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Mutational and haplotype analysis of *AGL* in patients with glycogen storage disease type III

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Abstract Glycogen storage disease type III (GSD III) is a rare autosomal recessive inherited disorder caused by a deficiency of the glycogen-debranching enzyme (AGL). We investigated two GSD III patients and identified four different mutations. Nucleotide sequence analysis revealed patient 1 of Chinese descent to be a compound heterozygote for a novel nonsense mutation, R34X, and the splicing mutation (IVS32−12A > G) reported in a Japanese patient. Patient 2 of Japanese origin was found to be compound heterozygous for a novel nonsense mutation, Y1148X, and the splicing mutation (IVS14+1G > T) that we had described previously. To determine whether splicing mutations occurred independently, we performed intense AGL haplotype analysis using 21 intragenic polymorphic markers plus a novel polymorphism IVS32−97 A/G in the vicinity of the IVS32 splicing mutation. Patient 1 of Chinese origin and the Japanese patient homozygous for the IVS32−12A > G were found to have different haplotypes, indicating the IVS32−12A > G mutation to be a recurrent mutation. This is the first recurrent mutation established by intense haplotyping in the *AGL* gene.

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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 (reviewed in Chen 2001). GSD III is caused by a deficiency in the glycogen-debranching enzyme (AGL), a key enzyme in the degradation of glycogen. AGL has two independent catalytic activities, oligo-1,4-1,4-glucantransferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33), on a single 160-KDa protein. The glycogen-binding site is assumed to be located in the carboxy terminus of its protein. Both activities and glycogen binding are required for complete function. In GSD III patients, AGL activities are virtually absent in affected organs.

The human *AGL* gene has been isolated and shown to be 85 kb in length and composed of 35 exons, encoding a 7.0-kb mRNA (Bao et al. 1996). Multiple tissue-specific isoforms of AGL mRNAs, differing only in the 5′-end, have been reported, and the predominant form, liver AGL, is predicted to have 1532 amino acids, deduced from mRNA isoform 1 (Bao et al. 1997). Over 20 different *AGL* mutations have been reported to date in GSD III patients (Human Gene Mutation Database; http://www.hgmd.org). Previous data suggest that each mutation is specific to each ethnic group (Shen et al. 1996, 1997a; Okubo et al. 1996, 1998, 1999, 2000a, 2000b; Parvari et al. 1997, 1998; Hadjigeorgiou et al. 1999; Fukuda et al. 2000; Uotani et al. 2000; Shaiu et al. 2000).

During our investigation of new GSD III cases, we identified the first recurrent mutation established by intense haplotyping in the *AGL* gene, together with two novel mutations.

Subjects and methods

Subjects

Patient 1. A 9-year-old Chinese girl was admitted to the Prince of Wales Hospital, Hong Kong, for a GSD III evaluation. Hepatomegaly had first been noted at the age of 1 year. Liver biopsy at 6 years of age showed portal fibrosis with excessive accumulation of abnormal glycogen. The patient's glycogen-debranching enzyme activity was undetectable in leukocytes. The patient had not shown any muscular manifestations. The patient was therefore diagnosed with GSD IIIb. There was no consanguinity in the patient's family. Both parents and two younger sisters had no signs of GSD III.

Patient 2. A 21-year-old Japanese male was admitted to Kanazawa University Hospital, Japan, for evaluation of GSD III. The patient had experienced hypoglycemia during childhood. Hepatomegaly was noted at 6 months of age. He was diagnosed as having GSD IIIa at 10 months of age on the basis of deficient AGL activity in both liver and muscle biopsy specimens. There was no consanguinity in the patient's family.

Mutation analysis of the *AGL* gene

Genomic DNA from patients and their family members was isolated from peripheral blood leukocytes after obtaining informed consent. The full coding exons, their relevant exon–intron boundaries, and the 5′- and 3′-flanking regions of the patients' and their parents' *AGL* genes were sequenced directly, as described previously (Okubo et al. 2000b) and compared with a control sequence (DDBJ/EMBL/GenBank accession no. SEG_AB03542S).

Point mutations identified in patients were verified using restriction fragment length polymorphism (RFLP). *Mnl* I and *Tfi* I RFLP analyses for detection of IVS14+1G > T and IVS32−12A > G, respectively, were performed as described previously (Okubo et al. 1996, 1998). To detect a C-to-T substitution at nucleotide 500 in exon 4, we used a sense primer (5′-taaaacatctgtttttcaatgtgg-3′) and an antisense primer (5′-TTTTCCCTGTAAAGTTGGGCCT GATC-3′) (the mismatched nucleotide is underlined) for polymerase chain reaction (PCR) analysis (lower case and upper case letters represent the intron and exon sequences, respectively). With the normal sequence (5′-CGATCA-3′), the 115-bp product was uncleaved by the restriction enzyme *Bcl*I, but in the presence of the C-to-T change (5′-**T**GATCA-3′), it was cleaved into 89- and 26-bp fragments. Restriction digests were analyzed on an 8% polyacrylamide gel.

To detect a C-to-G substitution at nucleotide 3844 in exon 27, we used a sense primer (5′-TTTAGCATTTGCG GGTACCC-3′) and an antisense primer (5′-CAGGCAA AGGAGCAGAATC-3′) for PCR. With the normal sequence (5′-CAATTG-3′), the PCR product was cleaved into 133- and 73-bp fragments by the restriction enzyme *Mfe* I, but in the presence of the C-to-G change (5′-**G**AATTG-3′), the 206-bp product was uncleaved. Restriction digests were analyzed on a 5% polyacrylamide gel.

After a mutation was detected, all other family members and 55 control subjects were examined by RFLP in the same manner.

Haplotype determination in the AGL gene

A total of 21 polymorphic markers in the *AGL* gene, five involving amino acid substitution, were genotyped according to previous reports (Shen et al. 1997b; Okubo et al. 2000a, 2000b; Horinishi et al. 2000). Furthermore, a novel IVS32−97 A/G polymorphism was revealed in this study and genotyped as follows: a sense primer (5′-tcagtatgtactaatttaaggttag-3′) and an antisense primer (5′-CATTGTCATAAATTCCACAGTAAACC-3′) were used for PCR, and the resulting PCR fragments were digested using the restriction enzyme *Ase* I. Allele A yields 130- and 73-bp fragments, whereas allele G results in one uncleaved 203-bp fragment. Each patient haplotype was determined by family study.

Results

We identified four different *AGL* mutations in patients 1 and 2. Direct sequencing analysis of patient 1 revealed an A-to-G transition at the –12 position at an acceptor splice site of intron 32 (IVS32−12A > G) and a C-to-T substitution at nucleotide 500 in exon 4 (Fig. 1a). The latter results in replacement of arginine by termination at codon 34 (R34X). Sequence analysis of patient 2 showed a G-to-T substitution at the +1 position at a donor splice site of intron 14 (IVS14+1G > T) and a C-to-G substitution at nucleotide 3844 in exon 27 (Fig. 1b), which results in replacement of tyrosine by termination at codon 1148 (Y1148X). No other nucleotide changes except for polymorphism described following were detected in the two patients' and their parents' *AGL* genes.

Mutational analysis of family 1 showed the IVS32−12A > G mutation to have been inherited from her father and the R34X mutation from her mother (Fig. 2a). Patient 1, her father, and sister-1 were verified to be heterozygous for the IVS32−12A > G mutation by *Tfi* I RFLP analysis (Fig. 2b). The R34X mutation was also verified by *Bcl* I RFLP, indicating that patient 1, her mother, and sister-2 were heterozygous for the mutation (Fig. 2c). Thus, patient 1 was a compound heterozygote for IVS32−12A > G and R34X. Mutation screening by *Bcl* I RFLP analysis showed that none of the 55 controls had the R34X mutation.

In Family 2, the IVS14+1G > T mutation was derived from the father and the Y1148X mutation from the mother (Fig. 2d). *Mnl* I RFLP analysis showed patient 2 and his father to be heterozygous for the IVS14+1G > T mutation

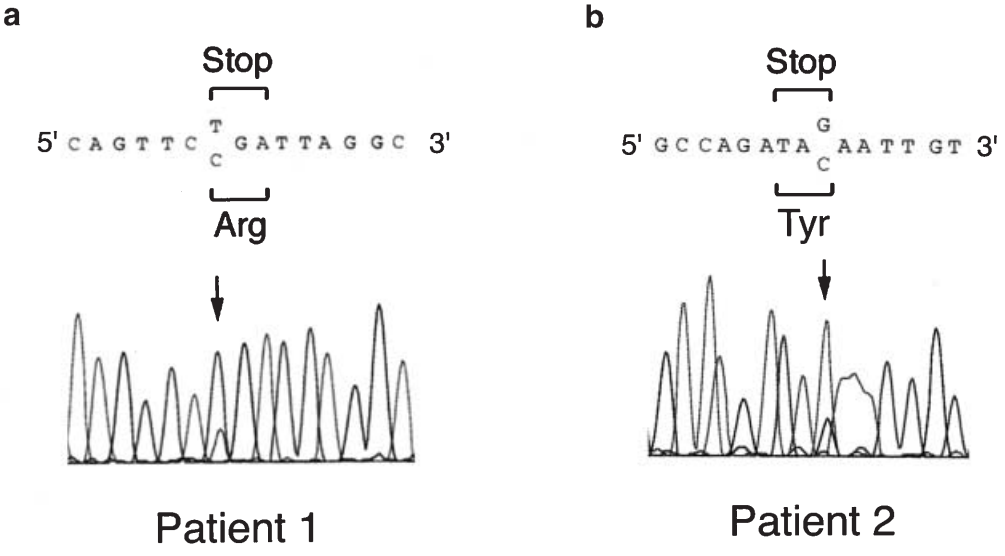

Fig. 1a,b. Sequence electropherograms corresponding to novel nonsense mutations in the *AGL* gene. **a** The sequence of patient 1 around codon 34 in exon 4 is shown. Patient 1 was heterozygous for CGA (Arg) and TGA (Stop). The point mutation of the first nucleotide at codon 34 is indicated by the *arrow*. **b** The sequence of patient 2 around codon 1148 in exon 27 is shown. Patient 2 was heterozygous for TAC (Tyr) and TGA (Stop). The point mutation in the third nucleotide at codon 1148 is indicated by the *arrow*

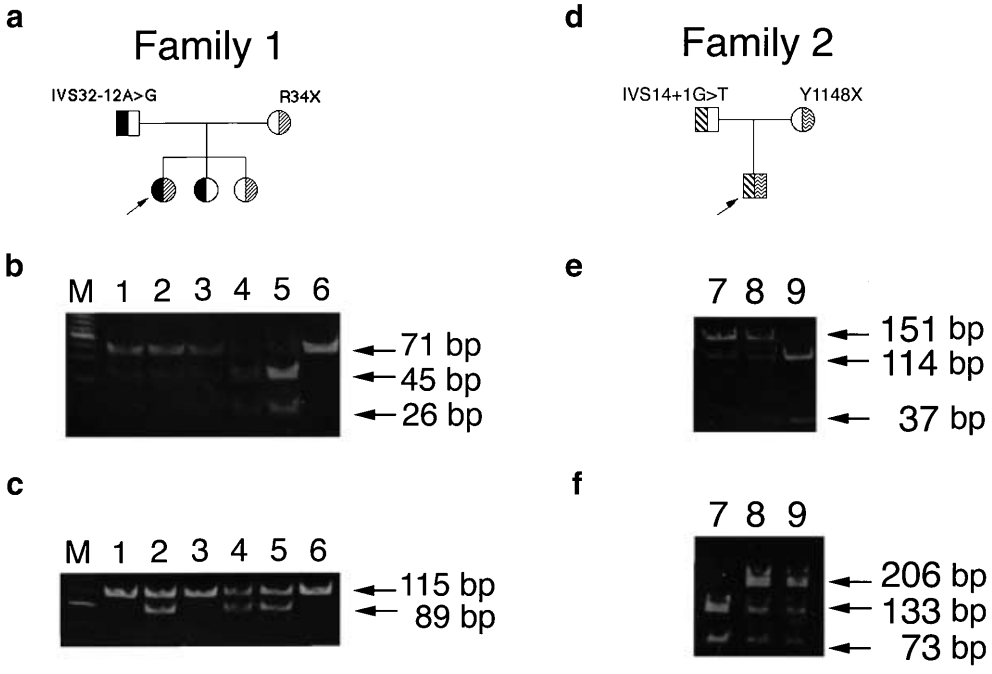

Fig. 2a–f. Mutational analysis of two families with GSD III. **a** Schematic representation of family 1. Patient 1 (indicated by the *arrow*) was a compound heterozygote for IVS32−12A > G inherited from her father and R34X from her mother. Electrophoretic analysis of polymerase chain reaction (PCR) products after digestion with *Tfi* I (**b**) and *Bcl* I (**c**). *Lane 1*, father; *lane 2*, patient 1; *lane 3*, sister-1; *lane 4*, sister-2; *lane 5*, mother; *lane 6*, a patient homozygous for IVS32−12A > G. **b** Patient 1, her father, and sister-1 had the 71-, 45-, and 26-bp fragments, showing heterozygosity for the IVS32−12A > G mutation. **c** Patient 1, her mother, and sister-2 had the 115- and 89-bp fragments, indicating heterozygosity for the R34X mutation. **d** Schematic representation of family 2. Patient 2 (indicated by the *arrow*) was a compound heterozygote for IVS14+1G > T inherited from his father and Y1148X from his mother. Electrophoretic analysis of PCR products after digestion with *Mnl* I (**e**) and *Mfe* I (**f**). *Lane 7*, father; *lane 8*, patient 2; *lane 9*, mother. **e** Patient 2 and his father had the 151-, 114-, and 37-bp fragments, confirming their heterozygosity for the IVS14+1G > T mutation. **f** Patient 2 and his mother had the 206-, 133-, and 73-bp fragments, indicating heterozygosity for the Y1148X mutation

(Fig. 2e). The Y1148X mutation was verified by *Mfe* I RFLP, indicating patient 2 and his mother to be heterozygous for the Y1148X mutation (Fig. 2f). Thus, patient 2 was a compound heterozygote for the IVS14+1G > T mutation and the Y1148X mutation. Mutation screening by *Mfe* I RFLP analysis did not detect the Y1148X mutation in any of the 55 controls.

We found a novel intronic polymorphism at the nearest 3′-end thus far reported, IVS32−97 A/G. This polymorphism is the proximate marker to the IVS32−12 A > G mutation. Its allelic frequency in Japanese controls is A 0.571/G 0.429 (n = 140 chromosomes). Intense *AGL* haplotyping of mutant alleles was conducted using 21 intragenic polymorphic markers plus IVS32−97 A/G (Fig. 3).

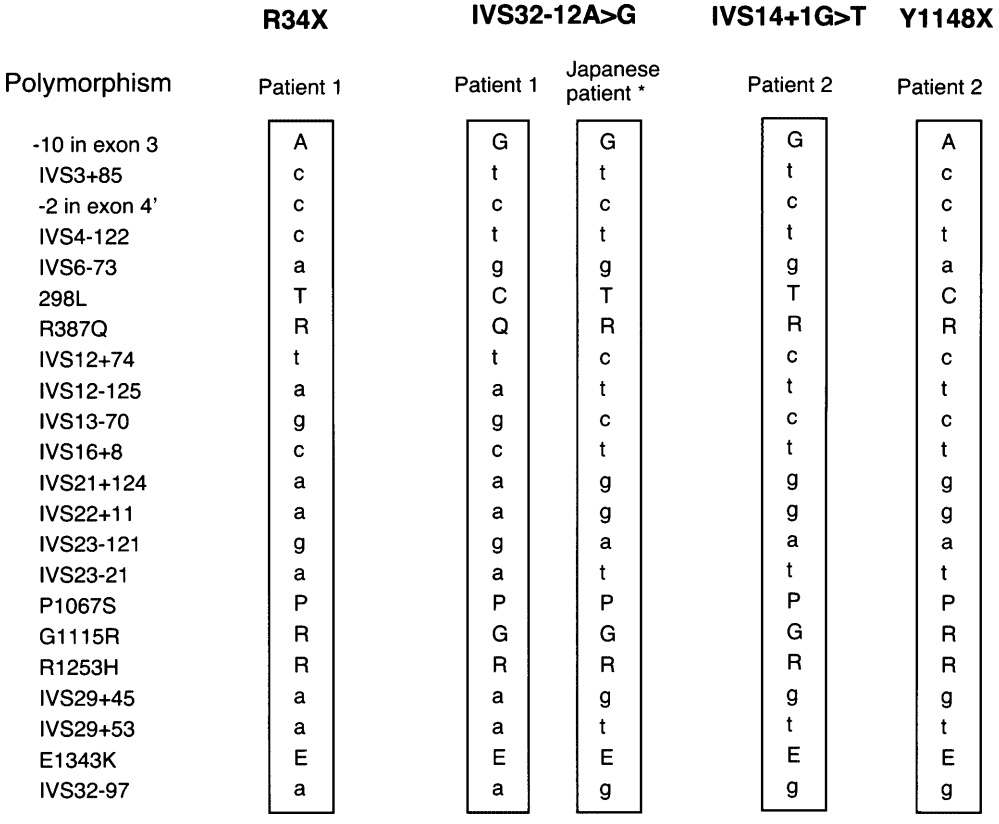

Fig. 3. Intense haplotyping of mutant *AGL* alleles using 22 intragenic polymorphic markers. A Japanese patient homozygous for the IVS32−12A > G mutation was described by Okubo et al. in 1998

The haplotype of the IVS32−12A > G mutation-bearing chromosome of patient 1 was different from that of a Japanese patient with the identical mutation. On the other hand, the haplotype of patient 2 on which the IVS14+1G > T mutation arose was identical to those of two Japanese patients with the same mutation.

Discussion

We found two GSD III patients to be compound heterozygotes for novel nonsense mutations (R34X and Y1148X) and splicing mutations (IVS32−12A > G and IVS14+1G > T), respectively, that were previously reported by us. The novel mutations cause premature termination at codons 34 and 1148, respectively, and the predicted proteins result in lack of a glycogen-binding domain indispensable for proper enzyme function. The splicing mutations also cause premature termination because of a frameshift, as we have previously shown. The IVS14+1G > T mutation results in skipping of exon 14 and the IVS32−12A > G mutation creates a new acceptor splice site and causes insertion of intron sequences into *AGL* mRNA (Okubo et al. 1996, 1998). Both predicted mutant enzymes were truncated by carboxyl amino acids as well. Four mutations found in patients 1 and 2 are most likely to be responsible for deficient enzyme activities.

We showed the IVS32−12A > G mutation to be a recurrent mutation in the *AGL* gene. This splicing mutation was first identified in a Japanese GSD IIIb patient (Okubo et al. 1998). Intense haplotyping of our patient 1 and the patient previously reported by us revealed different haplotypes, indicating that the mutations had occurred independently. Very recently, Shaiu et al. reported that their patients had the same mutation (Shaiu et al. 2000). However, whether or not their cases are recurrent remains unclear because haplotyping was not performed. Our intense haplotype analysis demonstrated that the IVS32−12A > G is the first recurrent *AGL* mutation to date.

As for the genotype/phenotype relationship in AGL, patients with an IVS32−12A > G mutation have mild or no muscular manifestations. Patient 1 has not shown any muscular involvement and neither has our previously reported case. Shaiu's two cases had milder muscular symptoms than other GSD III patients. The reason why this splicing mutation shows no or less intense muscular signs than other mutations remains unclear. It is possible that authentic splicing occurs and results in enough enzymatic activity to prevent accumulation of excess glycogen in muscles.

Patient 2 is the third reported Japanese patient with the IVS14+1G > T mutation. Intense haplotype analysis of three patients, who are unrelated, to our knowledge showed that they had an identical haplotype. We have experienced the same situation, i.e., overall heterogeneity but a limited number of frequent mutations in Japanese Wilson disease (Okada et al. 2000). We believe that the IVS14+1G > T mutation should initially be screened by *Mnl* I RFLP in Japanese patients suspected to have GSD III.

In summary, molecular defects in two GSD III patients have been characterized. Our mutation and haplotype

analyses provide evidence of recurrent mutation in the *AGL* gene.

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