Improved Method for Molecular Diagnosis of Myotonic Dystrophy Type 1 (DM1)

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Myotonic dystrophy type 1 (DM1) has been identified as the amplification of a polymorphic (CTG)_n repeat in the 3' untranslated region of a gene encoding a serine/threonine kinase (DMPK). The length of the CTG repeat correlates with clinical severity and the age at onset of the disease. Thus, the ability to perform quick and accurate molecular genetic diagnoses is of great importance to patients and their families. Polymerase chain reaction (PCR) and Southern blots are essential methods for such diagnoses. All previously published methods are based on Southern blots using

radioactive probes. Furthermore, the enzymes used for DNA digestion result in larger fragments, which makes it difficult to accurately determine the insert sizes. In this study, we report an improved method for molecular diagnosis of DM1. This method employs an initial screening with PCR followed by Southern blots using nonradioactive probes and *TaqI* as the enzyme, and provides a much better resolution of the labeled bands. *J. Clin. Lab. Anal.* 18:50–54, 2004. © 2004 Wiley-Liss, Inc.

Key words: DM1; molecular diagnosis; myotonic dystrophy; nonradioactive method

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is an inherited myopathic disorder. It progresses slowly, is autosomal dominant, and usually manifests itself in the third or fourth decade (although occasionally it appears early in childhood) (5). The genetic defect has been localized to chromosome 19q13.2–13.3, and encodes a protein kinase termed myotonin protein kinase (2). The trinucleotide repeats are located in the 3' untranslated region of the gene, consisting of a variable number of CTG trinucleotide repeats. Disease severity correlates with the number of repeats (4,8): normal subjects have five to 37 repeats, moderately affected individuals have 50–150 repeats, and severely affected patients may have several thousand repeats (11). An accurate molecular diagnosis of this disease is important not only to enable a differential diagnosis among neurological diseases, but also to predict the severity of the disease. To make such a diagnosis, one must be able to identify the insert sizes of the CTG-repeats as realistically as possible. Although previously published methods (2,3,6,10) can accurately determine the presence of the disease, the resolution of the bands in Southern blots is not sufficient to identify the insert sizes accurately, especially at higher molecular weights. Additionally, all of these methods are based on radioactivity, and thus have many disadvantages over nonradioactive methods in terms of user safety and cost-effectiveness.

Here we report an improved, nonradioactive method for molecular diagnosis of the trinucleotide expansions specific to the DM1 gene, which provides better resolution of the insert sizes.

MATERIALS AND METHODS

Genomic DNA was isolated from a proteinase K/SDS digest of blood samples. For detection of normal alleles, polymerase chain reaction (PCR) was performed in a total volume of 25 µL containing 100 ng genomic DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 10% DMSO, and 0.5 U *Taq* DNA polymerase, at an annealing temperature of 65°C, using previously described primers (2). The products were resolved on 2.5% metaphore agarose gels. With this procedure, only alleles up to 100 copies are reliably detectable. For alleles with more than 100 trinucleotide repeats, Southern blot hybridization was performed using the probe as described in the next section.

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We used semi-nested PCR to prepare a DIG-labeled probe specific for the DM1 gene. The strategy we used to design the probe is schematized in Fig. 1. First, 50 ng of genomic DNA and 0.5 pmol each of primers 1A (5'caaagtgcaggctttctgtcatgac 3') and 1B (5'ggctcta ggtggggacagacattaaatacc 3') in a total volume of 25 μ L were utilized to amplify the 646 base pair (bp) fragment. Second, 1 μ L of the first reaction product was amplified with 2A (5'ctccctgaaccctagaactgtcttcgactc 3') and 1B primers in a total volume of 50 μ L, in the presence DIG-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). The resulting DIG-11-dUTP-labeled amplification product was 295 bp long. The 2A and 1B primers were chosen from within the 773-bp-long fragment (as calculated for the most frequent normal allele with five CTG-repeats), which includes the repeat region after *TaqI* digestion as shown in Fig. 1.

Next, 15 μ g of genomic DNA for each individual were digested using *TaqI* restriction enzyme, run on 2.5% agarose gels, and transferred to positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany). Hybridization was performed at 48°C for 16 hr using the DIG-11-dUTP labeled probe

(10 ng/mL). Membranes were washed to a stringency of $0.1 \times$ SSC.

Nonradioactive labeling and chemiluminescence detection were performed according to the manufacturer's specifications (Genius[®] System User's Guide for Membrane Hybridization; Boehringer Mannheim, Mannheim, Germany). For comparison (Fig. 2), we included Southern blots from one healthy control and two patients (lanes 1–3), which were obtained by a method that utilizes *EcoRI* to digest genomic DNA prior to gel electrophoresis to diagnose DM1 (2).

RESULTS

For a molecular diagnosis of DM1, the standard method is to perform regular PCR to detect the two normal alleles of 127–220 bp, if the genotype is normal for DM1. This initial screening is simple and practical, and needs no further modification. For this screening, we routinely use the primers described by Brook et al. (2). Successful amplification, which must be done along with a positive control with both positive alleles, yields one of three possible results: two bands of normal allele

Fig. 1. Overview of the strategy used to design the probe. The arrows indicate sites for the *TaqI* restriction enzyme on genomic DNA. The $(CTG)_n$ repeat region is present within the 773 bp fragment (top of the figure). The estimation of the length of this fragment is based on the most frequently observed repeat size of $(CTG)_5$. The enlarged view of this fragment is shown at the bottom of the figure, which indicates the positions of the semi-nested primer pairs 1A and 1B for the first PCR, and 2A and 1B for the semi-nested PCR. The 295-bp-long probe (black bar) is located downstream of the $(CTG)_n$ repeat region.

Fig. 2. Southern blot analysis of genomic DNA using a nonradioactively labeled DM1 probe. As described in Materials and Methods, a DIG dUTP-labeled DM1-specific probe was used to hybridize genomic DNA digested with *EcoRI* and *TaqI* for comparison. Lane 1: Normal control. The molecular weight of the normal alleles in the *EcoRI*-digested genomic DNA is 9 kb and the *TaqI*-digested genomic DNA 773 bp long. This 773 bp long fragment includes 5 CTG repeats. Lanes 2 and 3: Patient samples with mutated alleles (insert sizes = 600 and 500 bp). The same samples were digested with *EcoRI* or *TaqI* prior to gel electrophoresis. Lanes 4 and 5: Two samples from adult patients with myotonic dystrophy as an example of mild (insert size = 450 bp) and moderate (insert size = 700 bp) disease severity. Lane 6: A sample from a juvenile patient illustrating the severe form of the disease (insert size = 1,400 bp).

sizes, one band of normal allele size, or no bands. Patients who are normal for this genotype are easily eliminated if two normal bands are observed. However, if there is only one normal band or no bands at all, Southern blots must be performed. We improved the accuracy and efficiency of the technique by using *TaqI* for digestion of the genomic DNA. Depending on the number of the CTG-repeats in general healthy population (between 5–36 CTG-repeats), this yielded a 773–866 bp fragment (as compared to 5–36 CTG repeats in a healthy population), which includes the repeat region. Additionally, we designed a 295-bp-long probe adjacent to this region within the fragment and labeled it using semi-nested PCR with DIG-dUTP, as described in Materials and Methods. Furthermore, we used a 2.5% agarose gel to obtain sharper bands, especially in cases of somatic mosaicism, which causes diffuse appearance of the bands as shown in Fig. 2. In this figure, Southern blots using both *EcoRI* and *TaqI* digestion of the genomic DNA are shown for comparison of the two methods. In lanes 1–3 the same samples are digested

with *EcoRI* or *TaqI*. Lane 1 shows the results from a healthy control subject. Lane 2 shows the results from a male patient who first showed typical clinical signs of myotonic dystrophy 7 years ago at the age of 31. The father of this patient was also clinically diagnosed at 20 years of age. A 10-year-old male patient clinically diagnosed with the disease is represented in lane 3. The family history of this patient indicates that the disease was transmitted from his grandmother (who only has cataract as a clinical manifestation) to the father of the index case, who manifested the clinical symptoms at 20 years of age. A comparison of both methods in the healthy subject (lane 1) shows no difference for the healthy alleles at lower molecular weights; however, the resolution of the larger alleles with inserts is clearly better with the new method. Those particular bands (top bands in lanes 2 and 3) are almost indistinguishable in the *EcoRI*-digested blots, whereas in the *TaqI*-digested blots the difference in size is clearly visible. The normal bands in the *EcoRI*-digested blots are approximately 9–9.5 kb, and the normal alleles and alleles with

CAG-repeats are very close together. The lower concentration of the gels (0.8%), which is required for separation of the digested fragments with *EcoRI*, along with the larger fragment sizes result in loss of resolution. Furthermore, as expected, the mosaicism is clearly identifiable in both lanes on the *TaqI*-digested blots, since it is prominent and distinguishable from the background. Lanes 4 and 5 show results from two adult patients with insert sizes of 450 and 700 bp, and lane 6 represents a juvenile patient with a 1,500 bp insert size. As with the other samples, the resolution of this high-molecular-weight band is good. Other methods using enzymes, which digest the genomic DNA into smaller fragments, have been used; however, none of these techniques produce fragments as small as those obtained by the new method. Furthermore, the use of a 2.5% agarose gel significantly improves the technique.

DISCUSSION

For the accurate diagnosis of DM1, careful selection of primers, good optimization of PCR conditions, and standardization of Southern blots are essential (both PCR and Southern blots are susceptible to false interpretations). One must be cautious in relying solely on long PCR methods. Especially at the highly expanded trinucleotide repeat regions, non-specific PCR amplification can limit the reliability of diagnosis. Furthermore, those additional bands can be confused with a possible somatic mosaicism (7). Initial screening of genomic DNA for the presence of the two normal alleles is routinely done by regular PCR, using primers previously described by Brook et al. (2). PCR analysis is less expensive and more practical than Southern blot analysis; however, PCR can not reliably amplify more than 100 repeats (11). Therefore, it is essential to obtain fast and accurate Southern blots if one or both of the alleles are not visualized after PCR. Very rarely, homozygote patients can be detected by a well-optimized Southern blot when no bands are observed after regular PCR, and when appropriate positive controls are used (1,9). A generally accepted procedure involves performing a Southern blot analysis of genomic DNA digested with a restriction endonuclease (*EcoRI*, *BamHI*, *NcoI*, *BglI*, *HindIII*, *PstI*, or *SacI*) to detect repeats > 100 (6,10). With the use of these enzymes, the resulting fragments (including the (CTG)_n insertion) range from 1,173 to 9,682 bp. For accurate molecular diagnosis using Southern blots, the test should be designed in such a way that the fragments under investigation are clearly visible on the blots, with minimal background staining. This is particularly difficult to accomplish when diagnosing diseases, such as DM1, that exhibit somatic mosaicism in addition to

the expected high-molecular-weight insertions in questionable genes (3,12).

We improved the technique by using *TaqI* for digestion of the genomic DNA, which yields a smaller fragment (791 bp) for normal alleles (including the repeat region). This improves the resolution of the bands that contain long inserts, which are expected for DM1-positive patients, as the smaller-sized bands are easier to differentiate on gels. Furthermore, by using semi-nested PCR for nonradioactive probe preparation, we obtained a probe that is highly specific for this repeat region.

Another improvement resulted from the increase of the percentage of the gels used for Southern blots (2.5%) to obtain sharper bands—especially in cases of somatic mosaicism, which causes diffuse appearance of the bands. Using this method, we achieved an improved resolution with a clear background (Fig. 2).

Overall, this is a cost-effective, efficient method. It is well standardized, since the nonradioactive probe can be made up and optimized once in large amounts, and can be stored at -70°C for up to a year for future use. Furthermore, the use of nonradioactive probes offers other advantages, such as less time required to visualize the bands (<1 hr), no health hazards to the user, and no additional requirements for protective equipment against radiation. More importantly, the length of the alleles and the insert sizes can be estimated more accurately compared to other methods.

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