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Glycogen-Storage Disease Type II (Acid Maltase Deficiency): Identification of a Novel Small Deletion (delCC482+483) in French Patients

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Glycogen-storage disease type II (GSD II, acid maltase deficiency, Pompe's disease) is caused by defects in the lysosomal acid α-glucosidase (GAA) gene. Clinically, patients with the severe infantile form of GSD II have muscle weakness and cardiomyopathy eventually leading to death before the age of two years. Patients with the juvenile or the adult form of GSD II present with myopathy with a slow progression over several years or decades. Apart from a common base substitution in intron1, designated IVS1(−13T→G) and resulting in the aberrant splicing of exon 2, the other mutations recently discovered in the GAA gene are rare and often unique to single patients. In this paper, we identified a two-base frameshift deletion in three unrelated adult-onset GSD II patients. This small deletion lies in the first coding exon (exon 2) and results in a premature stop codon at the very 5′ end of the coding sequence of the GAA gene. The three patients were compound heterozygotes and two of them had the common IVS1(−13G→T) mutation on the second allele. We speculate that this novel deletion may be relatively frequent among French patients, possibly leading to the severe infantile phenotype of GSD II if it occurs in homozygous form.

Glycogen-storage disease type II (GSD II) is the only lysosomal disease among the different glycogenoses and is caused by the deficiency of acid α-glucosidase (GAA; synonym, acid maltase, EC 3.2.1.20) which hydrolyses α-1,4 and α-1,6 glycosidic linkages (1, 2). This recessively inherited deficiency results in intralysosomal accumulation of glycogen in most tissues, but symptoms are mainly due to functional impairment of skeletal and heart muscle (3, 4). Infantile-onset patients with GSD II (Pompe's disease) have severe cardiomyopathy leading to death before the second year of life (5). Patients with the juvenile or the adult-onset form of GSD II have more or less progressive myopathy limited to skeletal muscle, death usually occuring by respiratory failure (6). This phenotypic variation of GSD II is related to different levels of residual enzyme activity, and is most likely explained by high degree of genetic heterogeneity (7, 8, 9, 10).

The GAA gene is located on chromosome 17q23 and sequences of both the structural gene as well as the cDNA have been characterised (11, 12, 13, 14, 15). In patients with GSD II, about 25 deleterious mutations have been discovered up to now, as pointed by recent review studies (10, 16). These mutations are found over most of the 20 exons of the gene. They represent a variety of molecular lesions which includes non-sense and missense mutations (17,18, 19, 20, 21, 22, 23, 24, 25, 26, 27), single base-pair and larger deletions (20, 24, 28, 29), and splice mutations (30, 31).

Among adult patients, the IVS1(−13T→G) mutation is the most common one (allele frequency 0.35), the other mutations being rare and often unique to each patient (30, 32). In this study, we report on the identification by sequence analysis of a mutant allele in 3 unrelated adult patients who are compound heterozygotes. This new deletion is predictably deleterious by leading to a stop codon at the very 5′ end of the coding sequence.

MATERIALS AND METHODS

Patients. Three female adult-onset patients from 3 unrelated families of French Caucasian ancestry, diagnosed between 1994 and

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1996, were examined. All of them were born to non-consanguineous parents. The age at onset of complaints varied between 19 and 37 years (mean, 30 years) and the presenting symptom was proximal weakness of the legs. The diagnosis was supported by depression in GAA activity. The residual enzyme activity was measured in the supernatant from homogenates of peripheral blood leukocytes. The results were 4.O, 5.0 and 8.0 nmol/h/mg protein, respectively (controls: 15-45 nmol/h/mg protein), using 4-Methylumbelliferyl-α-D-gluco-pyranoside (Koch-Light laboratories, Hoverhill, UK) as substrate at pH 4.0. In two cases, muscle biopsy was taken from the deltoid muscle and light microscopy showed characteristic vacuolar myopathy with accumulation of glycogen.

Isolation and analysis of genomic DNA. Genomic DNA was extracted from leukocytes according to established procedures.

Using the amplification refractory mutation system (ARMS), the patients were first tested for the most frequent mutation IVS1(−13T→G), as described (32).

Genomic DNA was subsequently used as template for polymerase chain reaction (PCR) in order to amplify all the 19 coding exons of GAA gene and their flanking intron-exon junctions. PCR amplification of each exon used an appropriate pair of sense and antisense oligonucleotide primers complementary to intron sequences of GAA gene (unpublished data). The reaction mixture contained 10-40 pM concentrations of each primer, 250 μM of each dNTP, 2 U of *Taq* polymerase and 1 × buffer (Boehringer, Mannheim, Germany) in a final volume of 1OO μl. Genomic DNA (0.2 μg) was amplified in 30-40 cycles using optimal denaturation, annealing and extension temperatures. For analysis of exon 2 (in which the novel mutation was found) two different pairs of specific primers (10 pM of each per reaction) were used in order to amplify all the exonic region in two overlapping fragments. The pair used for amplification of the 5′ end of exon 2 were 5′-TGTCTCAGAGCTGCTTTAGA-3′ in intron 1 (base pairs −78 to −59 from exon 2, sense primer) and 5′-TTGTCAGGG-GCGCAATCGAA-3′ in the coding sequence (base pairs 287 to 268,[2] antisense primer). Amplification of the second half of exon 2 was performed using the following primers previously obtained from Dr Arnold Reuser (Erasmus University, Rotterdam, The Netherlands): N86 5′-AGAGCAGTGCCCACACAGTG-3′ in the coding sequence (base pairs 226 to 245,[2] sense primer) and p20 5′-ACCCCAAGCTTG-TGAGGTGC-3′ in intron 2 (base pairs +84 to +65 from exon 2, antisense primer). PCR conditions were 1 cycle of 95°C for 5 min, 56°C for 5 min and 72°C for 5 min, then 40 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, the last cycle ending with extension at 72°C for 10 mn. DNA fragments were then purified using the QIAquick PCR Purification Kit as described by the manufacturer (Qiagen Inc., Chatsworth, CA).

The purified PCR products were sequenced in both the forward and reverse directions, with the same sense and antisense primers as described above, with Dye Deoxy terminators (Applied Biosystems, Inc.). Approximately 50 ng of the double-stranded DNA template was added to 80 μM Tris-HCl (pH 8.3) - 2 mM $MgCl_2$ - 20 μM each Dye Deoxy terminator - 15 μM oligonucleotide primer - 0.5 U of Taq polymerase. DNA sequencing was performed in an automated thermal cycler programmed for 25 cycles of denaturation at 96°C for 30 s, primer annealing at 50°C for 30 s and extension at 60°C for 4 min. The sample mixture was then precipitated in 0.3 M sodium acetate and vacuum dried. The amplified product DNA was added to 4 μl of 5:1 deionized fornamide - 50 μM EDTA and denatured at 90°C for 2 min. The Dye Deoxy-labeled sequencing reaction mixes were resolved by electrophoresis on a 6 % polyacrylamide gel and analysed with an Applied Biosystems 373A automated sequencing system.

RESULTS AND DISCUSSION

We report here the occurence of a new mutation in one allele of three adult-onset patients with GSD-II. In fact, these subjects were issued from a large series of 40 French GSD II patients (including infantile, juvenile and adult cases) we had started to screen for both known and unknown mutations. By using direct automated sequencing, our strategy initially consisted in the analysis of the three following regions which are essential for function of the enzyme: exon 2 (containing the translation initiation codon), exons 10 + 11 (containing the enzyme catalytic site) and exon 14 (corresponding to a highly conserved area of the protein). Then, we have initiated complete genotyping of the patients by sequencing all the remaining coding exons. So far, most of the new mutations we have discovered are point mutations unique to each patient (M. Nicolino, manuscript in preparation).

Interestingly, this approach allowed the detection of a deletion of two cytosine at position 482+483 in codon 161 of the first coding exon (exon 2) in three unrelated patients. This deletion delCC482+483 results in a reading frameshift and is predictably deleterious by creating an early stop codon just 41 base pairs downstream (Fig. 1). There are two short 5 nucleotides direct repeats (cccca) overlapping and flanking (immediately 5′, data not shown) the gene deletion, respectively. These repeats may generate the deletional mechanism by misalignement during DNA replication (33).

All the patients were heterozygous for this novel deletion. In two cases, we found the common IVS1-(−13T→G) mutation on the second allele. As previously described, this T to G transition in intron 1 leads to the aberrant splicing of exon 2, but a small amount of normal transcript is able to leak through from the −13 mutated allele (16, 31). This particular mechanism is responsible for the low but significant level of residual enzyme activity (13 to 26 % of the mean GAA activity in normal controls) observed in the two adult-onset patients heterozygous for the IVS1(−13T→G) mutation. In the last patient, the second allele is unidentified yet. This allele probably also carries a mild mutation allowing low GAA activity responsible for the similar adult-onset phenotype. Surprisingly, the deletion delCC482+483 was not found in patients with the infantile form of GSD II. Nevertheless, we can hypothesize that patients either homozygous for this mutation, or compound heterozygotes for delCC482+483 in combination with an other severe mutant allele, would have no GAA activity with the clinical appearance of Pompe's disease.

Different small deletions have previously been discovered in the coding sequence of the GAA gene (20, 24, 29). Interestingly, one of them occurs with a high frequency among Dutch patients. It is the deletion of a single base in exon 2 (ΔT525) which disrupts the reading frame and introduces a stop codon leading to premature termination of GAA synthesis. This deletion is found with an allele frequency of 0.35, 0.19 and 0.15 among infants, children and adults, respectively

[2] Counting from translation initiation site.

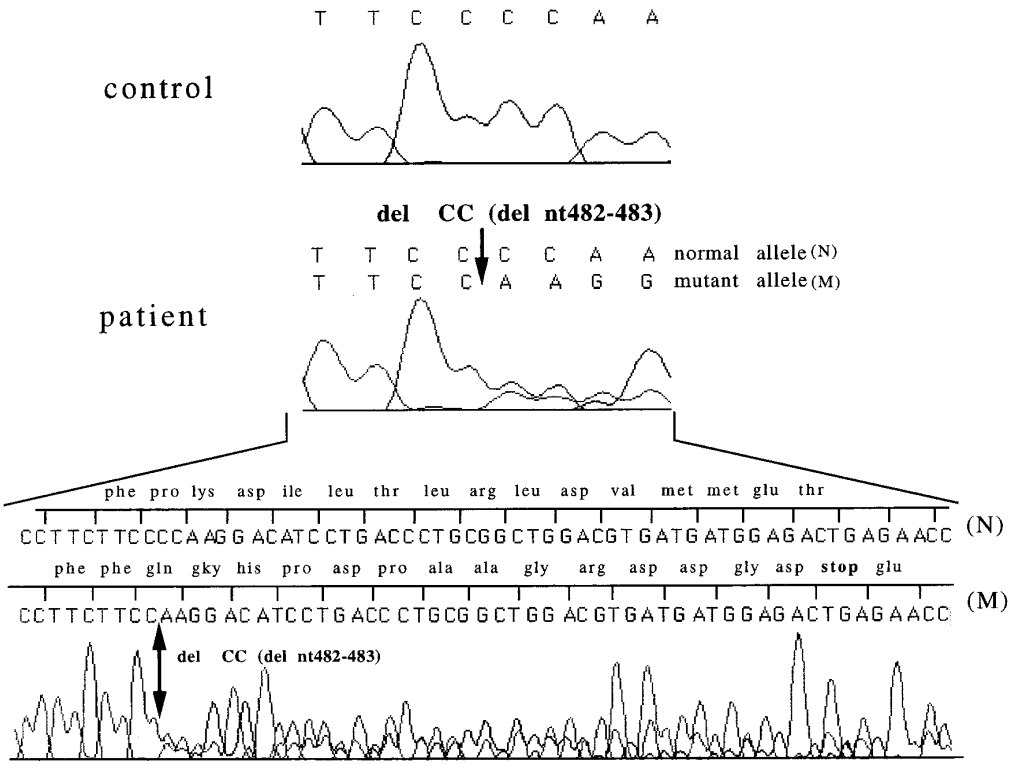

FIG. 1. Partial DNA sequence of a normal exon 2 and a mutant exon 2 with the deletion delCC482 + 483 in the GAA gene. Top, Chromatogram of sequence from a normal control. Bottom, Chromatogram of sequence from a GSD II patient heterozygous for delCC482 + 483. Automated sequencing is from a 5′ primer in the coding sequence and demonstrates absence of the second and the third nucleotides of codon 161 (Pro). The position of the deletion is indicated with arrows. The mutation generates a frameshift predicted to lead to a termination codon 41 bp downstream. Note the decrease in peak height due to overlapping of the two chromatograms, corresponding to the two different GAA gene alleles just downstream from the deletion. Chromatograms of sequence of deleted exon 2 were similar in the three cases studied.

(32). An other frequent mutation is the Δexon18 deletion which occurs with an allele frequency of 0.15 among infants (32, 34). In contrast, with the exception of IVS1(−13T→G) mutation, no common mutation has been reported in adult French patients. As observed in our study, the same delCC482+483 mutation was found in three unrelated cases in a total of 40 patients, including 23 adults, who were Caucasian patients originating from France. These data suggest this novel deletion may either have been propagated from a common ancestor or may have arisen independently in the different cases. The location of the mutation in a putative hotspot for deletion supports this last hypothesis. Further screening of patients needs to be done to confirm the possible increased frequency of delCC482+483.

Because of the high degree of molecular heterogeneity in GSD II, the majority of mutations causing the disease are expected to be new or rare mutations distributed over the 19 coding exons. Identification of molecular defects can therefore be difficult. Given delCC482 + 483 may be relatively frequent in our population, we propose that patients should be first tested for this deletion, like for the common IVS1(−13T→G) and Δexon18 mutations. So far, different attempts at therapy for GSD II were unsuccessful. Enzyme replacement is in progress (10), and we are exploring the feasibility of gene therapy using adenoviral vectors (35). With respects to either of these future experimental treatments, further insight in the genotype-phenotype correlations is essential. Our technical strategy, consisting in PCR amplification of the patient's genomic DNA followed by automated sequencing of the entire coding sequence may be adapted to efficient routine identification of mutations in a large number of GSD II patients. We anticipate that this simple approach will be the method of choice in clinical laboratories and will help to design the future protocols for treatment in GSD II patients.

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