

A founder splice site mutation underlies glycogen storage disease type 3 in consanguineous Saudi families

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BACKGROUND AND OBJECTIVES: Glycogen storage disease type 3 (GSD III) is an autosomal recessive disorder caused by genetic mutations in the gene *AGL*. *AGL* encodes amylo- α -1, 6-glucosidase, 4- α -glucanotransferase, a glycogen debranching enzyme. GSD III is characterized by fasting hypoglycemia, hepatomegaly, growth retardation, progressive myopathy, and cardiomyopathy due to storage of abnormally structured glycogen in both skeletal and cardiac muscles and/or liver. The aim of this study is to detect mutations underlying GSD III in Saudi patients.

DESIGN AND SETTINGS: A cross-sectional clinical genetic study of 5 Saudi consanguineous families examined at the metabolic clinic of the Madinah Maternity and Children Hospital.

PATIENTS AND METHODS: We present a biochemical and molecular analysis of 5 consanguineous Saudi families with GSD III. DNA was isolated from the peripheral blood of 31 individuals, including 12 patients, and the *AGL* gene was sequenced bidirectionally. DNA sequences were compared with the *AGL* reference sequence from the ensemble genome browser.

RESULTS: Genotyping and sequence analysis identified a homozygous intronic splice acceptor site mutation (IVS32-12A>G) in 4 families perfectly segregating with the phenotype. Complementary (c)DNA sequence analysis of the *AGL* gene revealed an 11-bp sequence insertion between exon 32 and exon 33 due to the creation of a new 3' splice site. The predicted mutant enzyme was truncated by 112 carboxyl-terminal amino acids as a result of premature termination.

CONCLUSION: Haplotype analysis revealed that the mutation arises as a result of founder effect, not an independent event. This is the first report of a genetic mutation in the *AGL* gene from Saudi Arabia. Screening for this mutation can improve genetic counseling and prenatal diagnosis of GSD III in Saudi Arabia.

Glycogen storage diseases (GSDs) comprise at least 16 different inherited conditions caused by genetic mutations in the genes encoding enzymes that regulate the synthesis or degradation of glycogen. Glycogen storage disease type 3 (GSD III) is an autosomal recessive metabolic disorder characterized by fasting hypoglycemia, hepatomegaly with increased transaminases, growth retardation, progressive myopathy, and cardiomyopathy with elevated creatine phosphokinase.¹ GSD III has 2 major subtypes: GSD IIIa and GSD IIIb. GSD IIIa affects both the liver and

muscles and accounts for 80% of GSD III cases, while GSD IIIb affects only the liver; the patient does not show any muscular manifestation. GSD IIIb accounts for approximately 15% of GSD III cases.² During childhood, it is difficult to differentiate between GSD IIIa and GSD IIIb, as the muscular manifestation starts together with liver disorders, or muscular symptoms arise in adulthood because of the progressive accumulation of glycogen in the muscles.^{3,4} There is no specific treatment for GSD, and GSD III patients require lifelong follow-up.⁵ Diet therapy improves symptoms (especial-

ly hypoglycemia), reduces the liver size, and assists with the resumption of overall growth and development.⁶

GSD III results from genetic mutations in the *AGL* gene. The *AGL* gene is located on chromosome 1p21.2, spanning 85 kb, and consists of 35 exons (UCSC genome browser). The *AGL* gene encodes amylo- α -1, 6-glucosidase, 4- α -glucanotransferase, a glycogen debranching enzyme (GDE) of 1515 amino acids in length. The GDE has 2 catalytic domains performing 2 independent catalytic activities: oligo1,4-glucotransferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33). Both catalytic sites are required for a complete action of the debranching enzyme.⁷ GDE is expressed mainly in the liver and muscles of the heart and skeleton. GDE deficiency causes an excessive accumulation of abnormal glycogen and results in the onset of GSD III. Homozygous knockout mice for GDE recapitulate the human GSD III phenotype, and it has been shown that repeated fasting is detrimental to these mice.⁸

Molecular studies have confirmed that GSD III is genetically highly heterogeneous with a large spectrum of mutations in the same population.^{4,9,10} Recurrent mutations have also been reported in populations with high consanguinity.¹¹

In Saudi Arabia, the rate of consanguinity is 56%, which contributes greatly to the high frequency of autosomal recessive disorders in the country,¹² but the prevalence of *AGL* mutations in GSD III is still unknown. We located 5 Saudi families with GSD III and performed a molecular study by screening the *AGL* gene in all affected and unaffected members of the families. We identified a homozygous splice site mutation in the *AGL* gene in 4 families. Complementary (c)DNA sequencing revealed an 11-bps insertion in the patients' messenger (m)RNA. Haplotype analysis identified that the splice site mutation is due to the founder effect and not an independent event.

PATIENTS AND METHODS

Families

In the present study, 5 consanguineous Saudi families (A, B, C, D, and E) originating from Madinah in western Saudi Arabia were investigated. Thirty-one individuals, including 12 affected, volunteered to participate in the study (Figure 1). The age of onset of the disease was 9 months to 3 years. Prior to the start of the study, approval was obtained from the ethical committee of Madinah Maternity and Children Hospital. Informed consent was obtained from all subjects participating in the study. All affected individuals showed typical features of autosomal recessive GSD IIIb with no other abnormalities,

and their growth conditions were normal.

Genomic DNA and Total RNA Isolation

Blood samples for DNA and RNA isolation were collected in ethylenediaminetetraacetic acid (EDTA)-containing Vacutainer sets (BD, Franklin, NJ, USA) and Tempus Blood RNA tubes, respectively (Applied Biosystems, Foster City, CA, USA) from both affected and unaffected individuals of the 5 families. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA). Total RNA was extracted using Tempus Spin RNA Isolation Kit (Applied Biosystems). To quantify DNA and RNA, a MaestroNano spectrophotometer (Green BioResearch, Los Angeles, CA, USA) was used, measuring optical density at 260 nm and diluted to 40–50 ng/ μ L for amplification by polymerase chain reaction (PCR). PCR products were resolved on 2% agarose gel stained with ethidium bromide.

Screening the *AGL* gene

It has been reported that mutations in the *AGL* gene result in GSD III. Therefore, all coding exons and adjacent sequences of intron–exon borders were amplified by PCR using gene-specific primers. PCR primers were designed using the Primer3 program (<http://frodo.wi.mit.edu/primer3>) and checked for specificity using a basic local alignment search tool (<http://genome.ucsc.edu/cgi-bin/hgBlat>). The amplification conditions used were 95°C for 1 minute, followed by 30 cycles of 95°C for 35 seconds, 60°C for 35 seconds, and 70°C for 3.5 minutes,

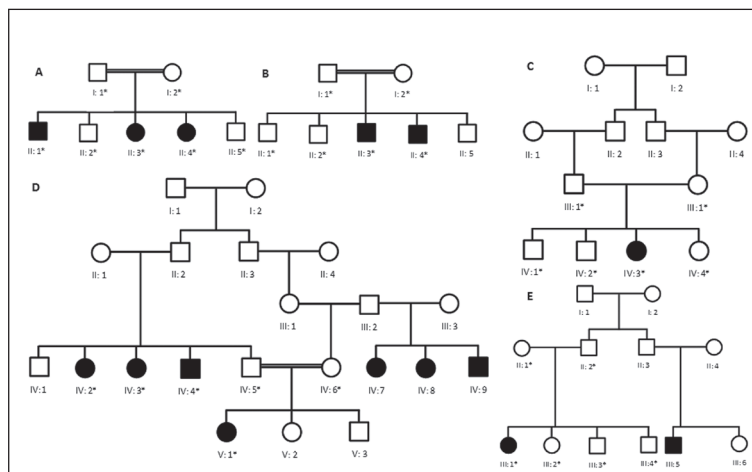


Figure 1. Pedigrees of five Saudi families segregating GSD III. Double lines are indicative of consanguineous union. Clear symbols represent unaffected individuals while filled symbols represent affected individuals. Symbols with asterisks indicate individuals who were clinically examined and for whom DNA samples were available for molecular analysis.

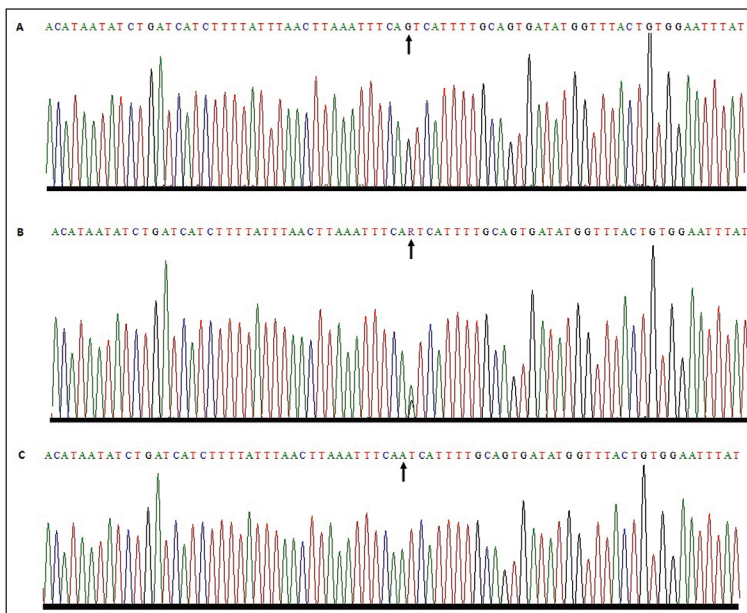


Figure 2. Sequence analysis of the splice site mutation identified in 4 families (A, C, D, E). The upper panel (A) represents the nucleotide sequences in the affected individuals, the middle panel (B) in the heterozygous carriers and the lower panel (C) in the affected individuals. Arrows in the panels indicate the position of the nucleotide change.

followed by a single incubation at 70°C for 10 minutes. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced bidirectionally following dideoxy chain termination chemistry using BigDye Terminator v3.1 Cycle Sequencing Kit on AB 3500 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions.

cDNA sequencing

cDNA was synthesized from total RNA using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA). Reverse transcription (RT) was carried out with 200 ng of total RNA. Hybridization of the oligo(dT) was realized by incubating the following mix for 5 minutes at 70°C: 3 µL of RNA; 2 µL of polyT oligo primers (dT) (10 mM, New England Biolabs); and 3 µL of H₂O (RNase free), followed by ice quenching. RT was then carried out for 60 minutes at 42°C after the addition of 2 µL of M-MuLV enzyme mix and 10 µL of M-MuLV reaction mix (New England Biolabs). For the subsequent PCR, 5 µL of the obtained cDNA mix was used.

Primers were designed to amplify exons 30–33 from cDNA to characterize the consequences of the splice acceptor site mutation. The reversed transcribed RNA was amplified with these primers (40 cycles) at a hybridization temperature of 57°C. Samples were then

sequenced on an ABI 3500 DNA sequencer (Applied Biosystems).

Genotyping with microsatellite markers

Four microsatellite markers (D1S2671, D1S1658, D1S2767, and D1S2896) flanking the AGL gene on chromosome 1 were used to trace the haplotype surrounding the AGL gene in all 5 families. PCR amplification conditions for microsatellite markers were the same as described earlier by Basit et al.¹³ The allele size for the respective microsatellite markers were determined using 05 bp, 10 bp, and 20 bp DNA ladders (MBI, Fermentas, York, UK).

RESULTS

Patients

The index case is a 9-year-old female (Figure 1A, II: 3). She was discovered and diagnosed as a GSD III patient at the age of 1 year by screening for hepatomegaly. Laboratory findings revealed fasting hypoglycemia and elevated transaminase (creatine phosphokinase 436 IU/L, alkaline phosphatase 409 IU/L). The lipid level was in the normal range (cholesterol: 3.9 mmol/L, triglycerides: 1.79 mmol/L). Hepatomegaly was confirmed with abdominal ultrasound. Cardiomyopathy was ruled out by echocardiography at the age of 8 months. Hypoglycemia was controlled using corn starch.

Affected individuals in all 5 families exhibited features of GSD IIIb. The ages of the affected members ranged from 2–50 years at the time of the study. Table 1 shows the clinical and biochemical findings of the affected individuals of the families. Mostly similar clinical features were observed in the affected members of all 5 families (Table 1). Affected individuals had no clinical and biochemical evidence of muscle involvement.

Sequencing the AGL gene in affected and normal individuals

The AGL gene located on chromosome 1p21.2 is the only gene known to be responsible for generating the GSD III phenotype when mutated. To search for the underlying mutation, all 35 exons of the AGL gene and their splice junctions were PCR amplified from the genomic DNA of affected and unaffected individuals of the family, using primers designed from intronic sequences of the gene. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and were sequenced in an ABI 3500 automated DNA sequencer using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster city, CA, USA). Sequence analysis of the PCR-amplified products revealed a homozygous splice acceptor site

Table 1. Clinical and biochemical features of GSD III patients.

Family Number	Age/Gender	Disease onset	Hypoglycemia	Hepatomegaly	Transaminases ALT/AST	CK
A, II: 1	5 Yr/M	3 Yr	Yes	Yes	99/108	834
A, II: 3	9 Yr/F	1 Yr	Yes	Yes	463/359	436
A, II: 4	11 Yr/F	3 Yr	Yes	Yes	54/78	–
B, II: 3	12 Yr/M	9 Mo	No	Yes	141/104	–
B, II: 4	8 Yr/M	9 Mo	No	Yes	202/152	232
C, IV: 3	2 Yr/F	1 Yr	No	Yes	355/487	61
D, IV: 2	36 Yr/F	2 Yr	Yes	Yes	–	–
D, IV: 3	42 Yr/F	3 Yr	Yes	Yes	768/877	3,120
D, IV: 4	44 Yr/M	2 Yr	Yes	Yes	–	–
D, IV: 7	44 Yr/F	3 Yr	Yes	Yes	–	–
D, IV: 8	46 Yr/F	2 Yr	Yes	Yes	–	–
D, IV: 9	50 Yr/M	4 Yr	Yes	Yes	657/766	3,420
D, V: 1	11 Yr/F	2 Yr	Yes	Yes	212/190	920
E, III: 1	4 Yr/F	1 Yr	Yes	Yes	–	–
E, III: 5	11 Yr/F	2 Yr	Yes	Yes	212/190	920

Notes: Five consanguineous Saudi families (A, B, C, D, and E) originating from Madinah in western Saudi Arabia were investigated. Abbreviations: GSD III: glycogen storage disease type 3; ALT: alanine transaminase; AST: aspartate transaminase; CK: creatine kinase; M: male; Yr: years; Mo: months.

mutation (VS32-12A>G) in all affected individuals of 4 families (A, C, D, and E). This mutation is present in a heterozygous state in parents (Figure 2). In family B, sequence analysis failed to detect any pathogenic variant that could be responsible for the GSD III phenotype.

Sequencing of cDNA of the AGL gene in affected and normal individuals

Amplification of exons 30 to 33 from the cDNA of affected individuals of 4 families (A, C, D, and E) was carried out using primers designed in the coding part of the AGL gene. Sequencing followed by alignment with the reference sequence revealed an insertion of 11 bps in the mRNA sequence of the AGL gene in affected individuals (Figure 2).

Genotyping and haplotype construction

Genotyping with 4 microsatellite markers flanking the AGL gene was carried out. Genotyping data and haplotype analysis revealed a similar homozygous pattern of the allele and an identical haplotype surrounding the AGL mutation in the affected individuals of 4 families (A, C, D, and E). Heterozygous haplotypes were obtained for the affected individuals of family B.

DISCUSSION

The diagnosis of GSD III requires the combination of clinical, biochemical, and molecular investigations. The broad spectrum of mutations in the AGL gene underlying GSD III and the large size of the AGL gene makes genetic diagnosis difficult. The genetic study of a specific population would help to identify inherited mutations due to the founder effect, generation after generation. We started to screen for the AGL gene in GSD III patients in Saudi Arabia to identify specific genetic variants common to this population.

In this study, we investigated 5 families with 16 GSD III patients. Most of them have manifested fasting hypoglycemia and/or hepatomegaly. Cardiomyopathy was excluded in most of the patients with echocardiography. Clinical and biochemical data of the patients presented here rule out muscular manifestations and thus confirm that these patients have GSD IIIb.

The homozygous IVS32-12A>G mutation found in 4 families segregating GSD IIIb has been previously reported in the Japanese population.³ The A to G transition mutation (IVS32-12A>G) found in our families gave rise to a new 3' splice site, and an 11-bp intronic sequence was inserted between exons 32 and 33 in the AGL mRNA (Figure 3). This is predicted to result in a

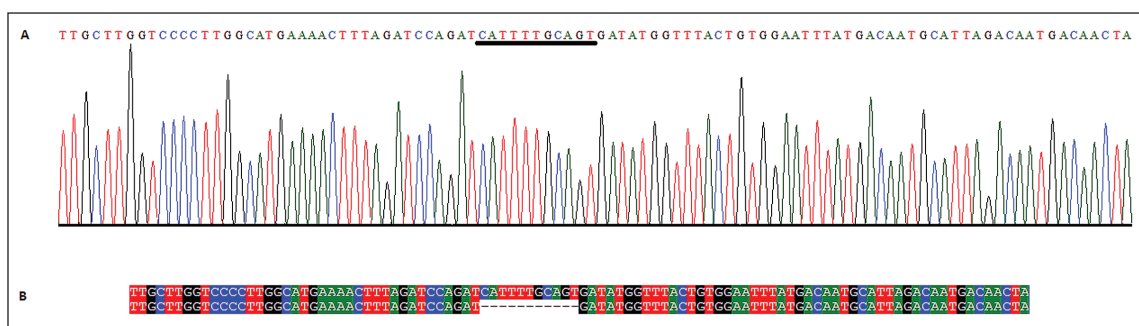


Figure 3. Sequence analysis of the splice site mutation using mRNA as a template. The upper panel (A) shows sequence chromatogram obtained by sequencing cDNA of the *AGL* gene. 11-bps insertion is underlined. The lower panel (B) shows cDNA sequence of the patient aligned with reference sequence.

truncated enzyme with the loss of 112 carboxyl-terminal amino acids due to premature termination. Genotyping with microsatellite markers and haplotype construction demonstrated that the splice acceptor site mutation IVS32-12A>G in 4 families (A, C, D, and E) appeared on very similar haplotypes, suggesting that the mutation in these 4 families was due to a single mutation event.

This is the first report of a mutation in GSD III patients from Saudi Arabia. The mutation was not detected in 70 control chromosomes. Nonetheless, with the high rate of consanguinity in Saudi Arabia, screening for this mutation can improve the molecular diagnosis of GSD III in the relatives of these and other GSD III families.

It was intriguing that in family B, the sequence analysis failed to detect any pathogenic variants in the entire *AGL* gene that could be responsible for the GSD III phenotype. The haplotype analysis showed heterozygous alleles for all 4 microsatellite markers surrounding the *AGL* gene, suggesting that the phenotype in family B is not linked with the *AGL* gene, thus ruling out the involvement of the *AGL* gene in

the disease pathogenesis in family B.

Several mutations in the *AGL* gene have been identified so far.¹⁴⁻¹⁸ It has been postulated that the mutations in exon 3 can give rise to GSD IIIb, and that GSD IIIa could be distinguished from GSD IIIb by analysis of exon 3.1. Our report, and the previous report of Okubu et al³ provide evidence that mutations in exons other than exon 3 could be responsible for the GSD IIIb phenotype.

In summary, we have identified the molecular basis of GSD III in 4 Saudi families without liver or muscle biopsies. The mutation identified will be useful for carrier detection and prenatal diagnosis of the disease.

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