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Absence of β-tropomyosin is a new cause of Escobar syndrome associated with nemaline myopathy

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ABSTRACT

While *TPM2* mutations identified so far in muscular diseases were all associated with a dominant inheritance pattern, we report the identification of a homozygous null allele mutation in the *TPM2* gene in a patient who presented with a recessive form of nemaline myopathy associated with a non-lethal multiple pterygium syndrome (Escobar-MPS MIM# 265000). The *TPM2* mutation led to a complete absence of the skeletal muscle isoform of β-tropomyosin not compensated by expression of other β-tropomyosin isoforms. Escobar syndrome has been recently described as a prenatal form of myasthenia associated with recessive mutations in genes of the neuromuscular junction (CHRNG, CHRNA1, CHRND, RAPSN). This observation expands the cause of Escobar variant-MPS to a component of the contractile apparatus. This first report of the clinical expression of the complete absence of TPM2 in human indicated that TPM2 expression at the early period of prenatal life plays a major role for normal fetal movements.

1. Introduction

Dominant mutations in the *TPM2* gene have been identified in these three allelic disorders: nemaline congenital myopathy (NEM4, MIM# 609285) [1], Cap disease [2,3] and distal arthrogryposis syndrome (DA1, MIM# 108120 and DA2B, MIM# 601680) [4,5]. Nemaline congenital myopathy is a clinically and genetically heterogeneous disorder characterized by nemaline rods within muscle fibres. Six subtypes of nemaline myopathy have been defined according to the severity and age of onset that range from lethal neonatal to mild adult onset expression [6]. Cap disease is characterized by incomplete sarcomeres with enlarged Z discs at the periphery of muscle fibres and presents many similarities with the typical form of nemaline myopathy [2]. Arthrogryposis syndromes are clinically and genetically heterogeneous disorders characterized by congenital contractures.

Tropomyosins (TM) are a family of closely related proteins that form dimeric structures interacting with actin. Multiple isoforms are expressed through the use of alternative promoters and alternative RNA splicing of four genes (*TPM1–TPM4*) [7–9]. In mammalian striated muscle, three TM-isoforms are expressed at a level that varies with muscle and fibre types: α-TM_{fast} (*TPM1*) in cardiac and fast skeletal muscles, α-TM_{slow} (*TPM3*) in slow skeletal muscles and β-TM (*TPM2*). They localise to sarcomeric thin filaments where they play a central role in the Ca^{2+}-dependent regulation of muscle contraction, in association with the troponin complex. *TPM2* gene is located on chromosome 9 p13.2-p13.1 and comprises 9 exons among which two are alternatively and exclusively spliced: exons 6b and 9a in isoform 1 and exons 6a and 9d in isoform 2. A third isoform contains alternative exons 1b spliced to 3 , 6a and 9d. In human, β-TM isoform 1 is expressed in both fetal and adult skeletal muscle [10], isoform 2 in smooth muscle and fibroblasts [11].

Recessive forms of multiple pterygium syndrome (MPS) are clinically and genetically heterogeneous. They may result from early-onset fetal akinesia and are traditionally classified into prenatally lethal MPS and non-lethal Escobar variant-MPS (EVMPS). MPS have been recently associated with mutations in genes encoding the CHRNG, CHRNA1 or CHRND acetylcholine receptor subunits and rapsyn, a protein that play a key role in the clustering of acetylcholine receptor [12–15]. EVMPS is a severe condition that presents mainly with inborn contractures, pterygia and respiratory distress (MIM# 265000).

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While *TPM2* mutations identified so far in muscular diseases were all associated with a dominant inheritance pattern, we report here involvement of the *TPM2* gene in an autosomal recessive form of nemaline myopathy. Noticeably this recessive form is associated with EVMPS.

2. Methods

2.1. Patients and samples

Blood samples from each member of the family were collected after an inform consent was obtained. Genomic DNA was extracted using standard procedures. For family members living in Algeria, blood samples were collected and shipped on FTA® cards (Transgenomic, USA).

2.2. Histological studies

Standard staining and histoenzymatic reactions were carried out on 10 μm cross sections originating from a quadriceps muscle biopsy performed at 2 years of age in the proband. Fine sections (0.07 μm) were stained with uranyl acetate and lead citrate.

2.3. Haplotyping analysis

Homozygosity mapping was used to screen for the main candidate genes involved in nemaline myopathy using the following set of microsatellite markers: D1S439, D1S2847 and D1S2631 for *ACTA1*, D2S2275 and D2S2299 for *NEB*. Fragment analysis was performed using an ABI 3100 DNA Analyser and the GeneMapper software (Applera, USA).

2.4. TPM2 mutation screening

The *TPM2* gene was amplified using standard conditions and was directly sequenced with primers chosen from genomic DNA (GenBank Accession No. NT_008413), and cDNA (GenBank Accession No. NM_003289). Total RNA was extracted from a proband's frozen muscle specimen, with the use of Trizol (Invitrogen, USA) and cDNA was synthesized using the Transcriptor System (Roche, Switzerland). Sequencing reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing v3.0 reaction kit and were analysed on an ABI 3100 DNA Analyser (Applera, USA). Direct sequencing of exon 6b was used to screen for the mutation in the proband's family.

2.5. TPM2 transcript quantification

Total RNA was reverse-transcribed with i-Script® and real-time PCR was performed using the I-Q SYBR Green Supermix (BioRad, USA). Two hundred and fifty five and 235 bp fragments encompassing, respectively, exon 9a (skeletal muscle β-TM isoform 1) and exon 9d (smooth muscle and fibroblast β-TM isoform 2 and non-muscle β-TM isoform 3) were amplified with primers selected from the cDNA reference sequence (GenBank Accession No. NM_003289).

2.6. Western-blot

Crude homogenates obtained from thin slices of frozen muscles were analysed by western-blot using anti-tropomyosin (TM311 mouse clone, Sigma, USA) and a peroxidase-conjugated anti-mouse secondary antibody. Monoclonal TM311 antibody is directed against exon 1a which shows very high homologies between α-$TM_{fast,}$ α-TM_{slow} and β-TM isoforms 1 and 2 [16] and can recognized the different proteins [17] . β-TM isoform 3 which contains exon 1b is not recognized by the antibody. Chemiluminescence was detected on a ChemiDoc XRS apparatus using the Quantity 1® software (BioRad, USA).

3. Results

3.1. Clinical and pathological report

The proband (III-12) belongs to an Algerian consanguineous family (Fig. 1. panel A). He was born at term of an uneventful pregnancy and presented at birth with severe hypotonia and arthrogryposis. The child was examined for the first time at one year of age. He presented with major hypotonia, distal amyotrophy and delayed acquisition of motor milestones. Skeletal signs included scoliosis, pes varus and proximal and distal joint contractures. Neither head nor sitting control was acquired. Multiple pterygia affecting neck, axilla and antecubital and popliteal area were associated with convergent squint, ptosis and cryptorchidism (Fig. 1, panels B and C). This association was characteristic of the non-lethal Escobar variant of multiple pterygium syndrome (EVMPS, MIM# 265000). No respiratory or swallowing difficulties were observed. At two years of age, cardiac control monitoring allowed the detection of a bifascicular ventricular block with right bundle block and left anterior hemiblock. No deteriorating evolution was observed after the electrocardiographic control performed at 5 years of age. In spite of a slowly improving evolution and surgical corrections of pterygia conferring joint limitations, erected posture was achieved but not independent walking at 6 years of age.

Both consanguineous parents (II-3, II-4) and siblings (III-8, III-9 and III-11) were healthy whereas three paternal cousins were also affected (III-3, III-4, III-7) (Fig. 1, panel A). Their mother (II-2), although not known to be related to the proband's family, originated from the same district in Algeria. Clinical presentation of the three affected cousins was comparable to the proband with a homogenous picture since birth of congenital hypotonia, delayed motor milestones, multiple congenital pterygia, distal amyotrophy, facial paresia, ptosis and a severe kyphoscoliosis (Fig. 1, panels D and E). Walking ability was acquired lately, around 6 years old, but they are still ambulatory at, respectively, 25, 22 and 16 years of age. Little or no progression of the disease was observed after birth. Since these patients did not receive specific medical care, this gave us incidentally some insights into the natural history of the disease. Clinical signs observed in all affected family members were thus consistent with the diagnosis of recessive congenital myopathy associated with the non-lethal Escobar variant of multiple pterygium syndrome.

A quadricipital skeletal muscle biopsy was performed in the proband at 2 years of age. Macroscopically, bundles of muscular fibres were difficult to find among fibro-adipose tissue. Even distribution of fibre types and size was observed at a microscopical level after ATPase or NADH staining (Fig. 2, panel A). On transversal muscle sections stained with Gomori trichrome (Fig. 2, panel B), rod-like structures were observed at the periphery or within fibres, there was no intranuclear rods. Quantification showed the presence of at least 1 rod in 14% ± 1% of the fibres ($n = 4730$ fibres analysed), with a slight predilection for type I fibres (60% type I versus 40 % type II). This level of nemaline bodies was in agreement with pathological findings reported in ACTA1-related nemaline myopathies in which rods were present in variable proportion of fibres ranging from 10% to virtually all fibres without clear correlation with disease severity [18]. The diagnosis of nemaline myopathy was confirmed by ultrastructural analysis demonstrating clusters of rods parallel to the longitudinal axis of the sarcomers