

A New Muscle Glycogen Storage Disease Associated with Glycogenin-1 Deficiency

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We describe a slowly progressive myopathy in 7 unrelated adult patients with storage of polyglucosan in muscle fibers. Genetic investigation revealed homozygous or compound heterozygous deleterious variants in the glycogenin-1 gene (*GYG1*). Most patients showed depletion of glycogenin-1 in skeletal muscle, whereas 1 showed presence of glycogenin-1 lacking the C-terminal that normally binds glycogen synthase. Our results indicate that either depletion of glycogenin-1 or impaired interaction with glycogen synthase underlies this new form of glycogen storage disease that differs from a previously reported patient with *GYG1* mutations who showed profound glycogen depletion in skeletal muscle and accumulation of glycogenin-1.

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Skeletal muscle relies on glycogen storage for contraction and relaxation. Defects in glycogen synthesis or degradation pathways provoke glycogen accumulation and/or depletion, leading to energy deficiency.¹

The 3 main enzymes involved in biosynthesis of glycogen are glycogenin-1, glycogen synthase, and branching enzyme.² Glycogenin-1 is a glycosyltransferase that forms a short glucose polymer of approximately 10 glucose residues by autoglucosylation. Glycogen synthase and branching enzyme allow further elongation and branching of the glucose polymer primer.²

Defects in various enzymes involved in glycogen metabolism cause glycogen storage diseases. Some of

these are characterized by accumulation of polyglucosan, which is variably resistant to digestion by alpha-amylase, and contains abnormally long and poorly branched glucosyl chains. Such diseases include branching enzyme (GBE1) deficiency, phosphofructokinase deficiency, adenosine monophosphate-activated protein kinase deficiency, and Lafora disease.^{1,3,4} Deficiency of RanBP-type and C3HC4-type zinc finger-containing 1 (RBCK1) has recently been shown to cause a polyglucosan storage disease with cardiomyopathy and skeletal myopathy.⁵

A specific mutation in the glycogenin-1 gene (*GYG1*) has been shown to cause an impaired priming of glycogen synthesis in 1 patient with cardiomyopathy and depletion of glycogen in skeletal muscle.^{6,7}

We describe a new polyglucosan body myopathy due to pathogenic variants in *GYG1*.

Patients and Methods

Patients

Seven adult patients with polyglucosan body myopathy from 7 unrelated families of various ethnic backgrounds were included in the present study. These patients belonged to a cohort of some 20 patients with polyglucosan body myopathy and/or cardiomyopathy negative for *GBE1* and *RBCK1* disease-causing variants explaining the phenotype. After informed consent, the patients underwent open biopsy of the deltoid or quadriceps femoris muscle for morphologic and histochemical analyses of fresh-frozen muscle tissue. Patients P5 and P6 have been previously reported.⁸

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TABLE . Clinical, Laboratory, and Genetic Findings

Characteristic	Patient						
	P1	P2	P3	P4	P5	P6	P7
Gender	M	F	M	F	M	F	F
Age at onset, yr	17	Probably childhood	39	65	62	61	49
Initial symptoms	Difficulty running	Trouble rising from squatting since childhood; progressive weakness after age 39 years	Difficulty walking	Difficulty climbing stairs and walking	Difficulty climbing stairs and walking	Loss of grip strength in both hands	Difficulty climbing stairs and loss of grip strength in right hand
Age at examination, yr	26	50	43	72	72	66	57
Clinical features	Hip girdle muscle weakness, atrophy, and fatigability	Shoulder, hip girdle, and distal anterior leg muscle weakness	Hip girdle, tibial, and peroneal muscle weakness	Shoulder and hip girdle muscle weakness	Hip girdle and thigh muscle weakness and atrophy; milder asymmetric weakness of shoulder girdle and upper arm muscles	Intrinsic hand muscle weakness and atrophy; mild extensor finger weakness	Shoulder and hip girdle muscle weakness; wrist and finger extensor, foot interossei, toe extensor and toe extensor paralysis
Serum CK	977U/l	Normal	Normal	Normal	Normal	Normal	Normal
Cardiac examination	Normal	Normal	Normal	Normal	Normal	Normal	Normal
EMG	Myopathic	Myopathic with fibrillation potentials and brief myotonic discharges	Myopathic	Myopathic	Myopathic and neurogenic, few positive sharp waves and fibrillation potentials	Myopathic, positive sharp waves and fibrillation potentials	Myopathic, polyphasic motor unit action potentials with early recruitment
Muscle pathology by light microscopy	Partially α -amylase-resistant, PAS-positive inclusions, desmin, SQSTM1, and ubiquitin positive	Partially α -amylase-resistant, PAS-positive inclusions	Partially α -amylase-resistant, PAS-positive inclusions; moderate interstitial fibrous and fat tissue	Partially α -amylase-resistant, PAS-positive inclusions, desmin, SQSTM1, and ubiquitin positive	Partially α -amylase-resistant, PAS-positive inclusions, desmin, SQSTM1, and ubiquitin positive; moderate interstitial fibrous and fat tissue	Partially α -amylase-resistant, PAS-positive inclusions, desmin, SQSTM1, and ubiquitin positive	Partially α -amylase-resistant, PAS-positive inclusions, desmin, SQSTM1, and ubiquitin positive; prominent interstitial fibrous and fat tissue

TABLE . Continued

Characteristic	Patient						
	P1	P2	P3	P4	P5	P6	P7
Muscle pathology by electron microscopy	Polyglucosan bodies	Polyglucosan bodies	Polyglucosan bodies	Polyglucosan bodies	Polyglucosan bodies	Polyglucosan bodies	Polyglucosan bodies
DNA analysis	Homozygous c.143+3G>C	Homozygous c.143+3G>C	Compound heterozygous c.304G>C, c.749G>A	Homozygous c.46G>C	Compound heterozygous c.143+3G>C, c.7G>C	Homozygous c.484delG	Compound heterozygous c.143+3G>C c.970C>T
RNA analysis	r.8_143del	r.8_143del	ND	r.46g>c	r.8_143del r.0	r.0	r.8_143del r.970c>u
Protein change	p.Asp3Glufs*4	p.Asp3Glufs*4	p.(Asp102His) p.(Trp250*)	p.Ala16Pro	p.Asp3Glufs*4 p.spl	p.Thr163Aspfs*5	p.Asp3Glufs*4 p.Arg324*

CK = creatine kinase; EMG = electromyography; F = female; M = male; ND = not determined; PAS = periodic acid-Schiff.

Molecular Genetic Analysis

Genomic DNA was extracted from blood or frozen skeletal muscle by standard methods, and Sanger sequencing was used to screen for *GYG1* (NM_004130) variants. Total RNA was isolated from frozen skeletal muscle using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). RNA was reverse-transcribed with the QuantiTect reverse transcription kit (Qiagen), and *GYG1* cDNA was analyzed by Sanger sequencing.

Morphological Analysis

Cryostat sections of fresh-frozen deltoid or quadriceps femoris muscle tissue were histochemically analyzed by standard techniques. For immunohistochemistry the following antibodies were used: SQSTM1 (D-3; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000), Anti-Ubiquitin (P4D1-A11; Merck Millipore, Billerica, MA; 1:100), and Anti-Human Desmin (D33; Dako Cytomation, Carpinteria, CA; 1:50).

Protein and Functional Analyses

Autoglycosylation was analyzed in vitro applying a cell-free protein expression system. The cloned sequence of glycogenin-1 (NM_001184720) was inserted downstream of the 6xHis tag in the pEXP5-NT/TOPO vector (Invitrogen Life Technologies, Carlsbad, CA). Variants identified in the patients were introduced using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers listed in the Supplementary Table. The recombinant glycogenin-1 was expressed using the FastLane *Escherichia coli* protein synthesis kit (RiNA, Berlin, Germany) for 1 hour at 37°C and allowed to autoglycosylate in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (50mM, pH7.5), MnCl₂ (5mM); dithiothreitol (0.5mM), and 0.1M uridine diphosphate (UDP) glucose (Calbiochem, San Diego, CA) for 1 hour at 30°C.^{7,9}

Western blot analysis was performed on protein extracted from skeletal muscle tissue as well as recombinant protein. Cryostat sections from biopsies were treated with 10μg/ml human alpha amylase (Sigma-Aldrich, St Louis, MO) in a total volume of 10μl phosphate-buffered saline, pH 6.5 for 1 hour at 37°C. Protein extracts were loaded and separated on 10% Bis-Tris gel or 3 to 8% Tris-acetate Gel (Novex; Life Technologies, Grand Island, NY) followed by electroblotting. The membranes were incubated with primary antihuman glycogenin-1 N-terminal antibody M07 clone 3B5 (Abnova, Taipei, Taiwan; 1:500) or C-terminal anti-GYG1 (Atlas Antibodies, Stockholm, Sweden; 1:500) or anti-GBE1 (Atlas Antibodies; 1:500) or anti-GYS1 (Atlas Antibodies). Western Breeze (Invitrogen) was used for antibody detection.

Results

Clinical Findings

A clinical summary is provided in the Table. Age at onset ranged from childhood to adult life. Muscle weakness was progressive in 1 patient and slowly progressive in the other patients. Four patients developed symmetrical proximodistal muscle weakness with variable

involvement of hip and shoulder girdle muscles, lower legs, and hand muscles. Two patients showed isolated proximal muscle weakness. One patient showed only hand and finger involvement starting in adulthood. Facial and ocular muscles were spared. Serum creatine kinase was elevated in 1 and normal in the other patients. Electromyography was invariably myopathic, but 1 patient showed in addition neurogenic involvement. Electrocardiography and cardiac ultrasound were normal in all patients. Cardiac magnetic resonance imaging was normal in P4. Myocardial biopsy was not performed.

Morphological Analysis

Morphological investigations by light- and electron-microscopy of muscle revealed characteristic alterations. Thirty to forty percent of the fibers, frequently in clusters, showed inclusions in the center and subsarcolemmal regions of the fibers (Fig 1). Periodic acid–Schiff (PAS)-positive material formed the main content of the inclusions. Some fibers presented inclusions surrounded by normal glycogen content, whereas other fibers showed depletion of normal glycogen around the inclusions. Alpha-amylase treatment showed a variable degree of digestion of the PAS-positive material. Inclusions were positively immunostained for desmin, ubiquitin-binding protein sequestosome-1 (p62/SQSTM1), and ubiquitin. Electron microscopy demonstrated presence of storage material in the subsarcolemmal and central regions of the fibers. The inclusions frequently formed a lobulated grape structure composed of oval subunits with the same electron density. A rim of normal glycogen particles and mitochondria surrounded the storage material. Smaller inclusions often contained central areas composed of less electron-dense filamentous material. Necrotic or regenerating fibers were not identified. Increase of interstitial connective tissue and fat was noted in P3 and P5, and more pronounced in P7.

Molecular Genetic Analysis

The identified *GYGI* pathogenic variants are summarized in the Table and Figure 2A. The most common variant was a single nucleotide substitution at the donor splice site in intron 2 (c.143+3G>C), either homozygous or compound heterozygous. Analysis of *GYGI* cDNA with this variant revealed aberrant splicing with skipping of exon 2 (r.8_143del) creating a frameshift p.Asp3Glufs*4 (see Fig 2B–E). P5 was compound heterozygous for the common splice site variant and a c.7G>C variant affecting the last nucleotide in exon 1, within the donor splice site. The corresponding transcript was not detected in cDNA, suggesting that this allele was not expressed. P7

was compound heterozygous for the common splice site variant and a C-terminal nonsense (c.970C>T, p.Arg324*) variant. P3 was compound heterozygous for a missense (c.304G>C, p.Asp102His) and a nonsense (c.749G>A, p.Trp250*) variant. P4 was homozygous for a N-terminal missense variant (c.46G>C, p.Ala16Pro) that was confirmed at the RNA level. P6 was homozygous for a single nucleotide deletion (c.484delG), and no transcript was detected in cDNA.

Protein and Functional Analyses

Expression of glycogenin-1 in skeletal muscle tissue was studied by Western blot analysis either without or with alpha-amylase treatment, to cleave off the glucose in the glycogen particles (see Fig 2F). Free autoglucosylated glycogenin-1 weighs approximately 1kDa more than the unglucosylated protein. In normal individuals, there is no free glycogenin-1. A weak band corresponding to glycogenin-1 was identified in P1 and P5 only after alpha-amylase treatment, demonstrating presence of some residual functional glycogenin-1. P4 and P6 did not show any glycogenin-1. Glycogenin-1 was detected both with and without alpha-amylase treatment in P7. Glycogenin-1 had a reduced molecular weight in P7, corresponding to a shorter protein produced by the truncating variant c.970C>T p.Arg324*. The gel shift induced by alpha-amylase treatment revealed that the protein was functionally active with regard to autoglucosylation. Material from P2 and P3 was not available for Western blot analysis.

A gel shift after alpha-amylase treatment was identified only in P7, lacking the C-terminal of glycogenin-1 (see Fig 2F), suggesting defective elongation of the glucose polymer primer. To further explore this hypothesis, an additional Western blot experiment of glycogenin-1 was performed. Samples from P7 and a patient lacking glycogen synthase due to *GYS1* nonsense variants were analyzed (see Fig 2G). In both cases, a gel shift of similar size after alpha-amylase treatment was demonstrated. These results suggest that elongation of the glycogen polymer, normally catalyzed by glycogen synthase, was impaired in P7 by loss of the glycogenin-1 C-terminal. Western blot analyses for glycogen synthase and branching enzyme showed no changes in the expression in the patients (see Fig 2H).

Assay of autoglucosylation in vitro of the *GYGI* variants resulting in p.Trp250*, p.Asp102His, and p.Ala16Pro did not show any gel shift after the addition of UDP glucose, suggesting nonfunctional glycogenin-1 (see Fig 2I). Proteins with the missense variants were detected with double bands even in the absence of UDP glucose, suggesting that the shift was due to a

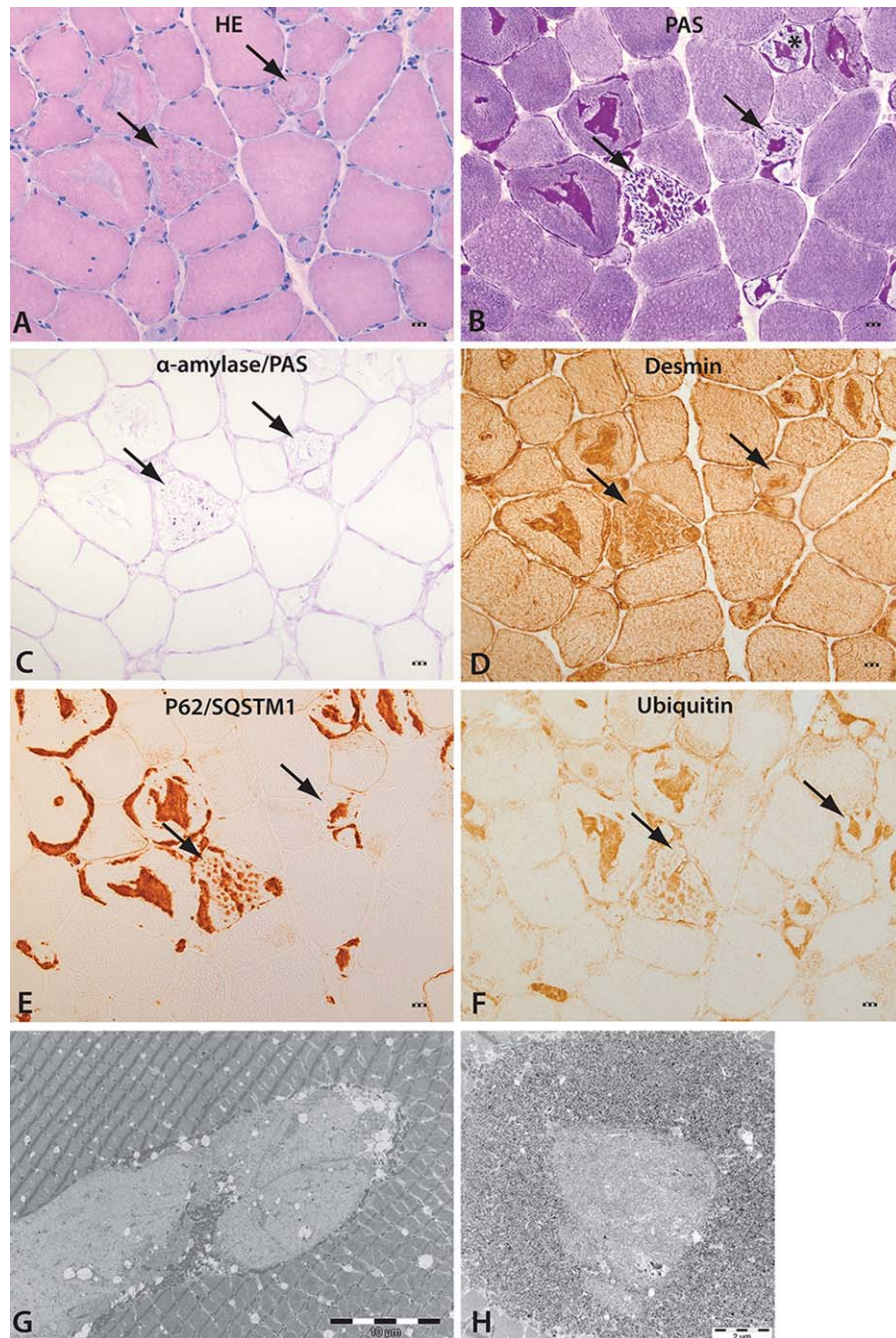


FIGURE 1: Characteristic morphological alterations of skeletal muscle. (A) Hematoxylin–eosin (HE) staining showing bluish inclusions in muscle fibers (arrows). (B) The inclusions contain periodic acid–Schiff (PAS)-positive material (arrows), and some fibers lack normal intermyofibrillar glycogen (asterisk). (C) The storage material is partially resistant to alpha-amylase treatment (arrows). (D–F) The storage material (arrows) is stained with antibodies for desmin, P62/SQSTM1, and ubiquitin. Scale bars in A–F correspond to 5 μm. (G, H) Electron microscopy demonstrating abnormal glycogen presenting with ovoid structures composed of partly filamentous material surrounded by a rim of more dense glycogen granules and mitochondria.

conformational change and not incorporation of glucose. The C-terminal nonsense variant p.Arg324* resulted in truncated protein with reduced molecular weight but preserved autoglucosylation, which was in accordance with the results from Western blot analysis of glycogenin-1 in patients' muscle tissue (see Fig 2F).

Discussion

In the present study, we report a new recessive muscle glycogen storage disorder caused by *GYGI* pathogenic variants. All patients presented a skeletal myopathy without cardiac involvement. Most patients manifested slowly progressive, adulthood onset, hip girdle, shoulder girdle,

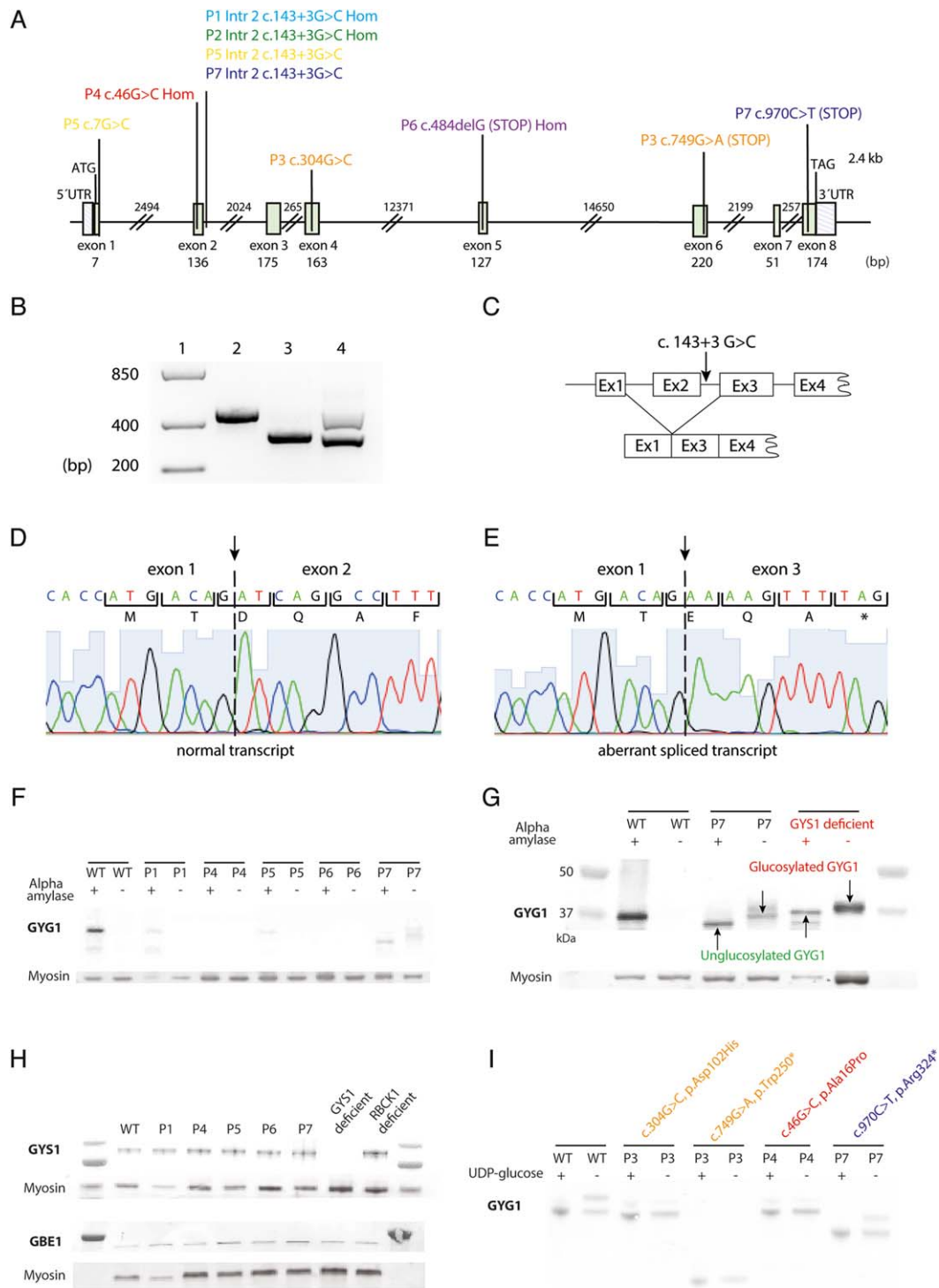


FIGURE 2: Genetic and protein analyses. (A) Schematic illustration of the coding and noncoding region of the gene *GYG1* (NM_004130). Detected variants are marked in different colors for each patient (P1, light blue; P2, green; P3, orange; P4, red; P5, yellow; P6, purple; P7, dark blue). (B) Reverse transcriptase polymerase chain reaction products covering exons 1 to 4. Lane 1: size ladder; lane 2: control sample with wild-type sequence; lane 3: patient homozygous for the *GYG1* c.143+3G>C variant resulting in transcripts with skipping of exon 2; lane 4: patient heterozygous for the *GYG1* c.143+3G>C variant, showing 2 bands, 1 of normal size and 1 with aberrant splicing with exon 2 skipping (lower band). (C) Schematic illustration of the consequences of incorrect splicing. The *GYG1* c.143+3G>C variant results in skipping of exon 2. (D) Normally spliced transcript. (E) Aberrantly spliced transcript with exon 2 skipping. (F) Results of Western blot analysis of glycogenin-1 in skeletal muscle from a normal control (WT) and patients (P1, P4, P5, P6, and P7) performed with (+) and without (-) alpha-amylase treatment. After alpha-amylase treatment, glycogenin-1 was detected in P1, P5, and P7, demonstrating that these patients produce some residual glycogenin-1. Without alpha-amylase treatment, free glycogenin-1 was detected in P7. (G) Western blot analysis of a normal control, P7, and a patient with glycogen synthase deficiency. The glycogenin-1 detected in P7 as well as in the glycogen synthase-deficient patient revealed a gel shift corresponding to approximately 1kDa, indicating that it was autoglucosylated. Glycogenin-1 in P7 is reduced in size due to the truncating variant. (H) Western blot analyses of glycogen synthase (*GYG1*) and branching enzyme (*GBE1*) in P1, P4, P5, P6, P7, 1 patient with *GYS1* deficiency, and 1 with *RBCK1* deficiency. No obvious upregulation or downregulation of these enzymes was evident in patients with pathogenic *GYG1* variants. (I) In vitro autoglucosylation demonstrated that glycogenin-1 in P3 and P4 was unable to autoglucosylate when uridine diphosphate (UDP) glucose was added (+). The truncated glycogenin-1 in P7 increased in size when UDP glucose was added, showing that it was autoglucosylated.

and/or hand and leg muscle weakness. One patient showed childhood onset, and 1 presented a peculiar phenotype characterized by late onset weakness of hands and fingers.

In the 7 patients, we identified different missense, nonsense, or frameshift *GYGI* pathogenic variants, distributed all over the gene. There was either reduced or complete absence of glycogenin-1 protein, in accordance with the deleterious effects of the variants. The most frequent variant was c.143+3G>C, identified in 4 patients from different ethnic backgrounds. This common splice site variant caused a complete or nearly complete alternative splicing, with profound reduction of wild-type glycogenin-1.

PAS-positive storage material was found in around 30 to 40% of muscle fibers. This picture had some similarities with polyglucosan body myopathies of other causes, especially branching enzyme or RBCK1 deficiency.^{5,10,11} However, the inclusions found in glycogenin-1-deficient patients appeared to be less alpha-amylase resistant. These were also more frequently found in fibers with apparently normal glycogen content compared to myopathy associated with *GBE1* and *RBCK1*. These findings suggest morphological heterogeneity in polyglucosan body myopathies and probably reflect the differences in primary gene defects. Polyglucosan body formation has in some cases been suggested to be caused by an imbalance between the activities of glycogen synthase and branching enzyme.^{12,13} We did not show any apparent upregulation or downregulation of glycogen synthase or branching enzyme in our glycogenin-1-deficient patients. However, analysis of glycogen synthase and branching enzyme activities were not available for our patients.

Unlike our patients, a previously reported patient with pathogenic *GYGI* variants showed cardiomyopathy with storage of abnormal glycogen in cardiomyocytes and glycogen depletion in skeletal muscle.⁶ This subject presented a different phenotype compared to our patients. He showed a cardiomyopathy at young age, whereas our patients developed late onset skeletal myopathy. His muscle biopsy demonstrated profound glycogen depletion, whereas our patients showed polyglucosan body myopathy. Glycogenin-1 was accumulated in the previous patients but depleted in ours. The type of pathogenic variant and other possible factors might explain these differences. Further studies may demonstrate a large variability with regard to cardiac and skeletal muscle involvement in patients with *GYGI* pathogenic variants.

Various amounts of normal glycogen were found in all patients despite depletion or absence of glycogenin-1. This observation raises the question how this glycogen is formed. Apparently, glycogenin-1 is not mandatory for glycogen formation in muscle, which may explain the

relatively late onset and the slow progression of the disease. Whether this is due to upregulation of another autoglucosylating protein, or alternative primers for glycogen synthase, remains to be investigated.

In P7, with glycogenin-1 lacking the C-terminal, we demonstrated free autoglucosylated glycogenin-1. This is similar to what is found in patients with glycogen synthase deficiency.¹⁴ Because glycogen synthase is known to bind the glycogenin-1 C-terminal,¹⁵ our results strongly indicate that this binding is essential for glycogen synthase function.

In summary, we have described a new muscle glycogen storage disorder characterized by polyglucosan bodies that is due to deficiency of glycogenin-1, and have shown that the C-terminal of glycogenin-1 is essential for glycogen synthase activity.

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Potential Conflicts of Interest

None.

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