Short Report

A c.3216_3217delGA mutation in AGL gene in Tunisian patients with a glycogen storage disease type III: evidence of a founder effect


Glycogen storage disease type III (GSD III) is an autosomal recessive disorder characterized by excessive accumulation of abnormal glycogen in the liver and muscles and caused by deficiency in the glycogen debranching enzyme, the amylo-1,6-glucosidase (AGL). In this study, we report the clinical, biochemical and genotyping features of five unrelated GSD III patients coming from the same region in Tunisia. The concentration of erythrocyte glycogen and AGL activity were measured by colorimetric and fluorimetric methods, respectively. Four CA/TG microsatellite markers flanking the AGL gene in chromosome 1 were amplified with fluoresceinated primers. The full coding exons and their relevant exon–intron boundaries of the AGL gene were directly sequenced for the patients and their parents. All patients showed a striking increase of erythrocytes glycogen content. No AGL activity was detected in peripheral leukocytes. Sequencing of the AGL gene identified a c.3216_3217delGA (p.Glu1072AspfsX36) mutation in the five patients which leads to a premature termination, abolishing the AGL activity. Haplotype analysis showed that the mutation was associated with a common homozygote haplotype. Our results suggested the existence of a founder effect responsible for GSD III in this region of Tunisia.

Conflict of interest
No competing financial interests exist and the authors declare the absence of conflict for each author.

Key words: AGL gene – amylo-1,6-glucosidase – founder effect – glycogen storage disease type III – haplotype study – mutation – Tunisian patients

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Glycogen storage disease type III (GSD III; MIM#232400) is an autosomal recessive inherited disorder characterized by fasting hypoglycemia, growth retardation, hepatomegaly, progressive myopathy, and cardiomyopathy (1). GSD III is caused by deficiency in the glycogen debranching enzyme; the amylo-1,6-glucosidase (AGL) which is indispensable in the glycogen degradation (1). AGL enzyme has two independent catalytic activities, oligo-1,4-glucantransferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33). The human AGL gene has been isolated since 1996, and has shown to be 85 kb in length (2). It is composed of 35 exons encoding a 7 kb mRNA. A lot of tissue-specific isoforms of AGL mRNAs, differing only in the 5-end, have been described. The predominant form, which is liver AGL, encodes for a 1532 amino acids protein (3).

Molecular analysis of GSD III has been performed in several ethnic populations (4) and over 70 different AGL mutations have been reported to date (Human Gene Mutation Database; http://www.hgmd.org). A number of these mutations are specific for some ethnic groups, such as 4455delT mutation in Jewish patients and R408X mutation in the Faeroe Islands (5–9). This
is generally due to the presence of a founder effect in these populations, confirmed by haplotype analysis. In the remaining cases, the causal mutations are very heterogeneous (4, 10, 11).

In this work, we studied five unrelated GSD III patients from the same region of Tunisia. We identified a p.Glu1072AspfsX36 deletion in the AGL gene associated with the same haplotype, which is in favor of a common ancestor.

Patients and methods

Studied population

Five Tunisian GSD III patients from five unrelated consanguineous families were investigated. They come from the same geographic region: it’s the region of ‘Kalaa Khira’ situated in the town of Sousse center-east of Tunisia. They were referred to us from biochemistry and pediatrics departments. Clinical data such as sex, age and liver dysfunction symptoms were obtained from patients. Their clinical and biochemical features are in favor of GSD III disease (Table 1). Blood samples were collected for measurement of glycemia, creatine phosphokinase and transaminases rates. The parents and sibs had no GSD III features. The study was performed on the patients and their families. Clear informed consent was obtained from all of them after a full explanation of the procedure undertaken.

Methods

Glycogen content of erythrocytes

Erythrocytes from 10 ml heparinized blood were washed three times in NaCl (0.155 mol/l). Glycogen concentration was measured in erythrocytes by colorimetric method (12). Briefly, after blood cells lysis, the suspension was homogenized with 5% cold trichloroacetic acid and centrifuged. The glycogen was dialyzed from the supernatant and its concentration was determined by the anthrone method (13).

Measurement of amylo1,6-glucosidase activity

The measurement of AGL activity in leukocytes was carried out as already described (14, 15). Briefly, the total leukocyte samples were prepared by polyvinyl pyrolidone sedimentation (16). Leukocyte debranching enzyme was determined fluorimetrically by coupling the production of glucose from ‘phosphorylase limit dextrin’ to the hexokinase and glucose S-phosphate dehydrogenates reactions. The formation of NADPH was measured continuously by a fluorimeter in 440 and 460 nm fluorescent light.

DNA extraction and single tandem repeat (STR) analysis

Genomic DNA was extracted from blood lymphocytes of patients and their relatives using WIZARD® Genomic DNA Purification Kit (Promega, Madison, WI 53711-5399, USA). Four CA/TG microsatellite markers including AmG24AC, D1S2671, D1S1658 and AmG29AC, flanking the AGL gene in chromosome 1, were amplified with universal fluorescent primers (17). The PCR was carried out in a total volume of 25 μl, containing 150 ng genomic DNA, 5 μl PCR buffer, 1.5 mM MgCl₂, 20 mM primer pair mix and 1 U AmpliTaq DNA polymerase (Promega). PCR conditions were a pre-incubation step at 95°C for 7 min, followed by 35 cycles of denaturation in 94°C for 30 s, annealing in 58°C for 30 s and extension in 72°C for 30 s, and a final extension in 72°C for 30 min. PCR products were electrophoresed on the ABI Prism Genetic Analyzer 310 (Applied Biosystems, USA). Obtained data were analyzed using the Genescan and Genotyper software (Applied Biosystems). The primer sequences were: AmG24AC (F: 5’-TCTTTCATCAAGATGTATAACAATATAAAG-3’, R: 5’-TTTTTCTCCCTCCCAGATT-3’), D1S2671 (F: 5’-TGAAGATCAACTACCCAAAGAA-3’, R: 5’-CTCTGC TTGCAGTCCTCA-3’), D1S1658 (F: 5’-GCCATGTCTATA TTAATTAGAGTGC-3’, R: 5’-TGGTCCGAGACTGCTG AA TATA-3’) and AmG29AC (F: 5’-CTGAGGTGGCA GGATCACTT-3’, R: 5’-TCTCCTGGGGGTGTGTTGTA T-3’). Microsatellite marker information and single nucleotide polymorphisms (SNP) data were available at UCSC Genome Database.

Sixteen SNPs in AGL gene were determined, as described previously (18). SNPs numbers were cited according to database of SNPs (dbSNP Build 130) available from http://www.ncbi.nlm.nih.gov/SNP/.

DNA sequencing

Genetic analysis of all exons and intron boundaries of the AGL gene was performed in all patients and their relatives. PCR was performed in a total volume of 25 μl, containing 1.5 mM MgCl₂ and 1 U Recombinant Taq DNA polymerase (Invitrogen). The temperature was programmed as follows: a pre-incubation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, and a final extension step at 72°C for 7 min. The amplified DNA was purified by Charge Switch®PCR Clean-Up Kit (Invitrogen, Carlsbad, CA, USA) and sequenced with the Big Dye Terminator Cycle Sequencing Kit. After a cleaning step by Wizard®MagneSil™ Sequencing Reaction Clean-Up System Kit (Promega, USA), the samples were electrophoresed on the ABI PRISM 310 genetic analyzer. Sequencing data were analyzed by SeqScape 2.0 software (Applied Biosystems). To confirm mutations, a second independent PCR amplification was performed and all sequencing reactions were carried out using the forward and the reverse primers. Sequence variants were designated according to Human Genome Variation Society recommendations (www.hgvs.org/rec.html) using the reference sequences (GenBank accession no. NM_000642).

Bioinformatical analysis

Alignment of amino acid sequences from various species was performed using ClustalW (http://www.ncbi.nlm.nih.gov/blast/).
Results

In our patients, the diagnosis of glycogenosis disease has been suspected by clinical features essentially the presence of hepatomegaly and hypoglycemia symptoms. Routine biochemical tests confirmed the hypoglycemia and showed elevated serum triglyceride, transaminase and with normal rates of uric acid and lactate. Glycogen content in the erythrocytes, after fasting, was elevated and no amylo-1,6-glucosidase activity was detected in peripheral leukocytes confirming the GSD III disease (Table 1).

A total of four microsatellite markers flanking the AGL gene were tested within the five families. Their segregation showed a common homozygote haplotype and the mutation found was always associated with this specific haplotype (Fig. 1). Direct sequencing of AGL gene found in all patients a small deletion of two nucleotides GA at position 3216–3217 in exon 25 it’s a c.3216_3217delGA mutation (p.Glu1072AspfsX36) (Fig. 2). We verified that this mutation was not found in 50 normal Tunisian controls (100 chromosomes). The sequencing of this exon for all other members of the five families showed a perfect segregation of the mutation with the disease (Fig. 1).

Moreover, comparison of amino acid sequences among various species showed that Glutamic acid and Isoleucine at codon 1072 and 1073 were well preserved during evolution (Fig. 3).

Discussion

In our study, we report five patients belonging to different consanguineous families from the region of Kalaa-Kbira (Sousse, Tunisia). They exhibited typical clinical and biochemical features of GSD III disease with large increase of transaminases [alanine aminotransferase (ALT) = 610.5 IU (normal range: 5–32) and aspartate aminotransferase (AST) = 450.5 IU (normal range: 7–35)], hypertriglyceridemia and hypoglycemia associated with normal rates of uric acid and lactate. However, these features could be associated to other GSD types; mainly GSD I. Nevertheless, the elevated glycogen content detected in erythrocytes as well as the absence of the AGL activity in leukocytes confirmed GSD III diagnosis (19).

The genotyping with four STR markers closely flanking the AGL gene allowed us to find a common haplotype for all the patients which suggested the existence of a founder effect in their region. Direct sequencing of AGL gene showed a homozogous deletion of the two nucleotides ‘GA’ at 3216 and 3217 positions in exon 25 (20). The parents of the patients were heterozygous for the mutation. Direct sequencing of the other members of the families showed a perfect segregation of the mutation with the disease. The deletion of these GA nucleotides in AGL gene is predicted leading to a premature stop in codon 1107 that drastically affects normal protein function leading to a severe phenotype that will probably be recognized as nonsense mediated decay. Moreover, Glutamic acid (at codon 1072) and

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**Table 1. Clinical and biochemical features of patients**

<table>
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<tr>
<th>Age at the time of diagnosis (months)</th>
<th>Sex</th>
<th>Hepatomegaly</th>
<th>Hypoglycemia</th>
<th>CPK &lt;185 IU/l</th>
<th>ALT/AST (10–60) IU/l</th>
<th>Triglyceride &lt;200 mg/dl</th>
<th>Cholesterol &lt;200 mg/dl</th>
<th>Lactic acid &lt;7.8 meq/l</th>
<th>Glycogen in Erythrocytes &lt;200 μg/ghb</th>
<th>Debranching enzyme activity in leukocytes (0.3–1.3 nmoles of glucose/min X mg protein)</th>
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<td>Yes</td>
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A c.3216_3217delGA mutation in AGL gene in Tunisian patients

Fig. 1. Pedigree of a Tunisian family affected by glycogen storage disease type III. AMG24AC, D1S2671, D1S1658 and AMG29AC represented the four microsatellite markers flanking the AGL gene. Their segregation showed a common homozygote haplotype (showed in a box). The c.3216_3217delGA mutation of the AGL gene was marked in red color.

Fig. 2. Electropherograms of exon 25 of the AGL gene: (a) normal subject, (b) patients with the 3216_3217delGA mutation, and (c) parent of patient (b). The position of the deleted sequence is indicated by arrows in (b).

Isoleucine (at codon 1073) are conserved amino acids across species (Fig. 3).

As it is a mutation that arose in a common ancestor and is found in unrelated individuals who share the same haplotype; it may have arisen many generations ago. Mutation carriers often live in same isolated geographic region. Such founder mutation is of great interest for genetic counseling of GSD. In fact, genotyping study can be used in the screening of AGL gene deficiency before targeting the causative mutation by direct sequencing.

Concerning clinical features, we were not able to observe a robust phenotype–genotype relationship. Although, all patients have the same mutation, the clinical features were different. This clinical heterogeneity could be explained by the variability of
the age at diagnosis in the studied patients. Moreover, it is well known that a suitable dietary regimen subsequent to an early diagnosis is very important because keeping hypoglycemic episodes under control and providing a protein-rich diet might be beneficial to patients (21).

Dependent on ethnic groups, the spectrum of AGL mutations in GSD III varies. This is the second mutation described in Tunisian patients. Previously, Lucchiari et al. reported a homozygous W1327X substitution located in exon 31 of AGL gene in Tunisian GSD III patient living in Italy (11, 22) which also have been reported in Egyptian patients (18).

Several studies revealed that the spectrum of some AGL mutations depends on ethnic groups. For example, the 4455delT AGL mutation is prevalent among the North African Jewish patients (23) and the R408X in the Faeroe Islands (9). Information on prevalent mutations would improve molecular diagnosis of GSD III in these populations and genetic counseling. However, in other ethnic groups, genetic heterogeneity has been widely reported (24). In Japan, 11 different mutations have been described (10). The same distribution has been explained in Caucasian population (25). Recently, using restriction fragment length polymorphism analysis, Aoyama et al. revealed 10 different mutations with five novel nonsense mutations (p.W373X, p.R595X, p.Q667X, p.Q1205X and p.Q1376X) in Turkish patients (26).

Our study confirmed previous reports by showing that haplotype analysis is a useful method for the first screening of patients coming from already characterized regions. Indeed, AGL is a big gene (35 exons) and other mutations responsible for GSD III were detected in center-east of Tunisia (data not shown). So haplotype analysis will help us in screening for mutations in this gene.

High frequency of a founder mutation for a serious disease in the general population could have an impact on medical care and genetic counseling.

Conclusion

In summary, molecular defects in five GSD III patients, coming from the same Tunisian region, have been characterized. The novel c.3216_3217delGA mutation (p.Glu1072AspfsX36) of AGL gene have been identified by direct sequencing and confirmed by haplotype analysis providing evidence for the existence of a founder effect in this region. The discovery of this founder mutation in ‘kalaa-Khira’ emphasizes the need to make a screening test for this mutation in every Tunisian individual at risk from this region. This test can be based on haplotype analysis and direct sequencing that improve the genetic counseling for GSD type III in Tunisia.

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References

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