Title:
A DM1 Patient with CCG variant repeats: Reaching the Diagnosis

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Abstract

We report the case of a male patient presenting in his 50s with ptosis, facial and distal limb muscle weakness, clinical and electrical myotonia, and a prior history of cataract extraction. He had a dominant family history in keeping with a similar phenotype. Myotonic dystrophy type 1 was clinically suspected. Triplet-primed polymerase chain reaction in a diagnostic laboratory did not identify a typical CTG repeat expansion on two separate blood samples. However, subsequent genetic testing on a research basis identified a heterozygous repeat expansion containing CCG variant repeats. Our case highlights the point that variant repeats are not detectable on triplet-primed polymerase chain reaction and result in a milder phenotype of myotonic dystrophy. It is crucial to maintain a high clinical index of suspicion of this common neuromuscular condition.

Keywords

Myotonic dystrophy; Triplet repeats; Variant repeats; Multisystem disorder

Abbreviations

DM(1/2)	myotonic dystrophy (type 1/2)
DMPK	dystrophy myotonic protein kinase
DMSO	dimethyl sulphoxide solution
EMG	electromyography
SNP	single nucleotide polymorphism
TP-PCR	triplet-primed polymerase chain reaction

Introduction

Myotonic dystrophy type 1 (DM1) is a progressive, multi-system disorder affecting skeletal muscles, the heart, gastrointestinal and uterine smooth muscle, eyes, central nervous and endocrine systems. The genetic basis is a repeat expansion of CTG

trinucleotides in the 3'-UTR of the *dystrophia myotonica* protein kinase (*DMPK)* gene. The expansion varies from 50 to several thousand repeats and inversely correlates with age at symptom onset [1-3]. The repeat tract is characterised by germline expansion, which contributes to genetic anticipation [4], and somatic expansion [5-6], which in turn contributes towards disease progression [7]. Over the past decade, it has become clear that a proportion of patients harbour variant repeats within the expanded repeat tract, including GGC, CTC and CCG motifs. These can sometimes be clinically suspected in cases with atypical clinical features or when symptoms are milder or later-onset than expected for their family structure or repeat size [7-15].

Triplet-primed polymerase chain reaction (TP-PCR) forms the basis for diagnosis of DM1 in UK-based diagnostic laboratories [16, 17]. A labelled PCR primer that binds in the flanking sequence is used in conjunction with a reverse primer within the CTG-repeat tract, generating fluorescently-labelled fragments that are detected by capillary electrophoresis. Since the repeat-binding primer binds in multiple places across the repeats, variable length fragments are produced, generating a ladder of fragments of increasing size up to the longest allele size, or the point at which PCR amplification efficiency limits detection (~100 repeats). Although TP-PCR does not provide an estimate of the size of an expanded allele, a ladder of fragments longer than 50 CTG repeats provides a positive molecular diagnosis. However, variant repeat interruptions within the CTG array may prevent the CTG-repeat primer binding, leading to gaps in the signal, and sometimes failure to amplify. The use of flanking primers sited at both ends of the repeat tract, in combination with an appropriate CTG/CAG reverse primer, is recommended [18] to maximise the detection of expansions, even in the presence of variant repeats that are usually located near the 3'-end of the CTG expansion.

However, these electropherograms can be difficult to interpret, and awareness of potential false negatives is important.

Case Report

A 54-year old employed man was referred by a physiotherapist who was treating him for shoulder injury and identified bilateral grip weakness. He had premature balding, reduced manual dexterity, and his legs tired easily. He had bilateral cataract extraction aged 47 and 49. He was not diabetic and did not complain of dysphagia, gastrointestinal symptoms or daytime somnolence. He had surgical treatment for testicular cancer aged 31. He had noted that his feet would tend to “flap” after walking for a distance. He had no respiratory or cardiac symptoms and no symptoms to suggest nocturnal respiratory compromise.

On examination, he had frontal balding, mild facial weakness affecting buccinators and risorius, and a transverse smile, with mildly dysarthric speech reflecting his facial weakness. He had bilateral ptosis with normal eye movements (Fig 1A and 1B). His upper limb musculature was thin with normal strength proximally, mild weakness of elbow flexion and extension, and severe distal weakness of finger flexion and extension bilaterally. Grip myotonia was detected; percussion myotonia was absent. Lower limb musculature was normal (Fig 1C), with preserved proximal muscle strength and distal weakness such that he was unable to rise on his toes.

His creatine kinase was 468 IU/L (normal <120 IU/L). His echocardiogram and ECG were normal (PR interval 164 ms). Respiratory function tests were normal. EMG studies, performed prior to referral to the neuromuscular clinic, showed electrical myotonia with myopathic changes.

His father, who died aged 75 from pneumonia, was clinically diagnosed with “muscular dystrophy” in his 40s. He was described as having frontal balding, cataract extraction in his 40s, distal weakness progressing over time to proximal weakness requiring full-time wheelchair use, and significant problems with dysphagia which did not require gastrostomy. His mother died aged 93, but was fully ambulant until her 90s, and had cataract extraction aged 89. His 57-year old sister denied symptoms but had bilateral cataracts extracted aged 49 and 50 years. His 7-year old son was well, with normal developmental milestones.

The initial clinical impression was of DM1 and genetic testing was requested. Standard PCR with two flanking primers revealed one allele with 13 repeats. TP-PCR at the 3’-end of the array did not detect the characteristic amplicon ladder (>50 repeats) that would indicate an expansion (Fig 2A). A fresh blood sample was obtained and re-tested but the same result was obtained: apparent homozygosity for a 13-repeat allele. Parental samples were unavailable to confirm homozygosity. DNA was therefore sent for analysis to the laboratory of our research collaborators.

In parallel with research testing, next generation sequencing of *DES*, *CRYAB*, *MYOT*, *ZASP*, *VCP* was carried out to investigate his distal myopathy and was normal. The *TTN* pathogenic variant c.95134T>C was excluded, as was DM2. Testing for facioscapulohumeral muscular dystrophy type 1 (unlikely in view of ptosis) showed no short 4q fragment and *POLG1* and *POLG2* next generation sequencing was normal. Repeat EMG showed typical myotonic discharges (Fig 3). Muscle MRI of lower limbs showed fatty infiltration of gluteus minimus, bilateral medial gastrocnemii muscles and diffuse early infiltration of bilateral peronei, with sparing of tibialis anterior. A muscle biopsy was not obtained.

In the research laboratory, small-pool PCR using primers that flank the CTG repeats, and Southern blot hybridisation with a repeat unit probe, was performed. Reactions were supplemented with 10% dimethyl sulphoxide solution (DMSO), to enhance melting and annealing of GC-rich variant repeat-containing templates, as described [12]. A repeat expansion was not detected (data not shown). Next, repeat-primed PCR and blotting were performed as described [15] using a 3'-flanking primer, and repeat unit primers that bound either CTG, multiple consecutive CCGs, or CCGCTG hexamer variant repeats. All three primer sets gave a weak signal. This inferred that an expansion interrupted by a complex pattern of CCG variant repeats was present (Fig 2B). However, amplification was very weak compared to the variant repeat-containing positive controls. In order to confirm or rule out the presence of a repeat expansion, nearby single nucleotide polymorphisms (SNPs) were sequenced. In a DM1 cohort, individuals with 11 to 13 repeat non-disease-causing alleles and a CTG-repeat expansion were all heterozygous at three SNPs, rs522769, rs672348 and rs16939, all within 10 kb of the CTG repeats revealing high levels of linkage disequilibrium between these SNPs and the CTG repeat (Monckton laboratory, unpublished data). Fragments that included each of these SNPs were PCR amplified and Sanger sequenced. In the patient's DNA sample, all three SNPs were heterozygous, highly consistent with the presence of one chromosome carrying 13 repeats, and a second chromosome carrying a repeat expansion (Fig 2C - data shown only for two SNPs).

Based on these findings, the patient's sister provided a blood DNA sample. By small-pool PCR, a repeat expansion with an estimated inherited repeat length of around 270 CTG repeats was detected. Small-pool PCR products were also digested by AciI, a restriction enzyme that cuts at CCG or CGG sequences (Fig 2D), confirming the existence of variant repeat interruptions. Her DNA sample was also subjected to SNP

sequencing and was also heterozygous at each of the three SNPs, as expected since her non-disease-causing allele was sized as 13 repeats in the diagnostic laboratory test (data not shown).

Discussion

Here we report the case of a DM1 patient who was heterozygous for 13 repeats and an expanded allele with variant repeats. The clinical and EMG picture, the history of early-onset bilateral cataracts, as well as the dominant family history, led to the initial clinical suspicion of DM1. Our patient is a high functioning, intellectual gentleman with no central nervous system symptoms, such as hypersomnolence, which we felt to be atypical for DM1. Alternative diagnoses were considered including myofibrillar myopathy, DM2 and *POLG*-related myopathy. The muscle MRI showed nonspecific changes. The index of suspicion for DM1 was high enough that we felt it was justified to repeat the genetic test, after the initial negative result, on a fresh blood sample in case of sample mix-up. At that point, the single allele size on the PCR raised the possibility of an expanded allele being present, since it is not possible to distinguish between an individual homozygous for a 13-repeat allele, and one where the second allele has failed to amplify.

Variant repeats, which occur in 3-8% of patients with DM1, may interfere with amplification of the repeat expansion, either directly due to the CTG-binding primer failing to bind to the variant repeats, or indirectly due to low efficiency of melting of the GC-rich interrupted allele, inhibiting amplification in general. Often, they are suspected by disruption of the characteristic ladder in TP-PCR results in the diagnostic laboratory [8] but this was not detected on our second test. When this test is used exclusively, a proportion of DM1 patients with variant repeats may be missed unless tested in a

research laboratory setting. Some variant repeat-containing expanded alleles only amplify in the presence of a PCR additive, such as DMSO. In over one third of the variant repeat-containing DNA samples in the OPTIMISTIC DM1 patient cohort, repeat expansions were detected only when small-pool PCRs were supplemented with DMSO [15].

Whilst the diagnosis in this case was made through research testing, another option would have been to test both parents (but both were deceased) to confirm that they did indeed both have a 13-repeat allele. Had only one parent carried a 13-repeat allele, it would have been apparent that the homozygous diagnostic laboratory result must be incorrect or that this was a case of non-paternity. If we had invited his sister earlier for testing, this would have allowed us to reach the diagnosis since her repeat expansion was detectable in the diagnostic laboratory.

Variant repeats reduce somatic instability and stabilise the expanded alleles in the blood resulting in a much milder phenotype [8, 10, 13, 19]. The lack of central features might be related to his variant repeats, since this has been noted in other variant repeats cases [9,11]. Our patient was treated for testicular cancer at a young age. DM1 patients have a higher incidence of various tumours including testicular cancer [20-22]. It is unclear, however, whether DM1 patients with variant repeats carry a similar risk. His muscle weakness is described as starting a decade later than it did in his father (who we presume had DM1) and he perceives himself as much more mildly affected. This could indicate a *de novo* variant repeat interruption and/or contraction arising during paternal transmission. Such instances of reverse anticipation have been previously reported in cases of *de novo* gain of variant repeats with patients being almost asymptomatic in contrast to their severely affected fathers [12]. The patient's sister also has variant repeats, however her expanded allele was amplifiable by small-

pool PCR using flanking primers, suggesting it contained a different pattern of variant repeats. If the variant repeats arose from a *de novo* insertion into a previously pure repeat expansion, two independent mutational events would be required. A second possibility is that the father's allele already contained variant repeats, which further mutated in the germline, resulting in different patterns in the two siblings (previously recognised - Monckton laboratory - unpublished).

This case underscores a few points. Firstly, it is important to extract a detailed family history. Secondly, some merit must be given to the "duck test" – if the phenotype points towards a specific diagnosis, even if not entirely typical, it often remains the likely diagnosis. Thirdly, there is mileage in pursuing a result even when it does not initially yield what is expected from it, including research laboratory avenues. Finally, this diagnosis raises challenges within genetic counselling since prognosis and progression of DM1 associated with variant repeats, and transmission of the expanded allele to future offspring may be different when compared to that with pure CTG repeats. DM1 remains the commonest genetic primary muscle disorder and it is important to be aware that routine diagnostic testing may fail to detect a repeat expansion.

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Legends

Fig. 1. (A) Photograph of our patient showing frontal balding, ptosis and transverse smile. (B) Upper limb musculature thin throughout with wasting of neck muscles too. (C) Normal lower limb musculature.

Fig. 2. The patient appears homozygous for a 13-repeat allele, but assays used in a research setting indicate that both he and his sister have interrupted repeat expansions not detected by the diagnostic TP-PCR assay. A. The three traces on the far left represent our patient's traces, showing one normal size range peak, indicating an allele in the normal range of 13 CTG repeats and no indication of TP-peaks priming beyond the size of those alleles, due to failure of the TP-PCR reactions as the CTG repeat tract is interrupted with non-CTG sequences. The middle three traces represent an unaffected control – one common normal size range peak indicating homozygosity for an allele in the non-disease associated range – similar to that in our patient. The three traces on the far right represent an affected individual with one non-disease associated allele peak and extension of TP-peaks in the typical laddered pattern in both 5' and 3' TP mixes. B. Repeat-primed PCR and blotting showing signal obtained using primers that bind to CTG, CCG and CCGCTG repeats, as well as in the 3'-flank. Estimated repeat length (rpts) based on the molecular weight marker are shown. The black arrows mark weak signals. The patient DNA sample (P) gives a much weaker signal than the variant repeat-containing positive control DNA (C). C. Sanger sequencing of the patient's DNA at the SNPs rs522769 (T/C) and rs672348 (A/C) near the DM1 mutation. The black arrows mark the positions of two overlapping peaks in the sequence traces, indicating his DNA is heterozygous at both SNPs. This pattern is seen in individuals with a 13 repeat allele and a repeat expansion. D. Small-pool PCR,

AciI digestion and Southern blotting shows that the patient's sister has an interrupted repeat expansion, since the long fragments detected using primers that flank the repeats become shorter following digestion. The control is a sample with known AciI-sensitive variant repeats. Estimated repeat lengths (rpts) based on the molecular weight marker are shown. The non-disease causing allele (N) is visible at the lower edge of the panel.

Fig.3. EMG studies showing evidence of electrical myotonia in tibialis anterior muscle.