Novel POMGNT1 point mutations and intragenic rearrangements associated with muscle-eye-brain disease

S. Saredi a, A. Ardissone b, A. Ruggieri a, E. Mottarelli a, L. Farina c, R. Rinaldi d, E. Silvestri c, C. Gandioli b, S. D’Arrigo b, F. Salerno a, L. Morandi a, P. Grammatico d, C. Pantalone b, I. Moroni b, M. Mora a,⁎

a Division of Neuromuscular Diseases and Neuroimmunology, Foundation Neurological Institute C. Besta, Milano, Italy
b Department of Child Neurology, Foundation Neurological Institute C. Besta, Milano, Italy
c Division of Neuroradiology, Foundation Neurological Institute C. Besta, Milano, Italy
d Division of Medical Genetics, Department of Molecular Medicine, “Sapienza” University, San Camillo Forlanini Hospital, Roma, Italy

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1. Introduction

Defects in genes coding for proteins involved in the glycosylation of α-dystroglycan (α-DG) are responsible for a heterogeneous group of diseases known as α-dystroglycanopathies, part of the wider group of congenital muscular dystrophies (CMD) [1]. Alpha-dystroglycanopathies are muscle diseases variably associated with central nervous system and eye abnormalities [1].

Dystroglycan (consisting of α-DG and β-DG) is an essential component of the dystrophin-glycoprotein complex that links the extracellular matrix to the actin-based cellular cytoskeleton; correct O-linked glycosylation with mannose, on the central mucin-like domain, is key to the maintenance of membrane stability [2]. Numerous studies on patients with α-dystroglycanopathies have demonstrated that correct glycosylation is vital for α-DG function [1,3,4].

Altered α-DG glycosylation has been associated with mutations in six genes (POMT1, POMT2, POMGnT1, fukutin, FKRP, and LARGE) that encode proven or putative glycosyltransferases. O-mannosyltransferase 1 and 2 (POMT1, POMT2) are required for the attachment of tetrasaccharides to α-DG. O-mannose beta-1,2-N-acetylgalactosaminyltransferase (POMGnT1) catalyzes the transfer of N-acetylgalactosamine (GlcNAc) to mannose O-linked to α-DG. Fukutin, fukutin-related protein (FKRP) and LARGE are also thought to be involved in glycosylation, but their exact function is unknown [3]. Recently, a defect in the POMT2 gene, coding for a DOL-P-Man synthase subunit, was associated with CMD in a patient with muscular dystrophy without mental retardation and greatly reduced α-DG staining on muscle biopsy [5]. Recently also, recessive mutations in DAG1 have been identified in a patient with reduced α-DG on muscle biopsy and a form of limb girdle muscular dystrophy (LGMD) characterized by severe mental retardation, but normal brain imaging and relatively mild muscular compromise [6].

Conditions associated with altered dystroglycan glycosylation range from mild LGMD without cognitive impairment, to muscle-eye-brain disease (MEB) and Walker–Warburg syndrome with severe brain and eye abnormalities [1,3–7]. However, no clear genotype-phenotype correlations have been found in α-dystroglycanopathies...
mutations in individual genes, for example MEB with POMGnTI [3,8], and microcephaly plus mental retardation with POMT1 and POMT2 [10]. We now report several new POMGnTI mutations in 3 patients and 2 foetuses with variable but severe MEB features, and evaluate protein expression in the 3 patients.

2. Patients and methods

Written, informed consent was obtained from subjects or their parents/legal guardians. The research was conducted according to protocols approved by the Institutional Review Board of the Besta Neurological Institute in compliance with the Helsinki Declaration and Italian legislation.

2.1. Clinical findings

Cases 1–3. Clinical features are summarized in Table 1. All were born from healthy, non-consanguineous Italian parents, after uneventful pregnancy (case 1), or after detection of brain malformation and hydrocephaly (case 2), or holoprosencephaly (case 3) on ultrasound. In cases 2 and 3 a ventricular-peritoneal shunt was inserted at 40 and at 20 days, respectively, for hydrocephaly.

All 3 cases had severe psychomotor delay; generalized epileptic seizures eventually controlled with antiepileptic drugs also developed in all. CK levels were moderately elevated. Cerebral magnetic resonance imaging (MRI) showed cerebellar cysts or cerebellar hypoplasia and widespread abnormalities in the cerebral cortex, in all 3 cases (Fig. 1). Muscle biopsy revealed increased mainly perimysial connective tissue, fibre diameter variability, and a few degenerating fibres, in all cases.

Cases 4 and 5 were a female (foetus 1) and a male foetus (foetus 2), from healthy non-consanguineous Italian parents originating from the same small village. The first pregnancy was terminated at gestational week 21 because foetal MRI at 20 weeks showed supratentorial hydrocephalus, thin cerebral cortex, undetectable corpus callosum, and hypoplastic and dysmorphic brainstem with posterior kink; the cerebellum was also slightly hypoplastic (Fig. 1). Subsequent autopsy confirmed these alterations and showed other severe brain abnormalities: type II lissencephaly with neurological ectopias in the posterior cerebral cortex and Sylvian aqueduct, cerebellar hypoplasia, dilatation of the vermis and agenesis of the culmen. Visceral malformations were not found.

The presence of an ectopic layer that was thinner than the cortical layer suggested, together with the MRI findings, MEB disease or Walker–Warburg syndrome and a POMGnTI mutation.

The second pregnancy was terminated at week 20 for ultrasound abnormalities (corpus callosum agenesis and lateral and third ventricular dilatation) similar to those observed in foetus 1. Autopsy of foetus 2 revealed type II lissencephaly with secondary hydrocephaly, frontotemporal neuronal ectopia, fusion of the cortical frontal lobes, and thinner ectopic layer than cortical layer, again suggesting a POMGnTI mutation. The brainstem was thin, with circumferential ectopia and disorganized fibres; the aqueduct was slightly dilated; the arachnoid was invaginated into the cerebellar folia, the vermis was hypoplastic, and the cerebellar cortex was dysplastic.

2.2. Immunohistochemistry

Muscle cryosections were solubilized in lysis buffer, boiled, and proteins were separated by SDS-PAGE (7.5% for α-DG and 4–15% for POMGnTI) and transferred to nitrocellulose membranes [11]. The membranes were probed with primary anti-α-DG antibody (Upstate Biotechnology, clone VIA4-1 and clone IIH6C4 from Upstate Biotechnology, Charlottesville, VA, USA), both diluted 1:5, then with biotinylated goat anti-mouse IgG or IgM, as appropriate, diluted 1:250 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and finally with Rhodamine-Avidin D 1:250 (Vector Laboratories, Burlingame, CA, USA). Sections were examined under a Zeiss Axiosplan fluorescence microscope.

2.3. Immunoblot

Muscle cryosections were solubilized in lysis buffer, boiled, and proteins were separated by SDS-PAGE (7.5% for α-DG and 4–15% for POMGnTI) and transferred to nitrocellulose membranes [11]. The membranes were probed with primary anti-α-DG antibody (Upstate Biotechnology, clone IIH6C4, 1:2000) or anti-POMGnTI (monoclonal from Santa Cruz, Santa Cruz, CA, USA; 1:100) together with anti-desmin (monoclonal from Dako Cytomation, Carpenteria, CA, USA; 1:400) or anti-dysferlin (Novocastra Laboratories, Newcastle upon Tyne, UK; 1:100) as indicators of muscle proteins. Membranes were then incubated in biotinylated goat anti-mouse IgG or IgM secondary antibodies (Jackson Labs), followed by streptavidin-HRP (Jackson Labs), and ECL detection (Amersham, Arlington Heights, IL, USA).

2.4. Molecular analyses

Genomic DNA was extracted by standard methods from peripheral blood lymphocytes, amniocytes or foetal skin. For mutation screening, primers were designed flanking the intron-exon junctions of each POMGnTI exon and the 3′ UTR, based on published sequences (GenBank accession number: POMGnTI NM_017739, GenBank reference POMGnTI NG_009205.1.gb). PCRs were performed with Mega Mix Double (Microzone, Haywards Heath, West Sussex, UK). The products were purified using microCLEAN (Microzone, Haywards Heath, West Sussex, UK) and sequenced directly with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequences were analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA) with Seqscape V2.5 software (Applied Biosystems).

2.5. Quantitative PCR (qPCR)

The intragenic deletion in patient 2 was analyzed by qPCR using primers for exons 1–9 and flanking regions. Amplification products were analysed with the 7000 Sequence Detection System, ABI Prism (Applied Biosystems) with SYBR Green (Applied Biosystems). As

| Patient/sex | Age last seen | Clinical features | Maximum motor achievement | Facial dysmorphisms | Mental retardation | Eye involvement | CK* (UI) |
|-------------|--------------|------------------|---------------------------|--------------------|-------------------|---------------|---------|
| 1/M         | 30 months    | Microcephaly, spastic tetraparesis | Head control | Rounded forehead, thin lips, short neck, micrognathia | Severe | Retinopathy | 1576 |
| 2/M         | 17 years, sudden death at 17 9 years | Failure to thrive, global hypotonia | Postural control not achieved | None | Severe | Myopia, cataracts, retinitis pigmentosa | 1215 |
| 3/F         |              | Macrocephaly, tetraparesis | Postural control not achieved | Frontal bossing, saddle nose, splayed nostrils, low-set ears, micrognathia | Severe, poor response to sounds and visual stimuli | No eye involvement | 702 |

* Normal <195 UI.
internal reference the TEX2 gene was amplified using a primer pair as described by Yanagisawa et al. [12]. gDNA from a healthy individual was sequenced as control. Results were expressed as normalized ratio of control to patient. Threshold values were set at 0.8–1.3 for normal, 0.45–0.74 for deletions and 1.6–1.8 for duplications. Experiments were replicated at least twice if a deletion/duplication was suspected.

2.6. Multiplex ligation-dependent probe amplification (MLPA)

MLPA was performed to screen for large POMGnT1 deletions according to the manufacturer’s instructions using the SALSA MLPA kit P061 Lissencephaly covering exons 2, 6, 17 and 20 (MRC-Holland, Amsterdam, The Netherlands).

2.7. cDNA

Total RNA was extracted from skeletal muscle using TRI Reagent (MRC, Cincinnati, OH). First-strand cDNA synthesis reaction from total RNA was catalyzed by Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). cDNA was amplified with specific primers using Mega Mix Double (Microzone, Haywards Heath, West Sussex, UK). The PCR products were purified and sequenced directly with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). When two PCR products were detected, the agarose gel bands were purified using QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and sequenced.

3. Results

Genomic DNA sequencing excluded mutations in POMT1 and POMT2 genes of case 1, and in FKRP, POMT1 and POMT2 genes of cases 2 and 3 (data not shown).

Sequence analysis of the POMGnT1 gene in case 1, showed two heterozygous null mutations: one maternally-inherited novel c.643 C>T (p.Arg215X) mutation in exon 7, and a previously reported (Italian; [8]) paternally-inherited c.931 C>T (p.Arg311X) mutation in exon 10 (Suppl. Fig. 1A). Arg215X was also present heterozygously in a maternal aunt of case 1. The Rescue–ESE software (http://genes.mit.edu/burgelab/rescue-ese/) for identifying candidate exonic splicing enhancers (ESEs), predicted that Arg215X would affect a native ESE and Arg311X would introduce a new ESE. cDNA amplification of a fragment encompassing exons 3–8 of POMGnT1 produced 2 bands, one of the predicted size and one of smaller size; direct cDNA sequencing of the 2 bands revealed in-frame skipping of exon 7 (Suppl. Fig. 1B). CDNA amplification of a fragment encompassing exons 7–12 showed that the Arg311X mutation did not affect mRNA. Immunohistochemical analysis showed absence of α-DG and normal expression of the laminin α2 chain (Fig. 2). POMGnT1 antibody did not work in our hands by immunohistochemistry. By immunoblot, the band corresponding to α-DG had altered mobility and reduced intensity, with 2 extra bands of lower molecular weight (Fig. 3A); immunoblot of POMGnT1 showed a band of normal molecular weight and intensity (Fig. 3B).

Sequence analysis of the POMGnT1 gene in case 2 showed an apparently homozygous single bp deletion (c.350delC) in exon 4, previously reported by us in another Italian case [9], affecting the evolutionarily conserved Thr117 residue and causing a premature stop codon 26 residues downstream (Thr117Stop26) (Suppl. Fig. 2A). POMGnT1 sequencing in the father showed the mutation to be heterozygous; however, sequencing of the mother DNA failed to reveal this alteration. The mutation was also present heterozygously in a healthy sister of the case. We therefore supposed that intragenic rearrangement had caused the proband to be compound heterozygous and that a second mutation was present. cDNA amplification of fragments encompassing exons 1–9, 1–5, 3–8, 1–12 failed to reveal any band of smaller size. Direct cDNA sequencing showed the presence of the point mutation, but no rearrangements. However qPCR analysis revealed that exons 2–8...
were absent from both the proband and his mother (Suppl. Fig. 2B). MLPA analysis confirmed a heterozygous deletion of exons 2 and 6, while exons 17 and 20 were normal (the commercial MLPA kit only contains probes for exons 2, 6, 17 and 20) (Suppl. Fig. 2C). Case 2 is therefore compound heterozygous for a point mutation in exon 4 and a large deletion of exons 2–8. By immunohistochemistry α-DG was absent and the laminin α2 chain was slightly reduced (Fig. 2). By immunoblot, the band corresponding to α-DG had altered mobility and reduced intensity (Fig. 3 A); immunoblot of POMGnT1 showed two bands of reduced intensity, one of normal (almost undetectable) and one of reduced molecular weight (approx. 47 kD) (Fig. 3B).

Sequence analysis of POMGnT1 in case 3 showed the same point mutation as case 2, paternally inherited, and a previously reported maternally inherited mutation [13], presumed to affect pre-mRNA splicing (c.1895 +1 G>T) (Suppl. Fig. 3A). PCR of the 16–22 exon cDNA fragment showed a band of higher molecular weight that, after extraction and sequencing, showed that the intron between exons 21 and 22 was retained (Suppl. Fig. 3B). By immunohistochemistry α-DG was absent and the laminin α2 chain was normally expressed (Fig. 2). Immunoblot of α-DG showed a band of altered mobility and reduced intensity (Fig. 3 A); immunoblot of POMGnT1 showed an almost undetectable band of normal molecular weight (Fig. 3 B).

Sequence analysis of POMGnT1 in cases 4 and 5 revealed a novel homozygous stop mutation in exon 21, c.1863delC (Leu622X) (Suppl. Fig. 3C). Sequencing of the mother and father’s DNA confirmed that the mutation was present heterozygously in both. We could not perform immunochemical analysis for lack of tissue.

Fig. 2. Muscle immunohistochemistry in control and patients 1, 2 and 3 with VIA4-1 (left column), IIH6C4 (central column), anti-α-DG and anti-laminin α2 chain (right column) antibodies, showing absence of α-DG in all patients, and normal laminin α2 in patients 1 and 3, and slightly reduced laminin α2 expression in patient 2. X200.

Fig. 3. α-DG (A) and POMGnT1 (B) immunoblots of muscle from control and the 3 patients showing variably altered mobility and reduced intensity of α-DG in all cases, and a POMGnT1 band of normal molecular weight and intensity in patient 1; 2 bands of reduced intensity, one of normal and one of reduced molecular weight (asterisk) in patient 2; and an almost undetectable band of normal molecular weight in patient 3.
4. Discussion

MEB was first described in 1989 in Finland by Santavuori [14] and was later shown to be caused by mutations in the POMGnT1 gene [15]. It is now known that genes involved in α-DG glycosylation can also cause MEB. MEB patients present as floppy infants with visual problems, severe mental retardation, dystrophic changes on muscle biopsy, and multiple structural abnormalities of the brain [4,7,13–15].

Numerous patients with POMGnT1 mutations have been identified worldwide, and the clinical spectrum ranges from severe conditions similar to Walker–Warburg syndrome or Fukuyama CMD, through classic MEB, to the relatively mild LGMD phenotype [4,7,13].

Our study on 3 Italian MEB patients and 2 foetuses has identified two new point mutations, one new large intragenic deletion, and a new intron retention resulting from a previously-reported point mutation. Our findings indicate that intragenic rearrangements of genes related to α-DG glycosylation may be relatively common [12,16] and provide further evidence that POMGnT1 mutations are usually associated with severe MEB-like features. Cases 1 and 3 were severely affected, case 2 survived until age 17, and both foetuses had very severe central nervous system defects.

Our study also provides further evidence that cerebral MRI is important for diagnosing CMD and α-dystroglycanopathies [17]. The combination of brainstem hypoplasia, diffuse polymicrogyria, white matter alterations, hydrocephalus, and cerebellar cysts, although present in patients with mutations in other α-DG-related genes, is more common in POMGnT1-mutated cases and should direct the genetic investigations accordingly [4,17]. Foetal pathological findings and MRI alterations were also highly suggestive of α-DG-related defects and, since POMT1 and POMT2 mutations in foetal cases are usually associated with the more severe Walker–Warburg phenotype [18], POMGnT1 was the first gene targeted in our genetic analysis.

To our knowledge, our study is the first to assess POMGnT1 protein expression in patient muscle. We found that the severity of the phenotype did not correlate with POMGnT1 protein expression. Thus case 1, with typical MEB phenotype, had apparently normal protein expression so the disease is almost certainly attributable to reduced enzyme activity. In fact, the Arg215Stop mutation, present in case 1, causes skipping of exon 27 to produce a protein lacking 40 amino acids in the stem domain crucial for enzyme activity [19,20]. The band of apparently normal molecular weight present on Western blot probably corresponds to this shortened protein: the short course of the gel was likely insufficient to resolve it from the normal protein. Furthermore, because the stem domain is also involved in enzyme localization, the mutated protein may not be transported to its normal location in the Golgi apparatus [20].

The previously described [8] second mutation in case 1, c.931 C>T in exon 10 produces a p.Arg311Stop mutation in the highly conserved GnT-1 domain of POMGnT1 that is thought to be essential for UDP-GlcNAc and Mn2+ binding. This amino acid is highly conserved in many species [21] and has been reported mutated in two other patients, suggesting mutational hot spot [8,21]. Biochemical studies on truncated mutant variants of the human enzyme have shown that this residue is involved in the regulation of its activity [2,20].

The two mutations in case 1 are therefore compatible with the patient’s severe clinical features and with the altered α-DG glycosylation shown by immunohistochemistry and Western blot data.

The c.350delC mutation producing Thr117fsX26 in case 2 is a novel stop mutation predicted to produce a truncated protein lacking the entire catalytic domain and part of the stem domain. We were unable to detect a band corresponding to this truncated protein by Western blot probably because it is unstable. The normal molecular weight band of greatly reduced intensity observed in this patient may have been produced by low levels of POMGnT1 transcript that either escaped nonsense-mediated decay [22] or underwent alternative translational initiation [23]. The second mutation in case 2 is an intragenic deletion encompassing exons 2 to 8. The predicted protein [20] would have reduced molecular weight (confirmed by Western blot), be soluble (transmembrane domain missing) and also retain enzymatic activity and perhaps retain some function.

Case 3 has the same stop mutation (c.350delC) as case 2, as well as the previously reported c.1895 + 1 G>T, which we have shown to affect RNA splicing causing retention of intron 22. We found a very faint POMGnT1 band of normal molecular weight on Western blot. The severe phenotype in this patient is probably related to almost total lack of POMGnT1 protein and hence massively reduced α-DG glycosylation.

In cases 4 and 5 with closely similar phenotype the new homozygous stop mutation Leu622X introduces a premature stop codon in exon 21, which is presumed to give rise to a protein lacking part of the catalytic domain and hence reduced or absent enzyme activity. Both foetuses had severe brain malformations and were aborted. Unfortunately no muscle tissue was available for analysis.

To conclude, our study provides further evidence that rearrangements of the POMGnT1 gene are relatively common. Importantly, they can be missed on standard sequencing of genomic DNA if heterozygous, as in our case 2. POMGnT1 protein analysis in 3 patients has shown that the severity of the phenotype does not correlate with protein expression. Cerebral MRI is confirmed as important for identifying MEB and α-dystroglycanopathy phenotypes in both children and foetuses and hence for directing the genetic analysis.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jns.2012.04.008.

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