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RESEARCH REPORT

Mutation Analysis in Glycogen Storage Disease Type III Patients in the Netherlands: Novel Genotype-Phenotype Relationships and Five Novel Mutations in the *AGL* Gene

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Abstract Glycogen Storage Disease type III (GSD III) is an autosomal recessive disorder in which a mutation in the *AGL* gene causes deficiency of the glycogen debranching enzyme. In childhood, it is characterized by hepatomegaly, keto-hypoglycemic episodes after short periods of fasting, and hyperlipidemia. In adulthood, myopathy, cardiomyopathy, and liver cirrhosis are the main complications. To determine the genotype of the GSD III patients ($n = 14$) diagnosed and treated in our center, mutation analysis was performed by either denaturing gradient gel electrophoresis or full gene sequencing. We developed, validated and applied both methods, and in all patients a mutation was identified on both alleles. Five novel pathogenic mutations were identified in seven patients, including four missense mutations (*c.643G>A, p.Asp215Asn*; *c.655A>G, p.Asn219Asp*; *c.1027C>T, p.Arg343Trp*; *c.1877A>G, p.His626Arg*) and one frameshift mutation (*c.3911delA, p.Asn1304fs*). The *c.643G>A*, *p.Asp215Asn* mutation is related with type IIIa, as this mutation was found homozygously in two type IIIa patients. In addition to five novel mutations, we present new genotype–phenotype relationships for *c.2039G>A, p.Trp680X*; *c.753_756delCAGA, p.Asp251fs*; and the intron 32 *c.4260-12A>G* splice site mutation. The *p.Trp680X* mutation was found homozygously in four patients, presenting a mild IIIa phenotype with mild skeletal myopathy, elevated CK values, and no cardiomyopathy. The *p.Asp251fs* mutation was found homozygously in one patient presenting with a severe IIIa phenotype, with skeletal myopathy, and severe symptomatic cardiomyopathy. The *c.4260-12A>G* mutation was found heterozygously, together with the p.Arg343Trp mutation in a severe IIIb patient who developed liver cirrhosis and hepatocellular carcinoma, necessitating an orthotopic liver transplantation.

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Introduction

Glycogen storage disease type III (GSD III; OMIM no. 233400) is an autosomal recessive disorder in which mutations in the *AGL* gene cause deficiency of amylo-1, 6-glucosidase and 1,4α-D-glucan 4-α-glycosyltransferase, also known as the glycogen debranching enzyme (GDE; EC no. 3.2.1.33 and 2.4.1.25). GDE catalyzes the last step in the conversion of glycogen to glucose, and GDE deficiency thus causes storage of an intermediate form of glycogen called limit dextrin (LD) (Smit et al. 2006). In the IIIa subtype, muscle and liver tissue are deficient in GDE, and this affects 85% of GSD III patients. Approximately 15% of the patients have type IIIb, in which only the liver is deficient in GDE (Shen et al. 1996). In the neonatal period and in infancy, the main features are hepatomegaly with elevated aspartate transaminase (ASAT) and alanine transferase (ALAT) values, keto-hypoglycemic episodes after relatively short periods of fasting, and hyperlipidemia.

Untreated neonates and children have developmental delay, growth retardation, and delayed puberty. In puberty and early adulthood, myopathy becomes the predominant feature of GSD III; the disease presents as a slowly progressive muscle weakness in which the proximal muscles of the shoulder and hip joints are affected. Clinical muscle weakness in the upper and the lower limb muscles can develop in later adulthood, which may be worsened by the development of peripheral neuropathy (Hobson-Webb et al. 2010; Wolfsdorf and Weinstein 2003). LD can also be stored in heart muscle, which causes a form of cardiomyopathy that resembles idiopathic hypertrophic cardiomyopathy on an echocardiogram (Lee et al. 1997; Akazawa et al. 1997).

GDE is composed of 1,532 amino acid residues and has two catalytic centers (Bao et al. 1996; Liu et al. 1991). Before GDE starts to act, a phosphorylase enzyme separates four glucose molecules from the glycogen molecule to form LD (Newgard et al. 1989). Then the first catalytic center of GDE, 1,4-glucan-4-D-glucosyltransferase, transports the three outer glucose molecules of LD to another chain. The second catalytic center, amylo-1,6-glucosidase, then releases the last glucose molecule (Ding et al. 1990). GSD III can be diagnosed biochemically by measuring GDE activity in skin fibroblasts and/or leucocytes. GDE activity and LD content can also be measured in liver and/or muscle biopsies (Wolfsdorf and Weinstein 2003). LD content can be measured in erythrocytes.

The *AGL* gene is located on chromosome 1p22 and contains 35 exons covering 85 kb of DNA (Yang-Feng et al. 1992). The full cDNA is 7 kb with a 4,596 bp coding region. A total of six mRNA isoforms are created by alternative splicing. Isoform 1 is the major isoform and widely expressed, including in liver and muscle tissue (Bao et al. 1996). Isoforms 2, 3, and 4 are present in muscle and cardiac muscle and are formed by alternative splicing or because of the difference in transcription start points. Isoform 1 contains exons 1 and 3. Isoforms 2, 3, and 4 start with exon 2. Isoforms 1 through 4 all contain exon 3, which includes the normal initiation codon for protein translation. Exons 4 through 35 are present in all isoforms (Bao et al. 1996, 1997). The glycogen-binding domain is encoded by exons 31–34. The 1,4α-D-glucan 4-α-glycosyltransferase catalytic site is encoded by exons 6, 13, 14, and 15. The amylo-1,6-glucosidase catalytic site is encoded by exons 26 and 27 (Shen and Chen 2002).

Molecular analyses of GSD III patients have been performed in several ethnic populations, and over 100 different *AGL* mutations have been described (Goldstein et al. 2010), but new mutations are still being reported (http://www.hgmd.org). No clear genotype-phenotype relationship has been established so far, although there is a relation between mutations in exon 3 and the IIIb subtype (Shen et al. 1996; Shen and Chen 2002). It is unclear, however, what mechanism enables patients with mutations in exon 3 to retain GDE activity in muscle tissue. A possible explanation was proposed by Goldstein et al. (2010) in which the exon 3 mutation is bypassed using a downstream start codon, thus creating an isoform without the exon 3 mutations.

To determine the genotype of the GSD III patients diagnosed and treated in our center ($n = 14$), the University Medical Centre Groningen (UMCG), the Netherlands, we performed mutation analysis by one of two methods. Denaturing gradient gel electrophoresis (DGGE) was applied on eight patients, and full gene sequencing was applied on six patients. We developed, validated, and applied both methods. Here we describe five novel mutations found in seven patients and their phenotypes, and evaluate the phenotype-genotype relationships in patients with previously described mutations.

Materials and Methods

Patients: For this study we analyzed 14 patients diagnosed with GSD III (seven females and seven males). They were diagnosed enzymatically by measuring GDE activity in leukocytes, fibroblasts, and/or liver tissue, and/or muscle tissue. All the patients were diagnosed and are being treated in the UMCG. Their clinical data was collected.

Mutation analysis by DGGE: Genomic DNA was isolated from EDTA blood (Miller et al. 1988). Primers were designed on the GenBank genomic reference sequence (NW_012865) to include at least 40 bp of intronic sequence on the front end of every exon and at least 20 bp at the back end. Analysis with NGRL Manchester's SNP database (http://ngrl.manchester.ac.uk/SNPCheckV2/snpcheck.htm) confirmed that no known single nucleotide polymorphisms were situated under the primers. The primer specificity was checked and verified in complete genomic sequences with NCBI's ePCR (http://www.ncbi.nlm.nih.gov/projects/e-pcr). We developed 50 primer sets to analyze 30 of the 33 coding exons by DGGE (primer sequences available upon request). The three remaining exons were sequenced directly, as designing DGGE primers with an appropriate melting curve was not possible. Per amplicon, the amplification mix consisted of 1.0 µl genomic DNA (40 ng/µl), 10 µl Amplitaq Gold ® Fast PCR Master Mix (Applied Biosystems, California, USA), 3.0 µl primer (3 pmol/µl), and 6.0 µl milliQ water; the reaction volume per sample was 20.0 µl. The samples were amplified by PCR in 96-well plates on a 96-well Gene Amp® 9700 PCR system (Applied Biosystems, California, USA).The PCR program used was as

Table 1 Sequences of the primer set designed according to the criteria of primer design used in conformation sensitive capillary electrophoresis (CSCE)[a]

Amplicon	Forward primer sequence 5′-3′	Reverse primer sequence 5′-3′	Size bp
3	CGAACATGTAAGTGCCGCTGTCA	AGAACACAGCACCATCTTTGCACAA	380
4	GTAGTGCCAAAACAGCATTAGGTTTGC	GCACTGCCATGGTTCATACAGTAACAT	457
5	TTCCATTAAGTTTTGTTGCAAC	CTGCAATGAGAGAATGGACTAATACAC	435
6	TGAACCCAAGTGTTTGACCTCTTTTCC	CCTTTCTCTTATTTGTGTGTATATGTG	432
7	AACTTTTCCTGTAACAGTATCATCG	AATACAGGTTCTAAGTAATTTTCAACC	429
8	GCACTTTGGCGTTTCTCCTGTGA	GACGTTACCCAAAAGAGAGTTTTCCCT	460
9	GGGAGGAGGTAGGAGGATAC	CACATATAGAAACATGGCCCACACACA	456
10	CTGTGTGTGGGCCATGTTTCTATATGT	TTCCCAAAAGGCAATTAACTGCCTGAA	409
11	CTGCATTTCTCCATCTGCTCTAGCAA	ATTTAAGAAATGTACTGAACTCACATG	440
12	CATCCTGCTAGATTTACTCAAAAAGCC	ACCAATAGACTAATGGGGAAGAAAATC	432
13	TTAAAAACCAGTGTTTCCTTGAAG	AATGCTTGTGTCCAACTAGC	381
14	TATGTCAAATCATGCCTCCTTTTGTC	GAAATGAGGTATCTTACCCCAAAGTAG	428
15	CCATTTCTCCAGTTAAGTTATGGG	TGGGTATGATTGTGACCAAGTGTCAGA	445
16	GGTCACAATCATACCCATATACTTC	AAACCACTGAAATCTGGACAAAGG	442
17	CTATGGCATGTTGTGCTAGTGGAAGT	TCCACATACACCTGAGAAGCAGAAAGA	433
18	AGGAGCTTGGAGCCAAGGGTTT	CCATCATACCTGGCCAAGTTACCAAA	447
19	GATTTGAAACCACTTTAGCCTTCC	TGTGGCAACTCCAGCTTGTTTAAC	340
20	TGGGACTCTCATCTTACTACTGTG	GCATGTGGATCAAGACTAACTCTG	340
22	TTGAAAACTTGTCTCCAGGAAGTG	TGGACCGTACTTTGAGTAGCAAGGAT	402
22	GAATGCTGAGTTCCTAAAACATACAC	TGCAACCCAAGTAGGCATACTCTGA	366
23	TTGTGGACTGGGTAGCCCTTGT	GAAGGAAGGAGGAAAATGGTTCAGGTT	397
24	CCTCCTTCCTTCATCATCTTTCAG	CTATCCACCTACAAGCCTTTTCAG	413
25	TGGGTGAAATGAAAGCAGTTTTG	AAAATCTTGAGTAGCATTACAAGC	458
26	ACCCCAGGTTTAGAGTAACTGTTC	CTACCTAAAGAAAATACAGCTCCC	323
27	CAAAAGTGACTGGTTTTTGTCTTC	GGTGCCAAATCAATACTGACATTTG	440
28	CTGGCCTCACCCCAATTCCTATTTC	ATTATATCGTGAGGTTTGGCACAC	352
29	CAAACTGAGCTTTAGAGTGGTTGTCCT	AGATGAAGGGAAGAAGGCAGGGAAAT	398
30	TTCATTACAATTGTTTACCGAATGCCC	GGGTTTTCCGATATTAGCTGATAG	301
31	CACATCTCAATTCAGACTGGCCACAT	AACAAATGGGAATAAGGAACTAAGC	441
32	GGCTTTCCTAACTTCTACGGCCAAAA	AGATGGCATCTCCTTTTGTTGCCC	407
33	TGCCGAGCTTATTCTGTAGAAGAC	AGGCCACAGCCACTCCTAAAAAAG	333
34	TCACCAAGGACCTGTAAGAATTTC	CCTAGGGCATACAGAAATCAATTC	350
35	CACTAGAAGGCAAAAATCACCAGGTCT	AACTTGAGCCTGTGCATATAAGGCATT	294

[a] Primer and amplicon criteria: Optimal primer length 20bp (minimum 18bp; maximum 27bp), optimal annealing temperature 58°C (minimum 52°C; maximum 64°C), optimal GC% 55 (maximum 70), optimal amplicon size 400bp (minimum 200bp; maximum 464bp), maximal amplicon GC% 73. A PT1 tail was added to every forward primer sequence (5′-3′; TGTAAAACGACGGCCAGT), and a PT2 tail was added to every reverse primer sequence (5′-3′; CAGGAAACAGCTATGACC)

follows: 3 min at 96°C, 45 cycles of 1 min at 96°C, 1 min annealing at multiple temperatures, 1 min elongation at 72°C, and a final extension step at 72°C lasting 5 min. The annealing temperatures were 5 cycles at 60°C, 5 cycles at 56°C, 5 cycles at 52°C, and 30 cycles at 50°C. The PCR products were analyzed by DGGE (Hayes et al. 1999). Amplicons showing an aberrant banding pattern were sequenced on an ABI 3730 automated sequencer (Applied Biosystems, California, USA) using specific primers. DNA samples from 38 GSD III patients with 34 known mutations were used to validate the DGGE system – and all mutations were detected in our system.

Sequence analysis: We designed 33 primer sets according to the criteria used in conformation-sensitive capillary electrophoresis (CSCE, Table 1) and applied them for sequencing all coding exons of the gene including flanking intronic sequences. Per sample, the amplification mix consisted of 2 µl genomic DNA, 5 µl Amplitaq Gold® Fast PCR Master Mix (Applied Biosystems, California, USA), and 3.0 µl primer (150 fmol/µl). The reaction

volume per sample was 10 µl; the samples were amplified by PCR in 384-well plates on a Veriti 384-well thermal cycler (Applied Biosystems, California, USA). The PCR program was as follows: 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min annealing at 60°C, 1 min at 72°C, and a final extension step at 72°C lasting 7 min, after which the samples were cooled down to 20°C. Five microliters of the PCR products were loaded with 5 µl loading buffer and run on a 2% agarose gel with a FastRuler Low Range DNA ladder (Fermentas, Vilnius, Lithuania) for comparison. The remaining PCR products were purified with ExoSAP-IT (Amersham Pharmacia Biotech, Piscataway, NY, USA) and subjected to direct sequencing on an ABI 3730 automated sequencer (Applied Biosystems, California, USA) using specific primers.

Analysis of the pathogenicity of novel mutations: The pathogenicity of novel mutations was assessed using six separate methods. One hundred control chromosomes from mixed ethnicity were checked for novel mutations. Conservation of the mutated nucleotide and amino acid was graded with Alamut Version 1.4 (©Interactive Biosoftware). The University of Harvard's PolyPhen program (http://genetics.bwh.harvard.edu/pph/) predicted the impact of an amino acid substitution on the structure and function of a human protein. The SIFT program (http://blocks.fhcrc.org/sift/SIFT.html) predicted whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids. Finally, we assessed whether the mutation was located in an exon encoding the glycogen binding domain (exon 31-34), or encoding a catalytic site (exon 6, 14 16, 26-27), and measured the GDE activity.

Results

Patients: Of our 14 GSD III patients, 4 were related to one other patient (two sisters, and two brothers), all other patients were unrelated. Patient 7 was born to consanguineous parents. Nine patients had type IIIa, two patients had type IIIb, and three pediatric patients were too young to be subtyped based on their clinical presentation. The clinical and biochemical characteristics of the patients are presented in Table 2.

Results of mutation analysis: The patients were fully analyzed and we detected two mutations in each patient (Table 3). Five novel mutations were identified in seven patients, including two sisters who were homozygous for *c.643G>A, p.Asp215Asn* and two brothers who were homozygous for *c.3911delA, p.Asn1304fs*. The other three patients were compound heterozygous with a novel missense mutation and a previously reported pathogenic mutation: *c.655A>G, p.Asn219Asp* in combination with *c.4529dupA, p.Tyr1510X*; *c.1027C>T, p.Arg343Trp* in combination with the splice mutation *c.4260-12A>G*; and *c.1877A>G, p.His626Arg* in combination with *c.1222C>T, p.Arg408X*.

Results of pathogenicity analysis: We consider the four new missense mutations to be pathogenic for several reasons. We did not detect the new mutations in 100 control chromosomes, and these mutations were not found in the NCBI SNP database. Highly conserved amino acids were mutated, and all mutations were predicted to affect protein function by the PolyPhen and SIFT programs. Additionally, three of the four new mutations (*Asp215Asn*, *p.Asn219Asp*, and *p.His626Arg*) were located in an exon encoding the 1,4α-D-glucan 4-α-glycosyltransferase catalytic site of GDE (Fig. 1). This makes a pathogenic effect of these mutations on GDE function very probable (Cheng et al. 2009). As the *c.3911delA, p.Asn1304fs* causes a frameshift with the new reading frame ending in a stop codon at position 10, this is considered to be a pathogenic mutation as well. Clinically, all the patients presented with a GSD III phenotype, and this was biochemically confirmed by enzymatic analysis that no GDE activity was measured to be present.

Discussion

Here we describe five novel pathogenic mutations in the *AGL* gene, four of which are missense mutations, very likely causing GSD III in seven patients. Missense mutations causing GSD III are scarce, as truncating mutations compose the majority of the pathogenic mutations. However, pathogenic missense mutations have been described. Cheng et al. showed that missense mutations located in the active sites produce a GSD III phenotype in which there is total or partial abolishment of GDE activity depending on the location of the mutation (Cheng et al. 2009).

GSD III is characterized by clinical and genotypical heterogeneity, and clear genotype-phenotype correlations are rare. We therefore present the clinical characteristics of the patients with novel mutations, and discuss the correspondence between their clinical presentation and that described in the literature for the mutations that have been previously described.

Genotype-phenotype analysis: Patients 1 to 4 all had type IIIa and were homozygous for *p.Trp680X*. The phenotype was similar in all four patients, with hepatomegaly, no or mild cardiac involvement, and mild skeletal myopathy with elevated CK values. This suggests a link between the *p.Trp680X* mutation and the type IIIa phenotype. The *p.Trp680X* mutation was described previously in a compound heterozygote patient, in whom it was linked to

Table 2 Demographic, clinical, and biochemical characteristics of the analyzed GSD III patients

Patient no.	Age (years)	Sex	Subtype	Ethnic origin	GDE residual activity (%)	Liver complications	Cardiologic complications	Skeletal muscle complications	Most recent CK value (U/L)
1	8	M	IIIa	Caribbean	0	Hepatomegaly	None	Proximal myopathy	1083
2	25	F	IIIa	Caribbean	0	Hepatomegaly	None	None	2232
3	26	M	IIIa	Caribbean	0	Hepatomegaly	Septal hypertrophy	None	893
4	40	F	IIIa	Caribbean	0	Hepatomegaly	None	None	3729
5	15	F	IIIa	Mediterranean	0	Hepatomegaly	None	None	662
6	20	F	IIIa	Mediterranean	0	Hepatomegaly	None	None	1342
7	32	F	IIIa	Mediterranean	0	Hepatomegaly	Severe symptomatic left ventricular and septal hypertrophy	Exercise intolerance, distal myopathy	1898
8	3	M	Too young	Caucasian	1	Hepatomegaly	None	None	133
9	30	F	IIIa	Caucasian	0	None	None	Exercise intolerance, distal myopathy	2257
10	30	M	IIIb	Caucasian	0	Hepatomegaly	None	None	68
11	41	F	IIIa	Caucasian	0	Hepatomegaly	None	Exercise intolerance	392
12	41	F	IIIb	Caucasian	0	None, the patient is post-OLT[c],pre-OLT[c] liver cirrhosis and hepatocellular carcinoma was present	None	None	70
13	3	M	Too young	Caucasian	0	Hepatomegaly	None	None	128
14	1	M	Too young	Caucasian	0	Hepatomegaly	None	None	466

M Male, *F* Female; *OLT* Orthotopic Liver Transplantation

the IIIb phenotype (Shen et al. 1996), which is in contrast to what we found in our patients. Interestingly, all our patients with the *c.2039G>A, p.Trp680X* mutation were from the same topographic region and ethnic origin, making a founder effect feasible, as seen for the *c.1222C>T, p.Arg408X* mutation on the Faroe Islands (Santer et al. 2001). However, in order to prove this, haplotyping for these patients would be required, which we have not done in this study.

Patient 5 (female, age 15 years) and patient 6 (female, age 20 years) were sisters (whose parents were from the Mediterranean) who had type IIIa, and were homozygous for the novel *p.Asp215Asn* mutation. Their phenotype was mild with hepatomegaly as the main finding, there was no myopathy or cardiomyopathy. In laboratory investigations ASAT, ALAT, and CK values were elevated, but there was no hypoglycemia or hyperlipidemia. The mild phenotype was probably due to their young age and the good metabolic control of these patients.

Patient 7 (female, age 32 years) had type IIIa and was found to be homozygous for *p.Asp251fs*. She had a severe IIIa phenotype, with hepatomegaly, and skeletal muscle involvement with severe exercise intolerance. There was also severe cardiac involvement with hypertrophy of the interventricular septum and left ventricle, necessitating pharmacological treatment and ICD placement. This mutation was previously described in a 3-year-old female, who was found to have hepatosplenomegaly and hypoglycemia but no myopathy or cardiomyopathy (Lucchiari et al. 2006).

In patient 8 (male, age 3 years), the novel mutation *p.Asn219Asp* was found to be heterozygous. The other mutation *p.Tyr1510X* was previously reported and associated with a severe IIIa phenotype (Shen et al. 1997). At first

Table 3 Mutation analysis results in 14 GSD III patients

Patient no.	Exon no.	Nucleotide change allele 1	Amino acid change allele 1	Mutation type	Exon no.	Nucleotide change allele 2	Amino acid change allele 2	Mutation type	Mutation analysis method
1	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	DGGE
2	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	DGGE
3	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	DGGE
4	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	Sequencing
5	6	c.643G>A	p.Asp215Asn	Missense	6	c.643G>A	p.Asp215Asn	Missense	DGGE
6	6	c.643G>A	p.Asp215Asn	Missense	6	c.643G>A	p.Asp215Asn	Missense	DGGE
7	7	c.753_756delCAGA	p.Asp251fs	Frameshift	7	c.753_756delCAGA	p.Asp251fs	Frameshift	DGGE
8	6	c.655A>G	p.Asn219Asp	Missense	35	c.4529dupA	p.Tyr1510X	Nonsense	Sequencing
9	35	c.4529dupA	p.Tyr1510X	Nonsense	35	c.4529dupA	p.Tyr1510X	Nonsense	DGGE
10	3	c.16C>T	p.Gln6X	Nonsense	3	c.16C>T	p.Gln6X	Nonsense	DGGE
11	11	c.1222C>T	p.Arg408X	Nonsense	15	c.1877A>G	p.His626Arg	Missense	Sequencing
12	9	c.1027C>T	p.Arg343Trp	Missense	Intron 32	c.4260-12A>G		Splice	Sequencing
13	30	c.3911delA	p.Asn1304fs	Frameshift	30	c.3911delA	p.Asn1304fs	Frameshift	Sequencing
14	30	c.3911delA	p.Asn1304fs	Frameshift	30	c.3911delA	p.Asn1304fs	Frameshift	Sequencing

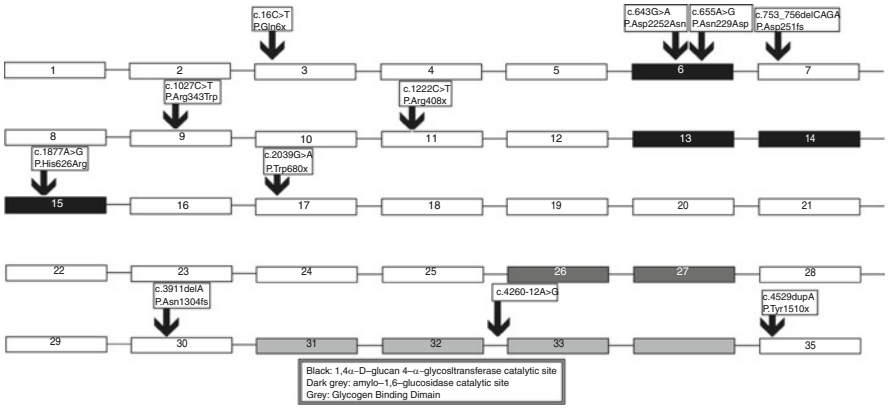

Fig. 1 The location of all found mutations in the gene, the catalytic sites of GDE are depicted as well; three of the four novel mutations (*Asp215Asn*; *p.Asn219Asp*; and *p.His626Arg*) were located in exons encoding the 1,4α-D-glucan 4-α-glycosyltransferase catalytic site

presentation (age 1.5 years), he had severe hepatomegaly extending 15 cm below the costal margin in the mediolavicular line, regular keto-hypoglycemic episodes and hyperlipidemia. These symptoms improved dramatically upon starting dietary treatment after diagnosis at the age of 1.5 years with frequent meals during the day and overnight gastric drip feeding. These findings are typical for GSD III patients in this age group, and the normal CK value does not exclude future muscle involvement, making it hard to assess the subtype or to determine the phenotype that goes with this novel mutation. Furthermore, the latter proves difficult because the patient was found to have compound heterozygous mutations.

Patient 9 (female, 30 years) had type IIIa and was found to be homozygous for *p.Tyr1510X*. She had a severe IIIa phenotype including distal myopathy and severe exercise intolerance with elevated CK (2257 U/L). Despite the absence of hepatomegaly, transaminases remained elevated (ASAT 237 U/L, ALAT 175 U/L). This is in concordance with a suggestion made by Shen et al. that this mutation is associated with a severe GSD IIIa phenotype (Shen et al. 1997).

Patient 10 (male, 30 years) had type IIIb and was homozygous for *p.Gln6X*. His phenotype was mild, with hepatomegaly, normal CK values, and no cardiac or skeletal muscle involvement. This mutation was previously described and is strongly linked with the GSD IIIb phenotype, as are other mutations in exon 3 (Shen et al. 1996; Shen and Chen 2002). Paradoxically, this patient first presented at the age of 2 years with proximal myopathy and severe hypotonia, but without cardiomyopathy or elevated CK values. His myopathy and hypotonia improved dramatically upon starting dietary treatment, and he has had no muscular symptoms since.

Patient 11 (female, age 41 years) had type IIIa and was compound heterozygous for the novel *p.His626Arg* mutation. Her other mutation, *p.Arg408X*, was previously described (Santer et al. 2001; Lam et al. 2004) and associated with the GSD IIIa phenotype. This corresponds with her phenotype: hepatomegaly and elevated transaminases. She has no proximal or distal myopathy but she does suffer from exercise intolerance and has elevated CK values.

Patient 12 (female, age 41 years) had type IIIb and was compound heterozygous for the novel *p.Arg343Trp* mutation and *c.4260-12A>G* in intron 32. *c.4260-12A>G* was previously described and associated with IIIb as well as IIIa (Okubo et al. 1998; Shaiu et al. 2000). This patients' phenotype was severe IIIb and a case report on her was published after she received an orthotopic liver transplantation after being diagnosed with liver cirrhosis (Haagsma et al. 1997). Hepatocellular carcinoma was found upon pathological examination of the excised liver. She has never had any muscle involvement, and CK values have always been normal.

Patients 13 (male, age 3 years) and 14 (male, age 1 years) were brothers and homozygous for the novel mutation *c.3911delA*, *p.Asn1304fs* in exon 30. Except for prominent hepatomegaly and the need for appropriate dietary requirements, both patients were generally well. There was no clinical muscle involvement, even though patient 14 had elevated CK values. As these are very young pediatric patients, it is not yet possible to assess the subtype based on the clinical findings.

Conclusions: We identified two separate mutations in each of our 14 GSD III patients. Five were novel pathogenic mutations considered to be causal. As we also analyzed parts of the intron sequences (40 bp on the front end, and 20 bp at the back end of every exon), we assume that the chance that we missed other disease-causing mutations is slim. The novel *c.643G>A*, *p.Asp215Asn* mutation is related with type IIIa, as this mutation was found homozygously in two patients both clinically presenting as type IIIa. We also established new genotype-phenotype relationships between *c.2039G>A,*

p.Trp680X and type IIIa; *c.753_756delCAGA, p.Asp251fs* and type IIIa; and the intron 32 *c.4260-12A>G* splice site mutation and type IIIb. The association between *c.4529dupA, p.Tyr1510X* and type IIIa complies with previous literature. However, as the GSD III subtype is not yet clear for every patient, it was not possible to establish a genotype-phenotype relationship for every novel mutation. There is still a large clinical and genotypical heterogeneity in GSD III, which makes establishing genotype-phenotype relationships for GSD III difficult. The fact that we found five new mutations in a relatively small number of GSD III patients further accentuates the need for more genotyping, and indicates that there are probably numerous unidentified mutations.

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Synopsis

GSD III is characterized by a large clinical and genotypical heterogeneity, which makes the establishment of clear genotype-phenotype relationships difficult.

Conflicts of Interest

None declared.

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