion/deletion mutations cause the synthesis of a truncated protein missing the two lysines at the carboxy-terminus necessary as a retention signal in the ER membrane [3] and therefore do not allow for glucose-6-phosphate transport. Overall, what is clear from our data and results coming from other labs [4,10] is that there is no correlation between the type and position of the genetic damage and the severity of the disease. For example, patient 41 has a possibly non-functional transporter protein because of an early truncation, but the clinical profile is quite good as in patients 38 and 39 who have an amino acid substitution instead of a truncated protein so that the transporter, though mutated, can still be inserted in the ER membrane. In addition to this, it should be considered that the same kind of mutation could be associated or not with clinical complications such as neutrophil impairment. We have already mentioned the 1211–1212delCT mutation that has been described either in patients with neutrophil deficiency or in patients not showing this type of alteration. Therefore, on the basis of the data actually available, it should be concluded that there must be other factors, unknown yet, influencing the appearance of symptoms such as neutropenia, dramatically influencing the clinical severity of the disease.

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