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Molecular and clinical delineation of 12 patients with glycogen storage disease type III in Western Turkey

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ABSTRACT

Background: 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. GSD III is caused by deficiency in the glycogen debranching enzyme (gene symbol: *AGL*). Molecular analyses of *AGL* have indicated heterogeneity depending on ethnic groups. In Turkey we reported 13 different *AGL* mutations from GSD III patients in the Eastern region; however, the full spectrum of *AGL* mutations in Turkish population remains unclear. Here we investigated 12 GSD III patients mostly from Western Turkey.

Methods: The full coding exons, their relevant exon–intron boundaries, and the 5′- and 3′-flanking regions of the patients' *AGL* were sequenced. *AGL* haplotypes were determined. Splicing mutations were characterized by RNA transcript analysis.

Results: Twelve different mutations were identified: 7 novel *AGL* mutations [69-base pair deletion (c.1056_1082+42del69), 21-base par deletion (c.3940_3949+11del21), two small duplications (c.364_365dupCT and c.1497_1500dupAGAG), and 3 splicing mutations (c.1736-11A>G, c.3259+1G>A and c.3588+2T>G)], along with 5 known mutations (c.1019delA, c.958+1G>A, c.4161+5G>A, p.R864X and p.R1218X). Transcripts of splicing mutations (c.1736-11A>G, c.3588+2T>G and c.4161+5G>A) were shown to cause aberrant splicing. *AGL* haplotype analyses suggested that c.1019delA and c.958+1G>A are founder mutations in Turkish patients, while p.R864X is a recurrent mutation.

Conclusions: Our study broadens the spectrum of *AGL* mutations and demonstrates that mutations in Western Turkey are different from those in the Eastern region.

1. Introduction

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 [1–3]. GSD III is caused by a deficiency in the glycogen debranching enzyme, which 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). Both activities are absent in the liver and muscles in most patients with GSD III, which is named as subtype IIIa. Approximately 15% of the patients lose both enzyme activities in solely the liver (subtype IIIb). Very rare patients have selective loss of one of the two enzyme activities: isolated transferase deficiency (subtype IIId) or isolated glucosidase deficiency [4].

At a molecular level, a gene encoding human glycogen debranching enzyme (gene symbol: *AGL*) was isolated [5] and mutational analyses of GSD III have been performed in several ethnic populations. Over 100 different *AGL* mutations have been reported to date [6] and in all 4 subtypes *AGL* mutations have been identified [7]. In Turkey we reported 13 different *AGL* mutations in GSD III patients from the Eastern region [4,8,9]. However, the full spectrum of *AGL* mutations in Turkish population remains unclear. In this study, in order to investigate further clinical and molecular characteristics in Turkey, we examined 12 new cases with Turkish GSD III mostly from the Western region of Turkey.

2. Materials and methods

2.1. Patients

Twelve Turkish GSD III patients from 11 unrelated families were investigated. Some of them were included in a clinical study described elsewhere [10]. Patients 8 and 9 were siblings in a family. The patients

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were confirmed as having deficient debranching enzyme activity in peripheral blood cells by the method of Shin [11]. Consanguinity was ascertained in 9 families except those of patients 1 and 3. The methods to evaluate cardiomyopathy were electrocardiography and echocardiography. The study was approved by local ethics committees and performed with the patients' and their families' informed consent.

2.2. Screening previously reported mutations in Turkey

Genomic DNA was isolated from peripheral blood leukocytes. In order to screen known *AGL* mutations in Turkey and frequently detected mutations across ethnic groups, PCR-restriction fragment length polymorphisms (RFLPs) were performed, as we described previously [4,8,9].

2.3. DNA sequence analysis of AGL

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 [12]. The nucleotides of *AGL* cDNA were numbered according to *AGL* isoform 1 (GenBank accession no. NM_000642). Point mutations identified in patients were verified using RFLP. Pairs of primers (Supplementary Table 1) were used for PCR and each specific restriction endonuclease was added to digest PCR products. Restriction digests were analyzed on a polyacrylamide gel. As long as DNA samples of parents were available for the investigation, they were tested by RFLP as well. A total of 50 Turkish control subjects were examined by RFLP in the same manner in order to rule out the possibility that they are mere polymorphisms in controls.

2.4. Haplotype determination in AGL

A total of 29 single nucleotide polymorphisms (SNPs) in *AGL* were determined, as described previously [4]. SNP numbers were cited according to Database of Single Nucleotide Polymorphisms (dbSNP Build 141) available from http://www.ncbi.nlm.nih.gov/projects/SNP/.

2.5. RNA transcript analysis

Total RNA was isolated from peripheral blood lymphocytes from patients using QIAamp RNA mini kit (Qiagen, Hilden, Germany), because both peripheral leukocytes and liver express *AGL* mRNA isoform 1 [13] and liver samples were unavailable for the study. Total RNA was reverse transcribed into single-strand cDNA with Superscript III (Life technologies, Carlsbad, USA). To analyze spliced transcripts, 3 pairs of primers were used for PCR (Supplementary Table 2). PCR products were electrophoresed on a 5% polyacrylamide gel, and PCR fragments were excised and sequenced directly.

3. Results

3.1. AGL mutation analysis

We identified all 24 mutant alleles in 12 Turkish patients and found 12 different *AGL* mutations (Table 1). Seven mutations were novel and five previously reported. All mutations were verified by RFLP analyses and not found in 50 normal Turkish controls (100 chromosomes). Accordingly, the frequency of nucleotide changes identified was less than 1% in Turkish controls. Furthermore, they were not listed in the NCBI SNP database Build 141. Nucleotide changes identified in our study were not deposited in three databases as well: the 1000 genomes browser (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/), NIH Heart, Lung and Blood Institute exome sequencing project exome variant server (http://evs.gs.washington.edu/EVS/), and Japanese SNP control database (http://gwas.biosciencedbc.jp/snpdb/snp_top.php).

Mutations in exon 3, reportedly associated with GSD IIIb, were not detected in any of 12 patients. Homozygosity was verified in 11 GSD III patients by both mutational and haplotype analyses, whereas patient 3 was the only patient who was compound heterozygous for two different mutations. Parents were heterozygotes for a mutation that their respective child possessed as shown in Supplementary Fig. 1. Thus, all *AGL* mutations were inherited from parents and there were no *de novo* mutations in the study patients.

3.2. Clinical features

Clinical features are shown in Table 1. All patients had hepatomegaly and patients who underwent liver biopsy showed excess accumulation of glycogen. Ege University hospital was the tertiary referral hospital and liver biopsies were performed in other hospitals. For example, patient 3 was pointed out hepatomegaly at the age of 8 months and a liver biopsy was performed in a local hospital in Turkey. She was then diagnosed as having GSD and was referred to Ege University at the age of 5 years. Laboratory data showed elevated liver enzymes and CK levels. An abdominal echogram indicated enlarged liver. Then glycogen debranching enzyme activity was measured and shown to be deficient.

All patients had either clinical muscle involvement or elevated plasma creatine phosphokinase (CK) concentrations, indicating that they are categorized as GSD IIIa. Geographic distribution of patients in Turkey was shown in Fig. 1.

3.3. AGL haplotype analysis

AGL haplotype analysis of 12 mutations was shown in Supplementary Table 3. Patient 7 with c.1019delA had the identical haplotype as another Turkish patients who had c.1019delA reported previously [IVS3+85 (rs663848) was misprinted in our previous report [4] and both were homozygotes for t]. Also, patient 11 with c.958+1G>A had the same haplotype as Turkish patients who had c.958+1G>A in our previous report [4] [P1067S (rs3753494) in exon 25 was misprinted in our previous report and both were homozygotes for S]. These results suggested that patients with the same mutations were descendants from a common ancestor in Turkey.

3.4. Aberrant splicing in three splicing mutations

The mutation c.1736-11A>G (IVS14-11A>G) in patient 2 was found to cause multiple aberrant splicing. Analysis of patient 2's *AGL* cDNA fragments revealed three PCR fragments different in size from the normal one (Fig. 2A). Sequencing analysis of the longest fragment 1 indicated the retention of 10 nucleotides (5′-TGCTCTGCAG-3′ located at −10 to −1 position in the acceptor splice site of intron 14) between exon 14 and exon 15. In the middle fragment 2, skipping of exon 14 (124 bp) was evident in addition to the retention of the 10 nucleotides. In the shortest fragment 3 skipping of both exons 14 and 15 was found. Thus, in patient 2 there was no normal splicing form that is present in the control.

In patient 5 with c.3588+2T>G (IVS27+2T>G), two fragments shorter than the normal PCR fragment were detected (Fig. 2B). The upper fragment 4 was a product of skipping of exon 27 (226 bp), and the lower 5 was that of skipping of exons 27 and 28.

Analysis of a patient 6's cDNA fragment 6 demonstrated aberrant splicing as well. In contrast to normal splicing, c.4161+5G>A (IVS31+5G>A) resulted in activation of a cryptic splicing site in exon 31, leading to skipping of 46 bp of the 3′ end of exon 31 (Fig. 2C). Unfortunately, peripheral blood samples from other patients with splicing mutations were unavailable for this study.

4. Discussion

In this study, we have shown the allelic heterogeneity of *AGL* mutations in Turkish patients: 12 different mutations were identified in 11 affected families of Turkish ancestry and 7 mutations are novel.