DNA-based diagnosis in Glycogen Storage Disease type Ia

Chapter 3

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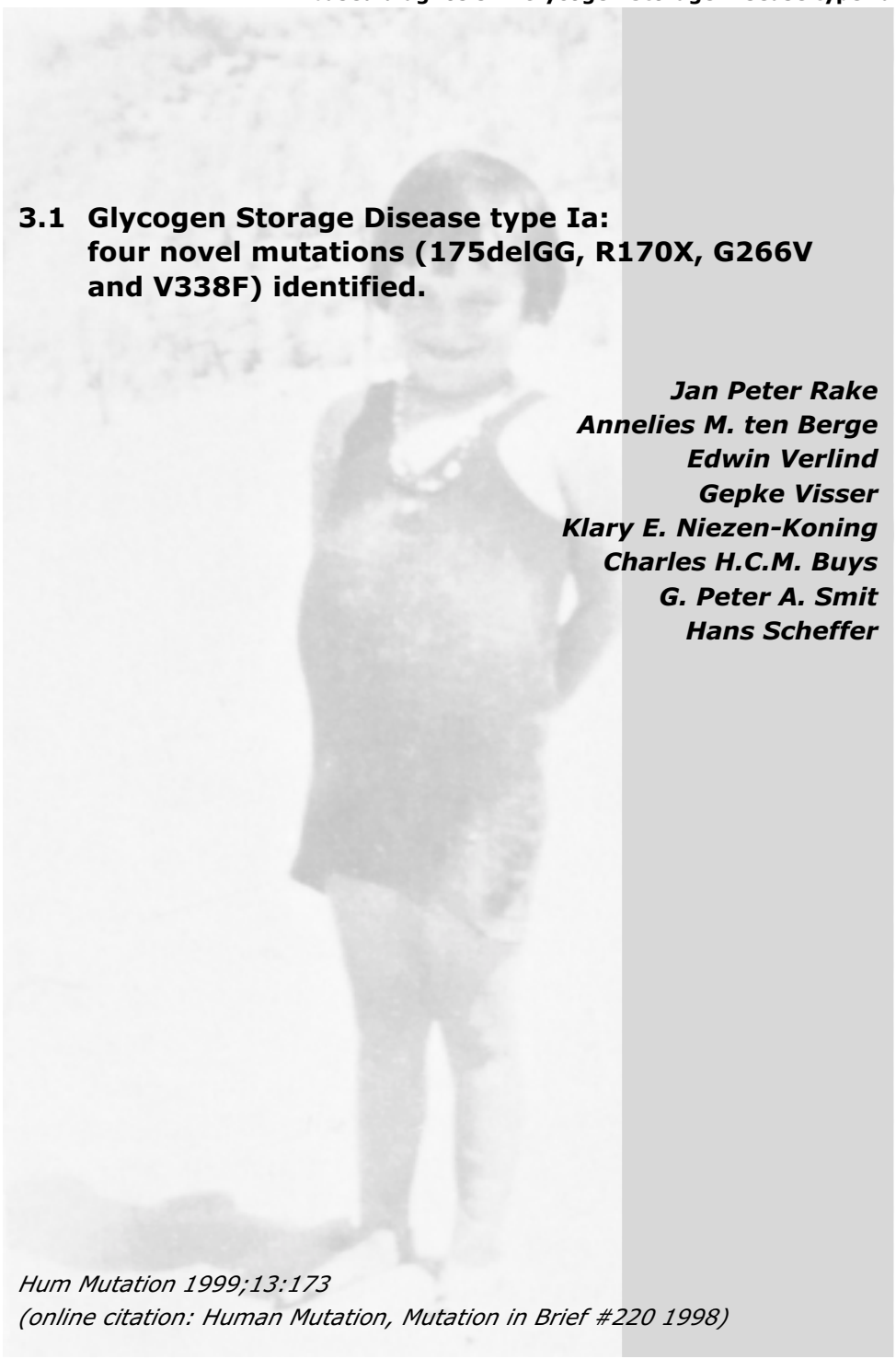

3.1 Glycogen Storage Disease type Ia: four novel mutations (175delGG, R170X, G266V and V338F) identified.

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Summary

Deficient activity of glucose-6-phosphatase (G6Pase) causes glycogen storage disease type Ia (GSD Ia). We analysed the G6Pase gene of 16 GSD Ia patients using single strand conformation polymorphism (SSCP) analysis prior to automated sequencing of exon(s) revealing an aberrant SSCP pattern. In all GSD Ia patients we were able to identify mutations on both alleles of the G6Pase gene, indicating that this method is a reliable procedure to identify mutations. Four novel mutations (175delGG, R170X, G266V and V338F) were identified.

Introduction

Glycogen storage disease type Ia (GSD Ia, McKusick 232200) which has a frequency among new-borns of 1 in 100.000 to 1 in 300.000, is caused by deficient glucose-6-phosphatase activity (G6Pase, E.C.3.1.3.9), the key enzyme in homeostatic regulation of blood glucose concentration by catalysing the terminal step in both glycogenolysis and gluconeogenesis. Severe fasting hypoglycaemia is the most striking feature of this autosomal recessive inborn error of metabolism. Other features are hyperlactacidaemia, hyperuricaemia, hyperlipidaemia, a protruded abdomen due to marked hepatomegaly, short stature, truncal obesity, a rounded doll face, hypothrophic muscles and bleeding tendency due to impaired platelet function. The aim of dietary treatment (frequent meals, nocturnal gastric drip feeding and oral administration of uncooked cornstarch) is to prevent hypoglycaemia in order to improve most biochemical and clinical abnormalities[7].

In 1993 the G6Pase gene (G6PC) has been identified[21,43]. It is located in band q21 of chromosome 17, consists of five exons, has a genomic length of 12.5 kb, and encodes a protein of 357 amino acids with a molecular mass of 35 kD. Using single strand conformation polymorphism (SSCP) analysis and DNA sequencing, Lei et al[22,23,24] characterised the G6Pase gene of 70 unrelated, enzymatically confirmed, patients with GSD Ia. Sixteen different mutations were identified. Twelve additional mutations were identified in a further 48 GSD Ia patients by other groups[4,13,14,33].

We analysed the G6Pase gene of 16 GSD Ia patients and identified four novel mutations.

Patients and methods

Mutation analysis of the G6Pase gene was performed in 16 unrelated GSD Ia patients. All the patients shared the clinical as well as the biochemical characteristics that are associated with GSD type Ia. Enzymological studies were performed in fresh liver biopsy according to Narisawa et al[29]. In all GSD Ia patients the activity of glucose-6-phosphate phosphohydrolase was deficient or greatly reduced in both intact and disrupted liver microsomes. Eight GSD Ia patients were of Dutch descent, five of German, two of Italian and one of Moroccan descent. Where possible the G6Pase gene of the parents was also analysed.

DNA was extracted from peripheral blood by the salting out method[28]. The coding regions and intron/exon borders were amplified by polymerase chain reaction (PCR) into six fragments (exon 5 into two overlapping fragments) according to a modification of the method described by Lei et al[21,24]. A new primer set for the exon 5-5' end was made to increase the

length of the overlapping segment: GSD5-5'S: 5'-CTTCCTATCTCTCACAG-3' (sense)[21], and GSD5-5'A: 5'-TACAATAGAGCTGAGGC-3' (antisense; nucleotides 963-979). The PCR amplified fragments were subjected to SSCP analysis[31] using two different conditions: (1) MDE nondenaturing gel (FMC BioProducts), according to the manufacturer's recommendations, and (2) 5 % polyacrylamide (PAA) gels containing 10 % glycerol. Both gels were run at room temperature at 2000V/60W in 0.5*TBE buffer for 5 h. PCR-amplified fragments showing an aberrant migration pattern were subjected to direct sequencing by an automated sequencer (ALF, Pharmacia). Forward primers were extended by an M13 oligonucleotide (CGACGTTG-TAAAACGACGGCCAGT) to enable the use of an identical M13 sequence primer in all reactions, except for the sequencing of the exon 5-5' fragment to which purpose the reverse primer was extended.

Results and discussion

In all GSD Ia patients we were able to identify mutations on both alleles by first using SSCP analysis prior to automated sequencing of exon(s) revealing an aberrant SSCP pattern. These mutations are summarised in Table 3.1.1.

The 100% detection rate indicates that the method used, is a reliable procedure to identify mutations in the G6Pase gene. The approach is less labour intensive compared to complete sequencing of the G6Pase coding sequence. However, if no mutations are identified by SSCP analysis, all five exons should be sequenced, since by relying on just SSCP analysis, theoretically mutations might be missed.

Four novel mutations were identified: 175delGG, R170X, G266V and V338F. 175delGG, identified in a compound heterozygous Dutch GSD Ia patient (175delGG/Q347X), is a deletion of 2 bp at nucleotides 175 and 176 in exon 1, creating a frame shift, which leads to a direct stopcodon at position 59. The protein product is therefore, only 58 amino acid residues long and is expected to be unstable at the cellular level. R170X, a nonsense mutation, identified in a homozygous German patient (170X/170X) and in two compound heterozygous unrelated Dutch patients (both 170X/F327del), is caused by a C to T substitution at nucleotide 587 in exon 4, which generates a stopcodon at position 170. The protein product is 169 amino acids long and is also expected to be unstable at cellular level. G266V, a missense mutation, identified in two compound heterozygous Dutch patients (158delC/G266V and G266V/F327del), is caused by a G to T substitution at nucleotide 876 in exon 5, leading to the substitution of a valine for a glycine at codon 266. Although both glycine and valine are amino acids with aliphatic non-polar

Table 3.1.1 Mutations in the G6Pase gene identified in 16 unrelated GSD Ia patients

		allele 1			allele 2				
	descent	exon	nucleotide change	mutation	exon	nucleotide change	mutation	father[1]	mother[1]
I	Dutch	2	326 C→T	R83C	5	1118 C→T	Q347X	Q347X	R83C
II	Dutch	1	158delC	158delC	**5**	**876 G→T**	**G266V**	158delC	**G266V**
III	Dutch	1	268 G→A	W63X	**5**	**1091 G→T**	**V338F**	W63X	**V338F**
IV	Dutch	**5**	**876 G→T**	**G266V**	5	1058delTTC	F327del	F327del	**G266V**
V	Moroccan	2	326 C→T	R83C	2	326 C→T	R83C	R83C	R83C
VI	Dutch	**4**	**587 C→T**	**R170X**	5	1058delTTC	F327del	n.a.	n.a.
VII	German	**4**	**587 C→T**	**R170X**	**4**	**587 C→T**	**R170X**	n.a.	n.a.
VIII	German	2	327 G→A	R83H	2	327 G→A	R83H	R83H	n.a.
IX	Dutch	1	158delC	158delC	1	158delC	158delC	n.a.	n.a.
X	German	2	326 C→T	R83C	5	1118 C→T	Q347X	n.a.	Q347X
XI	Dutch	**4**	**587 C→T**	**R170X**	5	1058delTTC	F327del	**R170X**	F327del
XII	Dutch	**1**	**175delGG**	**175delGG**	5	1118 C→T	Q347X	n.a.	n.a.
XIII	Italian	2	326 C→T	R83C	2	326 C→T	R83C	R83C	R83C
XIV	German	4	641 G→C	G188R[2]	5	1118 C→T	Q347X	n.a.	n.a.
XV	Italian	2	326 C→T	R83C	2	326 C→T	R83C	R83C	R83C
XVI	German	5	888 G→T	G270V	5	1118 C→T	Q347X	Q347X	G270V

The novel mutations identified in this study are in **bold** face.
n.a. not available.

1 Mutations identified on one of both alleles of the G6Pase gene of the parents. All parents were non-consanguineous, except for the parents of patient V

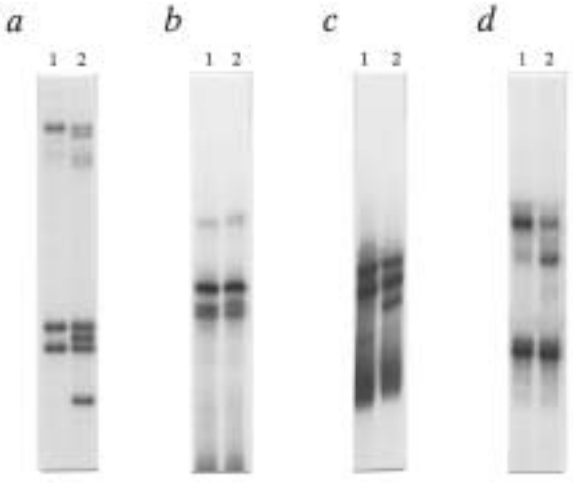
2 Besides leading to an amino acid change, this mutation disturbs most likely also the correct splicing of the G6Pase mRNA, as it is located at the last nucleotide of exon 4[4]

side chains, different arguments give reason to expect that G266V is a true mutation and not a sequence variation with minor effects on the activity of the gene product. One hundred and eight normal subjects were tested for this missense mutation and on none of the 216 alleles this substitution was found. Furthermore in mouse liver G6Pase, on the same position also a glycine is found and the direct environment of this amino acid is conserved, indicating that this part of the G6Pase protein is important for its functional activity. Finally, the segregation of this mutation through both families was as expected. Both mothers were heterozygous for G266V, whereas the other mutation in both patients was of paternal origin. V338F, a missense mutation, identified in a compound heterozygous Dutch GSD Ia patient (W63X/V338F), is caused by a G to T substitution at nucleotide 1091 in exon 5, leading to the substitution of a valine for a phenylalanine at codon 338. Valine is an amino acid with an aliphatic non-polar side chain, whereas phenylalanine contains an aromatic ring. Such a change could affect G6Pase activity or stability. Considering this, as well as similar arguments as discussed for the G266V mutation above, we conclude that V338F is a true mutation. The SSCP analyses and the sequence analyses of the novel mutations are shown in Figure 3.1.1 and Figure 3.1.2 respectively. The R170X, G266V and V338F

Figure 3.1.1 SSCP analyses of the four novel G6Pase mutations

lane 1 wild type SSCP pattern
lane 2 heterozygous mutant SSCP pattern

(a) exon 1, 175delGG
(b) exon 4, R170X
(c) exon 5-5′, G266V
(d) exon 5-3′, V338F



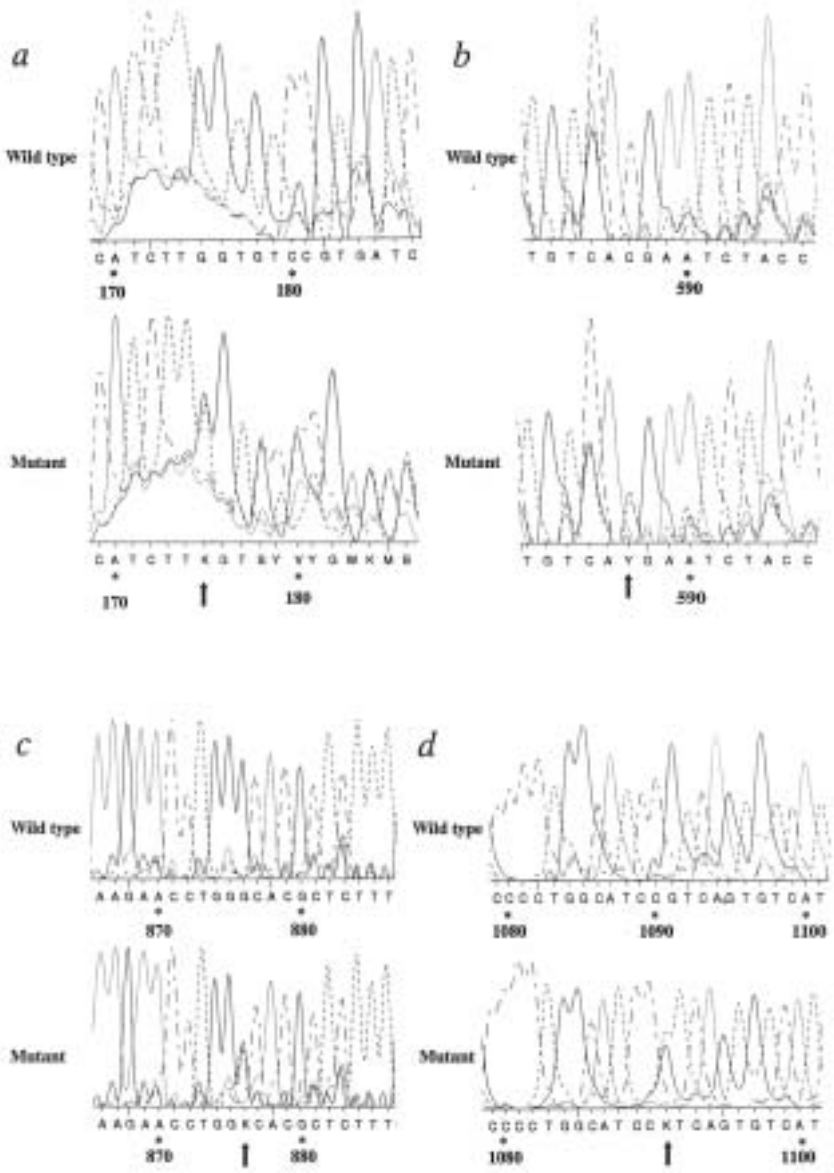
The exon-containing fragments were amplified by PCR and were analysed by electrophoresis on MDE gels (a,c,d) or 5% PAA gel containing 10% glycerol (b)

mutations can also be detected by restriction analysis of the amplification product of the exon in which they are contained; they create Nla III, Bsp1286I and Eco57I sites respectively.

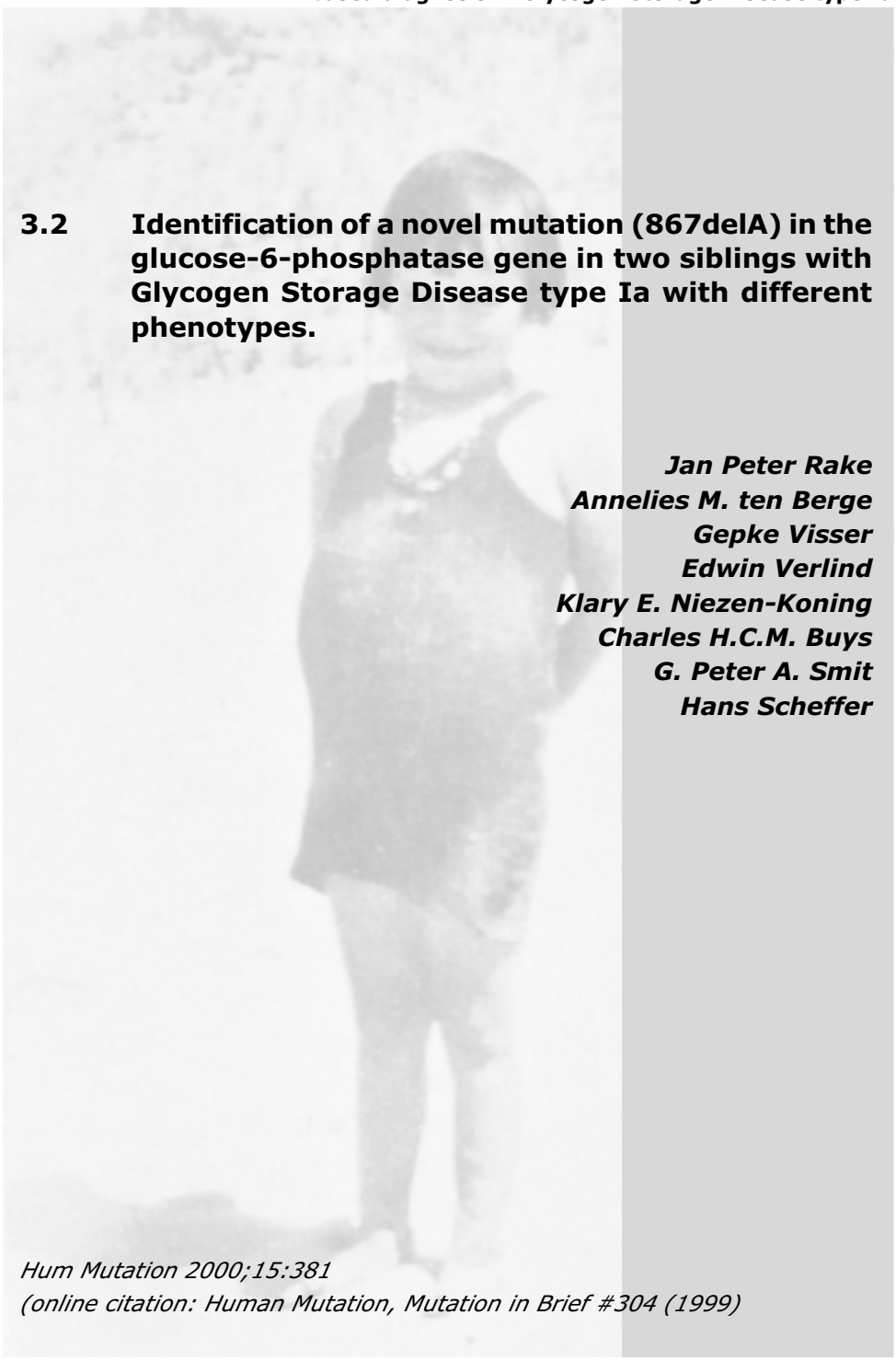
In both patients of Italian descent and in the patient of Moroccan descent, R83C (6/6) was homozygously present. In the 13 Dutch and German GSD Ia patients, 12 different mutations were identified. Most frequently Q347X was found (5/26). Eleven additional mutations account for the remaining 21 mutant alleles. This allelic heterogeneity in Caucasian patients and allelic homogeneity in patients from a more specifically defined ethnical and/or geographical origin, is in agreement with previous reports by other groups. In Caucasian patients from the USA and France most frequently Q347X (31%) and R83C (27%) were found, but 20 additional mutations account for the other mutant alleles. On the other hand in Jewish patients (R83C, 93%), Mediterranean patients (R83C, 71%; Q347X, 29%), Hispanic patients (459insTA, 50%; R83C, 28%), Chinese patients from the USA (R83H, 70%) and Japanese patients (727 T→G, 94%) one or two predominantly occurring mutations were found[4,14,24,33]. This opens the way to test for specific G6Pase gene mutations prior to a complete SSCP analysis in these patients.

Figure 3.1.2 Sequence analyses of the four novel G6pase mutations

Detection of the (a) 175delGG; (b) R170X; (c) G266V; (d) V338F mutations by direct sequencing of exon-containing PCR products. Normal and mutant sequences are shown. The position of the mutations is indicated by an arrow. Heterozygous nucleotides are indicated by a letter code: K means G and T; S means G and C;
M means A and C; B means C, G and T; W means A and T; Y means C and T



Characterisation of the human G6Pase gene and defining the mutational spectrum of this gene allows a DNA-based diagnosis in patients clinically suspected of having GSD Ia. However, if no alterations in the G6Pase gene can be identified, enzyme assays with specific substrates in intact and disrupted microsomes obtained by liver biopsy remain necessary. Furthermore, once mutations are identified in a family at risk for having an offspring with GSD Ia, prenatal DNA-based diagnosis in chorionic villus samples is possible[20,34,38].



3.2 Identification of a novel mutation (867delA) in the glucose-6-phosphatase gene in two siblings with Glycogen Storage Disease type Ia with different phenotypes.

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Summary

We identified a novel mutation (867delA) in the glucose-6-phosphatase gene of two siblings with glycogen storage disease type Ia. Although both siblings share the same mutations, their phenotype regarding adult height and hepatomegaly differs. In glycogen storage disease type Ia, substantial heterogeneity in genotype and phenotype is observed. At present, no evidence for a clear genotype-phenotype correlation has been found.

Introduction

Glycogen storage disease type Ia (GSD Ia, MIM# 232200) is a severe autosomal recessive inborn error of metabolism caused by microsomal glucose-6-phosphatase (G6Pase, E.C.3.1.3.9) deficiency. The disease phenotype is characterised by marked hepatomegaly, short stature, severe fasting hypoglycaemia, hyperlactacidaemia, hyperuricaemia and hyperlipidaemia. Life expectancy in GSD I has improved considerably, thanks to intensive dietary therapy including frequent meals, slowly released carbohydrates and continuous nocturnal gastric drip feeding. However, with ageing, numerous complications, such as liver adenomas, progressive renal disease, and osteopenia may develop[7].

After identification of the gene encoding the G6Pase catalytic unit (G6PC; GDB 231927)[21], a steadily growing list of mutations has been reported. In addition to our previous work[39], we report here a novel mutation in the G6Pase gene, identified in two siblings with different phenotypes.

Patients and methods

The affected index cases are a 24-year-old brother (sibling 1) and a 20-year-old sister (sibling 2), progeny of unrelated healthy Dutch Caucasian parents. In sibling 1 the diagnosis GSD Ia was suspected at the age of six months, in sibling 2 shortly after birth. Enzyme assays in liver tissue obtained by biopsy revealed 10% residual G6Pase activity in sibling 1 and no residual G6Pase activity in sibling 2. From early infancy both patients had been treated with frequent lactose-restricted feedings during day and night. At four years of age and one year of age respectively, continuous gastric drip feeding was introduced. At twelve years of age and eight years of age respectively, uncooked cornstarch during the day was introduced. The patients are raised in a family in which dietary treatment was maintained stringently for both.

At present, both patients are in good clinical condition. They share some of the disease phenotype characteristics: mild hypercholesterolemia, hypertriglyceridemia and hyperuricemia requiring a xanthine-oxidase inhibitor. Furthermore, both patients have renal hyperfiltration and microalbuminuria requiring an angiotensin converting enzyme inhibitor. Liver adenomas could not be detected in either sibling. Bone mineral density is reduced in both. However, the clinical phenotype regarding adult height and hepatomegaly differs. Sibling 1 has reached an adult height of 195.4 cm (+ 2.0 SDS), sibling 2 of 166.0 cm (- 0.4 SDS). In sibling 1, hepatomegaly (liver 2 cm palpable below costal margin in medioclavicular line) is less pronounced compared with sibling 2 (liver 9 cm).

Genomic DNA preparations, PCR amplification, single-stranded

conformation polymorphism (SSCP) and sequence analysis were performed as described earlier[39].

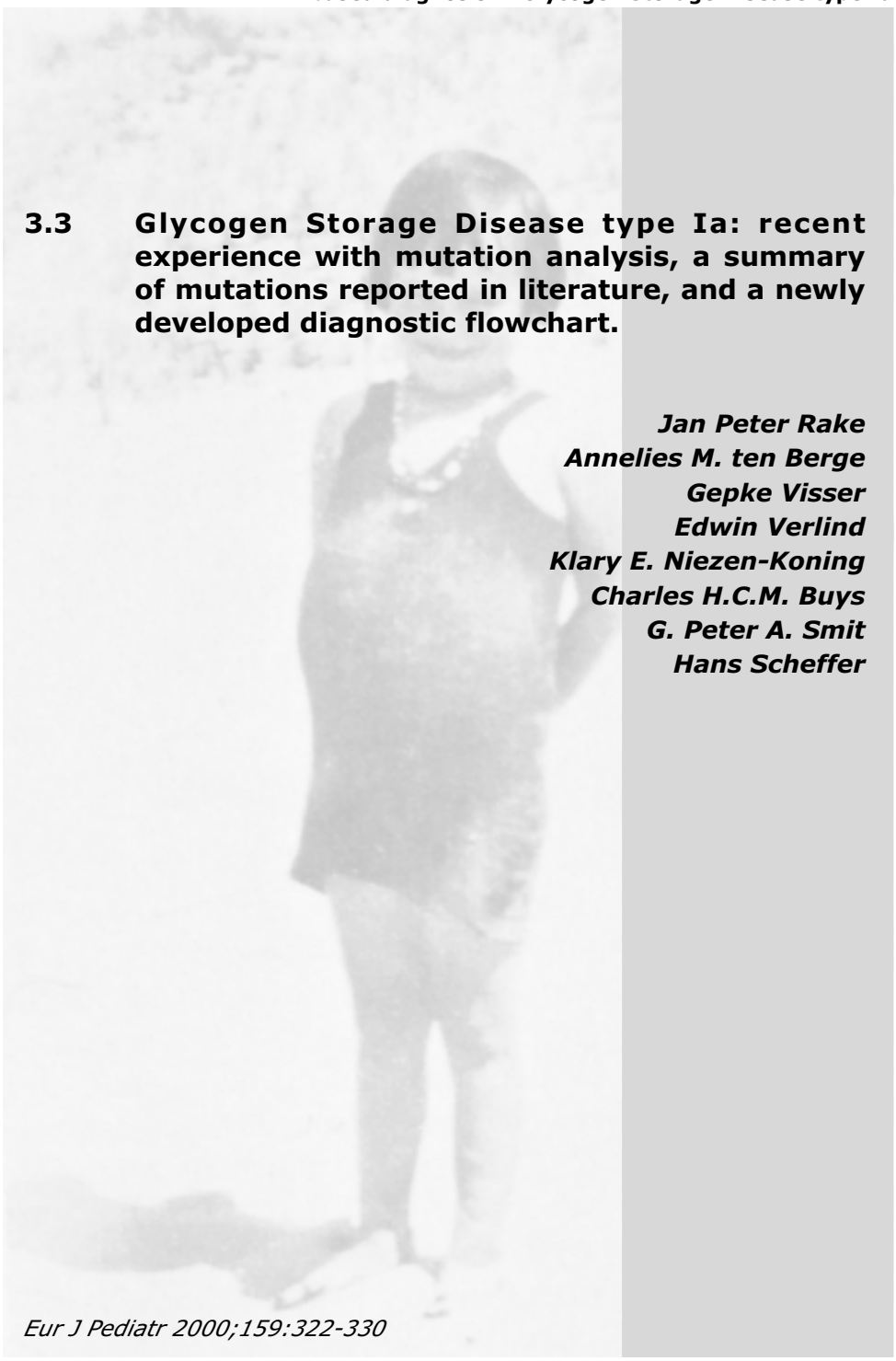
Results and discussion

In both siblings, SSCP analyses of exon 1 and exon 5-5'revealed aberrant migration patterns. Subsequent sequencing of the PCR-amplified exon 1 fragment revealed a known frameshift mutation, 175delGG[38]. Sequencing of the PCR-amplified exon 5-5'fragment revealed a deletion of an adenine at nucleotide 867 (867delA) resulting in a stopcodon at position 300. This frameshift mutation has not been described before. Although no transient expression analyses have been performed, most likely both mutations lead to protein products with completely abolished G6Pase activity. Lei et al[25] showed that only the 8 carboxyl-terminal amino acids (350-357) in human G6Pase are not essential for G6Pase activity or membrane retention. A stopcodon preceding these 8 residues causes loss of catalytic activity of G6Pase.

Both siblings share the same mutations in the G6pase gene. However, their phenotype regarding residual activity of G6Pase in liver tissue, adult height, and hepatomegaly differs. The difference in residual enzyme activity could reflect differences in quality of liver tissue (fat and glycogen storage displace normal liver tissue), differences in biopsy localisation (hepatic zonation of G6Pase activity) or analytical considerations (enzyme assays were performed in different laboratories). However, the in vitro observed variability could also reflect real difference in enzyme activity. The difference in phenotype regarding adult height and hepatomegaly could reflect this difference in residual enzyme activity, but also hepatic glycogen breakdown or glucose production by alternative pathways may play a role. Furthermore, it is possible that other modifying genes may be involved.

Since substantial heterogeneity in phenotype in GSD Ia is observed, a genotype-phenotype correlation may be very helpful to adjust dietary and pharmacological strategies. However, no evidence for a clear genotype-phenotype correlation has been found so far. Even of the presented siblings who share the same mutations in the G6Pase gene, phenotypes differ.

In summary, we identified a novel mutation (867delA) in the glucose-6-phosphatase gene of two siblings with glycogen storage disease type Ia. In glycogen storage disease type Ia, substantial heterogeneity in genotype and phenotype is observed. However, at present no evidence for a clear genotype-phenotype correlation has been found.



3.3 Glycogen Storage Disease type Ia: recent experience with mutation analysis, a summary of mutations reported in literature, and a newly developed diagnostic flowchart.

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Summary

We studied the glucose-6-phosphatase (G6Pase) gene of 30 unrelated glycogen storage disease type Ia (GSD Ia) patients using single strand conformational polymorphism (SSCP) prior to automated sequencing of exons revealing an aberrant SSCP pattern. In all patients we could identify mutations on both alleles of the G6Pase gene, indicating that this method is a reliable procedure. A total of 14 different mutations were identified. R83C (16/60), 158delC (12/60), Q347X (7/60), R170X (6/60) and ΔF327 (4/60) were found most frequently. Nine other mutations account for the other 15 mutant alleles. Two DNA-based prenatal analyses were performed successfully. At present, 56 mutations in G6Pase gene have been reported in 300 unrelated GSD Ia patients and an overview of these mutations is presented. Evidence for a clear genotype-phenotype correlation could be established neither from our data, nor from those in the literature. With increased knowledge about the genetic basis of GSD Ia and GSD Ib and the high detection rate of mutations, it is our opinion that the diagnoses GSD Ia and GSD Ib can usually be based on clinical and biochemical abnormalities combined with mutation analysis instead of enzyme assays in liver tissue obtained by biopsy. A newly developed flowchart for the diagnosis of GSD I is presented.

In conclusion, increased knowledge of the genetic basis of GSD I provides a DNA-based diagnosis, prenatal DNA-based diagnosis in chorionic villus samples and carrier detection.

Introduction

Glucose-6-phosphatase (G6Pase, E.C.3.1.3.9) is the key enzyme in the regulation of blood glucose homeostasis by catalysing the terminal step in both glycogenolysis and gluconeogenesis. Deficient G6Pase activity leads to glycogen storage disease type I (GSD I, McKusick 232200), an autosomal recessive inborn error of metabolism, which has a frequency among newborns of one in 100.000 to 1 in 300.000[3]. Based on the most plausible model, G6Pase is a multi component enzyme complex consisting of a catalytic subunit, with one or more membrane transporters. The catalytic subunit is situated on the luminal surface of the endoplasmic reticulum to which substrates gain access by the transporters. Based on kinetic studies in liver tissue four different subtypes of GSD I could be distinguished: GSD Ia which is caused by deficient activity of the catalytic unit, and GSD Ib, GSD Ic and GSD Id which are caused by defects of glucose-6-phosphate (G6P) translocase, phosphate/pyrophosphate translocase and a putative glucose translocase, respectively[51].

In 1993 the gene encoding the G6Pase catalytic unit was identified[21,43]. It is located in band q21 of chromosome 17, consists of five exons, has a genomic length of 12.5 kb and encodes a protein of 357 amino acids with a molecular mass of 35 kD. A steadily growing number of mutations in the G6Pase gene has been reported. More recently also the gene encoding the G6P transporter was identified[1,9]. It is located in band q23 of chromosome 11 and consists of nine exons. In GSD Ib and GSD Ic patients and in a GSD Id patient, allelic mutations have been identified, indicating that for those subtypes the basic defect is in the putative G6P translocase and that they should be reclassified as GSD Ib[49].

Following our previous reports about mutation analysis in GSD Ia patients[39,40], we report here the results of mutation analysis in 30 GSD Ia patients, including two DNA-based prenatal analyses. Furthermore, we present an overview of the mutations in the G6Pase gene reported in literature so far. Finally, we present a newly developed flowchart for the diagnosis of GSD Ia and GSD Ib.

Patients and methods

DNA analyses of the G6Pase gene in 30 GSD type Ia patients and their families was carried out. In patients 1 to 21, the clinical diagnosis GSD Ia had already confirmed by enzyme assay in biopsied liver tissue according to Narisawa et al[29]. In these patients, mutation analysis was performed to confirm the diagnosis at the molecular level. In patients 22 to 30, the diagnosis GSD Ia was suspected on the basis of both clinical and biochemical characteristics.

In these patients mutation analysis was performed to establish the diagnosis. In addition, in the families of patients 12 and 20, DNA-based prenatal diagnosis in chorionic villus samples (CVS) was performed. Furthermore, in the partners of patient 1 and 4, DNA analysis was performed to reduce the likelihood of being a carrier of a mutant G6Pase allele. In a newborn sibling of patient 25, postnatal mutation analysis was performed to exclude GSD Ia.

Genomic DNA was extracted from leukocytes from peripheral blood or from CVS obtained by transcervical biopsy in the first trimester of gestation. DNA was extracted from leukocytes by the salting out method[28] and from CVS by phenol/chloroform extraction. The coding regions and intron/exon borders were amplified by PCR into six fragments (exon 5 in two overlapping fragments) according to a modification of the method described by Lei et al[21,24]. A new primer set for the exon 5-5' end was designed to increase the length of the overlapping segment: GSD5-5'S: 5'-CTTCCTATCTCTCACAG-3' (forward)[21], and GSD5-5'A: 5'-TACAATAGAGCTGAGGC-3' (reverse: nucleotides 963-979). The PCR amplified fragments were subjected to single strand conformational polymorphism (SSCP) analysis[31] using two different conditions: (1) MDE nondenaturing gel (FMC BioProducts), according to the manufacturer's recommendations, and (2) 5% polyacrylamide (PAA) gels containing 10% glycerol. Both gels were run at room temperature at 2000V/60W in 0.5*TBE buffer for 5h. PCR-amplified fragments showing an aberrant migration pattern were subjected to direct sequencing by an automated sequencer (ALF, Pharmacia). Forward primers were extended by an M13 oligonucleotide (CGACGTTGTAAAACGACGGCCAGT) to enable the use of an identical M13 sequence primer in all reactions, except for the sequencing of the exon 5-5' fragment for which purpose the reverse primer was extended.

Results

In all 30 GSD Ia patients we were able to identify mutations on both alleles of the G6Pase gene (Table 3.3.1) providing a total of 14 different mutations.

Mutation analysis performed in the newborn sibling of patient 25 indicated that he had received at least one normal G6Pase allele, excluding GSD Ia. Subsequently, he developed no suspect clinical features.

Mutation analysis of DNA extracted from CVS in families of patients 12 and 20 indicated that both fetuses received at least one normal G6Pase allele. Both pregnancies were carried to term and healthy babies were born.

In the partners of patient 1 and 4 no aberrant SSCP patterns were detected.

Discussion

Table 3.3.1 Mutations in the G6Pase gene identified in 30 unrelated GSD Ia patients and their families (*n.a*. not available; *conf* diagnosis based on enzyme assays and confirmation by mutation analysis; *DNA* diagnosis based on mutation analysis; *cvs* DNA-based prenatal diagnosis in CVS; *c.det* carrier detection)

			allele 1			allele2				
patient		descent	exon	nucleotide change	mutation	exon	nucleotide change	mutation	father[a]	mother[a]
1	conf	Dutch	2	326C→T	R83C	5	1118C→T	Q347X	Q347X	R83C
2	conf/c.det[b]	Dutch	1	158delC	158delC	5	876G→T	G266V	158delC	G266V
3	conf/c.det[b]	Dutch	1	268G→A	W63X	5	1091G→T	V338F	W63X	V338F
4	conf	Dutch	5	876G→T	G266V	5	1058delTTC	F327del	F327del	G266V
5	conf/DNA[c]	Moroccan	2	326C→T	R83C	2	326C→T	R83C	R83C	R83C
6	conf	Dutch	4	587C→T	R170X	5	1058delTTC	F327del	n.a.	n.a.
7	conf	German	4	587C→T	R170X	4	587C→T	R170X	n.a.	n.a.
8	conf	German	2	327G→A	R83H	2	327G→A	R83H	R83H	n.a.
9	conf	Dutch	1	158del C	158delC	1	158del C	158delC	n.a.	n.a.
10	conf	German	2	326C→T	R83C	5	1118C→T	Q347X	n.a.	Q347X
11	conf	Dutch	4	587C→T	R170X	5	1058delTTC	F327del	R170X	F327del
12	conf/cvs[e]	Dutch	1	175delGG	175delGG	5	1118C→T	Q347X	n.a.	n.a.
13	conf	Italian	2	326C→T	R83C	2	326C→T	R83C	R83C	R83C
14	conf	German	4	641G→C	G188R[d]	5	1118C→T	Q347X	n.a.	n.a.
15	conf	Italian	2	326C→T	R83C	2	326C→T	R83C	R83C	R83C
16	conf	German	5	888G→T	G270V	5	1118C→T	Q347X	Q347X	G270V
17	conf	Dutch	1	175delGG	175delGG	5	867delA	867delA	n.a.	n.a
18	conf	Dutch	1	158delC	158delC	5	1118C→T	Q347X	n.a.	n.a.
19	conf	Dutch	1	158delC	158delC	1	158del C	158delC	n.a.	n.a.
20	conf/cvs[e]	Belgium	1	158delC	158delC	1	158delC	158delC	158delC	158delC
21	DNA	Dutch	2	326C→T	R83C	2	326C→T	R83C	n.a.	n.a.
22	DNA	Dutch	1	158delC	158delC	5	867delA	867delA	158delC	867delA
23	DNA	German	2	326C→T	R83C	2	326C→T	R83C	n.a.	n.a.
24	DNA	Dutch	4	587C→T	R170X	5	1058delTTC	F327del	F327del	R170X
25	DNA[f]	British	1	268G→A	W63X	5	1118 C→T	Q347X	W63X	Q347X
26	DNA	Austria	2	326C→T	R83C	2	326C→T	R83C	R83C	R83C
27	DNA	Austria	1	158delC	158delC	4	588G→A	R170Q	R170Q	158delC
28	DNA	Belgium	1	158delC	158delC	1	158delC	158delC	158delC	158delC
29	DNA	Belgium	1	268G→A	W63X	4	587C→T	R170X	n.a.	n.a.
30	DNA	Dutch	2	326C→T	R83C	2	326C→T	R83C	n.a.	n.a.

[a] Mutations identified on one of both alleles of the G6Pase gene of the parents: all parents were nonconsanguineous, except for the parents of patient 5 (third degree nephew-niece)

[b] No aberrant SSCP pattern could be detected in the partners of both patients

[c] Index patient diagnosed by enzyme assays and confirmed by mutation analysis. Next patient (father is a brother and mother is a third degree niece of index patient) diagnosed by mutation analysis

[d] Besides leading to an amino acid change, this mutation disturbs most likely also the correct splicing of the G6Pase mRNA, as it located at the last nucleotide of exon 4[4]

[e] Index patients diagnosed by enzyme assays and confirmed by mutation analysis. During next pregnancies DNA-based prenatal diagnosis: both fetuses received at least one normal G6Pase allele.

[f] Index patient diagnosed by mutation analysis. In newborn sibling DNA analysis to exclude GSD Ia, newborn sibling received at least one normal G6Pase allele.

We analysed the G6Pase gene of 30 unrelated GSD Ia patients. In all patients both mutations of the G6Pase gene were identified. The DNA analysis in patients 1 to 17 have been reported previously[39,40]. The mutations R83C (16 of the 60 alleles), 158delC (12/60), Q347X (7/60), R170X (6/60) and ΔF327 (4/60) were found most frequently. Nine other mutations account for the other 15 mutant alleles.

At present, 56 mutations in the G6Pase gene have been reported in 300 (600 alleles) unrelated GSD Ia patients[4,5,12-19,21-24,26,27,30,33,35-37,39-41,44-46,this study]. An overview of these mutations is given in Table 3.3.2. Among the 56 mutations, 11 are frameshift mutations, three are splice site mutations, seven are nonsense mutations, 34 are missense mutations and one is a codon deletion mutation. The frameshift mutations result in codons for different amino acids at the 3' side of the mutation. Eight of these eleven frameshift mutations, 97insTGAA, 158delC, 175delGG, 341delG, 459insTA, 518delA/518insTG, 813insC and 867delA create a stopcodon at positions 11, 35, 59, 101, 130, 203, 254 and 300, respectively. The other three, 79delC, 540delTTTTG and 813insG-822delC, do not create a premature stopcodon. Transient expression analyses of 459insTA and 813insG-822delC have shown abolished G6Pase activity[23,24]. It can be expected however, that all these frameshift mutations lead to protein products with abolished G6Pase activity since only the 8 carboxyl-terminal amino acids (350-357) in human G6Pase are not essential for G6Pase activity or membrane retention[25]. Three splice site mutations have been identified. 309+4 A→G results in the retainment of intron 1 in the mRNA[4]. 727G→T is thought to be the cause of a defective splicing of intron 4, resulting in a 91 nucleotide deletion of exon 5 and altering the reading frame resulting in a stopcodon at position 212[14]. Matsubara et al[27] identified in intron 1 a not further specified altered consensus splice acceptor site sequence, causing exon 2 skipping in ectopic mRNA. Seven nonsense mutations, W50X, W63X, W70X, R170X, Y172X, Q242X and Q347X, in which a single DNA base change results in a premature stopcodon, have been identified. These mutations lead to truncated protein products which all can be expected to be unstable. Transient expression analyses have shown this for W63X, Q242X and Q347X[22,24]. A total of 34 missense mutations, 33 in which a single DNA base change and one (L345R) in which two subsequent DNA base changes result in nucleotide triplets coding for different amino acids, have been identified. Among those missense mutations, G188S and G188R most likely also disturb the correct splicing of the G6Pase mRNA because these mutations are positioned at the last nucleotide of exon 4[4]. Furthermore, one codon deletion mutation, in which deletion of three subsequent DNA bases results in the deletion of an amino acid (ΔF327), has

been identified. Transient expression analyses of D38V, R83C, R83H, V166G, P178S, G188S, G270V, R295C, ΔF327 and L345R have shown abolished G6Pase activity, whereas transient expression analyses of E110Q, G222R, W236R have shown greatly reduced G6Pase activity[21,23,24,35,36]. So far, no transient expression assays have been performed for the other 22 missense mutations (Q20R, W63R, G68R, W77R, G81R, E110Q, E110K, P113L, A124T, V166A, R170Q, Y172X, G184E, G184V, G188R, L211P, P257L, L265P, G266V, S298P, V338F, I341N) but different arguments give reason to expect that these are true mutations and not sequence variations with no or only minor effects on the activity of the gene protein product. In large groups of normal subjects, who were tested for these missense mutations, these substitutions could not be identified in any of the alleles. Furthermore, normal sequence polymorphisms in the coding sequences of the G6Pase gene could not be identified by testing 180 G6Pase alleles[24]. Also the segregation of the different missense mutations through the families was as expected.

Recently, it was demonstrated that the topology of human G6Pase is rather a nine-transmembrane helical structure with the N-terminus and four loops localized on the luminal side of the endoplasmatic reticulum, than a six-transmembrane helical structure as was suggested previously[11,32]. According to the nine-transmembrane helical topology, 25 missense mutations and the codon deletion mutation result in amino acid changes in one of the transmembrane spanning segments, indicating the importance of the structural integrity of those segments. The other nine missense mutations result in amino acid changes in the N-terminal domain (one mutation), loop 1L (five mutations in three different codons), loop 2L (one) and loop 3L (two in two), all located on the luminal side of the endoplasmic reticulum. Till so far no mutations have been identified in loop 4L and the four cytoplasmic loops, suggesting that those loops are less important for phosphohydrolase activity of G6Pase.

Among the 56 different mutations identified in 600 alleles of 300 unrelated GSD Ia patients, the two missense mutations R83C and Q347X and the splice site mutation 727G→T account for more than 60% of all mutant G6Pase alleles (32.5%, 14.3% and 11.3% respectively). No other mutation account for almost 5%; 28 mutations are even 'private' or single allele mutations. An overview of the prevalence of the different mutations among different geographical/ethnical groups is given in Table 3.3.2. Striking is the allelic heterogeneity in Caucasian patients from the USA and from North-West Europe and the allelic homogeneity in patients of some specifically defined ethnic and/or geographical origin. In Caucasian patients from the USA and North-West Europe, R83C and Q347X account for 25.2% and 22.4% of all mutant

Table 3.3.2 Prevalence of mutations in the G6Pase gene among different geographical and/or ethnic groups

nucleotide change	mutation	first reported	country/ethnic group										
			USA	USA	USA T, HK	USA, I	Japan	NWE	SE	EE, TR	M	total (600)	%
			Caucasian	Hispanic	Chinese	Jewish							
		reference	[24]	[24]	[13,18,19,24]	[24,35,38]	[14,15,27,30]	[4,16,26,46] this study	[4,45] this study	[5,12,17,36]	[4,35,37,41] this study		
79delC	FS	[17]								1/44		1	
97insTGAA	FS, 11X	[45]							1/114			1	
A138G	Q20R	[26]						2/162				2	
158delC	FS, 35X	[24]	4/96					18/162				22	3.7
175delGG	FS 59X	[39]						2/162				2	
A192T	D38V	[4,36]						7/162	3/114	1/44		11	1.8
G228A	W50X	[26]						1/162				1	
T266C	W63R	[45]							1/114			1	
G268A	W63X	[24]	2/96					6/162				8	1.3
G281A	G68R	[41]									2/16	2	
G289A	W70X	[26]						1/162				1	
T308C	W77R	[4]						1/162				1	
altered splice acceptor site		[27]					1/74					1	
309+4 A→G	SS	[4]						1/162				1	
G320A	G81R	[26]						1/162				1	
C326T	R83C	[21]	32/96	5/18	1/32	41/44		33/162	55/114	22/44	6/16	195	32.5
G327A	R83H	[24]	1/96		12/32		1/74	5/162				19	3.2
341delG	FS, 101X	[18,26]			1/32			1/162				2	
G407C	E110Q	[36]								1/44		1	
G407A	E110K	[4]						1/162				1	
C417T	P113L	[26]						1/162				1	
G449A	A124T	[4]						1/162				1	
459insTA	FS, 130X	[21]		9/18								9	1.5
518delAinsTG	FS, 203X	[17]							1/114			1	
540delTTTTG	FS	[45]								1/44		1	
T576G	V166G	[33]									4/16	4	
T576C	V166A	[17]								1/44		1	
C587T	R170X	[26,27,39]					1/74	8/162				9	1.5
G588A	R170Q	[12]						1/162		2/44		3	

nucleotide change	mutation	first reported	country/ethnic group										
			USA	USA	USA T, HK	USA, I	Japan	NWE	SE	EE, TR	M	total (600)	%
			Caucasian	Hispanic	Chinese	Jewish							
		reference	[24]	[24]	[13,18,19,24]	[24,35,38]	[14,15,27,30] this study	[4,16,26,46] this study	[4,45] this study	[5,12,17,36]	[4,35,37,41]		
C595A	Y172X	[45]							1/114			1	
C611T	P178S	[24]		1/18								1	
G630T	G184V	[45]							1/114			1	
G630A	G184E	[4]						2/162				2	
G641C	G188R,SS	[4,46]						14/162				14	2.3
G641A	G188S	[24]	2/96									2	
T711C	L211P	[4]						1/162				1	
G727T	SS	[14]			3/32		65/74					68	11.3
G743A	G222R	[45]							2/114			2	
G743C	G222R	[23]	1/96							1/44		2	
T785A	W236R	[24]		1/18								1	
C803T	Q242X	[24]	3/96									3	
813insG -822delC	CEQP245-248WRAA	[24]	1/96									1	
813insG	FS, 254X	[4]						1/162				1	
867delA	FS, 300X	[40]						2/162				2	
	P257L	[27]					1/74					1	
	N264K	[16]						1/162				1	
T873C	L265P	[26]						1/162				1	
G876T	G266V	[39]						2/162				2	
G888T	G270V	[24]	1/96					4/162	1/114	1/44	2/16	9	1.5
C962T	R295C	[21]	2/96						2/114			4	
T971C	S298P	[44]							3/114			3	
1057delTTC	ΔF327	[23]	4/96					6/162				10	1.7
G1091T	V338F	[39,45]						1/162	1/114			2	
T1101A	I341N	[19]			1/32							1	
T113G/C1114A	L345R	[24]	1/96									1	
C118T	Q347X	[22]	29/96			2/44		29/162	24/114		2/16	86	14.3
unidentified			13/96	2/18	14/32	1/44	5/74	7/162	18/114	13/44		73	12.2

abbreviations:
EE Eastern Europe (Hungary (2 patients), Croatia (1), Czech Republic & Slovakia (9)); *FS* frame shift mutation; *HK* Hong Kong; *I* Israel; *M* miscellaneous (North-Africa (2), Israeli Muslim Arab (4), Brasil (1), Marocco (1)); *NWE* North-West Europe (United Kingdom (1), The Netherlands (15), Belgium (3), Germany (39), Austria (2), France (21)); *SE* Southern Europe (Italia (56), Portugal (1)); *SS* splice site mutation; *T* Taiwan; *TR* Turkey; *USA* United States of America

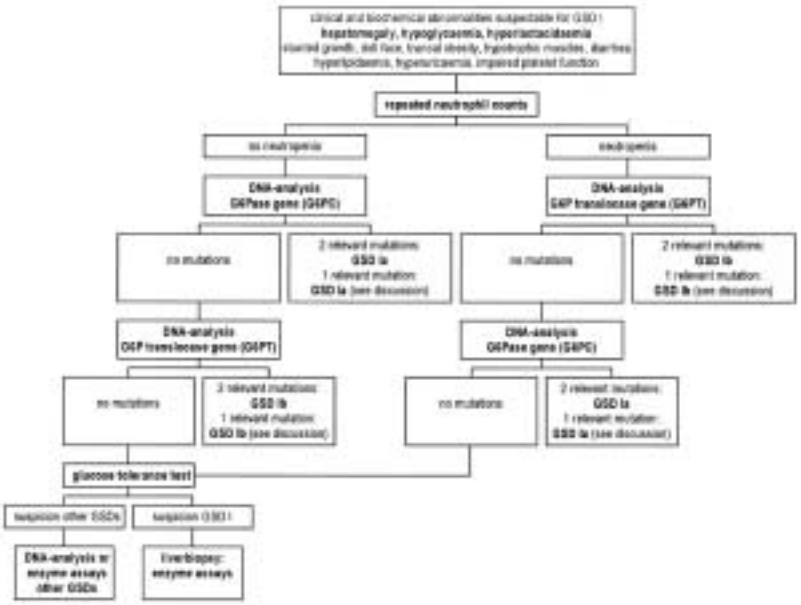
alleles respectively, but 34 additional mutations have already been identified. On the other hand, in Jewish patients (R83C, 93%), Chinese patients from the USA (R83H, 70%), Hispanic patients (459insTA, 50% and R83C, 28%), Japanese patients (727G→T, 88%), patients from South-Europe (R83C, 48% and Q347X, 21%) and Turkish patients (R83C, 60%), one or two predominantly occurring mutations are found. This opens the way to test for specific G6Pase gene mutations in patients with these background prior to a complete analysis.

We were able to identify mutations in the G6Pase gene on both alleles of all patients by using SSCP prior to automated sequencing of the exons revealing an aberrant SSCP pattern. This indicates that this method is a reliable procedure to identify mutations in the G6Pase gene. The high detection rate of mutations in the G6Pase gene is in line with published data (an overall detection rate of 87.8%). Because of this high detection rate, we feel that this approach is also adequate for carrier detection in partners of a known G6Pase mutation carrier. We analysed the G6Pase gene of the partners of patient 1 and 4 and found no aberrant SSCP patterns; however one should keep in mind that with SSCP analysis, mutations in the 5'UTR and 3'UTR and mutations in the control regions of the G6Pase gene will not be identified.

Identification of mutations on both G6Pase alleles of a GSD Ia index-case allows reliable prenatal DNA-based diagnosis in CVS by restriction enzyme analysis[38,47], sequencing[20] or SSCP analysis[34]. We successfully performed prenatal DNA-based diagnosis in families 12 and 20 by direct sequencing.

With increased knowledge of the genetic basis of GSD I and the high detection rate of mutations in the G6Pase and G6P translocase genes, it is our opinion that the diagnoses GSD Ia and GSD Ib can be based on clinical and biochemical abnormalities combined with mutation analysis instead of enzyme assays in liver tissue obtained by biopsy. Marked hepatomegaly, severe fasting hypoglycaemia and hyperlactacidaemia are the most striking abnormalities in GSD I. Other abnormalities are stunted growth, a rounded doll face, truncal obesity, hypothrophic muscles, diarrhoea, hyperlipidaemia, hyperuricaemia and bleeding tendency due to impaired platelet function[7]. Patients with GSD Ib may suffer from recurrent bacterial infections and inflammatory bowel disease in addition, due to neutropenia and neutrophil dysfunction[10]. A diagnostic flowchart to the diagnosis of GSD Ia and GSD Ib is presented in Figure 3.3.1. If patients suffer from neutropenia or neutrophil dysfunction, mutations in the G6P translocase gene should be excluded first. However, one should keep in mind that especially in younger GSD Ib patients, these specific features are not obligatory[50]. If two mutations in the G6Pase gene or in the G6P translocase gene are identified, enzyme assays in liver

Figure 3.3.1 Flowchart for the diagnosis of GSD Ia and GSD Ib



tissue obtained by biopsy are not necessary to establish the diagnosis GSD Ia or GSD Ib. If mutations neither in the G6Pase gene nor in the G6P translocase gene are identified, a glucose tolerance test should be performed. A marked decrease of blood lactate concentration from an elevated level at zero time is observed in patients with GSD I. This pattern is also observed in (other) disorders of gluconeogenesis. An increase of blood lactate concentration is observed in other glycogen storage diseases[8]. If the suspicion of GSD I remains, enzyme assays in liver tissue should be performed. These assays can be simplified however, because it is only necessary to measure G6Pase activity in intact and disrupted liver microsomes to establish the diagnosis GSD Ia or GSD Ib[49]. DNA-based diagnosis requires two mutations. Consequently, the diagnosis GSD Ia or GSD Ib may not be established officially if only one mutation in the G6Pase gene or in the G6P translocase gene is identified. However, with one mutation in the G6Pase gene or in the G6P translocase gene, the likelihood of having a different disease resembling GSD Ia or GSD Ib is virtually zero. Therefore, one should discuss for each individual patient with only one mutation identified whether a liver biopsy is needed to establish the definite diagnosis. For DNA-based prenatal diagnosis in the mother's possible next pregnancy, identification of the second mutation

is prerequisite.

As a large heterogeneity in phenotype in GSD Ia is observed, a genotype-phenotype correlation may be very helpful to adjust dietary and pharmacological strategies[6]. However, evidence for a clear genotype-phenotype correlation could be established neither from our data, nor from data in the literature.

In conclusion, using SSCP prior to automated sequencing of the exons revealing an aberrant pattern, we were able to identify mutations in the G6Pase gene on both alleles of all 30 GSD Ia patients we studied. The increased knowledge of the genetic basis of GSD Ia and GSD Ib allows DNA-based diagnosis instead of enzyme assays in liver tissue obtained by biopsy. Furthermore, it allows prenatal DNA-based diagnosis in CVS and carrier detection.

Addendum

In the period between acceptance and publication of this manuscript, three more molecular genetic studies concerning GSD Ia were published[2,42,48]. Bruni et al [2] reported that transient expression analyses of four previous identified missense mutations, W77R, A124T, G184E and L211P, showed totally abolished G6Pase activity. Seydewitz et al[42] reported five novel mutations: Q20R (A138G), W50X (G228A) and G81R (G320A) were mentioned earlier in an appendix to a study of the same group[26]; W156L (G546T), a missense mutation in exon 4, and G188D (G642A), a missense mutation in exon 5 and most likely also disturbing the correct splicing of the G6Pase mRNA, were not reported previously. Finally, Trioche et al[48] reported 3 novel mutations: Q54P (A240C), a missense mutation in exon 1, W70X (G288A), a nonsense mutation in exon 1 and previously reported[26], and T108I (C402T), a missense mutation in exon 2.

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