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Incidence of amplification failure in *DMPK* allele due to allelic dropout event in a diagnostic laboratory

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ABSTRACT

Background: Myotonic dystrophy type 1 (DM1) is caused by an expanded CTG repeat in the non-coding 3' UTR of the *DMPK* gene. PCR and Southern Blot Analysis (SBA) of long-range PCR represent the routine molecular testing most widely *used* for DM1 diagnosis.

However, in these conventional methods artifacts such as allele dropout (ADO) represent a risk of misdiagnosis for DM1. Subjects, who show a single product by conventional methods, require a complementary technique such as triplet repeat primed PCR (TP-PCR).

Object: To estimate and minimize the incidence of allele dropout event in our diagnostic molecular laboratory by the use of new kit TP-PCR-based.

Methods: We retrospectively studied 190 *DMPK* alleles, on blood samples from to ninety-five subjects, previously genotyped by traditional methods to validate a new assay. The pedigree of a DM1 family was used to expand our analysis.

Results: TP-PCR assay correctly identified all 95/95 (100%) subjects and these results were in agreement with the other molecular laboratory. By conventional methods the amplification failure due to allele dropout in our cohort was in 12/190 (6.3%) *DMPK* alleles analyzed. When these 12 alleles were detected and solved by new assay, we found that the 2.6% was caused by primer sequence-dependent and the remaining 3.6% by polymerase-hindering secondary structures.

Conclusions: Allele dropout could be considered as a potentially important problem in DM1 diagnosis that may lead to the attribution of a wrong genotype with long-term consequences for both proband and family.

1. Introduction

Myotonic dystrophy type 1 (DM1) is a multisystemic disease and the most common form of muscular dystrophy among adults [1]. The symptoms and clinical findings of this dominantly inherited disease include myotonia, muscle wasting, cataracts, myalgia, cognitive dysfunctions, mental retardation and cardiac conduction defects [1,2]. DM1 phenotypes may be classified as congenital, infantile, juvenile, classical, or late onset [1,2]. DM1 is due to a CTG-repeat expansion (> 50 repeats) in non-coding 3′ UTR of the *DMPK* gene, which in turn leads to a global deregulation of gene expression in affected individuals [3–5].

DM1 have a particular feature known as anticipation in which the penetrance of the disease is increased in subsequent generations and it is correlated with a larger expansion of CTG-repeats. This phenomenon is a non-Mendelian genetic process and the mechanisms responsible for the increase of CTG number are unknown.

On the other hand, the intergenerational contractions is a phenomenon responsible for the decrease in the CTG repeat size during transmission from parents to child and can also occur in about 7.4% of transmissions, most frequently during paternal transmissions [6].

Recently, Musova et al. [7] describe for the first time the

Abbreviations: DM1, myotonic dystrophy tipe 1; *DMPK* gene, dystrophia myotonica-protein kinase gene; TP-PCR, triplet-repeat primed polymerase chain reaction; SBA, Southern Blot analysis

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identification of different patterns of CCG and CTC interruptions in the *DMPK* CTG repeat tract that display unique intergenerational instability. Currently, these *DMPK* interrupted alleles have been called "variant" or "atypical" DM1 expanded alleles. The location of the interruptions change dramatically between generations and the tract expanded expansions tend to contract. Commonly, the phenotype of these patients corresponds to the mild form of the disease but in some cases, the muscular dystrophy is absent.

Currently, conventional methods such as PCR and SBA represent the techniques most used for molecular diagnosis of DM1. However, one problem associated at these PCR-based methods is the occasional amplification failure of one of the two allele. This phenomenon termed allele dropout is well known in microsatellite diseases and can cause mistaken assignment of heterozygous genotypes as homozygotes. Allele dropout also occurs in *DMPK* gene and may be due to either sequence independent factors (DNA extraction quality, presence of PCR inhibitors) or for the presence of allele-specific sequence variations (polymorphisms, interrupted sequences). Several studies have found that although different alleles at the same locus have similar probabilities of dropping out, loci with longer alleles tend to have higher dropout rates than those with shorter alleles. Allelic dropout is an insidious problem that is difficult to recognize, therefore half of genetic information is missing [8–11].

Newer techniques as well as allele-specific oligonucleotide PCR (ASO-PCR) or TP-PCR-based assays are used to reanalyze discordant results before issuing the laboratory report [12,13].

Here, we investigate the incidence rate of allele dropout in a large cohort of DM1 patients in our molecular laboratory and we took advantage of the capacity of a new kit TP-PCR-based to detect this event. The pedigree of a DM1 family were used to expand and to validate our analysis. Neuromuscular and cardiac data were collected to clinically describe DM1 individuals and support the results.

2. Material and methods

2.1. Patient enrollment

A total of 95 individuals, with clinically suspected DM1, were collected from December 2015 to December 2017. All subjects were of Italian nationality and evenly distributed by sex and age: 37 female (average age 45 ± 21 yrs) and 58 male (average age 48 ± 18 yrs). Subjects were enrolled because they presented the typical clinical features of DM1disease or for family history.

In addition, the clinical data of a DM1 Italian Family, afferent at the Neuromuscular Center of Neurological Department of IRCCS Policlinico San Donato, was collected.

This study has been approved by the local Ethic Committee. All patients included in the study have been informed about the study and a written informed consent (protocol number M-DS-C73-c) was obtained for the use of samples for research purposes before undergoing blood collection.

2.2. DNA extraction and analysis

Genomic DNA was extracted from white blood cell, using "High Pure PCR preparation kit Template" Roche. The quality and quantity of the extracted DNAs were determined by a spectrophotometer (NanoDrop). All 95 patients were tested: a) by conventional methods (PCR and long-range PCR associated with SBA) performed according to Valaperta et al. [14] and b) by a new diagnostic kit TP-PCR-based ("Myotonic Dystrophy type 1 GC kit-FL") (Experteam s.r.l, Venezia, Italy).

2.3. TP-PCR reaction

Components of this assay, named "Myotonic Dystrophy type 1 GC kit-FL" are: DM1 forward Master Mix, DM1 reverse Master Mix, DM1 GC DNA Polymerase. Each master mix is composed by: 10 µl of DM1 Forward or Reverse master mix, 0.5 µl of DM1 GC DNA Polymerase, 0.25 µl of DMSO, 10 µl of each DNA patient (150–250 ng) and 4.25 µl of DNAse-free water, for a final volume 25 µl. PCR conditions were: one cycle of 12 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C; and finally 10 min at 72 °C. The amplifications have been performed by MyCycler instrument (BioRad). If, amplification's products are not used immediately, they should be stored in the dark at −20 °C. The sequences of the primers are designed using the mapping kit of the human DMPK gene (NM_004409.3; NG_009784.1), as described by Warner et al. [15].

2.4. Capillary electrophoresis

The analysis of the amplicons is performed by capillary electrophoresis. The reaction mix for each sample consists of 14.5 µl of HiDi formamide, 0.5 µl of Standard Gene Scan-500 ROX ™ and 2.5 µl of amplified. The reaction is then denatured for 3 min at 94 °C and then loaded on the analyzer. The assay has been designed and developed on analyzer of fragments Applied Biosystems.

2.5. Neuromuscular and cardiac parameters

Each patient underwent neurological examination included clinical history collection and evaluation of muscle involvement, scored by Muscular Impairment Rating Scale (MIRS) and Medical Research Council scale (MRC). Myotonia was quantified using an arbitrary 4-point self-assessment scale in five different body parts (tongue, eyes, jaw muscles, hands and lower limbs).

All patients underwent also a full cardiac evaluation including standard ECG, 24-hour ECG-Holter monitoring and 2D-echocardiography. ECG was considered abnormal when PR interval and QRS duration were ≥120 ms and ≥210 ms respectively [16]. Wall thickness, cavity diameters and ejection fraction (EF) were measured on echocardiograms. Left ventricular (LV) systolic dysfunction was diagnosed when EF was lower than 50%.

3. Results

One hundred-ninety alleles from ninety-five blood samples genotyped for *DMPK* expansion alleles were investigate by conventional methods. Each genotype characterized by these methods was compared to the results obtained by a complementary technique TP-PCR-based.

On the total samples, 75/95(79%) subjects were equally identified by all molecular tests used in this study, such as PCR, SBA and TP-PCR. Of these 75 subjects: 22 (23.2%) subjects were heterozygotes in the normal size range and 53 (55.8%) were heterozygotes for the CTG expansion. Of these 53 pathologic individuals, 38 (71.7%) presented an expansion ranging between 125 and 455 CTG repeats and the remaining 15 (28.3%) between 622 and 890 CTG repeats.

The remaining 20 of 95 (21%) individuals were undetermined by conventional method of analysis, because presented a single product, corresponding to the normal allele.

After TP-PCR characterization, we obtained that: 12 of 20 samples resulted to be false-negative individuals, showing the presence of large expanded alleles and the remaining 8 (8.4%) samples were classified as true homozygotes for 5 CTG repeats.

Of the 12 expanded genotypes obtained by TP-PCR analysis: 5 individuals showed atypical *DMPK* alleles, in particular of these: 3 subjects presented interruptions in the 3′-end of expansion and 2 subjects in the 5′-end of expanded tract. The manifestation of the 5 interruption patterns, within 3′ and 5′ end, has been confirmed by direct sequencing of TP-PCR products. The sequencing established the presence of the contiguous stretches of the CTC-CCGCTGCTG-CCG repeats. On the other side, the last 7 subjects were indicated to be pathological