

## Molecular analysis of the *AGL* gene: heterogeneity of mutations in patients with glycogen storage disease type III from Germany, Canada, Afghanistan, Iran, and Turkey

Yoriko Endo · Asako Horinishi · Matthias Vorgerd · Yoshiko Aoyama · Tetsu Ebara · Toshio Murase · Masato Odawara · Teodor Podskarbi · Yoon S. Shin · Minoru Okubo

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**Abstract** Glycogen storage disease type III (GSD III) is an autosomal recessive disorder characterized by excessive accumulation of abnormal glycogen in the liver and/or muscles and caused by deficiency in the glycogen debranching enzyme (*AGL*). Previous studies have revealed that the spectrum of *AGL* mutations in GSD III patients depends on ethnic grouping. We investigated nine GSD III patients from Germany, Canada, Afghanistan, Iran, and Turkey and identified six novel *AGL* mutations: one nonsense

(W255X), three deletions (1019delA, 3202–3203delTA, and 1859–1869del11-bp), and two splicing mutations (IVS7 + 5G > A and IVS21 + 5insA), together with three previously reported ones (R864X, W1327X, and IVS21 + 1G > A). All mutations are predicted to lead to premature termination, which abolishes enzyme activity. Our molecular study on GSD III patients of different ethnic ancestry showed allelic heterogeneity of *AGL* mutations. This is the first *AGL* mutation report for German, Canadian, Afghan, Iranian and Turkish populations.

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Y. Endo · A. Horinishi · Y. Aoyama · T. Ebara · T. Murase · M. Okubo (✉)  
Okinaka Memorial Institute for Medical Research,  
2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan  
e-mail: QFG00550@nifty.ne.jp

M. Vorgerd  
Department of Neurology, Ruhr University Bochum,  
Bochum, Germany

M. Odawara  
Third Department of Internal Medicine,  
Tokyo Medical University, Tokyo, Japan

T. Podskarbi  
Molecular Genetics and Metabolism Laboratory,  
Munich, Germany

Y. S. Shin  
Department of Pediatrics, University of Munich,  
Munich, Germany

M. Okubo  
Department of Endocrinology and Metabolism,  
Toranomon Hospital, Tokyo, Japan

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### Introduction

Glycogen storage disease type III (GSD III; MIM #232400) is an autosomal recessive inherited disorder caused by deficiency in the glycogen debranching enzyme, a key enzyme in the degradation of glycogen. The enzyme has two independent catalytic activities, oligo-1,4-1,4-glucoantransferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33), on a single 160 kDa protein. Both activities and glycogen binding are required for complete function. The glycogen-binding site is assumed to be located in the carboxyl terminal of its protein. In GSD III patients the enzyme's activities are virtually absent in affected organs. Deficiency in the enzyme results in an excessive accumulation of abnormal glycogen. Typical clinical manifestations are hepatomegaly, pro-

gressive myopathy, cardiomyopathy, and fasting hypoglycemia (Chen 2001). Most patients have both liver and muscle involvement (GSD IIIa), but approximately 15% of patients have solely liver involvement without any muscular manifestations (GSD IIIb). These subtypes have been explained by differences in tissue expression of the deficient enzyme.

The human *AGL* gene (*AGL*) 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). Liver glycogen debranching enzyme, a predominant form, is predicted to have 1,532 amino acids, deduced from mRNA isoform 1 (Bao et al. 1997). Molecular analyses of GSD III have been reported, mainly in Jewish, Japanese, Caucasian, and Italian populations (Shen and Chen 2002). Those studies revealed that the spectrum of *AGL* mutations in GSD III patients depends on ethnic grouping. For example, specific mutations were prevalent in Jewish GSD III patients (Parvari et al. 1997) and in an isolated region such as the Faroe Islands (Santer et al. 2001) because of founder effect, while heterogeneous mutations were found in Japanese patients (Horinishi et al. 2002; Okubo et al. 1996, 1998, 1999, 2000a, b). In Caucasian and Italian populations a few mutations accounted for a quarter of GSD III patients, but the rest of the mutations were heterogeneous (Lucchiari et al. 2002a, b; Shen and Chen 2002). To date, reports on other ethnic groups have been limited, and molecular analysis on patients from Germany, Canada, Afghanistan, Iran, and Turkey has yet to be performed.

Here, we investigated GSD III patients from Germany, Canada, Afghanistan, Iran, and Turkey and identified nine mutations, including six novel ones.

## Materials and methods

### Patients

Nine GSD III patients from eight unrelated families were investigated. They were German (patients 1, 2, and 3), Canadian (patient 4), Afghan (patient 5), Iranian (patient 6), and Turkish (patients 7, 8, and 9). Patients 7 and 8 were siblings whose parents were first cousins. The parents of patient 6 were first cousins as well. All patients were confirmed as having deficient debranching enzyme activity in peripheral red blood cells by the method of Shin (1990) and had been diagnosed with GSD IIIa. The study was approved by local ethics committees and performed with the patients' and their families' informed consent.

### DNA sequence analysis of the *AGL* gene

Genomic DNA was isolated from peripheral blood leukocytes. The full coding exons, their relevant exon–intron boundaries, and the 5'- and 3'-flanking regions of the patients' *AGL* gene were sequenced directly as has been described previously (Okubo et al. 2000b). The nucleotides of *AGL* cDNA were numbered according to *AGL* isoform 1 (GenBank accession no. NM\_000642).

### Mutation analysis of the *AGL* gene

Mutations identified in patients were verified using restriction fragment length polymorphism (RFLP). A pair of primers (listed in Table 1) was used for the polymerase chain reaction (PCR), and each specific restriction endonuclease was added to digest the PCR products. Restriction digests were analyzed on a polyacrylamide gel. Fifty Caucasian, 55 Japanese and 50 Turkish control DNA samples were examined by RFLP in the same manner to eliminate the possibility that they were mere polymorphisms in controls. Unfortunately, control DNA samples from individuals of Afghan and Iranian descent were not available in this study.

### Haplotype determination in the *AGL* gene

Twenty-three polymorphic markers in the *AGL* gene were genotyped in accordance with previous reports (Horinishi et al. 2000, 2002; Okubo et al. 2000b; Shen et al. 1997).

## Results

We identified nine mutations, six novel and three previously reported ones, in eight affected families. Results are summarized in Table 2. Fourteen mutated alleles out of 16 in the patients' *AGL* genes were revealed. Sequence electropherograms are shown in the electronic supplementary material (S1).

Patient 1, of German descent, had a G-to-A substitution at nucleotide 765 in exon 7, leading to the replacement of tryptophan by termination at codon 255 (W255X). RFLP analysis with *Hga* I showed patient 1 to be heterozygous for W255X (Fig. 1a). The other allele has yet to be identified, though all 35 exons, their relevant exon–intron boundaries, and the 5'- and 3'-flanking regions were sequenced.

Patient 2, of German ancestry, was shown to be homozygous for a nonsense mutation. Sequencing

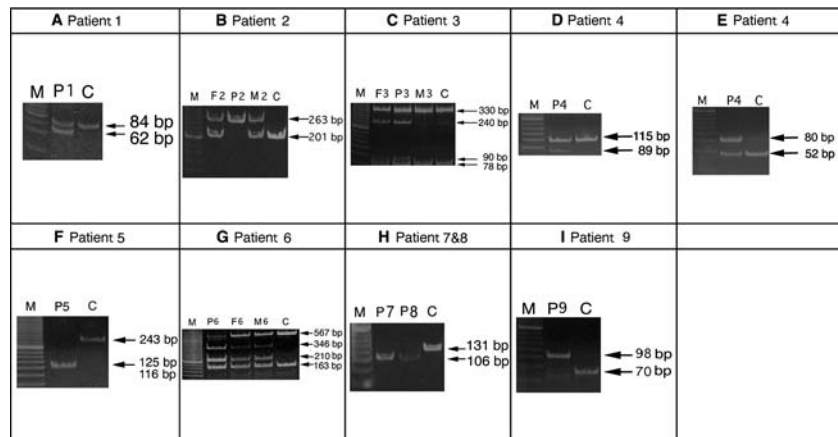
**Table 1** PCR primers using RFLP detection for *AGL* mutations. Exon sequences are represented by *uppercase letters* and introns by *lowercase letters*. Mismatch nucleotides are *underlined*. *F* forward, *R* reverse

Location	Mutation	PCR primer (5' → 3') for RFLP detection	Restriction enzyme	Fragment size (bp)
Exon 7	765G > A [W255X]	F: CTT GTG AAT TCT CCA CAC TTA AAA CCT GCC TGG R: TTT CCC TTC TGC AAC ATC ACA GGA GAA <u>GCG</u>	<i>Hga</i> I	Normal: 84 Mutant: 62 + 22
Intron 7	IVS7 + 5G > A	F: CTT AGA CAG <u>AGC</u> ACT TTG GC R: ttt agt gtg ata ctc etc <u>tgt</u> aaa ta	<i>Ssp</i> I	Normal:131 Mutant:106 + 25
Exon 9	1019delA	F: CGA GTA ACC AAG <u>TCT</u> GAT CC R: ATC TAC AGT ACA GCC AAA CCG TCT <u>GAA</u> T	<i>Eco</i> RI	Normal: 52 + 29 Mutant: 80
Exon 16	1859–69del 11-bp	F: ctc ttc ctg gac ata aat aat g R: aga aac cac tga aat ctg gac	<i>Alu</i> I	Normal: 567 + 163 Mutant: 346 + 210 + 163
Exon 21	2590C > T [R864X]	F: GAA TTT GAA AAC TTG TCT CCA G R: GCA TTG TCA ACA GCT AGG CTG	<i>Taq</i> I	Normal:201 + 62 Mutant:263
Intron 21	IVS21 + 1G > A	F: CAT CTG ACA CAA TTC AGT CC R: ggt aaa ggg tat gag gta tgc	<i>Msl</i> I	Normal: 330 + 78 Mutant: 240 + 90 + 78
Intron 21	IVS21 + 5insA	F: TCC TAT ATT AAA AAT TCC TTT TGC TTC <u>tta</u> R: ctg ctt aat tca gtc tag tgg	<i>Afl</i> II	Normal: 70 + 27 Mutant: 98
Exon 25	3202–3delTA	F: TTT GTT CAG AAT GGT TCA ACC R: ttt cct ata act ttt ctt ccg c	<i>Dde</i> I	Normal: 243 Mutant: 125 + 116
Exon 31	3980G > A [W1327X]	F: AAG GCT ATA AAG GTC TCA TAT GAT GTC T R: CCA GAT TTG GAT GCT TTT CAT	<i>Xba</i> I	Normal:115 Mutant: 89 + 26

**Table 2** *AGL* mutations and single nucleotide polymorphisms (SNPs) in patients with GSD III. Novel mutations are in *bold type*. *ND* not determined

Patient	1	2	3	4	5	6	7, 8	9
Ethnic group	German	German	German	Caucasian from Canada	Afghan	Iranian	Turkish	Turkish
Mutation 1	<b>W255X</b>	R864X	IVS21 + 1G > A	<b>A1019delA</b>	<b>3202–3delTA</b>	<b>1859–69del 11-bp</b>	<b>IVS7 + 5G &gt; A</b>	<b>IVS21 + 5insA</b>
Mutation 2	ND	R864X	ND	W1327X	<b>3202–3delTA</b>	<b>1859–69del 11-bp</b>	<b>IVS7 + 5G &gt; A</b>	<b>IVS21 + 5insA</b>
Consanguinity						Yes	Yes	
Polymorphism								
–10 in exon 3	gg	aa	aa	ag	gg	gg	aa	aa
IVS3 + 85	tt	tt	cc	cc	tt	tt	cc	cc
–2 in exon 4'	cc	tt	ct	cc	cc	cc	cc	cc
IVS4-122	ND	tt	tt	tt	tt	ND	ND	tt
IVS6-73	gg	aa	aa	aa	gg	gg	aa	aa
298L	tt	tt	ct	ct	tt	tt	ND	cc
R387Q	RR	RR	RR	RR	RR	RR	ND	RR
IVS12 + 74	ND	cc	ct	cc	cc	tt	cc	cc
IVS12-125	ND	tt	tt	tt	tt	tt	ND	tt
IVS12-44	ga	aa	aa	aa	aa	aa	ND	aa
IVS13-70	ND	cc	cg	cc	cc	gg	ND	cc
IVS16 + 8	cc	tt	ct	tt	tt	cc	ND	tt
IVS21 + 124	ND	aa	ga	gg	ND	ND	gg	ND
IVS22 + 11	aa	aa	aa	gg	aa	aa	ND	ND
IVS23-121	gg	aa	aa	aa	aa	gg	ND	aa
IVS23-21	aa	tt	tt	tt	tt	aa	ND	tt
P1067S	cc	tt	ct	cc	tt	cc	cc	cc
G1115R	GG	GG	GR	GG	GG	GG	ND	GG
R1253H	RR	RR	RR	RR	RR	RR	RR	RR
IVS29 + 45	ND	gg	ag	gg	gg	gg	gg	gg
IVS29 + 53	ND	tt	at	tt	tt	tt	tt	tt
E1343K	EE	EE	EE	EE	EE	EE	EE	EE
IVS32-97	aa	gg	gg	gg	gg	gg	gg	gg

**Fig. 1** Mutational analysis for GSD III patients. PCR products after digestion with restriction endonucleases (listed in Table 1) were electrophoresed. *M* DNA marker, *P1–P9* patients 1–9, *C* control, *F2, 3, 6* father of patient 2, 3, 6, *M2,3,6* mother of patient 2, 3, 6



analysis showed a C-to-T transition at nucleotide 2590 in exon 21, resulting in substitution of arginine at codon 864 by termination (R864X). Restriction analysis with *Taq* I confirmed that patient 2 was a homozygote and that the parents were heterozygotes (Fig. 1b).

Patient 3 was heterozygous for a G-to-A substitution at +1 position of the donor splice site of intron 21 (IVS21 + 1G > A). Restriction analysis with *Msl* I showed that patient 3 was heterozygous for IVS21 + 1G > A as well as the father (Fig. 1c). The other allele has yet to be identified.

Patient 4, of Caucasian heritage from Canada, had a G-to-A substitution at nucleotide 3980 in exon 31, which replaces tryptophan by a termination at codon 1327 (W1327X). RFLP analysis with *Xba* I showed that patient 4 was heterozygous for W1327X (Fig. 1d). Further sequencing analysis revealed a deletion of A at nucleotide 1019 in exon 9 (1019delA), leading to a premature termination because of frame shift. RFLP analysis with *Eco* RI showed that patient 4 was a heterozygote for 1019delA (Fig. 1e). Thus, patient 4 was compound heterozygous for W1327X and 1019delA.

Patient 5, from Afghanistan, was homozygous for a 2-bp deletion in exon 25. Sequence analysis for patient 5 showed deletion of TA at nucleotides 3202 and 3203, causing premature termination because of frame shift. RFLP analysis with *Dde* I verified that patient 5 was homozygous for the 2-bp deletion (Fig. 1f).

Patient 6, from Iran, had deletion of 11 bp at nucleotides 1859–1869 in exon 16. RFLP analysis with *Alu* I verified that patient 5 was homozygous for the 11 bp deletion, while the parents were heterozygous (Fig. 1g).

Sequencing analysis of patients 7 and 8, from a Turkish consanguineous family, revealed a G-to-A substitution at +5 position of the donor splice site of intron 7 (IVS7 + 5G > A). RFLP analysis using *Ssp* I

showed homozygosity of the mutation in both patients (Fig. 1h).

Patient 9, of Turkish decent, was homozygous for a 1 bp insertion at +5 position of the donor splice site of intron 21 (IVS21 + 5insA). RFLP analysis with *Afl* II confirmed that patient 9 was a homozygote for the mutation (Fig. 1i).

These nine mutations were not found in 50 Caucasian and 55 Japanese control DNA samples. Neither of the splicing mutations found in the Turkish patients was detected in 50 Turkish controls.

Determination of single nucleotide polymorphisms (SNPs) in the *AGL* gene showed that the three German patients had different *AGL* haplotypes (Table 2). In patient 4, the *AGL* haplotype (–10 in exon 3/IVS3 + 85/–2 in exon 4'/IVS4-122/IVS6-73/298L/R387Q/IVS12 + 74/IVS12-125/IVS12-44/IVS–70/IVS16 + 8/IVS21 + 124/IVS22 + 11/IVS23-121/IVS23-21/P1067S/G1115R/R1253H/IVS29 + 45/IVS29 + 53/E1343K/IVS32-97) associated with W1327X was (?/c/c/t/a/?/R/c/t/a/c/t/g/g/a/t/c/G/R/g/t/E/g) (? : we could not determine two SNPs in –10 in exon 3 and 298L, because DNA samples from the parents were not available for segregation analysis). We have described an Egyptian patient with the same W1327X elsewhere (Endo et al. 2005). The Egyptian patient had (a/c/t/t/a/c/R/c/t/a/c/t/g/g/a/t/c/G/R/g/t/E/g), indicating that at least one SNP, i.e., –2 in exon 4', was different from that of patient 4. Patients 5–9 were homozygous for SNPs, which we determined, although some SNPs were not examined because further DNAs were not available for genotyping.

## Discussion

We have identified heterogeneous mutations in GSD III patients from ethnic groups in which no molecular

studies have been reported. Of nine mutations identified in this study, six were novel and three had been previously reported. Novel mutations were one nonsense (W255X), three deletions (1019delA, 3202–3203delTA, and 1859–1869del11-bp), and two splicing mutations (IVS7 + 5G > A and IVS21 + 5insA). All nine mutations are predicted to lead to premature termination, which abolishes enzyme activity. Nonsense mutation W255X is predicted to cause a truncated enzyme lacking the glycogen-binding site in the carboxyl terminal. Three deletions (1019delA, 3202–3203delTA, and 1859–1869del 11-bp) lead to a premature stop codon because of frame shift, resulting in truncated proteins as well. Alternatively, those premature termination codons will probably be recognized by nonsense-mediated decay, leading to absence of *AGL* mRNA. Moreover, two mutations in donor splice sites (IVS7 + 5G > A and IVS21 + 5insA) are likely to cause aberrant splicing, because no *AGL* mRNA was detected in patients' peripheral leukocytes (data not shown). These results are consistent with the fact that the majority of the mutations reported are nonsense mutations, deletions, insertions, and splicing mutations (Shen and Chen 2002). Only six missense mutations have been reported thus far (Lucchiari et al. 2002b, 2003; Okubo et al. 1999).

Three mutations (R864X, IVS21 + 1G > A, and W1327X) have been previously reported in other ethnic groups. Nonsense mutation R864X in patient 2 has been shown to account for approximately 10% of the Caucasian GSD III mutant alleles in the United States of America (Shaiu et al. 2000; Shen et al. 1996). Splicing mutation IVS21 + 1G > A in patient 3 from Germany has also been reported in Italian patients (Hadjigeorgiou et al. 1999; Lucchiari et al. 2002a, b) and a Japanese patient (Uotani et al. 2000). However, haplotype data have not been reported in the literature, and we could not compare ours with theirs. Presumably, IVS21 + 1G > A may be a recurrent mutation, but further *AGL* haplotyping studies will be needed.

W1327X is, to our knowledge, the third recurrent mutation reported in the *AGL* gene. Two recurrent *AGL* mutations have been verified by *AGL* haplotyping. We demonstrated that splicing mutation IVS32–12A > G occurred in a Japanese patient and a Chinese patient, independently, by showing that they were on different haplotypes (Horinishi et al. 2002). Lam et al. reported that a Chinese patient and Faroese patients had the same nonsense mutation R408X on different haplotypes (Lam et al. 2004). As far as W1327X is concerned, Lucchiari et al. have described a Tunisian GSD III patient heterozygous for the mutation (Lucchiari et al. 2002a), but the haplotype has not been reported. Very

recently, we identified an Egyptian patient homozygous for W1327X (Endo et al. 2005). In order to determine whether the mutation had occurred independently, we compared the *AGL* haplotypes of two of the patients we studied. Patient 4 had a different haplotype from that of the Egyptian patient; the Canadian had cc in –2 in exon 4', while the Egyptian had tt. Our finding indicates that W1327X is the third recurrent mutation. Accordingly, *AGL* haplotyping is useful to determine whether mutations are recurrent or not.

In summary, we have identified nine *AGL* mutations, including six novel ones. Our molecular study on GSD III patients of different ethnic ancestry showed allelic heterogeneity of the *AGL* mutations. This is the first *AGL* mutation report for German, Canadian, Afghan, Iranian, and Turkish populations.

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