A Molecular Protocol for Diagnosing Myotonic Dystrophy

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Myotonic dystrophy (DM) is an autosomal dominant genetic disease caused by an unstable CTG repeat sequence in the 3′ untranslated region of the myotony protein kinase gene. The CTG repeat is present 5–30 times in the normal population, whereas DM patients have CTG expansions of 50 to several thousand repeats. The age of onset of the disorder and the severity of the phenotype is roughly correlated with the size of the CTG expansion. We developed a molecular protocol for the diagnosis of DM based on an initial polymerase chain reaction screen to detect normal-sized alleles and small expansions, followed by an improved Southern protocol to detect larger expansions.

Indexing Terms: inherited disorders/muscle disease/polymerase chain reaction/DNA expanded repeats

Myotonic dystrophy (DM) is an autosomal dominant, multisystem disorder characterized by myotonia, muscle weakness, cataracts, defects in cardiac conduction, mental retardation, frontal balding, and testicular atrophy (1). It is the most common form of adult muscular dystrophy, with an estimated incidence of 1 in 8000. Because of its highly variable expression, the diagnosis of DM may not be straightforward in many cases. Moreover, the late age of onset often makes genetic counseling difficult, since the affected individual may not be diagnosed until after having had children. Genetic anticipation, the occurrence of symptoms at progressively earlier ages in successive generations, has been observed in DM (1, 2). A severe congenital form occurs among offspring of affected mothers.

The DM mutation was recently identified as an unstable CTG triplet repeat located in the 3′ untranslated region of the myotony protein kinase gene (3–5). This CTG repeat is polymorphic in the normal population, having a range of 5 to 30 repeats. In DM-affected patients, the repeat number expands from 50 to several thousand (3, 9). As in Fragile X syndrome, the copy number tends to increase in successive generations, accounting for the anticipation phenomenon (5, 6, 10–14), and the expanded repeats are often both meiotically and mitotically unstable (4, 15). Recombinant DNA techniques are now being routinely used to confirm the diagnosis of DM in affected individuals (16). The goal of this study was to develop a new molecular-based protocol for the diagnosis of DM.

Materials and Methods

We examined six families affected by DM—21 affected and 17 unaffected individuals—and 17 unrelated cases. All procedures performed were in agreement with the ethical standards of The Ohio State University Human Subjects Committee. Diagnosis of DM was based on clinical symptoms. The diagnosis was made at The Ohio State University Hospitals.

Genomic DNA was extracted from leukocytes harvested from whole blood anticoagulated with EDTA or acid citrate/dextrose. The polymerase chain reaction (PCR) was initiated with ~1 μg of DNA in the presence of 150 ng of the oligonucleotide primers and 3 U of Taq polymerase (Cetus Perkin-Elmer, Norwalk, CT) in a final volume of 50 μL containing 3 mmol/L MgCl2, 300 mmol/L deoxynucleotide triphosphates, 67 mmol/L Tris (pH 8.8), 16.6 mmol/L ammonium sulfate, 6.7 μmol/L EDTA, 10 mmol/L 2-mercaptoethanol, 50 mg/L bovine serum albumin, and 100 μg/L dimethyl sulfoxide. Amplification was carried out for 10 cycles at 68°C for 1 min for annealing and 95°C for 5 min for denaturation, followed by 20 cycles of 62°C for 2 min for annealing, 72°C for 5 min for extension, and 95°C for 1 min for denaturation in a thermal cycler (Ericomp, San Diego, CA). On the final cycle, extension was performed for 10 min at 72°C. To optimize the PCR conditions, we used a new forward primer (5′GCTGAAGGGTCTTGTTAGCC3′) coupled with the reverse primer (5′GGGGTGCGTGAGGATGGAA3′) published by Mahadevan et al. (8).

For Southern transfer, 10 μg of genomic DNA was digested with the appropriate restriction enzyme and separated by electrophoresis on a 8 g/L agarose gel. DNA fragments were transferred to a nylon membrane (Hybond-N+; Amersham, Amersham, Bucks, UK) by the Southern procedure with 0.75 mol/L NaCl and 0.075 mol/L sodium citrate. Conditions for hybridization, washing, and autoradiography have been previously described (17). The original cDNA probe was a 1.4-kb BamHI fragment containing the CTG repeat (pMDY1) identified and cloned by Fu et al. (4). Our laboratory also developed a new probe derived from pMDY1 but lacking the CTG repeat. Probes were radiolabeled with [32P]dCTP by using random primers (Amersham).

Results and Discussion

All patients’ samples were initially screened by PCR. Under the conditions described in Materials and Methods, myotonic PCR has been shown to be extremely sensitive and reproducible for products <500 bp. This
enabled us to clearly visualize ethidium bromide-stained amplified product up to ~100 repeats. Moreover, by using Metaphor agarose gel (FMC Bioproducts, Rockland, ME), we obtained about the same degree of resolution of a polyacrylamide gel.

Although larger expansions (>100 repeats) cannot be detected by the PCR amplification, this initial screen allows us to identify unaffected individuals, demonstrating two alleles in the normal range and small expansions often observed in minimally affected cases (e.g., individuals showing cataracts as the only symptom at a very late age). Fig. 1 shows the PCR products from three different patients. The 32-year-old woman represented in lane 1 presented with mild myotonia and marginal muscle weakness. The PCR shows two bands (equivalent to 7 and 15 CTGs) within the normal range. The result indicated that the patient was not affected with DM and other neuromuscular diseases were considered. The 74-year-old man represented in lane 2 presented with mild myotonia and facial weakness. PCR analysis clearly showed that the patient had a mutant allele of ~75 repeats, thus confirming the suspect case of DM. Last, a 56-year-old man, represented in lane 3, had several of the DM classic symptoms: characteristic facies, muscle weakness in the neck and arms, and difficulty in walking. This patient showed only one PCR allele within the normal range. The most likely explanation, that the other allele failed to amplify because of the high number of repeats, was confirmed by Southern analysis.

Since the heterozygosity frequency for the CTG repeats is ~75% in the normal human population (4, 13), ~25% of unaffected individuals will be homozygous for a given allele. Therefore, the presence of a single PCR band does not confirm the diagnosis of DM. All single bands require a Southern confirmation. Although the PCR does not have a specificity of 100%, it has several advantages, including fast turnaround time (24 h), no isotope requirement, and use of minimal quantities of DNA. The PCR is also a cost- and labor-effective initial screen, which allows us to quickly rule out DM in many patients with DM-like symptoms and in at-risk relatives who demonstrate two normal-range alleles.

For all patients demonstrating one PCR band, further analysis by Southern transfer is required. The original Southern test for DM was performed on EcoRI- or HindIII-digested DNA hybridized with a 1.4-kb BamHI cDNA (pMDY1) fragment (3, 4). Since there is a polymorphic 1-kb insert in the human population, two distinct HindIII fragments (8.5 and 9.5 kb) containing the CTG region can be obtained, with the larger allele being in linkage disequilibrium with the mutation (6). Similar results are obtained with EcoRI (3, 5, 6). Expansions of >1 kb are easily detected; however, the Southern protocol is not sensitive enough to pick up smaller expansions. To increase the sensitivity, we perform a double digestion with HindIII and BglII, which gives a CTG-containing restriction fragment of ~2.2 kb. By reducing the size of the restriction fragment, resolution is improved and expansions as small as ~70 repeats can be detected.

The original cDNA probe (pMDY1) included the CTG repeat region (4). Since these repeats are present elsewhere in the human genome, pMDY1 often produces high background when hybridized to the genomic DNA and thus requires blocking techniques. The background may interfere with the detection of expanded alleles since the expanded alleles often appear as smears because of somatic instability of the mutation. To reduce the background and increase the sensitivity of the Southern test, we cut the BamHI fragment of pMDY1 with SaeII, releasing a 1-kb fragment located 5' of the CTG repeat. The new probe is highly specific for the myotonia gene. Fig. 2 shows a comparison of a family study done with the original Southern protocol, HindIII and pMDY1, and with our new Southern protocol; all other conditions were identical. The proband II-3 in this family (lane 3) was a 40-year-old man who presented with muscle weakness in both distal upper extremities, ptosis, facial weakness, percussion myotonia, and myotonia of the tongue. The parents I-1 and I-2 (lanes 1 and 2) were both asymptomatic, whereas the 18-year-old daughter III-4 of the proband (lane 4) already exhibited facial weakness with ptosis, and a profound tongue myotonia. Although we were able to detect the large expansion of the proband and his daughter by either Southern protocol, only the new digestion allowed us to clearly distinguish the minimally expanded allele (~80 repeats) displayed by the father (lane 2). Furthermore, with the new probe, which does not contain the CTG repeat, the background was significantly reduced.

The ability of the DNA analysis to determine the origin of the mutation, when family members are asymptomatic or mildly affected, has important implications for genetic counseling. No new mutations have been described in DM, which is consistent with the linkage disequilibrium data. Thus, it is essential to identify on which side of the family the mutation is segregating to provide accurate risk assessment to all at-risk individuals in a family.

With our new Southern procedure, we are able to more accurately determine the size of the larger expansions in affected patients. Since the mutation is now on a 2.2-kb restriction fragment, rather than a 9.5-kb restriction fragment, it is possible to approximate the size

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Fig. 1. PCR analysis of trinucleotide expansion in DM.
Lane 1, two normal-range alleles; lane 2, one normal-range allele and one mutant allele of ~75 repeats; lane 3, one allele in the normal range and an expanded allele that failed to amplify (confirmed by Southern analysis).
of most expanded repeats within ±50 repeats. We believe that this level of accuracy is sufficient for two reasons: Because of the mosaicism that often occurs with the larger mutations, the bands appear as smears and it becomes difficult to determine exact repeat sizes with a high degree of confidence. Most importantly, although there is an overall positive correlation between clinical onset and repeat size, the correlation is not linear or absolute (18). We have observed large expansions in many asymptomatic and mildly affected adults. There have also been cases of severe congenital DM with small expansions (12). As a result of the somatic heterogeneity seen in DM, genotype/phenotype associations derived from leukocytes may not be as accurate as the measurement of the repeat size in the affected tissue (muscle, heart, or others). In a recent study, CTG expansions were 2- to 13-fold greater in DNA isolated from skeletal muscle than in DNA from leukocytes in 10 of 11 DM patients (19).

Figure 3 shows the Southern analysis of one of the larger family studies we have performed. Shown are results for seven of the affected and one of the unaffected individuals of this family. The interesting feature is that clear clinical symptoms of DM were present only in individual I-2 (age 65), whereas individuals I-1 (age 68) and I-3 (age 62) presented with questionable symptoms of DM. Southern analysis not only confirmed expansions in individuals I-1, I-2, and I-3 but also showed significant expansions in four asymptomatic individuals I-5, I-6, II-1, and II-2 (ages 32 to 54). There were reductions in the repeat size between I-3 and II-1 and between I-5 and II-2, which may have contributed to the absence of phenotype in II-1 and II-2. Some evidence suggests that a decrease in allele size may be accompanied by a later onset (20).

This family study shows the importance of the direct DNA analysis. It is now possible to detect DM patients who are asymptomatic or show few of the classical signs of the disease. DNA testing should not be limited to family members with clinical symptoms, but should be extended to all members (symptomatic or not). It is not unusual for asymptomatic and mildly affected individuals with small expansions to have severely affected offspring with very large expansions. As a result of the identification of the gene defect and the ability to perform a direct DNA test, one can now provide families with more accurate risk estimates. Through genetic counseling, family members can make family planning decisions with information that was not available a short time ago. Although closely linked restriction fragment length polymorphism markers have been available for several years (21), linkage analysis is not as accurate as direct testing for the mutation, especially when there is an uncertain diagnosis or when key family members are not available. Also, linkage results provide no information regarding the severity of the disease.

Figure 4 outlines an algorithm for the molecular diagnosis of DM. Obviously the diagnosis can often be
made clinically, so this protocol will be most helpful for clinically questionable cases and those whose family history is negative. According to the results of the initial PCR test, we proceed in different directions. If the patient shows two normal alleles, we notify the physician that studies for other neuromuscular diseases should be considered. If we detect a normal and a slightly expanded allele in the patient, the diagnosis is confirmed. Most importantly, since many of these patients with small expansions are asymptomatic, they can now be provided with accurate risk estimates and other family members can also be offered testing. If only one band is detected by PCR, we then perform our Southern analysis. As described in Fig. 4, there are two possible outcomes. The first is that only one band will be present upon hybridization. In this case the patient is ruled homozygous for the normal allele, and again other neuromuscular diseases should be considered. The second outcome is when an expanded allele is detected, thus confirming the diagnosis of DM.

Since our recent development of this direct DNA analysis protocol for DM, we have been able to rule out a DM diagnosis in 41% of the cases with the PCR screen, thus directing the physicians towards other diagnoses. In 90% of the remaining cases we have confirmed the diagnosis of DM with our Southern test; the remaining 10% were shown to be homozygous for the normal PCR allele. Our protocol is both labor- and cost-effective, since we have reduced the number of Southern transfers being performed. Also, by eliminating the repeat from the probe and using a double digest, we have improved the quality of the Southern procedure.

References