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Egyptian glycogen storage disease type III – identification of six novel *AGL* mutations, including a large 1.5 kb deletion and a missense mutation p.L620P with subtype IIId

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Abstract

Background: Glycogen storage disease type III (GSD III) is caused by mutations in *AGL* which encodes for a single protein with two enzyme activities: oligo-1, 4-1, 4-glucantransferase (transferase) and amylo-1, 6-glucosidase. Activity of both enzymes is lost in most patients with GSD III, but in the very rare subtype IIId, transferase activity is deficient. Since the spectrum of *AGL* mutations is dependent on the ethnic group, we investigated the clinical and molecular characteristics in Egyptian patients with GSD III.
Methods: Clinical features were examined in five Egyptian patients. *AGL* was sequenced and *AGL* haplotypes were determined.
Results: Six novel *AGL* mutations were identified: a large deletion (c.3481–3588+1417del1525 bp), two insertions (c.1389insG and c.2368insA), two small deletions (c.2223–2224delGT and c.4041delT), and a missense mutation (p.L620P). p.L620P was found in a patient with IIId. Each mutation was located on a different *AGL* haplotype.
Conclusions: Our results suggest that there is allelic and phenotypic heterogeneity of GSD III in Egypt. This is the second description of a large deletion in *AGL*. p.L620P is the second mutation found in GSD IIId.
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Introduction

Glycogen storage disease type III (GSD III; MIM #232400) is an autosomal recessive inherited disorder characterized by hepatomegaly, growth retardation and fasting hypoglycemia (1, 2). GSD III is caused by a deficiency in the glycogen debranching enzyme, a key enzyme in the degradation of glycogen. The enzyme has two independent catalytic activities, oligo-1, 4-1, 4-glucantransferase (EC 2.4.1.25) (transferase) and amylo-1, 6-glucosidase (EC 3.2.1.33) (glucosidase), located on a single 160-kDa protein. Activity of both enzymes is virtually absent in affected organs. Most patients have both liver and muscle involvement (GSD IIIa), but ~15% of the patients have liver involvement only and without any muscular manifestations (GSD IIIb). Rarely, patients have been categorized with selective loss of one of two enzyme activities (3, 4). Selective transferase deficiency is named GSD IIId. However, the molecular basis for GSD IIId has not been fully elucidated.

A gene coding human glycogen debranching enzyme (gene symbol: *AGL*) has been isolated and shown to be 85 kb in length and composed of 35 exons, encoding a 7.0-kb mRNA (5). Molecular analyses of GSD III have been performed in several ethnic populations, and over 60 different *AGL* mutations have been reported to date in GSD III patients (Human Gene Mutation Database; http://www.hgmd.cf.ac.uk/ac/index.php). A majority of *AGL* mutations have been point mutations, small deletions, insertions, or splicing mutations. However, a large deletion has been reported in the single case (6). Since eight missense mutations have been reported in *AGL* (7–10), whether these are responsible for GSD III remains unclear. Only G1448R has been characterized in vitro (11).

The spectrum of *AGL* mutations in GSD III depends on the ethnic group. In an ethnic group with high rate of consanguinity, prevalent mutations have been reported (12, 13). In Egypt, the incidence of consanguineous marriages in the general population is reported to be ~29% (14). However, three different *AGL* mutations have been identified in three patients from the Delta region in Egypt (15). To investigate the

molecular characteristics further, we examined five new cases and detected a total of six novel mutations, including the second large deletion and a novel missense mutation with selective loss of transferase activity.

Materials and methods

Patients

Five Egyptian GSD III patients from five families from Cairo and Alexandria, Egypt were investigated. The patients were confirmed as having deficient debranching enzyme activity in peripheral blood cells by the method of Shin (16). This method assessed a reverse incorporation of isotope-labeled glucose into glycogen, whereby measured activity reflects primarily that of glucosidase. Because IIId has residual glucosidase activity, this method enabled us to distinguish IIId from IIIa or IIIb (2). Tissue samples from liver or muscle were unavailable for use in this study. Consanguinity was ascertained in three out of five families. The study was approved by the Local Ethics Committees and performed with the patients' and their families' informed consent.

DNA sequence analysis of *AGL*

Genomic DNA was isolated from peripheral blood leukocytes.

The full coding exons, their relevant exon-intron boundaries, and the 5′- and 3′-flanking regions of the patients' *AGL* were sequenced directly as described previously (17). In brief, 11 DNA fragments to cover *AGL* were amplified from genomic DNA by LA-PCR. LA-PCR products were recovered and subjected to direct sequencing with internal sequencing primers. The nucleotides of *AGL* cDNA were numbered according to *AGL* isoform 1 (GenBank accession no. NM_000642).

For the detection of the large deletion around exon 27, LA-PCR was performed for 30 cycles of denaturation and annealing/extension at 94°C for 30 s and 68°C for 5 min, respectively. The fragments were then electrophoresed on a 0.9% agarose gel.

Point mutations that were identified in patients were verified using restriction fragment length polymorphism (RFLP) analyses. A pair of primers (listed in Table 1) was used for PCR, and each specific restriction endonuclease was added to digest PCR products. Restriction digests were analyzed on polyacrylamide gel.

Fifty Egyptian control subjects were examined with LA-PCR and RFLP analyses in the same manner in order to rule out the possibility that there are mere polymorphisms in controls.

Haplotype determination in AGL

A total of 30 single nucleotide polymorphisms (SNPs) in *AGL* were determined as described previously (18).

RT-PCR analysis of the *AGL* cDNA

Total RNA was isolated from whole blood samples from patient 1 and a normal control using QIAamp RNA blood mini kits (QIAGEN, Düsseldorf, Germany), and reverse transcribed into single strand cDNA using random hexamers and Superscript II (Invitrogen, Carlsbad, CA, USA). *AGL* cDNA fragments were amplified by PCR and electrophoresed on 0.9% agarose gel as described previously (19). In addition,

Table 1 PCR primers using LA-PCR and RFLP detection for *AGL* mutations.

Location	Mutation	PCR primer (5′→3′)	Restriction enzyme	Fragment size, bp
Exon 27	c.3481−3588+1417del1525 bp	F: TTA GCA TTT GCG GGT ACC CTG AGG CAT GGT CTC R: CTT CGT CCT TCA TGT TTC GAT CTA TCT GGG GAC	None	Normal: 2149 Mutant: 624
Exon 12	c.1389insG	F: CTG ATG GCA CAC AAT GGA TCC GTA ATG R: tta ttt tga cct tgc cat gaa c	*BslI*	Normal: 123 Mutant: 98+26
Exon 18	c.2223–2224delGT	F: TGA TGA AGA CAT AGT GGC AG R: acC AGG GAT GCA CAT TTG AG	*MslI*	Normal: 136 Mutant: 83+51
Exon 15	c.1859T>C [p.L620P]	F: GCC TTT AAT GCC AGC TAT TGC ACA AGG CC R: gat tgt gac caa gtg tca gag ag	*StuI*	Normal: 120+27 Mutant: 147
Exon 19	c.2368insA	F: atg att tga aac cac ttt agc c R: ACT GTG ATA TCT GGT GTT CC	*PsiI*	Normal: 184 Mutant: 139+46
Exon 31	c.4041delT	F: AAG GCT ATA AAG GTC TCA TAT G R: aaa tgg gaa taa gga act aag c	*DraI*	Normal: 190+92 Mutant: 281

Exon sequences are represented by uppercase letters and introns by lowercase letters. Mismatch nucleotides are underlined. F, forward; R, reverse; RFLP, restriction fragment length polymorphism.

PCR was performed using a sense primer in exon 26 (5′-CTT ACC TCA TTT TTC TTC TGG-3′) and an antisense primer in exon 29 (5′-CAT GTG CCA CAA TTG AAA CG-3′) to examine whether aberrant spliced cDNA was detected in patient 1.

Bioinformatical analysis

Repetitive sequences, such as *Alu* elements were searched using RepeatMasker software by Smit AFA, Hubley R and Green P (http://repeatmasker.org). Nucleotide sequences of intron 27 in *AGL* were retrieved from *Homo sapiens* chromosome 1 genomic contig NT_032977. Alignment of amino acid sequences from various species was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) (20).

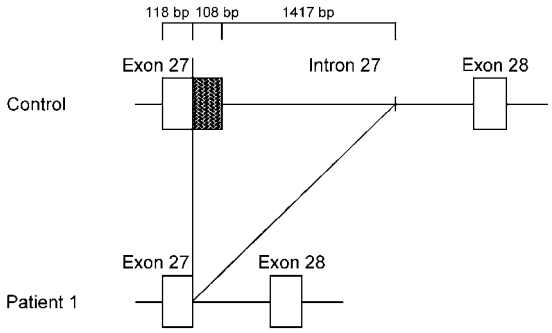

Figure 2 Schematic representation of 1525 bp deletion in patient 1 (c.3481–3588+1417del1525 bp).
The 3′-part of exon 27 plus the 5′-part of intron 27 were deleted.

Results

We identified six *AGL* mutations in five Egyptian patients. All were novel mutations and none were found in the 50 Egyptian controls. Four patients were homozygotes and patient 2 was a compound heterozygote. None of the patients had mutations in exon 3, indicating that none were IIIb. Sequence electropherograms and RFLP analyses are shown in Figure 1.

Patient 1 was homozygous for a large deletion involving exon 27. LA-PCR fragment encompassing exons 25–28 was shorter than that from a control (data not shown). To further narrow the location, we designed a forward primer in exon 27 and a reverse primer in exon 28 as shown in Table 1. The LA-PCR fragment (exon 27 to exon 28) from patient 1 was shorter than that from a control (Figure 1A). Sequence analysis showed a deletion of 1525 bp (g.70,338,229–70,339,753 in NT_032977). One hundred and eight bp of the 3′-part of exon 27 (c.3481–3588) plus 1417 bp of the 5′-part of intron 27 (c.3481–3588+1417del1525 bp) was deleted, as illustrated in Figure 2. The deletion contained 108-bp coding sequences in exon 27 and presumably caused an in-frame deletion of 36

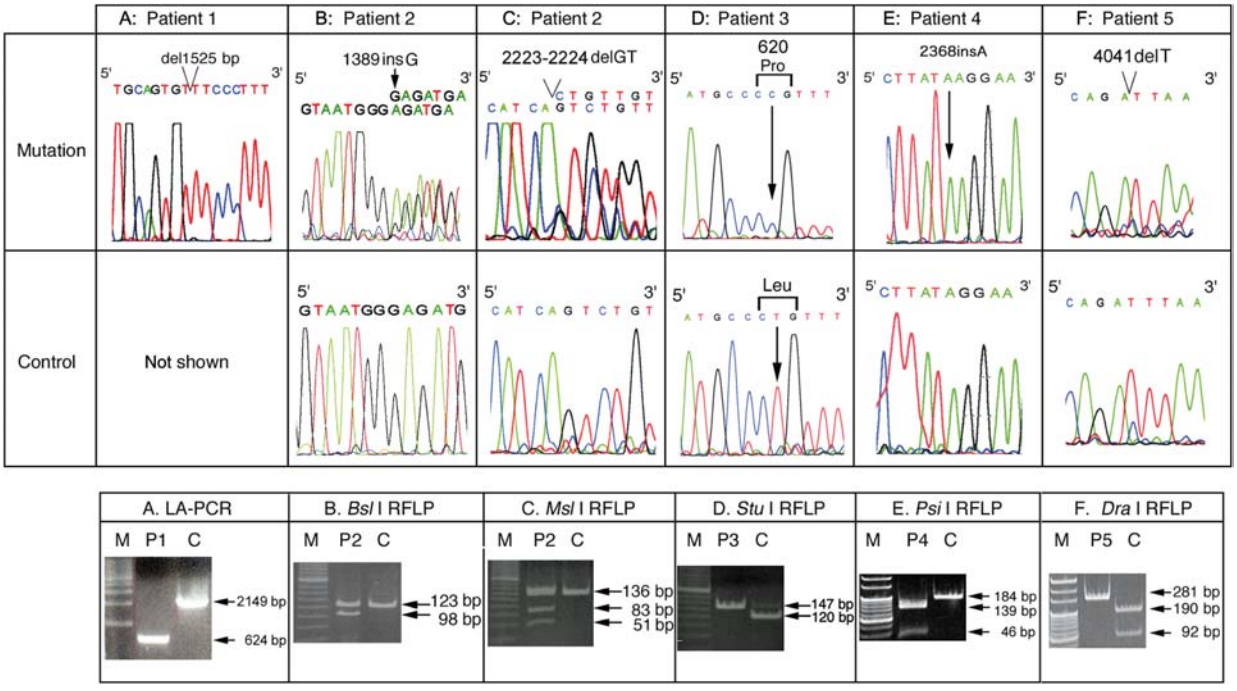

Figure 1 Identification of six novel *AGL* mutations.
Upper panel: Sequence electropherograms of patients 1–5, and those of a control. Lower panel: Mutational analysis. LA-PCR products were electrophoresed directly on agarose gel. In RFLP analyses, PCR products following digestion with restriction endonucleases were electrophoresed on polyacrylamide gels. M, DNA marker; P1–P5, patients 1–5; C, control. (A) Patient 1 had a shorter 624-bp fragment in LA-PCR around exon 27, showing that patient 1 was homozygous for the large deletion (c.3481–3588+1417del1525 bp). (B) In *Bsl*I RFLP analysis, patient 2 had both normal 123- and mutant 98-bp fragments, indicating that patient 2 was heterozygous for c.1389insG. (C) Patient 2 had normal 136- and mutant 83- and 51-bp fragments in *Msl*I RFLP analysis, verifying that patient 2 was also heterozygous for c.2223–2224delGT. (D) In *Stu*I RFLP analysis, patient 3 had an uncleaved 147-bp fragment, indicating that patient 3 was homozygous for p.L620P. (E) In *Psi*I RFLP analysis, patient 4 had mutant 139- and 46-bp fragments, showing that patient 4 was homozygous for c.2368insA. (F) Patient 5 had a mutant 281-bp fragment in *Dra*I RFLP, verifying that patient 5 was homozygous for c.4041delT.

amino acid residues. In order to examine aberrant splicing which could be caused by this deletion, RT-PCR using a pair of primers in exons 26 and 29 was performed. No PCR fragments were detected from patient 1, while the expected 516-bp fragment was obtained from a control sample (data not shown). A nucleotide homology search showed little homology between breakpoint flanking sequences. In addition, no *Alu* repetitive elements were found in intron 27 by the RepeatMasker software.

Sequence analysis of patient 2 showed an insertion of G at nucleotide 1389 in exon 12 (c.1389insG). Further sequencing analysis for patient 2 revealed a deletion of GT at nucleotides 2223 and 2224 in exon 18 (c.2223–2224delGT). Thus, patient 2 was a compound heterozygote for c.1389insG and c.2223–2224delGT.

Patient 3 was homozygous for a missense mutation in exon 15. Sequence analysis of patient 3 showed a T-to-C substitution at nucleotide 1859 in exon 15. This replaces leucine by proline at codon 620 (p.L620P). No mutations, except p.L620P, were found in patient 3. Comparison of amino acid sequences among glycogen debranching enzymes from various species showed that leucine at codon 620 was well preserved during evolution, as shown in Figure 3. In addition, leucine at codon 620 was located in the consensus sequence four in the α-amylase superfamily (21, 22), suggesting that this amino acid is important for enzyme activity.

Patient 4 had an insertion of A at nucleotide 2368 in exon 19 (c.2368insA). Patient 5 had a deletion of T at nucleotide 4041 in exon 31 (c.4041delT). Haplotype analysis of six mutant alleles demonstrated that each mutation was located on a different *AGL* haplotype (Table 2).

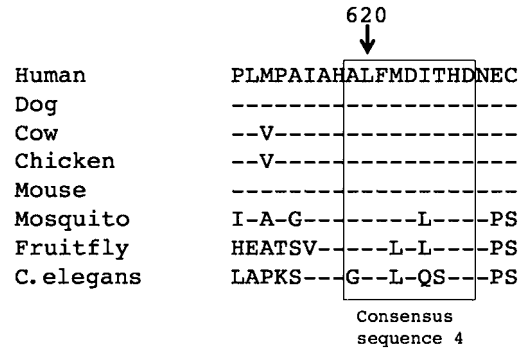

Figure 3 Comparison of amino acid sequences around the 620th amino acid of glycogen debranching enzymes from various species.
Identical amino acids are represented as – – –. An arrow indicates the human leucine at codon 620 and the consensus sequence four in the α-amylase superfamily are boxed. The species and the NCBI accession numbers are as follows: human (*Homo sapiens*), NP_000019; dog (*Canis familiaris*), XP_537057; cow (*Bos taurus*) predicted protein, XP_595566, chicken (*Gallus gallus*) predicted protein, XP_422317; mouse (*Mus musculus*), NP_001074795; mosquito (*Anopheles gambiae*), XP_321957; fruitfly (*Drosophila melanogaster*), NP_726062; *Caenorhabditis elegans*, hypothetical protein, NP_496984.

Clinical features and enzyme activities in patients are shown in Table 2. All patients had hepatomegaly and deficient enzyme activity. They did not show muscle weakness, which was consistent with the previous finding that muscle weakness is usually minimal during childhood in patients with GSD IIIa (1). Of note, patient 3 had residual enzyme activity and no episodes of hypoglycemia. This may be consistent with glucose release by glucosidase not being completely absent. A liver biopsy proved accumulation of glycogen and liver enzyme activities were increased in patient 3: alanine aminotransferase (ALT) 438 IU/L (normal range: 6–50 IU/L) and aspartate aminotransferase (AST) 363 IU/L (normal range: 11–38 IU/L).

Discussion

Our genetic analysis of five Egyptian patients revealed six novel *AGL* mutations, including the second large deletion and p.L620P. In addition, haplotype analysis demonstrated that each mutation was located on a different *AGL* haplotype. These findings support the notion that heterogeneous mutations are responsible for Egyptian GSD III patients.

A large deletion in *AGL* was identified in patient 1. The large deletion (c.3481–3588+1417del1525 bp) results in a missing 108-bp of 3′-part of exon 27, which could not alter the reading frame for amino acids, if the resulting exon 27 was spliced and combined with exon 28. However, RT-PCR did not detect any fragments from patient 1, suggesting that neither authentic nor cryptic splicing occurred and that there were no detectable *AGL* mRNA due to the deletion. Some of the large deletions were caused by *Alu* repetitive elements mediated recombination, as we described previously (23), but this was not the case in patient 1. In this patient, no repetitive elements were identified around the junction. Since there was little homology between breakpoint flanking sequences in exon 27 and intron 27, a mechanism for this large deletion could be non-homologous end-joining, which is thought to be responsible for deletions in genetic diseases (24–26). Very recently, another large deletion has been reported in an Egyptian patient (6). Thus, our case is the second large deletion in *AGL*.

Two novel small deletions (c.2223–2224delGT and c.4041delT) and two novel insertions (c.1389insG and c.2368insA) are predicted to lead to premature termination due to a frame shift which completely abolishes enzyme activity. These premature stop codons are likely to be recognized by non-sense-mediated mRNA-decay machinery, leading to absence of *AGL* mRNA.

A novel missense mutation, p.L620P, was found in patient 3. The leucine at codon 620 in glycogen debranching enzymes is well preserved among various species and was located in the consensus sequence four in the α-amylase superfamily. An amino acid substitution (corresponding to amino acids in exon 15 in the human *AGL*) results in loss of transferase activity, but glucosidase activity remains in a

Table 2 Clinical and genetic features in 5 Egyptian patients.

Patient	1	2	3	4	5
Clinical features					
Age, years/sex	4/Male	3/Male	4/Female	5/Female	9/Female
Hepatomegaly	+	+	+	+	+
Hypoglycemia	+	+	–	+	+
Muscle weakness	–	–	–	–	–
Enzyme activity[a]	0	0	0.05	0	0
Subtype	IIIa	IIIa	IIId	IIIa	IIIa
Consanguinity	–	–	+	+	+
Mutation	Large deletion	Insertion, deletion	Missense	Insertion	Deletion
Mutation 1[b]	c.3481–3588+1417del1525 bp	c.1389insG	c.1859T>C (p.L620P)	c.2368insA	c.4041delT
Mutation 2		c.2223–2224delGT			
	Homozygote	Compound heterozygote	Homozygote	Homozygote	Homozygote
Exon	27	12, 18	15	19	31
SNPs					
–1246	aa	aa	aa	aa	cc
–787	aa	aa	aa	aa	aa
–10 in exon 3	gg	ag	aa	gg	gg
IVS3+85	tt	tt	cc	tt	tt
–2 in Ex4′	cc	ct	cc	cc	cc
IVS4–122	tt	tt	tt	tt	tt
IVS5–53	cc	cc	gg	cc	cc
IVS6–73	gg	ag	aa	gg	gg
L298L	tt	tt	cc	tt	tt
IVS8–18	aa	aa	gg	aa	aa
R387Q	RR	RR	RR	RR	RR
IVS10+15	tt	tt	tt	tt	cc
IVS12+74	tt	cc	cc	tt	tt
IVS12–125	aa	at	tt	aa	aa
IVS12–44	aa	aa	aa	gg	gg
IVS13–70	cc	cg	cc	gg	gg
IVS14+38	gg	gg	gg	gg	gg
IVS16+8	cc	ct	tt	cc	cc
IVS18+215	cc	cg	cc	cc	cc
IVS21+124	aa	ag	gg	aa	aa
IVS22+11	aa	gg	gg	aa	gg
IVS23–121	gg	ag	aa	gg	gg
IVS23–21	aa	at	tt	aa	aa
P1067S	PP	PP	PP	PP	PP
G1115R	GG	GG	GG	GG	GG
R1253H	RR	RR	RR	RR	RR
IVS29+45	gg	ag	gg	aa	gg
IVS29+53	tt	at	tt	aa	tt
EI343K	EE	EE	EE	EE	EE
IVS32–97	gg	ag	gg	aa	gg

[a]mmol/min/gHb, normal range: 0.6–3.5. [b]Nucleotide numbering starts at the initiation codon.

yeast glycogen debranching enzyme (22). Furthermore, we expressed p.L620P mutant and normal *AGL* proteins in COS cells transiently, and measured both glucosidase and transferase activities. The p.L620P exhibited complete loss of transferase activity, but retained glucosidase activity compared with wild type *AGL* (27). Thus, p.L620P was responsible for GSD IIId in patient 3.

According to the available literature, GSD IIId patients are very rare and only one mutation has been reported: a 4-bp deletion in exon 10 (28). This deletion was probably associated with skipping of the following exon (98 bp) in the mRNA level, leading to an in-frame mutation with loss of the corresponding 34 amino acids in exon 11. However, this was not verified with in vitro expression analysis. An amino acid homology search predicted that a putative active site for enzyme activity was located in exons 6, 13, 14, and 15 (5). Presumably, missense mutations or in-frame deletions near these exons are more likely to cause GSD IIId, but this needs to be verified by further studies. Accordingly, p.L620P is the second mutation associated with GSD IIId.

In summary, we identified six novel *AGL* mutations, including the second case of the large deletion in *AGL* and the second mutation in GSD IIId, and showed allelic and phenotypic heterogeneity in Egypt.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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