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Identification, molecular characterization and segregation analysis of a variant *DMPK* pre-mutation allele in a three-generation Italian family

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DM1 is an autosomal dominant multisystemic disease caused by an unstable CTG repeat expansion in the 3'-untranslated region (UTR) of the *DMPK* gene. The complex variant *DMPK* expanded the alleles containing CAG, CCG, CTC and/or GGC interruptions repetition sequences have been reported in 3-8% of DM1 patients. To date, very few information is available about the frequency and clinical consequences of pre-mutated *DMPK* variant allele. In this study, we describe a three-generation Italian family showing the segregation of an interrupted *DMPK* allele within the premutation range. TP-PCR with primers complementary to CCG repetitions and direct sequencing allow us to identify a hetero-triplet $(CTG)_6(CCGCTG)_{15}(CTG)_5$ repeat structure. The haplotype analysis demonstrated that this variant allele is associated with the European founder DM1 haplotype. The pyrosequencing analysis of the CpG islands contained in the flanking regions of the CTG array, did not show the presence of a *cis effect* of the CCG interruptions on the methylation profile of the DM1 locus. The analysis of both meiotic transmissions, one maternal and one paternal, revealed the intrafamilial stability of the DM1 premutation among relatives. Our findings further support the hypothesis of a stabilizing effect of CCG interruptions on the mutational dynamics of the DM1 locus, also in intermediate *DMPK* alleles.

Key words: *DMPK* variant alleles, premutation, TP-PCR analysis, methylation

Introduction

Myotonic dystrophy type 1 (DM1, OMIM #160900) is the most common form of adult muscular dystrophy, with a prevalence of 12.5/100,000 and an autosomal dominant mode of inheritance [1,2].

Patients with DM1 show a progressive multisystemic disease affecting mainly skeletal muscle, heart and the central nervous system [3]. DM1 is caused by the expansion of an unstable CTG trinucleotide repeat located in the 3′ untranslated region of the *DMPK* gene, on chromosome 19q13.3 [4,5]. The number of CTGs is polymorphic in the general population, with a range of 5 to about 37 repeats, a premutation range from 38 to 49 triplets

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Conflict of interest
The Authors declare no conflict of interest

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and increases to 50 and up to many thousand in patients [6]. Germline instability is the major factor determining the pronounced anticipation seen in DM1 and depend on the sex of the transmitting parent. Intermediate alleles can be stably inherited for several generations, especially if transmitted by the mother, passages through male germline almost invariably lead to a large increase into the full disease range [7,8]. On the contrary, alleles longer than 80 CTGs tend to expand when transmitted through affected mothers and, depending on the mutation size, may lead to the congenital form of the disease. In the last years, variant (CAG)n, (CCG)n, (CTC)n and (CGG)n repeats interspersed within the CTG array have been reported, with an overall frequency of about 3-8% in DM1 patients [9-13]. These variant alleles greatly alter the mutational dynamics and the phenotypic manifestation of the disease, leading to important practical consequences on DM1 genetic testing and counseling. Interruptions of the repeated tract have been observed in normal and intermediate alleles of other trinucleotide repeats (TNRs) diseases, such as spinocerebellar ataxia 1 (SCA1) and fragile X syndrome, where one or more interruptions must be lost before expansion can occur. In DM1, the search for variant repeats in a large set of *DMPK* normal alleles did not reveal any interruptions, which instead have been detected only within the intermediate alleles of four individuals with discordant clinical phenotypes [14-17]. The analysis of a larger set of individuals is therefore warranted to assess the frequency and the possible causal or modifying effect on DM1 phenotype of variant *DMPK* intermediate alleles.

In this work we describe a three-generation Italian family in which a single 41 repeats interrupted allele showing a $(CTG)_6(CCGCTG)_{15}(CTG)_5$ configuration segregates. Interestingly, the length and interruption pattern of this allele remained stable through either paternal and maternal transmissions, with no apparent consequences on the phenotype. The haplotype and methylation analysis of the DM1 locus demonstrated its association with the European founder DM1 haplotype and the absence of *in cis* epigenetic effects on the genomic region surrounding the CTG array.

Materials and methods

Patients collection and DNA extraction

Samples were obtained from an Italian family referred to the Genetic Unit of the Children's University Hospital "A. Meyer", Florence for DM1 and PWS genetic testing. Major clinical data available from patients and their pedigree are summarized in Figure 1. Genomic DNA was extracted from peripheral blood leukocytes us-

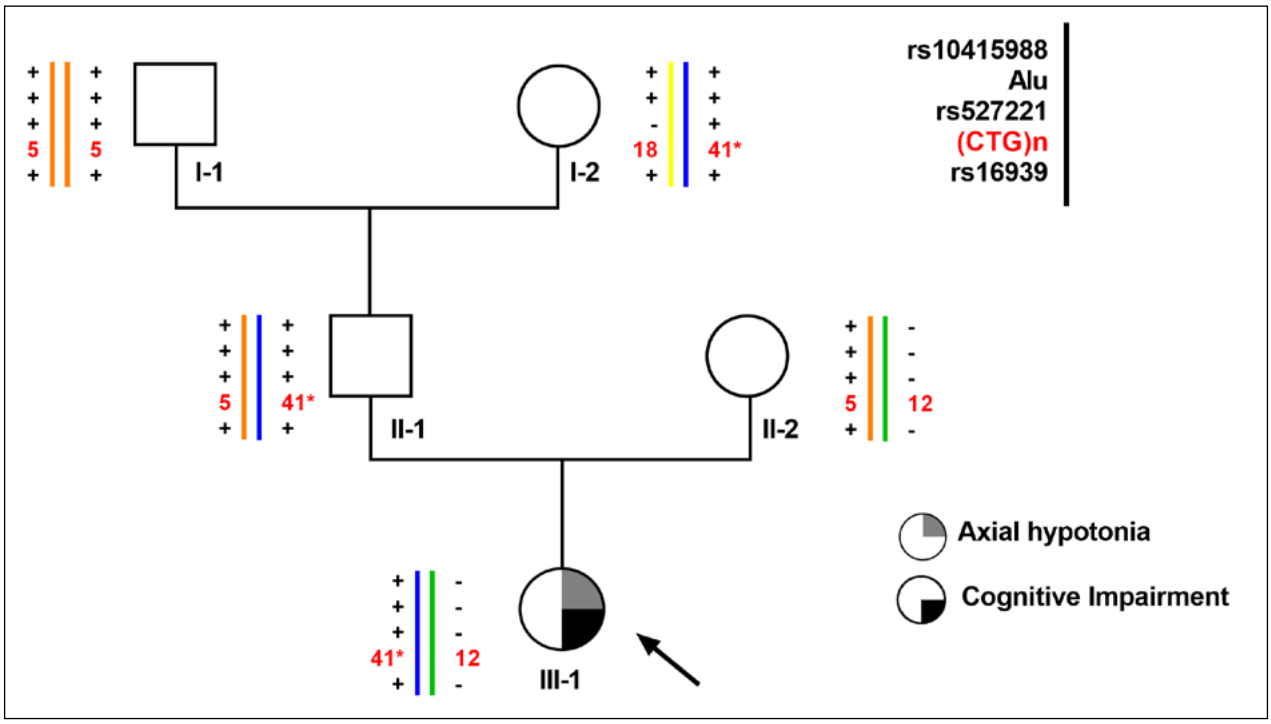

Figure 1. Pedigree and haplotype analysis of the DM1 locus of our three-generation Italian family. The proband is marked with an arrow.* This value corresponds to the apparent number of CTG repetitions.

ing EZ1 DNA Blood Kit (Qiagen, Germany) according to standard procedures. Informed consents were obtained from all individuals participating to this study (Ethical Approval register number: CS_02/2019).

Short range PCR (SR-PCR), triplet repeated-primed PCR (TP-PCR) and direct sequencing of DMPK alleles

DNA of each patient was analyzed and characterized using SR-PCR and TP-PCR with synthetic fluorescently labeled primers flanking and within the CTG repetitions, as described [16]. The interruptions of the CTG array were detected with P4 internal primers, specific for the variant motif (CCGCTG)n, according to published protocols [14]. The characterization of the interruption motifs was obtained using Sanger direct sequencing of gel-purified (Gel Extraction kit, Qiagen, Germany) SR-PCR products corresponding to the premutated *DMPK* alleles. Direct sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit and the sequences were analyzed with ABI 3130xl Automated Sequencer (ThermoFisher Scientific, Massachusetts, USA).

Haplotype analysis

The haplotype analysis of the DM1 locus was performed using four biallelic polymorphic markers as previously described [18]. The presence of a 1-kb *Alu* insertion/deletion polymorphism was typed by PCR using a three-primers protocol [19]. The following additional 3 single base pair polymorphic markers rs10415988 [Taq1] (T/C) in 15kbCEN, rs527221 [Bpm1] (G/C) in *DMPK* exon 10 and rs16939 [HinfI] (T/G) in intron 9 were typed by PCR and Sanger direct sequencing.

DMPK methylation analysis

Bisulfite conversion DNA (1 µg in 20 µl) has been obtained using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The genomic DNA was quantified by the Qubit® 2.0 Fluorometer (ThermoFisher Scientific, Massachusetts, USA).

The methylation study was carried out on *DMPK* genomic region as previously described [20]. A 189 bp fragment (10 CpG sites) in 5' end region of CTG array was amplified by PCR from bisulfite-treated DNA using CTCF-1F (5'- GGAAGATTGAGTGTTCGGGGTA -3') and CTCF-1R (5'- Biotinylated -GGGTTTTTGTAGTCGGGAATG -3') primers. For 3' end region, a 173 bp fragment (6 CpG sites) was amplified using CTCF-2F (5'- TAAATTGTAGGTTTGGGAAG -3') and CTCF-2R (5'-biotyinylated- GGGAAATTTGTTTTTGTTAAA -3') primers. PCR conditions: 95°C for 5 min, followed by 50 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec with final extension of 5 min at 72°C. The pyrosequencing analysis was performed on a PyroMark Q24 (Qiagen, Germany) with following sequencing primers: CT1-S (5'- GGGTTTTCGTTTAGTTTTAGTTTTG -3') for 5' end and CTCF-2F for 3' end regions. The methylation percentage at each CpG sites was quantified by the PyroMark Q24 software, version 2.0.7 (Qiagen, Germany).

Results

Phenotypes

The proband (Subject III-1) is the 16 year-old-second-born female of a couple of healthy and unrelated parents. The perinatal period was characterized by a marked axial hypotonia, minor hypertonia in the lower limbs, respiratory insufficiency, poor sucking, and frequent apneas. She had facial hypotonus and micro-retro-gnathia. A child-neuropsychiatrist evaluation diagnosed a medium/severe cognitive impairment, with relational and learning problems at 6 years of age. No myotonic phenomenon was elicited in the proband nor in her parents on physical examination. Her father (II-1) is a 54 years old ex-sportsman (still practicing amateur marathon) and has a normal electromyography (EMG). The paternal grandmother (I-2) is 84 years old and reported only a senile cataract with no other signs of DM1. According to the proband's clinical phenotype, the genetic testing for Prader-Willi syndrome (PWS) – and DM1 as differential diagnosis – was requested for III-1. The PWS/AS-region analysis showed a maternal uniparental heterodisomy (UPD) of chromosome 15 (data not shown), confirming the diagnosis of PWS in the proband.

Detection of DMPK premutated alleles and characterization of variant non CTG interruptions

As SR-PCR analysis of the proband's DNA showed a 12 and an apparent 41 CTG alleles at the DM1 locus, the *DMPK* molecular analysis was extended to all the available family members. The results (see Figure 1), indicated that only the proband's father and the grandmother (II-1 and I-2 respectively) were carriers of the 41 triplets *DMPK* allele. In order to exclude the presence of a DM1 expansion not detectable with SR-PCR analysis, a bi-directional TP-PCR, with primers P4-CTG (3'- end of CTG array) and P4-CAG (5'- end of the CTG array) was then performed. TP-PCR analysis did not reveal the presence of a pathological CTG expansion of the *DMPK* gene in subjects I-2, II-1 and III-1. However, the electrophoresis profiles were atypical, characterized by gaps in the continuous 3-base-pairs ladder signal, strongly suggesting an atypical interruption

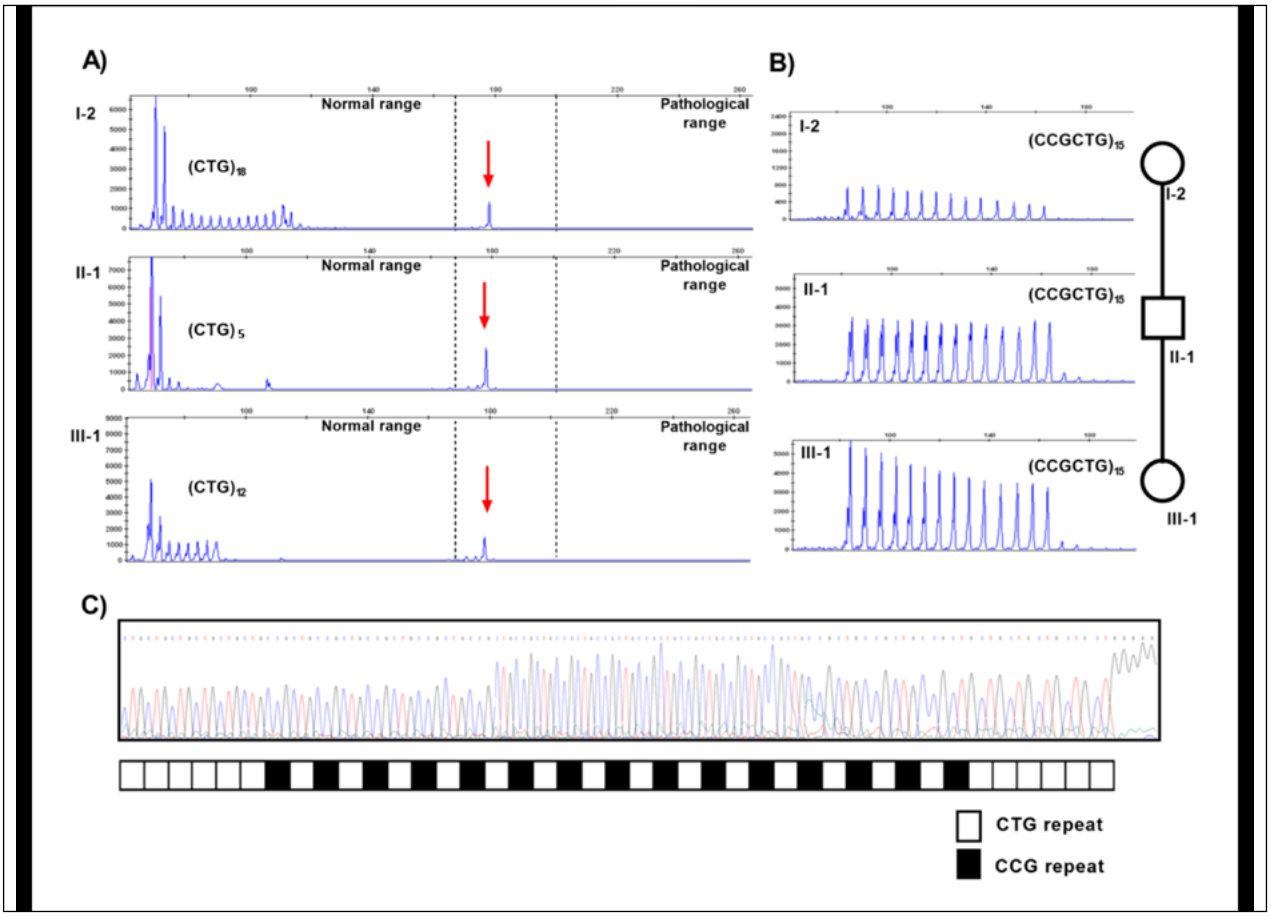

Figure 2. Molecular characterization of the DM1 locus: TP-PCR profiles of *DMPK* alleles of I-2, II-1 and III-1 with primers complementary to the CTG (A) and CCG (B) repetitions. Red arrows indicate the amplification of the apparent (CTG)41 *DMPK* allele. C) Sequencing analysis and structure of *DMPK* premutated interrupted alleles. Each square represents a single CCG repeat.

within the CTG repeated array (Figure 2A). A second round of TP-PCR, using P4-CCGCTG primer, confirmed the presence of atypical CCG interruption which remains stable through the meiotic transmissions (Figure 2B). The Sanger sequencing of the SR-PCR products confirmed a $(CTG)_6(CCGCTG)_{15}(CTG)_5$ (HGVS nomenclature: NM_001081563.2: c.*224_*283CTG[6]CCGCTG[15] CTG[5]) interruption motif in the proband (III-1), in her father (II-1) and in the grandmother (I-2) (Figure 2C).

Haplotypes analysis

To determine the haplotype in linkage disequilibrium with the DM1 interrupted premutated alleles, all family members were analyzed with 4 genetic markers closely flanking the (CTG)n repeat: rs16936 in intron9 [HinfI], rs527221 in exon10 [BpmI], rs10415988 [TaqI] and 1-Kb Alu insertion/deletion. Their polymorphisms were used to construct compound haplotypes as follow: TaqI site present (+)/absent (-); 1-kb Alu insertion (+)/deletion (-); BpmI site present (+)/absent (-) and HinfI site present (+)/absent (-).As a result, three possible haplotype combinations were identified in this family: haplotype (+ + + +) linked to the $(CTG)_5$ and $(CTG)_6(CCGCTG)_{15}(CTG)_5$ *DMPK* alleles, haplotype (- - - -) linked to $(CTG)_{12}$ allele, and haplotype (+ + - +) linked to the $(CTG)_{18}$ allele (Fig. 1).

CpG methylation profile

In order to test a possible *in cis* effects of the CCG interruptions on the *DMPK* locus already reported in DM1 patients [20], we performed a methylation analysis on two CpG islands flanking the unstable CTG tract. The first CpG island, that contains 10 CpG sites, was localized in the upstream region of the (CTG)n expansion, while the second CpG island was in the downstream region of CTG array including 6 CpG sites (Fig. 3).

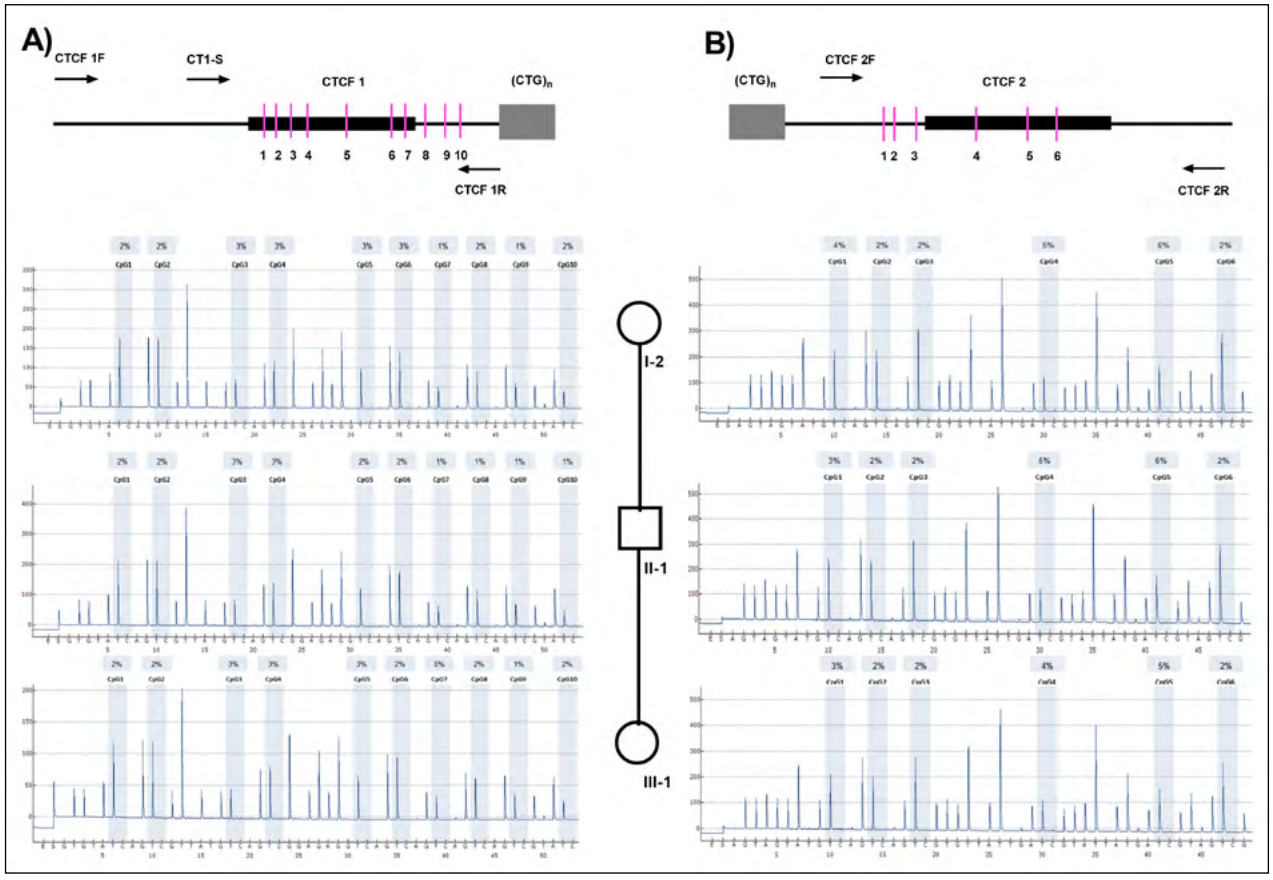

Figure 3. Methylation analysis of regions 5' and 3' to the CTG array. Up-panel: genomic structure upstream (A) and downstream (B) of the CTG repeat in the *DMPK* gene. The CpG islands are represented as bars, CTCF1 binding site as black box, CTG repeat region as gray box and the PCR primers used in this study are indicated as arrows. Down-panel: Pyrosequencing profiles of I-2, II-1 and III-1 samples, respectively 5' (A) and 3' (B) regions to the CTG array.

By pyrosequencing analysis, a homogeneous 2-4% hypomethylation level with no significant differences among I-2, II-1 and III-1 samples was found in the upstream region (Fig. 3A). Equally, in the downstream region of the (CTG)n expansion an average methylation level of 3% with no significant differences in I-2, II-1 and III-1 individuals was found (Fig. 3B). These results indicate that the presence of CCG interruptions in the DM1 locus do not influence the methylation levels of the genomic regions flanking the (CTG)n array.

Discussion

The presence of atypical interruptions in the DM1 locus can influence the phenotype in several simple repeat expansion disorders [11]. To date, very few patients have been described not carrying CTG interruption in DM1 alleles containing more than 35 repetitions and the effects on the mutational dynamics and phenotypic outcome are still subject of debate. The first *DMPK* interrupted allele was described by Leeflang et al. [17], in a sperm donor carrying apparent 37 CTG repetitions and a $(CTG)_4(CCGCTG)_{16}(CTG)$ hetero-triplet structure. Musova et al. [14], reported three individuals with a similar repeated structure in intermediated *DMPK* alleles. However the contribution to the phenotype remains unclear because of the simultaneous occurrence of other neuromuscular conditions [14]. Our proband has been also referred for DM1 genetic testing because of a neuromuscular phenotype, which was associated to a maternal uniparental heterodisomy of PWS/AS critical region on chromosome 15q, confirming the diagnosis of PWS. The molecular characterization of the DM1 locus revealed the presence of a premutated *DMPK* allele with apparent 41 CTG repetitions in a heterozygous state. The combined use of SR-PCR and bidirectional TP-PCR allows us to detect CCG interruptions of the CTG tract with a $(CTG)_6(CCGCTG)_{15}(CTG)_5$ structure. The extension of the molecular analysis in all the available family members established that the premutated interrupted *DMPK* allele was paternally inherited and derived from the proband's grand-

mother. The interruption we have defined may explain the anomalous meiotic stability of this allele through both maternal and paternal transmission, as demonstrated by direct sequencing of SR-PCR products in II-1 and I-2 DNA samples. The linkage analysis also showed that the $(CTG)_6(CCGCTG)_{15}(CTG)_5$ *DMPK* allele (HGVS nomenclature: NM_001081563.2: c.*224_*283CTG[6]CCGCTG[15]CTG[5]) is associated with the same chromosomal haplotype as pathogenic alleles present in affected DM1 patients (defined A-haplotype) [21]. We can speculate that the hexamer at the DM1 locus originated from at least one mutation of the CTG to CCG, combined with subsequent slippage of the hexamer. The novelty of our work report is that no clinical signs of DM1 have been detected in the proband's father and grandmother, in an apparent normal clinical status despite their advanced age. This allow us to conclude that the *DMPK* $(CTG)_6(CCGCTG)_{15}(CTG)_5$ premutated allele does not have phenotypic consequences in the analyzed individuals. Our family enlarges the set of individuals so far described who carry the variant *DMPK* premutations and may help to assess the frequency and the possible clinical effects of these very rare alleles.

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