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One Hundred Twenty-One Dystrophin Point Mutations Detected from Stored DNA Samples by Combinatorial Denaturing High-Performance Liquid Chromatography

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Duchenne and Becker muscular dystrophies are caused by a large number of different mutations in the dystrophin gene. Outside of the deletion/duplication "hot spots," small mutations occur at unpredictable positions. These account for about 15 to 20% of cases, with the major group being premature stop codons. When the affected male is deceased, carrier testing for family members and prenatal diagnosis become difficult and expensive. We tailored a cost-effective and reliable strategy to discover point mutations from stored DNA samples in the absence of a muscle biopsy. Samples were amplified in combinatorial pools and tested by denaturing high-performance liquid chromatography analysis. An anomalous elution profile belonging to two different pools univocally addressed the allelic variation to an unambiguous sample. Mutations were then detected by sequencing. We identified 121 mutations of 99 different types. Fifty-six patients show stop codons that represent the 46.3% of all cases. Three non-obvious single amino acid mutations were considered as causative. Our data support combinatorial denaturing high-performance liquid chromatography analysis as a clear-cut strategy for time and cost-effective identification of small mutations when only DNA is available. ***(J Mol Diagn 2010, 12:65–73; DOI: 10.2353/jmoldx.2010.090074)***

Duchenne (DMD [MIM 310200]) and Becker muscular dystrophies (BMD [MIM 300376]) are allelic inherited disorders of muscle. They affect males in >99% of cases, being transmitted as X-linked recessive traits.[1] The *DMD* gene spans 2.2 million bp of genomic DNA on the X chromosome, and the 14-kb transcript encodes a full-length protein (dystrophin) of 427 kd (Dp427m). Both DMD and BMD arise due to mutations at the dystrophin gene locus, which comprises 79 exons and eight tissue-specific promoters. The most common mutations are large intragenic deletions or duplications, encompassing one or more exons, but point mutations are about 15 to 20% of cases, with the major group being premature stop codons.[2–9]

Patients and their families confer great value to mutation detection for genetic counseling, but also for therapeutic options, since there are claims of novel mutation-targeted treatments.[10–12] Unfortunately, very often muscle biopsies are not possible because the affected family member is deceased. We have tailored a cost-effective and reliable strategy to discover point mutations from DNA samples. Based on the sensitivity of denaturing high-performance liquid chromatography (DHPLC) to detect mutations, especially in A/T-rich sequences, such as the dystrophin gene,[6,7] we developed a combinatorial DHPLC approach to screen pooled samples.

Materials and Methods

Patients

We used archive DNA samples from six different centers: Laboratory of Molecular Biology, Scientific Institute E. Medea, Lecco; Department of Neurological and Psychiatric Sciences, University of Padua; Institute of Neurology, Catholic University, Policlinico Gemelli, Rome; Muscular and Neurodegenerative Disease Unit, Giannina Gaslini Institute, University of Genova; Depart-

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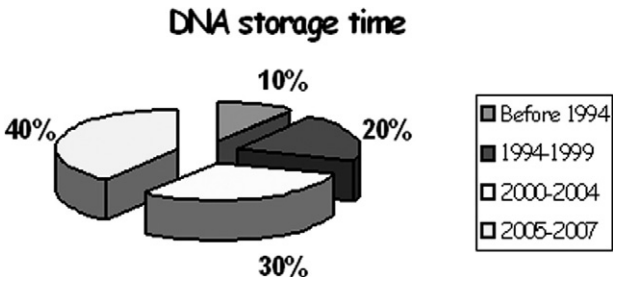

Figure 1. Extraction dates of DNA samples.

ment of Experimental Medicine, Cardiomyology and Medical Genetics, Second University, Naples; and Centro de Estudos do Genoma Humano, Instituto de Biociências Universidade de São Paulo, Brasil. Diagnosis was determined by clinical features consistent with DMD or BMD, along with an X-linked family history. Informed consent was obtained from patients, when possible, according to the guidelines of Eurobiobank or Telethon.

Archive Samples

One hundred fifty-three DNA archive samples were stored in Tris-EDTA at 4°C. Fifteen were extracted by phenol-chloroform before 1994, whereas 31 were extracted from 1994 to1999, and 46 from 2000 to 2004 (Figure 1). More recent samples (from 2005 to 2007) were extracted using a FlexiGene DNA kit (Qiagen, Hamburg, Germany). Old samples were often recovered as dry pellets. In this case, we rehydrated the pellet. We evaluated the DNA integrity by 0.6% agarose gel electrophoresis. We did not re-precipitate any of the samples. When required, we performed a preamplification step using the GenomiPhi HY DNA amplification kit (GE Healthcare, Chalfont St. Giles, UK), according to the manufacturer's instruction. This kit provides microgram quantities of DNA from nanogram amounts of starting material in only a few hours. The limit of polymerase chain reaction (PCR) product size using this archived DNA was about 1000 bp.

Sample Optimization

Each DNA sample was diluted to a final concentration of 30 ng/μl, and 1 μl was used in each pool. To control for the possibility of unequal PCR product yield, short tandem repeat (STR) polymorphic markers *DXS8015*-HEX and *DXS1204*-FAM (Table 1) were amplified from single and pooled DNA templates, in a final reaction volume of 20 μl, by using 0.5 μmol/L each marker primer, buffer LB 10× [200 mmol/L Tris; 100 mmol/L Hepes; 25 mmol/L MgSO4 × 7 H_2O; 100 nm KCl; 100 mmol/L $(NH4)_2$ SO4], 0.25 μmol/L each dNTP, 0.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA).

Primer Design

Genomic sequence for Dp427m, the main dystrophin isoform found in muscle, was obtained from GenBank (NM 004006.1). Its exon 1 encodes a unique N-terminal MLWWEEVEDCY amino acid sequence and is expressed in the skeletal muscle and heart.

For each dystrophin exon and muscular promoter a primers pair was designed using the Primer 3 software package with the following criteria: product size between 200 and 400 bp, primer size between 24 and 28 nucleotides, and melting temperature between 58°C and 62°C (Table 2).

Primer pairs were chosen to include flanking-intron sequence. Primer sequences were checked by BLASTn to avoid matching with repeated human sequences or covering single nucleotide polymorphisms in the vicinity of exon sequences. Only in the case of exon 26, we designed two primer pairs that split it into two overlapping fragments. Following these requirements, we created a series of amplicons, all with the same melting characteristics. All were amplified using the same PCR conditions. Primers were synthesized by MWG Biotech AG, Ebersberg, Germany. All PCR share the same conditions (95°C 30 seconds, 60°C 90 seconds, 68°C 90 seconds for 33 cycles).

Amplification of Genomic DNA

PCR reactions were set up semiautomatically using an automatic liquid handling Eppendorf epMotion and 384/96-well plates. DNA was amplified in a final reaction volume of 18 μl by using 30 ng of genomic DNA for each pool, buffer LB [20 mmol/L Tris; 10 mmol/L Hepes; 2.5 mmol/L MgSO4 × 7 H_2O; 10 nm KCl; 10 mmol/L $(NH4)_2$ SO4], 1.5 mmol/L MgCl2, 0.25 μmol/L each dNTP, 0.5 μmol/L each primers, 0.5 U AmpliTaq Gold (Applied Biosystems).

WAVE System DHPLC Analysis

The dystrophin exons and flanking intronic sequences and the muscular promoter were analyzed using high-throughput denaturing high-performance liquid chromatography (HT-DHPLC). PCR products were directly analyzed. Using pooled samples a preliminary annealing step is not required. The system is based on DHPLC. The WAVE DHPLC system is an ion-pair, reverse-phase HPLC

Table 1. STR Markers

DXS1204-FAM	DXS8015-HEX
F Primer: 5′-ATGAACCCTTAACTCATTTAGCAGG-3′ R Primer: 5′-AGCNTGCACCAACATGCC-3′ Length: 237–251 bp	F Primer: 5′-AGTCTTCTCAGGCCAGAGC-3′ R Primer: 5′-AGGACCAACTTTCACATGC-3′ Length: 174–190 bp

F, forward; R, reverse.