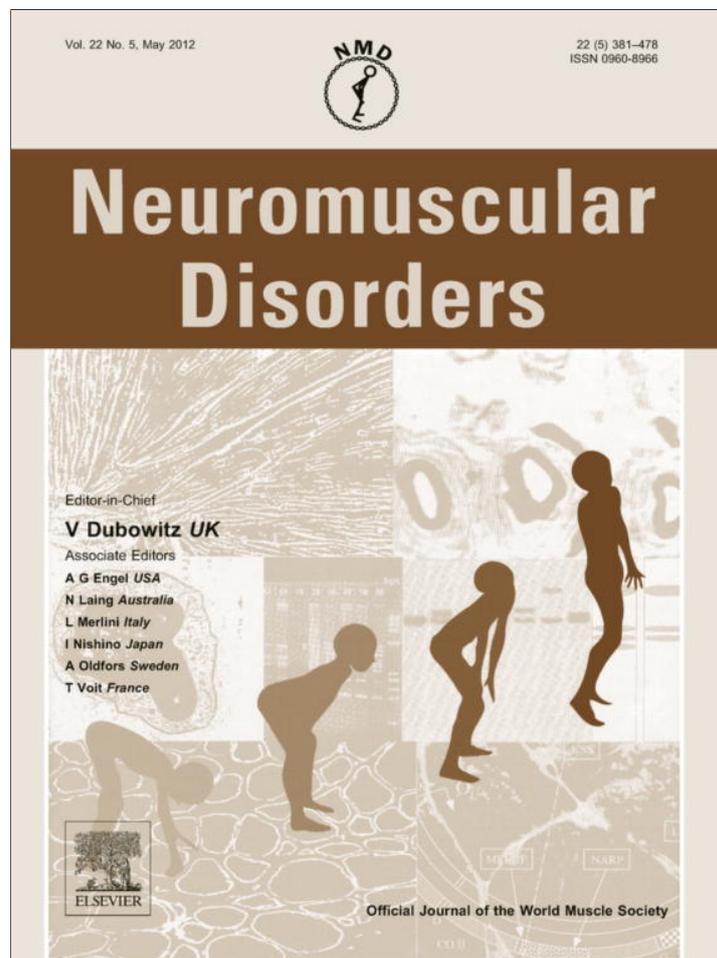


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## Case report

## X-linked myotubular myopathy due to a complex rearrangement involving a duplication of *MTM1* exon 10

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### Abstract

X-linked myotubular myopathy is a predominantly severe congenital myopathy with central nuclei on muscle biopsy due to mutations in the *MTM1* gene encoding myotubularin. We report a boy with typical features of X-linked myotubular myopathy. Sequencing of the *MTM1* gene did not reveal any causative mutations. Subsequent MLPA analysis identified a duplication of *MTM1* exon 10 both in the patient and his mother. Additional quantitative fluorescent PCR and long-range PCR revealed an additional large deletion (2536 bp) within intron 10, 143 bp downstream of exon 10, and confirmed the duplication of exon 10. Our findings suggest that complex rearrangements have to be considered in typically affected males with X-linked myotubular myopathy.

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**Keywords:** X-linked myotubular myopathy (XLMTM); Myotubularin (*MTM1*) gene; Rearrangement; mRNA; Duplication

### 1. Introduction

X-linked centronuclear (“myotubular”) myopathy (XLMTM) is a predominantly severe congenital myopathy in males characterized by numerous central nuclei on muscle biopsy (for review, [1]). XLMTM is due to hemizygous mutations in the *MTM1* gene on chromosome Xq28 [2] encoding myotubularin, a dual-specificity 3-phosphoinositide phosphatase with an important role in the regulation of signalling pathways involved in growth and

differentiation. Dominant mutations in the dynamin 2 (*DNM2*) gene [3] as well as recessive mutations in the amphiphysin 2 (*BINI*) [4] and skeletal muscle ryanodine receptor (*RYR1*) gene [5], respectively, have been implicated in autosomal forms of centronuclear myopathy (CNM), in the majority of patients associated with milder clinical features and easily distinguishable from XLMTM. However, there is clear overlap with some patients harbouring *MTM1* mutations, especially those at the milder end of the clinical spectrum.

Molecular genetic analysis of the *MTM1* gene is now widely available as a routine diagnostic service and disease-causing mutations have been identified in more than 400 patients [2,6–14]. Maternal carrier state is confirmed in around 85% of affected families [8,14] and germ cell mosaicism has been reported in several instances [8,15,16]. Causative *MTM1* mutations include deletions/insertions, nonsense, missense and splice mutations, with

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approximately equal distribution of the specific mutation classes [8,14]. Although three substitutions account for 15% of all *MTM1* mutations [7], most *MTM1* mutations are private or have been reported in few families only. *MTM1* mutations localize most frequently (in descending order) to exons 12, 4, 11, 8 and 9 [8–10,14,17–21] but there are no clear mutational hotspots.

Although routine *MTM1* molecular analysis, typically involving sequencing of all exons with inclusion of exon–intron boundaries, will detect the majority of causative *MTM1* mutations, more complex rearrangements such as duplications will remain undetected applying this approach [22]. Here we report a patient with typical clinical and histopathologic features of XLMTM due to a complex rearrangement involving a duplication of *MTM1*.

## 2. Case report

This premature male infant (35 + 1 weeks gestation) presented shortly after birth with severe hypotonia and respiratory insufficiency. In the family history he was the only affected member of a healthy non-consanguineous Caucasian couple who also had two healthy daughters and one healthy son. Three years later another healthy daughter was born. There was no family history of neuromuscular or neurological disorders; his mother had eight brothers who were all healthy. On examination he was profoundly hypotonic with markedly reduced antigravity movements. There was no obvious extraocular muscle involvement. He did not have any contractures and no scoliosis. Deep tendon reflexes were absent. He had cryptorchidism and elongated fingers and toes. CK and liver enzymes were normal. Specific genetic testing for chromosomal abnormalities, SMA, myotonic dystrophy and Prader–Willi syndrome was negative. He had a normal MRI of the brain and a normal cardiac ultrasound. Abdominal ultrasound showed kidney stones in the right kidney but was otherwise normal. His further course was characterized by progressive respiratory deterioration and he subsequently died at 1 month of age from respiratory failure.

Muscle biopsy taken in the neonatal period from the quadriceps showed numerous centrally located nuclei on H&E. On oxidative stains there was central accumulation of stain and peripheral halos compatible with a diagnosis of centronuclear/myotubular myopathy. Respiratory chain enzyme studies were normal.

## 3. Molecular genetic studies

A DNA sample from the patient was received and screened for mutations in the *MTM1* and, subsequently, *BINI*, *DNM2* and *RYR1* genes by routine DNA sequencing, all of which were negative. Haplotype analysis of the patient and his healthy brother showed different haplotypes around the *MTM1* gene. Because of clinical and pathological features highly suggestive of XLMTM, MLPA analysis of the *MTM1* and *MTMRI* genes was then performed

(SALSA MLPA kit P309-A1 *MTM1*, MRC, Holland) revealing a duplication of *MTM1* exon 10. Testing of the patient's mother showed her to be a carrier of the same duplication.

In order to confirm the MLPA result, quantitative fluorescent PCR (QF-PCR) was performed by a two-stage PCR using fluorescently-labelled primers complementary to a tag sequence incorporated into the exon-specific primers (binding sites approximately 100 bp either side of exon 10) in a multiplex reaction (full details available upon request), however, this assay failed to replicate the duplication result in either the patient or his mother.

In order to investigate this unexpected finding further, PCR primers that lie within exon 10 (close to the MLPA probe binding site) were designed and the QF-PCR repeated. A duplication of exon 10 in both the patient and his mother was observed, confirming the initial result and indicating that only a small region of the *MTM1* gene was duplicated.

To establish the nature of the duplication, long-range PCR (LR-PCR) was performed using KAPA LongRange kit (Boston, Massachusetts, United States) to amplify a 12 kb region including exons 9–11 of *MTM1* and the products were separated by agarose gel electrophoresis. A single product was amplified from the patient that was approximately 2 kb smaller than normal controls. Two products were amplified in the mother, indicating the wild-type and mutant alleles. The product from the patient was purified using AMPure (Agencourt, Beckman Coulter Inc., Brea, CA) and sequenced using a number of sequencing primers designed within intron 10 (10 µl reaction volumes using BigDye v3 sequencing chemistry, Applied Biosystems Inc., Foster City, CA). Sequencing products were cleaned-up using CleanSEQ (Agencourt Bioscience Corp). Products were analysed on an ABI 3730 DNA analyzer (Applied Biosystems) and visualised using Sequence Scanner software v1.0 (Applied Biosystems). Sequencing revealed a large deletion (2536 bp) within intron 10, 143 bp downstream of exon 10 and a duplication of exon 10 (see Fig. 1). The duplicated region (305 bp) was present 377 bp downstream of the deletion and included exon 10 and flanking intronic sequence (82 bp of intron 9 and 37 bp of intron 10). The size of the duplication was therefore concordant with the inability to detect the duplication in the initial QF-PCR experiment since the primer-binding sites were outside of the duplicated region.

Primers were designed to amplify across the region containing the deletion of intron 10 and duplicated exon 10 (P1 and P2, see Fig. 1) in order to establish if the smaller product amplified by LR-PCR in the mother represented the affected X chromosome carrying the same rearrangement as in her son. A 710 bp product was amplified in both the patient and his mother, but not in a normal control, confirming the presence of the complex rearrangement in both the patient and his mother.

To investigate the effect of the rearrangement on the *MTM1* transcript, RNA was extracted from muscle biopsy

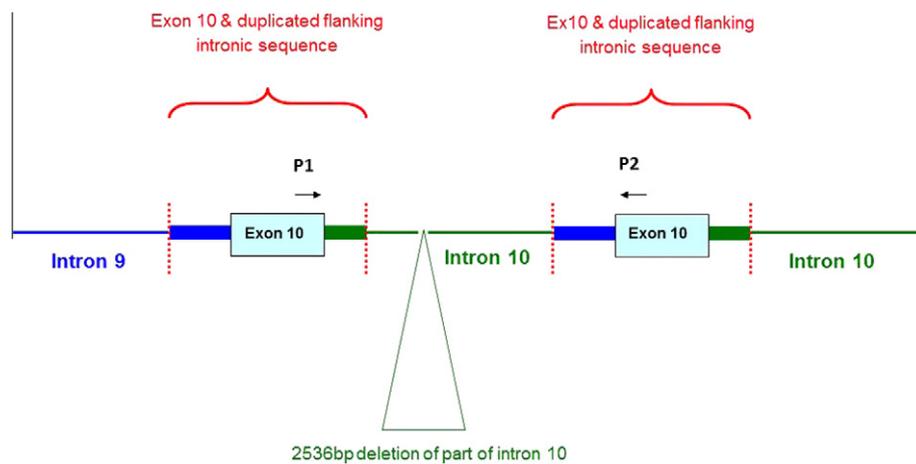


Fig. 1. Schematic diagram showing the complex rearrangement of the *MTMI* gene found in the patient and his mother. Exon 10 and flanking intronic sequence (highlighted in bold and shown by dashed red lines) is duplicated and inserted downstream of exon 10 in intron 10. In addition, there is a large deletion (2536 bp) of intron 10 upstream of the duplication. P1 and P2 show the location of the primers that were used to amplify a 710 bp product across the rearranged region in the patient and his mother. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

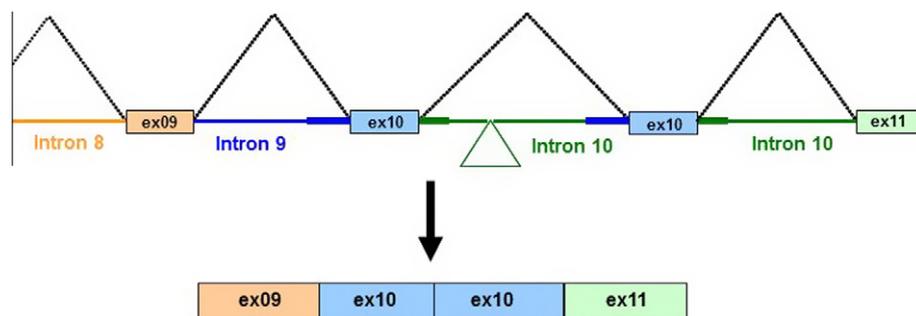


Fig. 2. Schematic diagram showing splicing of the mutant *MTMI* genomic DNA and the resulting *MTMI* transcript with a tandem duplication of exon 10.

from the patient using the RNeasy fibrous tissue mini kit (Qiagen) and reverse transcribed using the Superscript III first strand synthesis super mix (Invitrogen) and a gene specific primer designed against exon 11. This cDNA template was then subject to a two stage nested PCR reaction using primers flanking exon 10. The products were sequenced as described above.

The sequencing products showed a 186 base-pair insertion in the *MTMI* transcript the sequence of which corresponds to a tandem duplication of exon 10 at the RNA level (Fig. 2). This demonstrated that the duplicated *MTMI* exon 10 at the DNA level maintains the necessary intronic sequence for recognition by the spliceosome. The transcript containing the duplicated exon 10 retains the reading frame of the wild type transcript and is therefore expected to generate a mutant polypeptide, although this product could be targeted for degradation by the proteasome.

#### 4. Discussion

Here we reported a male infant with characteristic clinical and histopathologic findings of X-linked myotubular

myopathy (XLMTM) in the context of a complex genetic background involving duplication of exon 10 of the myotubularin (*MTMI*) gene.

Currently no specific mechanism is hypothesised for the complex rearrangement identified in this family. Non-allelic homologous recombination (NAHR) between low copy repeats (LCRs) is proposed to be a frequent mechanism for recurrent rearrangements and non-homologous end joining (NHEJ) is associated with non-recurrent rearrangements with breakpoints showing micro-homology or micro-insertion [23,24], however, neither of these mechanisms are clearly implicated in this case. Although repetitive MIR and Alu elements, associated with NHEJ-mediated rearrangement [23], do flank exon 10 of the *MTMI* gene, their locations do not coincide with the breakpoints in this case and are therefore unlikely to be involved other than in a more complex mechanistic manner.

In most cases of typical X-linked myotubular myopathy (XLMTM) disease-causing *MTMI* mutations will be identified on conventional sequencing including all exons and exon–intron boundaries. However, the limitations of this approach with regards to deep intronic mutations has been recently emphasized [22] and resulted in the

recommendation of a more complete molecular diagnostic strategy including myotubularin detection and RNA analysis. Detection of reduced myotubularin levels by Western blotting [25] in particular is a useful tool to select cases with suggestive clinico-pathological features but without molecular confirmation for further RNA analysis, however, some cases will remain unresolved even applying this more comprehensive approach [22]. Although a mutation in another gene regulating myotubularin expression is a possibility to explain this discrepancy, a rearrangement interfering with primer sites would be another plausible explanation, as illustrated in our case. To date, *MTM1* mutations are the only known cause of the X-linked form of myotubular myopathy, however, around 10% of typically affected males remain currently genetically unresolved. Although possible locus heterogeneity had been suggested [26] soon after the identification of the *MTM1* locus on Xq28, that was subsequently refuted [27] and is probably unlikely, considering that most cases without detectable *MTM1* mutations do have substantially reduced myotubularin levels [22]. Whilst it would have been ideal to measure the amount of myotubularin protein in our case, unfortunately we were unable to do this due to unavailability of sufficient muscle tissue for further protein extraction. Some mutations in the known genes for autosomal forms of CNM may occasionally mimic the phenotypical appearance of XLMTM, but on the whole give rise to milder phenotypes and are unlikely to account for all affected males with suggestive features but no *MTM1* mutation identified. Another unusual genetic mechanism, exonisation of a conserved intronic sequence due to an intronic single nucleotide substitution, has been reported in a boy with XLMTM recently [22], indicating, together with our own findings, that more complex rearrangements ought to be considered in XLMTM patients where none of the more common mutations has been identified. Complex rearrangements as the cause of neuromuscular disease are rare but have been, for example, reported in few instances of boys affected by Duchenne muscular dystrophy [28–30].

Our findings emphasize the importance of a careful search for uncommon *MTM1* variations applying complementary molecular strategies in boys with clinico-pathological features of XLMTM where no *MTM1* mutation has been identified on routine sequencing. In addition to RNA analysis and myotubularin detection, we suggest also screening for pathogenic copy number variants in the evaluation of these cases, to increase diagnostic yield and to allow appropriate diagnosis and genetic counselling for affected families. MLPA analysis used in the present study was successful in identifying the rearrangement. However, the increasing availability of microarray capture approaches will probably make this the method of choice for identification of copy number variations in the *MTM1* gene in future, particularly as this technique will explore more thoroughly intronic and regulatory regions not covered with the MLPA approach.

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