Prediction of Dystrophin Phenotype by DNA Analysis in Duchenne/Becker Muscular Dystrophy

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Allele-specific molecular diagnosis of Duchenne and Becker muscular dystrophies (DMD and BMD) has been largely dependent upon muscle biopsy for dystrophin protein assay. We performed lymphocyte DNA mutation analysis by polymerase chain reaction on 14 boys presenting with a clinical picture compatible with DMD or BMD. DNA analysis revealed that 12 of 14 boys had a deletion of the dystrophin gene, thus establishing the diagnosis of DMD/BMD. Furthermore, genotypes for 9 of 12 deletion patients permitted prediction of the specific allelic disorder (i.e., DMD or BMD). Subsequent dystrophin testing confirmed all of the DNA-based diagnoses. We propose that DNA mutation analysis be included in the initial evaluation of patients suspected of having DMD/BMD, thus potentially eliminating the need for muscle biopsy in the majority of patients.

Specht LA, Beggs AH, Korf B, Kunkel LM, Shapiro F. Prediction of dystrophin phenotype by DNA analysis in Duchenne/Becker muscular dystrophy. Pediatr Neurol 1992;8:432-6.

Introduction

Diagnosis of Duchenne and Becker muscular dystrophies (DMD and BMD), allelic disorders characterized by progressive skeletal muscle weakness, has improved dramatically since the identification of the underlying molecular defect [1-3]. Immunoblotting of muscle tissue for the DMD/BMD gene product, dystrophin, has permitted definitive identification of patients with DMD, the milder BMD, and non-DMD/BMD conditions on the basis of absent, altered, or normal dystrophin, respectively [4]. This molecular-based approach has resulted in improved diagnostic accuracy which has been particularly important in distinguishing sporadic DMD/BMD patients from clinically similar conditions and in identifying the severity of the phenotype (i.e., DMD versus BMD) early in the disease course [5]. As a result, molecular diagnosis has eliminated the need for less specific diagnostic tests (e.g., electromyography) in confirmed patients with DMD or BMD; however, a relative disadvantage of diagnosis by dystrophin testing is its dependence on muscle biopsy, an invasive, expensive, and time-consuming procedure.

An alternate approach for molecular diagnosis of DMD/BMD is lymphocyte DNA mutation analysis. At least 65% of affected patients have demonstrable mutations of the dystrophin gene [6-10]. Approximately 95% of these mutations are intragenic deletions of one or more exons, while 5% are duplications [8]. The remaining patients (i.e., those without demonstrable mutations) are believed to have point mutations affecting protein coding sequences or splicing of dystrophin RNA. Recent advances in our understanding of the genotypic basis for DMD/BMD have permitted the correlation of the type of mutation or genotype with dystrophin phenotype and/or clinical severity. For the vast majority of patients studied, mutations of the DMD/BMD gene that disrupt the translational reading frame or include the promoter sequences result in a deficiency of dystrophin leading to the DMD phenotype [11-14]. Conversely, mutations that do not disrupt the reading frame or the promoter result in an altered and partially functional dystrophin protein, and the milder BMD phenotype [9,11-14]. A small percentage of DMD/BMD mutations, namely those affecting exons 3-7 and those spanning 30 or more exons, do not conform to this general rule [12,15].

Two recent developments permit the clinical application of DNA diagnosis for DMD/BMD. First, polymerase chain reaction (PCR) allows amplification and analysis of crude DNA obtained from lymphocytes, providing a rapid approach to DNA analysis [16]. Second, several sets of PCR primers have been designed to span the dystrophin gene, so that multiple exons can be analyzed simultaneously [17-20]. This multiplex PCR approach to DNA mutation analysis detects approximately 98% of mutations iden-

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Received March 25, 1992; accepted July 1, 1992.

tified by the original, and far more labor intensive, Southern blotting approach [19].

Previous reports of molecular diagnostic technologies have been retrospective studies conducted in the course of test development [4,6-9,12-15,17-20]. To demonstrate the efficacy of these tests in the clinical setting, we conducted a prospective clinical study to predict dystrophin phenotype, and thus the clinical allele-specific phenotype, by lymphocyte DNA mutation analysis. Multiplex PCR mutation analysis using peripheral blood samples was performed prior to dystrophin testing of muscle biopsy from suspected DMD/BMD patients presenting to our pediatric neuromuscular clinic. Diagnoses predicted from mutation analysis were compared to diagnoses provided by dystrophin protein analysis.

Methods

Patient Selection and Clinical Features. All patients presenting to the Children's Hospital Neuromuscular Disease Clinic over a 2-year period from September, 1989 to September, 1991 and suspected of having DMD/BMD by clinical criteria [21] were included in this prospective study. Fourteen boys were studied, ranging in age from 8 months to 9 years (median: 4 years). Except for 2 boys younger than 18 months of age who presented because of a positive family history, all had evidence of proximal weakness with or without pseudohypertrophy. Three boys had family histories of presumptive DMD. All boys had markedly elevated creatine phosphokinase (CK) values, ranging from 2,000-33,000 IU/L (median: 12,000 IU/L; normal: < 50 IU/L). None of the patients or their family members had previously undergone dystrophin or DNA testing.

Case Reports

Patient 4. This 8-month-old infant was the product of a 26-week twin gestation whose perinatal course was complicated by multiple organ failure. Follow-up at 8 months of age for neonatal liver failure revealed a CK of 8,000 IU/L. Parents reported a maternal uncle with DMD. Motor development and neuromuscular examination were within normal limits.

Patient 5. This 3-year-old boy ran poorly, fell frequently, and had difficulty with climbing stairs. Motor milestones had been delayed. Neuromuscular examination revealed proximal muscle weakness. CK was 18,000 IU/L. No family history of neuromuscular disease existed. His mother was in the eighteenth week of a subsequent pregnancy.

Patient 8. This 3-year-old boy in foster care had difficulty with climbing stairs and running. Developmental history was unavailable. Neuromuscular examination demonstrated proximal weakness and pseudohypertrophy. CK was 27,000 IU/L. Uncertainty regarding legal guardianship delayed muscle biopsy for 4 months.

DNA Mutation Analysis. Peripheral blood (5-15 ml) was collected into EDTA at the first Neuromuscular Clinic visit. DNA was usually isolated from lymphocyte nuclei as described [19], but occasionally rapid analysis was performed on boiled blood as described by Kunkel et al. [20]. PCR deletion analysis of the dystrophin gene was performed with the primer sets of Chamberlain et al. [17,18] and Beggs et al. [19] using conditions as described. Chamberlain's primer set was sometimes supplemented by the addition of new primers that amplify a 148 base pair portion of exon 46. Their sequence is, 46F = 5'-GCT AGA AGA ACA AAA GAA TAT CTT GTC-3' and 46R = 5' CTT GAC TTG CTG AAG CTT TTC TTT TAG-3'. Occasionally, deletions were further characterized using selected primers described by Kunkel et al. [20], or by Southern blotting using dystrophin cDNA probes [6]. Using the detailed dystrophin exon map with exon borders published by Koenig et al. [12], defined deletions (i.e., those in which both the 3' and 5' extent of the mutation could be determined) were determined to be either in- or out-of-frame.

Dystrophin Analysis. Muscle biopsy for dystrophin testing was scheduled at the initial clinic visit; Patients 11 and 14 provided samples of muscle biopsy obtained prior to presentation to Children's Hospital. Dystrophin analysis was performed on biopsied quadriceps muscle by Western blotting according to standard methodology [4]. Samples from Patients 1, 2, and 4 were analyzed by E. Hoffman at the Children's Hospital; the remaining samples were tested by Genica Pharmaceuticals, Worcester, MA.

Results

The results of DNA mutation analysis and correlative dystrophin testing are summarized in Table 1. In each of the 14 patients, DNA data were available prior to dystrophin immunoblot results from muscle biopsy, sometimes within 24 hours of the initial clinic visit. Representative PCR deletion analysis results for 2 patients are depicted in Figure 1. Twelve patients had a demonstrable deletion of the DMD/BMD gene by PCR analysis; no patient had a duplication. Results from 2 patients were not informative because there was no detectable mutation in one (Patient 10) and the other had an ambiguous result (Patient 9). In the latter patient, the PCR product for exon 44 was not reproducible, suggesting the possibility of a point mutation or polymorphism affecting the primer binding site. PCR-defined deletions were confirmed by Southern blotting for Patients 1, 4, 5, 8, and 11; the 3' end of the deletion in Patient 5 was defined only by Southern blotting.

For 9 of 12 deletion patients, the genotype permitted prediction of the specific allelic phenotype. Eight patients were predicted to have DMD based on out-of-frame (6 patients) or promoter (2 patients) deletions. One boy was predicted to have BMD based on the presence of an in-frame deletion. For the remaining 3 deletion patients, the specific allelic phenotype (i.e., DMD versus BMD) could not be predicted because the 3' end of the deletion was not determined by PCR.

Dystrophin analysis of muscle biopsy demonstrated the absence of detectable dystrophin, indicating the diagnosis of DMD, for 13 patients. The one patient with an in-frame deletion had a smaller molecular weight (390 kD versus normal 400 kD) and less abundant (10% normal levels) dystrophin consistent with the DNA-based diagnosis of BMD.

Discussion

It is crucially important to establish with certainty the diagnosis of DMD/BMD in the clinical neuromuscular setting. Other phenotypically similar disorders (e.g., dermatomyositis, limb girdle and Emery-Dreifuss muscular dystrophies) have quite different therapeutic, prognostic, and genetic implications. With molecular diagnostic testing, distinctions can be made between DMD/BMD and unrelated conditions. Within the DMD/BMD category, molecular testing can also allow the distinction of DMD from the ultimately milder BMD. This distinction is essential for optimal family and genetic counselling, but is often

Table 1. Results of DNA deletion and dystrophin analyses and comparison of DNA- and dystrophin-based diagnoses

Pt. No.	Age (yrs)	FH	DNA Deletion	DNA Diagnosis	Dystrophin Phenotype	Dystrophin Diagnosis
1	3	No	Exons 44-47*	DMD	Absent	DMD
2	4	No	Exons 48-50*	DMD	Absent	DMD
5	3	No	Exons 8-9*	DMD	Absent	DMD
8	3	No	Exons 46-47*	DMD	Absent	DMD
11	4	No	Exon 44*	DMD	Absent	DMD
13	6	No	Exons 46-48*	DMD	Absent	DMD
4	0.7	Yes	Pb, Pm, and exon 2	DMD	Absent	DMD
6	5	No	Pb and Pm	DMD	Absent	DMD
14	9	No	Exons 45-47†	BMD	390 kD/ 10%‡	BMD
3	1	Yes	Exons 48-52 >	DMD or BMD	Absent	DMD
7	4	No	Exons 45-52 >	DMD or BMD	Absent	DMD
12	4	No	Exons 45-60 >	DMD or BMD	Absent	DMD
9	5	No	Indeterminate	Uninformative	Absent	DMD
10	6	Yes	None	Uninformative	Absent	DMD

* Out-of-frame.
† In-frame.
‡ Molecular weight in kD/quantity (% of normal).
> 3′ end of deletion not identified.

Abbreviations:
FH = Family history
Pb = Brain promoter
Pm = Muscle promoter

not readily apparent from nonmolecular clinical and laboratory findings early in the disease course of sporadic patients [5].

Seventy-five percent (9 of 12) of the PCR-detected deletion patients in this study had genotypes that permitted accurate prediction of the dystrophin phenotype, and thus the clinical phenotype. Eight boys were predicted to have absent dystrophin, and thus DMD, because of out-of-frame (6 of 8) or promoter (2 of 8) deletions; all 8 predictions were confirmed by dystrophin testing. One boy was predicted to have an altered dystrophin phenotype and, thus, BMD because of an in-frame deletion; dystrophin testing confirmed the presence of a smaller molecular weight, less abundant dystrophin protein. As PCR primers for more dystrophin gene exons become available, the proportion of patients for whom frame-shift determinations can be made will increase. In the interim, Southern blotting will often define the deletion endpoints in patients for whom PCR is not informative (e.g., Patient 5).

The presence of a dystrophin gene deletion is sufficient to establish a diagnosis of DMD/BMD. The sensitivity of DNA mutation analysis in this study (i.e., the incidence of deletion detection in patients with abnormal dystrophin protein by Western blotting) was 86%, which is consistent with other series in our clinic [9,10] (and unpublished data). Series that report a lower rate of mutation detection, approximately 65%, are based on detection of mutations in clinically-ascertained, rather than dystrophin-ascertained, populations [6-8,13]; the inadvertent inclusion of clinically similar, but non-DMD/BMD, patients (e.g., limb-girdle dystrophy) may explain this lower rate of mutation detection. The one ambiguous PCR result (Patient 9) was unusual in that this was the only such finding in over 150 samples examined to date (unpublished data). In

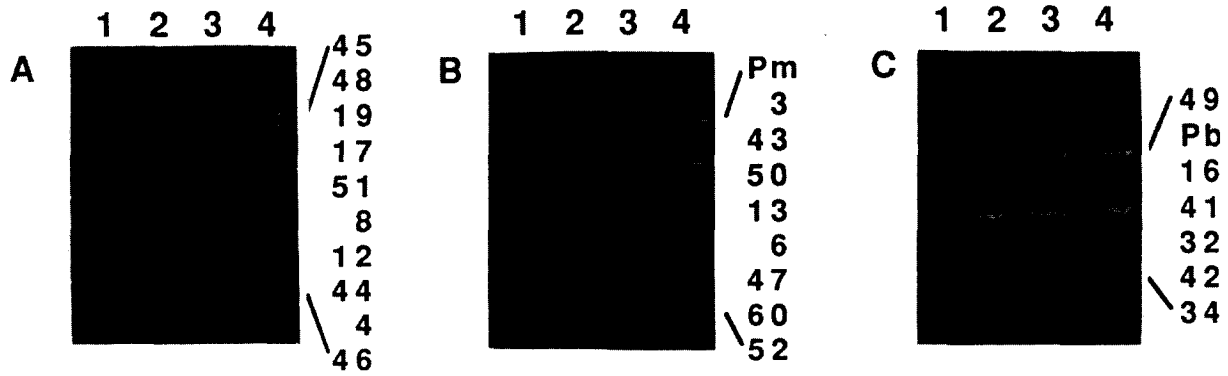

Figure 1. Representative PCR results. Purified lymphocyte DNA was amplified with primer sets described by Chamberlain et al. [17,18] with the addition of primers for (A) exon 46, (B) Beggs et al. [19], and (C) Kunkel et al. [20]. Exon numbers are indicated at the right, Pm = muscle promoter, Pb = brain promotor. Each of the 3 panels has the same 4 DNA samples: lane 1 = no DNA control; lane 2 = Patient 7; lane 3 = Patient 6; and lane 4 = normal male control. Note that the deletion in Patient 6 is only detectable by the use of the alternative primer sets in Figures 1B and 1C.

this case, a definitive diagnosis of DMD was made based on Western blot results in the same manner as for other patients with no demonstrable mutations.

Despite the findings of this study, some degree of caution regarding DNA diagnosis must be expressed. In particular, two types of deletions represent exceptions to the reading frame rule: out-of-frame deletions of exons 3-7 are associated with a variable clinical phenotype [12,15], and large, in-frame deletions spanning more than 30 exons are associated with DMD [12]. Therefore, identification of one of these genotypes would be insufficient to establish the allelic phenotype. When the severity of the phenotype is not apparent from the family history, we recommend dystrophin assay for the precise diagnosis of DMD versus BMD. Although none was identified in this small sample, these genotypes represent approximately 5% of all DMD/BMD deletions [12].

A few additional exceptions to the reading frame rule have been reported [13,14]. Such reports, however, are difficult to interpret because they lack dystrophin protein data and instead rely on correlations between genotype and clinical diagnosis of the specific allelic disorder; identification of DMD versus BMD by traditional clinical diagnostic criteria is somewhat arbitrary and does not always accurately predict the dystrophin phenotype [22]. Further studies of large numbers of DMD/BMD patients are necessary to clarify the relationship between the dystrophin genotype, protein phenotype, and clinical phenotype. Such a study is currently underway in our institution.

The obvious advantages of diagnosis by DNA mutation analysis versus dystrophin testing are speed and lack of a need for muscle biopsy. The latter, although a benign procedure, is invasive and costly. The cost of outpatient open muscle biopsy with dystrophin testing is almost 10-fold that of DNA analysis of peripheral lymphocytes from venipuncture. The noninvasive nature of DNA analysis permits diagnosis in patients in whom muscle biopsy is delayed, as for Patient 8. Because DNA mutation analysis can establish the diagnosis within a matter of hours, the approach is particularly advantageous in certain timely clinical situations, such as for Patient 5, in whom there was an ongoing pregnancy in a potential DMD carrier.

On the basis of this small pilot study, we propose that DNA mutation analysis be included in the initial evaluation of patients suspected of having DMD/BMD. Muscle biopsy for dystrophin protein analysis could be reserved for (1) patients who do not have a detectable mutation or (2) patients who have a detectable deletion but for whom the specific allelic phenotype is not apparent from family history, clinical course, or nature of the deletion. Of course, the molecular data must always be interpreted in the context of other clinical and family history information. Application of DNA mutation analysis to DMD/BMD diagnosis underscores the clinical relevance of the study of molecular mechanisms of heritable disorders.

The authors thank Dr. Eric Hoffman for performing the dystrophin assay for 3 patients in this study. This work was supported by the National Institutes of Health Grants NS01254, NS23740, HD18658, and AR37318, by the Howard Hughes Medical Institute, and by the Muscular Dystrophy Association.

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