Clinical features and molecular characterization of a patient with muscle-eye-brain disease. A novel mutation in the *POMGNT1* gene.

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Abstract

Muscle-eye-brain (MEB) disease is a congenital muscular dystrophy characterized by structural brain and eye defects. Here, we describe a 12-year-old boy with partial agenesis of corpus callosum, ventriculomegaly, flattened brainstem, diffuse pachygyria, blindness, profound cognitive deficiencies and generalized muscle weakness, yet without a clear dystrophic pattern on muscle biopsy. There was no glycosylation of α-dystroglycan and the genetic screening revealed a novel truncating mutation, c.1545delC (p.Tyr516Thrfs*21), and a previously identified missense mutation, c.1469G>A (p.Cys490Tyr), in the protein O-mannose beta-1,2-N-acetylglucosaminyltransferase 1 (*POMGNT1*) gene. These findings broaden the clinical spectrum of MEB to include pronounced hypotonia with severe brain and eye malformations, yet with mild histopathological changes in the muscle specimen, despite the absence of glycosylated α-dystroglycan.

Keywords: Dystroglycan; Dystroglycanopathy; Muscle-eye-brain disease; POMGNT1; Glycosylation.

Introduction

Muscle-eye-brain disease (MEB, MIM 253280) belongs to a group of rare muscular dystrophies with autosomal recessive inheritance known as dystroglycanopathies.[1] These diseases have a wide clinical spectrum, ranging from congenital muscular dystrophy (CMD) associated with severe brain and eye malformation [Walker Warburg syndrome (WWS, MIM 236670), MEB and Fukuyama congenital muscular dystrophy (FCMD, MIM 253800)][2, 3] to mild forms of muscle weakness with later onset and without brain involvement [Limb-girdle muscular dystrophy type 2O (LGMD2O, MIM 613157)].[4, 5] MEB patients usually present muscle weakness and hypotonia at or soon after birth, and only some patients acquire independent ambulation. The most common cerebral and ocular features are pachygyria-type cortical neuronal migration disorder with hypoplasia of the brain stem and cerebellum, severe myopia, cataracts and retinal hypoplasia. Severe mental retardation and epilepsy are also constant clinical features.[1]

It is known that aberrant O-glycosylation of α-dystroglycan (α-DG) underlies the pathogenesis of these diseases since the interaction between α-DG and extracellular matrix (ECM) proteins is mediated by O-linked glycans. This protein is the central component of the dystrophin-glycoprotein complex (DGC), and one of the best known O-mannosyl glycosylated proteins.[6] There are two main O-mannosyl glycans on the mucin-like domain of α-DG that are critical for ligand binding activity. The first O-mannosyl glycan of α-DG, Neu5Acα3Galβ4GlcNAcβ2Man-O-Ser/Thr, was described by Chiba *et al* for bovine peripheral nerve.[7] Recently, a novel phosphorylated O-mannosyl glycan required for laminin binding was identified on recombinant α-DG.[8]

MEB is caused almost exclusively by mutations in the gene encoding the POMGNT1 glycosyltransferase (protein O-mannose beta-1,2-N-

acetylglucosaminyltransferase 1), which catalyzes the transfer of N-acetylglucosamine to O-mannose residues.[9]

The clinical phenotype of MEB is heterogeneous, ranging from severe brain, eye and muscle anomalies to mild brain and muscle defects accompanied by severe ocular deficits.[10]

Here we describe the clinical, histopathological and molecular genetic characterization of an unusual MEB patient with severe neurological and ocular abnormalities, yet with minimal myopathological changes. Immunohistological examination revealed a hypoglycosylated α-DG with a highly reduced laminin binding ability. The *POMGNT1* gene of this patient suffered a novel frameshift mutation, giving rise to a truncated protein. These findings highlight the wide clinical spectrum of MEB and further emphasize the role of the *POMGNT1* gene as the worldwide predominant cause of MEB.

Methods

Patient

A patient with muscle-eye-brain disease that was associated with abnormal α-DG glycosylation, as witnessed in a muscle biopsy, was studied.

Histological analysis and immunohistochemistry

Two biopsies of the quadriceps muscle were performed (at 6 and 12 months of age) and analyzed by classical histology and immunohistochemistry. Ten µm-thick cryosections of muscle specimens from the patient and a control individual were stained with hematoxylin and eosin[11] and mouse monoclonal antibodies against different glycan epitopes of α-DG (VIA4, Millipore Corporation, Billerica, MA, USA) and β-DG

(Novocastra, Newcastle-on-Tyne, UK) as described previously.[12] Also, antibodies against dystrophin (DYS 1, 2 and 3), spectrin (both from Novocastra) and laminin-α2 (MAB1922, Chemicon, Millipore Corporation) were used.

Immunoblot analysis and laminin overlay assay

Total protein extracts were obtained from frozen tissue samples and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7% for α-DG and 10% for β-DG) following the procedures described previously.[13] For laminin overlay assay the polyvinylidene difluoride (PVDF) membrane was blocked using a laminin binding buffer (LBB: 10mM triethanolamine, 140 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.6) containing 5% non-fat dry milk and incubated overnight with merosin (Sigma Aldrich, St Louis, MO, USA) at 4 ºC in LBB. The membrane was then washed and incubated with anti-laminin antibody (Sigma Aldrich) followed by horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the bound proteins were visualized by chemiluminiscence (ECL Plus, GE Healthcare, UK).

Mutation analysis

Genomic DNA from the patient and his parents was isolated from total blood samples using standard techniques. The coding exons, flanking intronic regions and polyadenylation sites of the *POMT1*, *POMT2*, *POMGNT1*, *FKTN, FKRP*, *LARGE* and *DAG1* genes were screened. A 5'-upstream region of approximately 650 bp (promoter region) was also screened for each gene. Polymerase chain reactions were performed using BIOTAQ™ DNA Polymerase (Bioline, London, UK) and specific primers available under request.

Results

Clinical findings

This 12-year-old boy was the first child of non-consanguineous parents and there were no noteworthy features in his family medical history. An ultrasound scan in the third trimester revealed ventricular enlargement associated with midline anomalies, and a brain malformation was suspected. The child was born at term without any complications, weighing 2700 g and with an Apgar score of 8/9. From birth he exhibited marked axial and appendicular hypotonia with generalized muscle weakness and fixed knee flexion contractures, although no breathing or feeding difficulties were observed. He showed global hyporreflexia. The baby had poor visual alertness and abnormal eye movements. A neonatal ophthalmological examination revealed the persistence of the hyaloid artery and anomalous choroidal vascularization with retinal dysplasia. Biochemical evaluation demonstrated moderate elevation of serum creatine kinase (CK) levels (857 units/L: normal <175 units/L). The child had a normal 46, XY karyotype. Cranial magnetic resonance imaging (MRI) revealed partial absence of the corpus callosum, with ventriculomegaly and colpocepahly. There was also flattening of the brainstem and diffuse pachygyria predominantly affected the frontal lobes (Figure 1A and B).

In a follow-up examination the patient exhibited severe global developmental delay, and the axial and limb weakness persisted although the initial hypotonia gave rise to appendicular spasticity by age 3. The child's ocular problems were exacerbated by bilateral glaucoma and cataracts. CK levels reached 3000 units/L. A new cranial MRI (at age 5) revealed periventricular white matter hyperintensities in T2 and fluid attenuated inversion recovery (FLAIR) sequences, in addition to the previous observations. At 7 years of age, the patient suffered a generalized tonic-clonic seizure

while sleeping. Conventional electroencephalography revealed background slowing, with slow waves in the temporo-occipital lobe of the left cerebral hemisphere. On examination at age 12, the patient was blind and suffered profound cognitive deficiencies, with no verbal language and he only reacted to tactile stimuli. He had spastic tetraplegia with hyperreflexia 3+ in the bilateral knees and Achilles tendon as well as fixed contractures with equinovarus foot. Moreover, he had not acquired unsupported sitting and his head control was incomplete.

At that age the family declined to perform any further muscular studies.

Histological analysis and immunohistochemistry

Two biopsies of the quadriceps muscle were performed at 6 (Figure 1D) and 12 months of age (Figure 1E) and analyzed by classical histology and immunohistochemistry. In both cases histological examination revealed mild myopathic changes with variation in fibre size and the presence of hyper-contracted fibers. The muscle architecture was preserved without endomysial fibrosis or fat tissue replacement. There were not necrotic or regenerating fibers. The diameter of the fibers varied generally between 10 and 50 microns. Muscle cryosections were stained for myofibrillar adenosine triphosphatase (ATPase) at pH 4.3 (Figure 1G) showing a slight predominance of type II fibers and the atrophic fibers were mostly type II. There were no obvious intermediate stain type 2C fibers.

However, when glycosylation state of α-DG was evaluated by immunohistochemistry, there was no staining in the muscle biopsy revealed with the VIA4 antibody (Figure 1I), whereas immunostaining with anti-β-DG antibody showed positive membrane staining (Figure 1K). Merosin, dystrophin and β-spectrin immunostaining was normal (data not shown).

Immunoblot analysis and laminin overlay assay

The absence of functionally glycosylated α-DG was confirmed in immunoblots probed with the VIA4 antibody and this result was corroborated in a laminin overlay assay that revealed no detectable laminin binding (Figure 1L).

Mutation analysis

DNA sequencing revealed two heterozygous mutations in the *POMGNT1* gene. One mutation was a novel 1-bp deletion in exon 18, c.1545delC (p.Tyr516Thrfs*21), which led to a frameshift and created a premature stop codon at amino acid 536 (Figure 2A, right panel). This alteration was absent in 200 healthy control individuals. Sequence analysis of the corresponding exons in the parents' *POMGNT1* gene confirmed that this mutation was inherited from his father. The second mutation was a previously reported missense mutation located in exon 17, c.1496G>A (p.Cys490Tyr), which was carried by the patient's mother (Figure 2A, left panel).

Discussion

The Spanish MEB patient described here presented a combination of severe ocular and cerebral malformations associated with marked hypotonia, muscle weakness and elevated serum CK levels but with almost normal muscle histology.

From birth he exhibited hypotonia with generalized muscle weakness and severe global developmental delay. Functional capacity was limited as the patient was unable to acquire a sitting position or maintain head control unaided. These are common features of severe MEB, whereas motor abilities may be acquired in mild MEB presentations.[14]

Histological examination of the patient's muscle revealed only mild myopathic changes and minimal variation in muscle fibre diameter, somewhat surprising given the pronounced phenotypic severity exhibited by the patient. Muscular dystrophy is present in all dystroglycanopathy patients, as reflected by biochemical parameters such as elevated serum CK levels and the appearance of necrosis, fibrosis and regenerative fibres in muscle biopsies.[15] It has been described that in MEB dystrophic changes develop more slowly and become evident with increasing age. However, necrotic and regenerative fibers are a common feature at infancy (0-5 years), and architectural changes, such as fat infiltration become evident during later years.[16] In contrast, our patient did not present necrotic and regenerating fibers at 1 year of age. It was not possible to monitor the progress of myopathological changes beyond 1 year of age since a muscle biopsy is invasive for the patient and a repetitive extraction is not always feasible.

Also, mild dystrophic changes have been described in mild MEB patients with increased functional capacity that achieve independent ambulation.[14] It is intriguing that despite severe brain and eye anomalies exhibited by the patient, only mild muscle histopathological alterations with absent glycosylated a-DG were found.

The absence of the glycosylated form of α-DG was consistent with the correlation between the loss of α-DG glycosylation and the severity of the phenotype in patients carrying mutations in *POMT1*, *POMT2* and *POMGNT1*.[17] Our observations also confirm the correlation between the hypoglycosylation of α-DG and motor ability, whereby glycosylated α-DG is not detected in patients who never acquire an independent sitting position and there is stronger α-DG staining in individuals that achieve independent ambulation than in those who never walk.[18]

The patient carried a novel heterozygous mutation in the *POMGNT1* gene, c.1545delC (p.Tyr516Thrfs*21), which generates a frameshift and an early stop codon and results in the deletion of 125 amino acids within the catalytic domain. The second mutation, c.1469G>A (p.Cys490Tyr), has been described previously in several dystroglycanopathy patients and in a few foetal cobblestone lissencephaly cases.[10, 19-21] The two mutations affect residues spanning the catalytic domain of POMGNT1 (Fig. 2B). While the novel mutation p.Tyr516Thrfs*21 gives rise to a truncated protein, the Cys 490 residue, which according to the crystal structure of GnTI (N-acetylglucosaminyltransferase I) forms a cysteine bridge with Cys[421], is critical for the catalytic activity of POMGNT1. Indeed, the recent biochemical characterization of various POMGNT1 mutants containing Cys490Tyr mutation and nonsense mutations affecting residues within the catalytic domain such as Trp590X and Arg580X revealed no enzymatic activity, which allows postulating that the two alterations reported may strongly impair the enzyme activity.[22]

We have characterized a severe dystroglycanopathy patient with brain and eye abnormalities typical of MEB but with very mild histological muscular changes. Severe muscle weakness, hypotonia and elevated serum CK levels were detected, along with the absence of glycosylated α-DG. Our findings broaden the clinical spectrum of MEB and suggest that analysis of *POMGNT1* gene mutations may be useful in patients with severe structural brain and ocular defects, mental retardation and congenital hypotonia, independent of the severity of the dystrophic pattern in skeletal muscle. Given the overlapping clinical spectra of dystroglycanopathy patients, further understanding of the different phenotypes of MEB and other dystroglycanopathies will aid the establishment of gene priority in genetic analysis of these patients.

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Author contributions

M. Raducu and RPC designed and performed the molecular genetic studies. AL conducted the histological analysis and M. Rubio helped with the interpretation of molecular genetic data. RS and AC cared for the patient. M. Raducu, RS, AC and AL wrote the manuscript. JC supervised the work and revised the manuscript.

Declaration of conflicting interests

All the authors declared no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

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Ethical approval

This work was conducted according to the protocols approved by the Institutional Review Board of the Universidad Autónoma de Madrid and the Hospital Universitario 12 de Octubre. Written informed consent was obtained from the parents of the patient.

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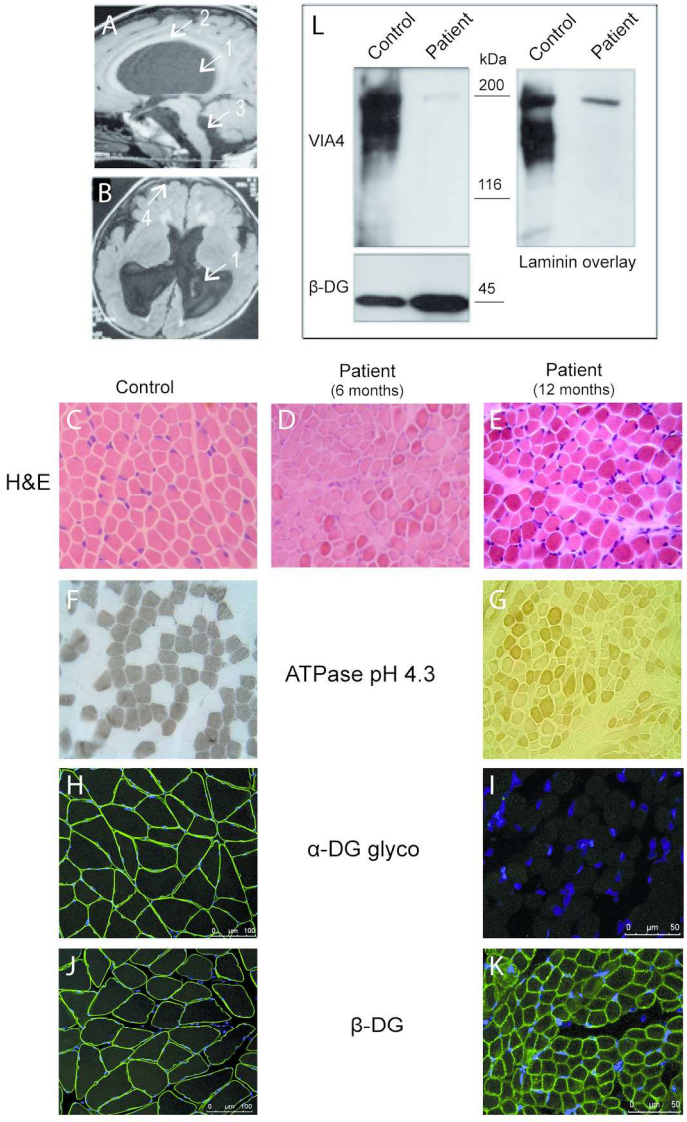
Figure legends

Figure 1. Brain magnetic resonance imaging and muscle biopsy analysis. (A) Sagittal T1-weighted image showing ventricular enlargement [1] and partial absence of the corpus callosum [2]. The pons is thinned with a concave posterior border [3]. (B) Axial FLAIR-weighted image showing ventriculomegaly [1], pachygyric changes in frontal cortex [4] and periventricular white matter hyperintensities. (C, D and E) Hematoxylin and eosin staining of patient's muscle at 6 months (D) and 12 months of age (E) showing mild variation in fibre size compared to a muscle sample from a healthy control infant (C) of around one year of age. (F and G) Histological staining for ATPase at pH 4.3 indicates the presence of type II atrophic fibers and no intermediate stain type 2C fibers were noted. (H and I) Immunostaining for α-dystroglycan of skeletal muscle from the patient and from an adult healthy individual, using an antibody against the glycosylated epitope (VIA4 antibody). (J and K) Immunostaining for β-dystroglycan. Objectives: 40X (C, D, E, F, G, I and K) and 20X (H and J). (L) Western blot and laminin overlay reveal the absence of glycosylated α-dystroglycan in the MEB patient using the VIA4 antibody and they demonstrate a complete inability to bind laminin. A band at around 200 kDa representing the endogenous laminin was detected in both control and patient lanes. The samples were analyzed for β-dystroglycan, which also served as a loading control.

Figure 2. (A) Mutation analysis of the *POMGNT1* gene confirmed that this patient was heterozygous for the missense mutation p.Cys490Tyr in exon 17 (left upper panel), and the truncating mutation p.Tyr516Thrfs*21 in exon 18 (right upper panel). His father was heterozygous for the mutation p.Tyr516Thrfs*21 mutation (right middle panel) and his mother heterozygous for the p.Cys490Tyr mutation (left bottom panel). Nucleotide

changes are indicated by asterisks (*). (B) Scheme of the POMGNT1 protein indicating the mutations found in the patient (in bold and red) together with all the other mutations reported to date in association with the mutation p.Cys490Tyr. The interrupted line connects heterozygous mutations, while an asterisk (*) indicates the homozygous forms. The cytoplasmic domain is shown in green, the transmembrane domain in black, the stem domain in blue, and in violet the catalytic domain, divided into the UDP-GlcNAc/Mn^{+2} binding region (300 to 530 amino acids) and the substrate specific region (531 to 660 amino acids).



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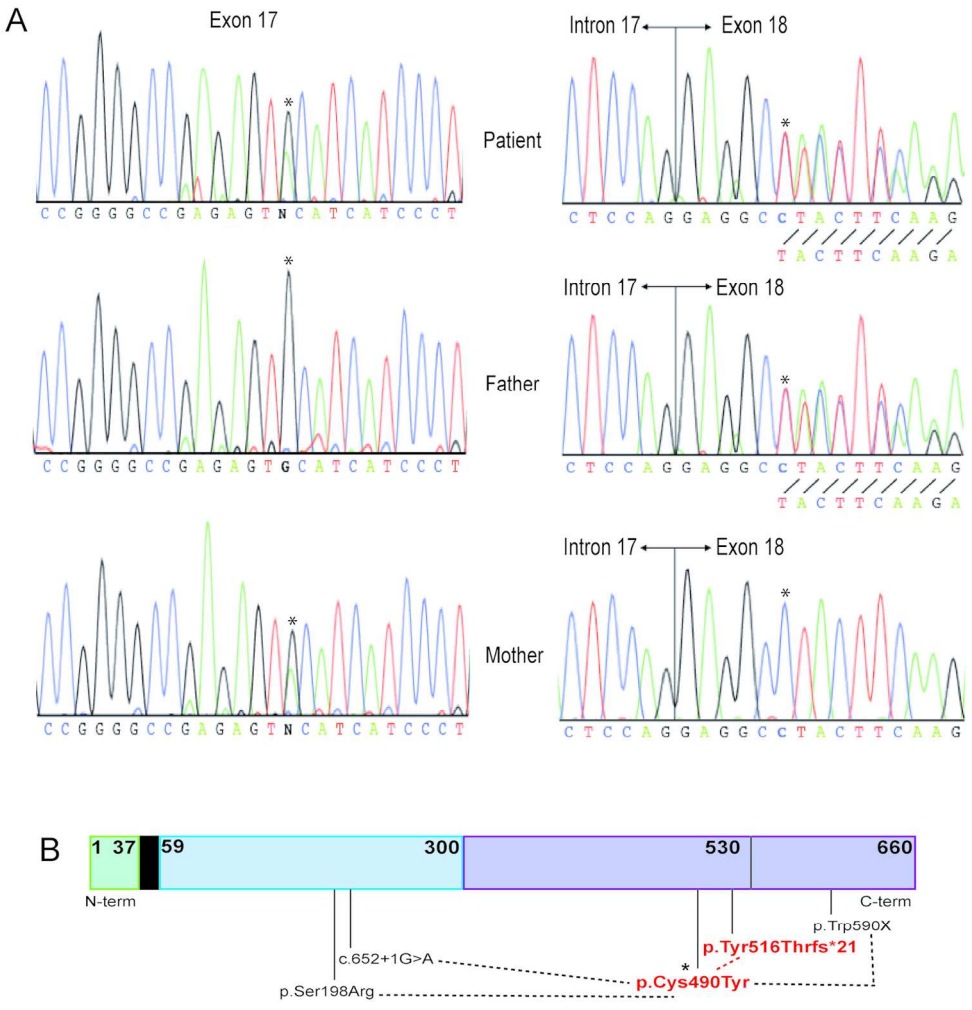

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