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Quality Assurance for Duchenne and Becker Muscular Dystrophy Genetic Testing

Development of a Genomic DNA Reference Material Panel

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Duchenne and Becker muscular dystrophies (DMD/BMD) are allelic X-linked recessive disorders that affect approximately 1 in 3500 and 1 in 20,000 male individuals, respectively. Approximately 65% of patients with DMD have deletions, 7% to 10% have duplications, and 25% to 30% have point mutations in one or more of the 79 exons of the dystrophin gene. Most clinical genetics laboratories test for deletions, and some use technologies that can detect smaller mutations and duplications. Reference and quality control materials for DMD/BMD diagnostic and carrier genetic testing are not commercially available. To help address this need, the Centers for Disease Control and Prevention–based Genetic Testing Reference Material Coordination Program, in collaboration with members of the genetic testing and the DMD/BMD patient communities and the Coriell Cell Repositories, have characterized new and existing cell lines to create a comprehensive DMD/BMD reference material panel. Samples from 31 Coriell DMD cell lines from male probands and female carriers were analyzed using the Affymetrix SNP Array 6.0 and Multiplex Ligation-Dependent Probe Amplification (MRC-Holland BV, Amsterdam, the Netherlands), a multiplex PCR assay, and DNA sequence analysis. Identified were 16 cell lines with deletions, 9 with duplications, and 4 with point mutations distributed throughout the dystrophin gene. There were no discordant results within assay limitations. These samples are publicly available from Coriell Institute for Medical Research (Camden, NJ) and can be used for quality assurance, proficiency testing, test development, and research, and should help improve the accuracy of *DMD* testing. *(J Mol Diagn 2011, 13:167–174; DOI: 10.1016/j.jmoldx.2010.11.018)*

Duchenne muscular dystrophy (DMD) is a severe progressive neuromuscular disorder that is manifested in early childhood. Patients with this disorder reach milestones such as sitting and walking later than expected, experience progressive symmetric muscular weakness throughout childhood, and are usually wheelchair-dependent by age 13 years. In addition, these patients can have cognitive impairment and involvement of other organs including the heart. Becker muscular dystrophy (BMD) is similar to DMD but with later onset and less severity. Patients with BMD usually become wheelchair-dependent at a later age than those with DMD. Both DMD and BMD result in a shortened lifespan, and most affected individuals die before their third or fourth decade. DMD is relatively common, occurring in 1 in 3500 live male births worldwide.[1] Both disorders exhibit X-linked inheritance and are fully penetrant in male patients. Female carriers are at risk for X-linked cardiomyopathy.

DMD and BMD, and DMD-associated cardiomyopathy are caused by mutations in the dystrophin (*DMD*) gene, which is located at X-p21.2[2,3] and encodes the 427-kDa dystrophin protein[4] (1988 Leiden Open Variation Data-

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MRC-Holland BV donated reagents for this project.

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base, muscular dystrophy, *http://www.dmd.nl/DMD_deldup.html,* accessed May 18, 2010). *DMD* has 79 exons and covers nearly 2400 kb[5] (1988 Leiden Open Variation Database, muscular dystrophy, *http://www.dmd.nl/DMD_deldup.html*, accessed May 18, 2010; and GeneCards database, *http://www.genecards.org/cgi-bin/carddisp.pl?gene=DMD,* accessed May 19, 2010). *DMD* is believed to be one of the largest genes in the human genome. Deletions, duplications, and point mutations account for approximately 65%, 7% to 10%, and 25% to 30%, respectively, of mutations in patients with DMD.[6,7] Point mutations may occur throughout the gene. Deletions and duplications frequently occur in two "mutational hot spot" regions of the gene, located between exons 2 to 20 or exons 44 to 53, but may occur throughout the gene. Deletions and duplications are much less common in the 3′ end of the gene (Leiden Open Variation Database, muscular dystrophy, *http://www.dmd.nl/DMD_deldup.html,* accessed May 18, 2010).

Treatment for DMD and BMD is currently limited to steroids to prolong and improve quality of life. In addition, several therapies are being developed for muscular dystrophy that target particular *DMD* mutations. Pharmaceutical agents such as aminoglycosides[8] and PCT124[9] allow translation of certain nonsense mutations, enabling production of a full-length functional protein. Morpholino antisense oligonucleotides that mediate exon skipping[10] are under development. This experimental therapy would preserve the reading frame of *DMD* with frame-shifting deletions and enable generation of potentially functional dystrophin molecules. These experimental therapies are targeted to patients with specific types of mutations or mutations in certain exons. Thus, it is vitally important for patients with DMD or BMD to have accurately characterized *DMD* mutations so that those eligible for these new therapies or future therapies will be able to receive them. Accurate testing is also important for patient management and carrier testing.

A number of molecular diagnostic methods are used to detect mutations in the *DMD* gene.[7] Targeted multiplex PCR can be used to quantitatively detect exon and promoter deletions in male patients. Quantitative PCR analysis can be used to identify DMD exon duplications, and multiplex ligation-dependent probe amplification (MLPA) can be used to detect both deletions and duplications in all 79 exons in both affected male and heterozygous female individuals. In addition, newer chip-based technologies such as array comparative genomic hybridization and single nucleotide polymorphism (SNP) chips can enable identification of deletions and duplications in *DMD*. Smaller deletions, duplications, splice-site mutations, and point mutations may be identified in patients with no identified deletion or duplication using mutation scanning or DNA sequence analysis. There are currently no US Food and Drug Administration–approved assays for DMD genetic testing; all testing is performed using laboratory-developed tests.

Clinical laboratories use characterized reference materials for a variety of quality assurance purposes including test development, test validation, quality control, and proficiency testing. Ideally, reference materials should resemble a patient sample and all variant alleles or mutation types that the assay is designed to detect.[11] Use of reference materials for laboratory quality assurance is also mandated by regulatory requirements and professional guidelines[12–16] (Washington State Legislature, *http://www.doh.wa.gov/hsqa/fsl/lqa_home.htm*, accessed August 30, 2010; New York State Clinical Laboratory Evaluation Program, *http://www.wadsworth.org/labcert/clep/clep.html*, accessed July 15, 2010; College of American Pathologists, *http://www.cap.org/apps/docs/laboratory_accreditation/checklists/molecular_pathology_sep07.pdf*, accessed August 30, 2010; and American College of Medical Genetics, *http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm*, accessed July 15, 2010). For genetic testing of DMD and BMD, assays should be developed and evaluated using reference materials that represent deletions, duplications, and point mutations throughout the *DMD* gene. Reference materials should also include both male and female samples, and should be thoroughly characterized using methods different from the laboratory's routine clinical assay. Use of a characterized and comprehensive reference material panel will help to ensure proper design and function of the clinical assay.

Reference materials for clinical *DMD* genetic testing are not commercially available. Laboratories commonly use genomic DNA from residual patient specimens or from publicly available cell lines such as those in the Coriell Cell Repositories (Camden, NJ) as reference materials. To help address the need for publicly available, renewable, and characterized genomic DNA reference materials, the Centers for Disease Control and Prevention coordinated the Genetic Testing Reference Material Coordination Program (GeT-RM), in collaboration with members of the genetic testing community and the Coriell Institute for Medical Research, have identified and characterized the mutations in existing DMD cell lines. In addition to exon deletions, some of the cell lines had duplications or point mutations, which are *DMD* mutation types that had not previously been identified in the Coriell collection. The GeT-RM has also established a collaboration with a DMD patient registry, DuchenneConnect, to collect blood from consenting patients with DMD with mutations not yet represented at Coriell for cell line development. In the present study, these new and existing cell lines were characterized using a variety of methods to create a comprehensive publicly available reference material panel for *DMD* genetic testing.

Materials and Methods

Cell Line Selection

Reference material needs for DMD and BMD genetic testing were identified through discussions with clinical laboratory directors. Twenty-four cell lines from the Coriell collection were selected for study based on the sex of the donor and the exons expected to be deleted or duplicated based on preliminary characterization studies. Seven cell lines from patients with DMD with no identified deletion or duplication were selected for DNA sequence analysis.

Anonymous Blood Collection from Consenting Patients with DMD

Patients with mutations considered important for creation of a comprehensive reference material panel by the committee of laboratory directors were selected from a de-identified list of patients and their previously determined *DMD* genotype provided by the DuchenneConnect Patient Registry. These patients were issued written requests for participation by genetic counselors from DuchenneConnect, explaining the project and its goals. If the family wished to participate, DuchenneConnect obtained informed consent using the Coriell Model Consent document and sent a code representing each consenting patient along with the shipping address and telephone number (required by the shipping service) to the data manager of the National Institute of General Medical Science Human Genetic Cell Repository, part of the Coriell Cell Repositories. Prepaid blood collection kits were mailed to each family, with blood collection tubes coded as directed by the registry. When the patient visited his or her physician for a regular visit, blood was drawn and sent to the Coriell Repository using the return shipping label and materials provided; the Repository has no record of the patient name or other identifying information. When the blood specimens were received, each was assigned a new Repository number and prepared for cell line establishment. Once the cell line was established, the data manager requested the mutation and clinical information from the DuchenneConnect Patient Registry using the appropriate sample code.

Establishment of New Cell Lines

Whole-blood samples collected from consenting patients or their families were sent to the Coriell Cell Repositories for Epstein-Barr virus transformation of B lymphocytes, as previously described.[17,18] All samples were placed in culture and expanded to yield approximately 2×10^8 total viable cells. The culture medium was antibiotic-free to increase the likelihood that contamination would be readily detected. The cell suspension was dispersed into forty 1-ml ampules containing 5×10^6 viable cells each. Cultures were cryopreserved in heat-sealed borosilicate glass ampules and stored in liquid nitrogen (liquid phase). Successful cultures were free from bacterial, fungal, and mycoplasma contamination and were viable after cryopreservation in liquid nitrogen, as evidenced by doubling of the cell number within 4 days of recovery.

DNA Preparation

Approximately 2 mg of DNA was prepared from cultures of each of the selected cell lines by the Coriell Cell Repositories using the Gentra Autopure system (Qiagen Corp., Gaithersburg, MD) per the manufacturer's instructions or previously described methods.[19]

Testing Laboratories

Four clinical genetic laboratories volunteered to participate in the study. Laboratories were solicited based on their current *DMD* assay methods. Thus, each of the DNA samples was tested using a variety of platforms. All of the laboratories are located in the United States and are accredited by the College of American Pathologists. All samples were also tested in the Cytogenetics Laboratory at the Coriell Cell Repositories.

Protocol

Each of the four volunteer testing laboratories received one 50-μg aliquot of DNA from each of the cell lines they were asked to test. The two laboratories using MLPA analysis received all 24 samples to test. The laboratory using the PCR assay, which is not designed to test samples from female carriers or to detect duplications, received only the 10 samples from male probands expected to exhibit deletions. The laboratory that performed DNA sequence analysis received the seven samples in which deletions or duplications were not detected using the other assay methods. The expected mutation in each of the samples was not revealed to the laboratories. The laboratories genotyped each DNA sample using their standard assay methods. Each laboratory performed one assay, except for the Coriell Cytogenetics Laboratory, which performed both the Affymetrix Human SNP Array 6.0 and 2.7 assays (Affymetrix, Santa Clara, CA). The results were sent to the study coordinator (L.K.), who examined the data for discrepancies.

Assays

Targeted Multiplex Assay for Qualitative Detection of Deletions in Male Patients

This assay, as previously described,[20] amplifies 24 exons (exons 3, 4, 6, 8, 12, 13, 16, 17, 19, 32, 34, 41 to 52, and 60) and two promoter regions of *DMD*, which comprise the gene regions in which most mutations are identified. The multiplex PCR reactions enable detection of as many as 10 different exons in a single reaction. Three separate reactions are performed per sample. Collectively, the PCR assays detect greater than 98% of deletions.[20] For deletions of more than a single exon, the absence of missing PCR products in more than one reaction provides assurance that an actual deletion has occurred in the patient. Furthermore, the end points of many deletions can be identified, which can guide prediction of disease severity (eg, DMD versus BMD) based on maintenance or loss of the translational reading frame. Predictions based on the reading frame hypothesis, however, are inaccurate in approximately 10% of the cases, and should be applied with caution.[21] Detection of a deletion is based on the absence of a PCR product after electrophoresis on an agarose gel stained with ethidium bromide. This quantitative targeted multiplex PCR assay determines the presence or absence of an amplicon, which reflects the presence or absence of a specific exon. Thus, this assay is only suitable for use in male patients, who have a single X chromosome. Female patients would demonstrate amplicons for all tested exons from their unaffected X chromosome. This method is not appropriate for female carrier or prenatal screening and is not designed to detect exon duplication or point mutations.

Multiplex Ligation-Dependent Probe Amplification

The multiplex ligation-dependent probe amplification assay, which detects duplications and deletions in all DMD exons, was performed in two of the participating clinical laboratories. Both laboratories used the same procedure. MLPA reagents were developed and manufactured by MRC-Holland BV (Amsterdam, the Netherlands). The specific MLPA kits (PO34 and PO35) used for amplification of *DMD* screen for the copy number of all 79 exons in two multiplex reactions. In brief, 100 ng of target DNA was denatured for 5 minutes at 98°C, after which 3 μL of the probe cocktail was added. The mixture was heated at 95°C for 1 minute, and incubated at 60°C overnight (16 hours). Ligation was performed with the temperature-stable ligase-65 enzyme for 15 minutes at 54°C. After inactivation of the ligase, the ligated products were amplified using PCR according to the manufacturer's protocol using one primer labeled with 6-carboxyfluorescein. PCR was performed for 35 cycles (30 seconds at 95°C, 30 seconds at 60°C, and 60 seconds at 72°C), and the resulting DNA products were fractionated by size using a capillary electrophoresis instrument (model 3130; Applied Biosystems, Inc., Foster City, CA). Fragment mobility was analyzed using GeneMapper Fragment Analysis software (Applied Biosystems, Inc.). The sizes of exon-specific peaks were identified according to their migration relative to size standards. Further data analysis was performed using Coffalyser Software (MRC-Holland BV) or MLPA analysis–specific software (SoftGenetics LLC, State College, PA). Exon dosage was calculated for samples from affected male patients using averaged peak heights generated during the run from characterized normal male control individuals. Exon dosage for samples from affected female patients was calculated similarly using characterized normal female control individuals. Approximate dosage with no exon deletion is 1 (range, 0.65 to 1.35) for both male and female patients. Exon deletions result in no signal for male patients, and reduced signal for female patients (~0.5). A duplication in a male patient is detected as a dosage of approximately 2 (range, 1.65 to 2.35), and in a female patient is approximately 1.5.

Affymetrix Human SNP Array 6.0

The Affymetrix Human SNP Array 6.0 assay (Affymetrix, Inc.) detects deletions and duplications but is not designed specifically for DMD genetic testing. In brief, 250 ng of genomic DNA was digested with either *Nsp*1 or StyI (New England Biolabs, Inc., Ipswich, MA). A universal adaptor oligonucleotide was then ligated to the digested DNA. The ligated DNA was diluted with water, and three 10-μL aliquots from the StyI digestion and four 10 μL aliquots from the *Nsp*1 digestion were transferred to fresh 96-well plates. PCR master mix (Titanium DNA Amplification Kit; Clontech Laboratories Inc., Mountain View, CA) was added to each well, and the reactions were cycled as follows: 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 68°C for 15 seconds; 68°C for 7 minutes; and 4°C hold. After PCR, the 7 reactions for each sample were combined and purified using Agencourt SNPClean beads (Beckman Coulter Genomics; Brea, CA). The purified PCR products were quantified using spectrophotometry to ensure a yield of at least 4 μg/μL. Forty-five microliters (≥180 μg) of each PCR product was digested using DNase-1 to produce fragments of fewer than 185 bp. The fragmented PCR products were then end-labeled with a biotinylated nucleotide using terminal deoxynucleotidyl transferase. The end-labeled PCR products were combined with a hybridization cocktail, denatured at 95°C for 10 minutes, and incubated at 49°C. Two hundred microliters of each mixture was applied to an SNP 6.0 array and hybridized with rotation at 60 rpm overnight at 50°C. After 16 to 18 hours of hybridization, the arrays were washed and stained using the GenomeWideSNP6-450 fluidics protocol (Affymetrix) with the appropriate buffers and stains. After washing and staining, the arrays were scanned on a GeneChip Scanner 3000 (Affymetrix, Inc., Santa Clara, CA). Analysis was performed using Affymetrix Genotyping Console software (version 3.0). All samples achieved recommended thresholds for both copy number and SNP data quality. Data from both SNP and copy number probes were used to identify copy number aberrations when compared with an internal copy number reference set created from samples as determined at the Coriell Genotyping and Microarray Center.

Affymetrix Cytogenetics 2.7M

The Affymetrix Cytogenetics 2.7 array (Affymetrix, Inc.) detects deletions and duplications but is not designed specifically for DMD genetic testing. This assay was used to resolve a discrepancy in one sample (GM23127) but was not used to test the other samples. One hundred nanograms of each genomic DNA sample was prepared for whole-genome amplification. Each sample was treated in 1× denaturing solution for 3 minutes at room temperature, followed by incubation on ice and the addition of 1× neutralization solution. Amplification master mix was added to the DNA, and the mixtures were incubated at 30°C for 16 hours, followed by inactivation at 65°C for 3 minutes. The amplified DNA products were purified by binding to magnetic beads (Affymetrix, Inc.), washing twice with cytosol washing buffer and eluting with cytosol elution buffer. The purified products were quantified using spectrophotometry. A DNA concentration of more than 55 μg/μL indicated successful amplification. Fragmentation and labeling master mix was added to the amplified DNA, and the mixtures were incubated at 37°C for 2 hours, followed by inactivation at 95°C for 10 minutes. Cyto Hyb buffer was added to the fragmented labeled DNA, and the mixtures were denatured at 95°C for 5 minutes, incubated at 50°C for 15 minutes, and held at 50°C. The hybridization mixtures were applied to the Affymetrix Whole-Genome 2.7M Array (Affymetrix, Inc.) and allowed to hybridize with rotation at 60 rpm overnight at 50°C. After 16 to 19 hours of hybridization, the arrays were washed and stained using the Cytogenetics-Array-450 fluidics protocol with the buffers and stains supplied with the reagent kit. The arrays were then scanned on a GeneChip Scanner 3000 (Affymetrix, Inc.).

DNA Sequence Analysis

DMD sequence analysis was performed essentially as described.[22] PCR was performed under standard conditions using primers designed to amplify promoter and exonic regions of the gene, with 92 amplicons ranging in size from 1.2 to 1.4 kb. PCR products were separated at capillary gel electrophoresis using a Qiaxcel system (Qiagen Corp), which enabled visualization of amplicons and detection of deletions. Purification of PCR products was performed using the Ampure method (Beckman Coulter) on the Biomek NX (Beckman Coulter). Bidirectional sequencing was performed on each amplicon using terminator chemistry (Big Dye, version 3.1; Applied Biosystems). Reactions were purified with size-exclusion columns (Performa; Edge Biosystems, Inc., Gaithersburg, MD), and sequencing products were separated using capillary electrophoresis (AB3730; Applied Biosystems). Sequence electropherograms were analyzed electronically using Sequencher 4.9 (GeneCodes Corp., Ann Arbor, MI) or Mutation Surveyor (SoftGenetics, Inc.). Any suspected pathogenic *DMD* mutations were confirmed via a second independent PCR reaction.

Results

The goal of the present study was to create a comprehensive panel of publicly available genomic DNA reference materials for *DMD* genetic testing. Input about needed reference materials was obtained through discussions with clinical laboratory directors who perform *DMD* testing. The clinical laboratory directors decided that the reference materials panel should include *DMD* deletions, duplications, and point mutations covering as many of the *DMD* exons as possible. Both large and small deletions, duplications ranging in size from a single exon to many exons, and samples from female *DMD* deletion and duplication carriers should be represented in the panel.

The National Institute of General Medical Sciences Repository at Coriell maintains a large collection of DMD cell lines, many with known deletions. There were, however, no known DMD lines in the Coriell collection with duplications or point mutations, and no cell lines were known to have deletions in the 3′ end of the gene. Cell lines from male and female donors with deletions that fit the recommendations of the clinical laboratory directors were selected for further study. In addition, DNA samples from DMD cell lines without known mutations were sent to a clinical *DMD* testing laboratory for MLPA analysis. Data were also obtained about these and other cell lines as part of an independent effort by the Coriell Cell Repositories to characterize their collection using the Affymetrix Human SNP Array 6.0 copy number assay. These analyses resulted in identification of a number of DMD cell lines with duplications, as well as previously unidentified deletions. No deletions or duplications were identified in DNA samples from seven of the cell lines using MLPA or Affymetrix Human SNP Array 6.0 analysis.

To create new cell lines with duplications and deletions that were not available in the Coriell collection, a collaboration was established with a DMD patient registry, DuchenneConnect, to collect blood from consenting patients with DMD with mutations for cell line development. A protocol was developed for anonymous contact, consent, and blood collection from patients with DMD, and was approved by the Coriell Institutional Review Board. Patients with deletions and duplications in parts of *DMD* that were not represented in the Coriell collection were selected from a list of deidentified patients with previously identified *DMD* mutations provided by the registry. The families of the selected patients were contacted by genetic counselors with the DuchenneConnect Patient Registry and asked to participate. If the patient or family agreed, they were provided information about the study and asked to sign a consent form. Blood collection kits with prepaid mailers were provided to the consenting patients, who obtained and submitted blood samples during their routine medical care. In some cases, mothers and/or siblings of the proband also provided blood samples. Through this process, 11 blood samples were received and 10 new cell lines were created.

Based on preliminary data from MPLA and Affymetrix Human SNP Array 6.0 analysis and information from DuchenneConnect, 16 cell lines with exon deletions, 8 with exon duplications, and 7 with no identified deletion or duplication in the *DMD* gene were selected for further characterization. Cell lines were selected to create a comprehensive reference material panel with exon deletions and duplications throughout the *DMD* gene. Seven of the selected cell lines are from female deletion or duplication carriers.

All of the DMD cell lines with deletions or duplications selected for the panel were analyzed using MLPA analysis in two laboratories and Affymetrix Human SNP Array 6.0 analysis in one laboratory. The 10 samples from male probands with expected deletions were also analyzed using a targeted multiplex PCR assay (Table 1).

The results obtained using the various assays fully agreed with each other when the limitations of the assay methods were considered. The MPLA results from both laboratories using this assay were identical for the 24 lines with deletions or duplications. Results for MLPA and the Affymetrix Human SNP Array 6.0 were identical for all but two of the 24 samples, GM10283 and GM23127. In both cases, MLPA detected a deletion that was one exon larger than that detected by the Affymetrix Human SNP Array 6.0. Analysis of the array data suggested that limited probe coverage for exons 28 and 72 resulted in the smaller deletion call in these two samples. Sample GM23127 was also tested using the Affymetrix Cytogenetics 2.7M array. Similar to the Affymetrix Human SNP Array 6.0, this test detected a duplication in exon 27 but was unable to detect duplication in exon 28 because of lack of probe coverage in this exon. Results from the targeted multiplex PCR assay agreed with results from the other assays. While this assay was designed to detect deletions in 24 of the 79 exons, it cannot reliably detect duplications or mutations in female carriers because amplicons would be produced for all tested exons from the unaffected X chromosome. Only samples for which preliminary data suggested a deletion in a male sample were tested using this method. When results of this assay were compared with the data obtained from MLPA and Affymetrix Human SNP Array 6.0 analyses, discrepant data were found in samples GM07691, GM05089, GM04981, GM04364, and GM10283 (Table 1). In all cases, the deleted exons not de-

Table 1. Genotype of DMD Cell Lines

Coriell cell line	Sex	Preliminary data source*	Consensus genotype	Characterization method used				
				Affymetrix SNP array 6.0	MLPA†		Targeted multiplex‡	DNA sequence analysis
					Laboratory 1	Laboratory 2		
GM07691	Male	Coriell catalog	del 5′ end-18	del 5′ end-18	del 5′ end-18	del 5′-18	del pb-17	ND
GM07692	Female	Coriell catalog	del 5′ end-18	del 5′-18	del 5′-18	del 5′-18	ND	ND
GM03782	Male	Coriell catalog	del 3–17	del 3–17	del 3–17	del 3–17	del 3–17	ND
GM05089	Male	Coriell catalog	del 3–5	del 3–5	del 3–5	del 3–5	del 3–4	ND
GM05170	Male	Coriell catalog	del 4–43	del 4–43	del 4–43	del 4–43	del 4–43	ND
GM23094	Female	Registry	del 35–43	del 35–43	del 35–43	del 35–43	ND	ND
GM04315	Female	Present study	del 44	del 44	del 44	del 44	ND	ND
GM05115	Male	Coriell catalog	del 45	del 45	del 45	del 45	del 45	ND
GM05117	Female	Present study	del 45	del 45	del 45	del 45	ND	ND
GM04981	Male	Coriell catalog	del 45–53	del 45–53	del 45–53	del 45–53	del 45–52	ND
GM03929	Male	Coriell catalog	del 46–50	del 46–50	del 46–50	del 46–50	del 46–50	ND
GM05159	Female	Coriell catalog	del 46–50	del 46–50	del 46–50	del 46–50	ND	ND
GM04100	Male	Coriell catalog	del 49–52	del 49–52	del 49–52	del 49–52	del 49–52	ND
GM04099	Female	Present study	del 49–52	del 49–52	del 49–52	del 49–52	ND	ND
GM04364	Male	Present study	del 51–55	del 51–55	del 51–55	del 51–55	del 51–52	ND
GM10283	Male	Present study	del 72–79	del 73–79§	del 72–79	del 72–79	ND	ND
GM23086	Male	Registry	dup 2–30	dup 2–30	dup 2–30	dup 2–30	ND	ND
GM23087	Female	Registry	dup 2–30	dup 2–30	dup 2–30	dup 2–30	ND	ND
GM09981	Male	Present study	dup 2–4	dup 2–4	dup 2–4	dup 2–4	ND	ND
GM04327	Male	Present study	dup 5–7	dup 5–7	dup 5–7	dup 5–7	ND	ND
GM23099	Female	Registry	dup 8–17	dup 8–17	dup 8–17	dup 8–17	ND	ND
GM23159	Male	Registry	dup 17	dup 17	dup 17	dup 17	ND	ND
GM23127	Male	Registry	dup 27–28	dup 27§	dup 27–28	dup 27–28	ND	ND
GM05124	Male	Present study	dup 45–62	dup 45–62	dup 45–62	dup 45–62	ND	ND
GM05263	Male	Present study	ND	No del/dup	No del/dup	ND	ND	c.7893delC (p.Q2632Sfs6)
GM05127	Male	Present study	ND	No del/dup	No del/dup	ND	ND	c.5533G>T (p.E1845X)
GM02298	Male	Present study	ND	No del/dup	No del/dup	ND	ND	No mutation
GM04569	Male	Present study	ND	No del/dup	No del/dup	ND	ND	No mutation
GM04619	Male	Present study	ND	No del/dup	No del/dup	ND	ND	c.8713C>T (p.R2905X)
GM04978	Male	Present study	ND	No del/dup	No del/dup	ND	ND	c.5893C>T (p.Q1965X)
GM05082	Male	Present study	ND	No del/dup	No del/dup	ND	ND	No mutation

del, deletion; dup, duplication; MLPA, multiplex ligation-dependent probe amplification; ND, not determined.

*Coriell catalog: deletion in this line was listed in the Coriell catalog when the present study was initiated. The present study: the mutation in this line was identified in a preexisting Coriell line during the study. Registry: this cell line was created by donation of blood from consenting patients for the present study.

†MRC-Holland BV, Amsterdam, the Netherlands.

‡This assay does not detect all deleted exons.

§The Affymetrix SNP Array 6.0 is unable to detect mutations in exons 28 and 72 because of lack of probes in these regions. A duplication was identified in GM23127 using the Affy Cytogenetics 2.7 mol/L array, which is also unable to identify mutations in exon 28 because of lack of probe coverage.

tected using the PCR method were not included in the design of the assay.

No deletions or duplications were identified in DNA samples from seven existing DMD cell lines at MLPA and Affymetrix Human SNP Array 6.0 analysis during preliminary testing. DNA from these cell lines was analyzed using DNA sequence analysis in one laboratory to identify smaller mutations. Point mutations were found in four of the seven samples. Three of the mutations were translation termination codons in exons 39, 41, and 59, and one sample had a one-bp deletion that resulted in a frame shift in exon 54 (Table 1). No mutations were identified by sequence analysis in the other three samples. It is possible that these samples had mutations in introns or other regulatory regions; however, these parts of the gene were not analyzed in this study.

A schema of the *DMD* gene is shown in Figure 1. The drawing depicts the locations of the deletion, duplication, and point mutations in each cell line, and the sex of the donor.

Discussion

A number of assays are used in clinical laboratories to detect mutations in *DMD*. Historically, targeted PCR assays were designed to detect gene deletions in a subset of the 79 *DMD* exons (representing deletion hotspots), and were unable to detect exon duplications or point mutations, which together account for about 35% of mutations detected in patients with DMD.[7] With the availability of a commercial MLPA assay, widespread use of quantitative PCR, and DNA sequencing and array-based analyses, identification of duplications and point mutations in *DMD* is becoming more feasible and commonly available in testing laboratories. The ability to detect point mutations and to precisely map deletions and duplications is important to identify patients who could benefit from several new treatments currently under development.

Clinical laboratories need access to reference materials containing the spectrum of mutations present in their clinical

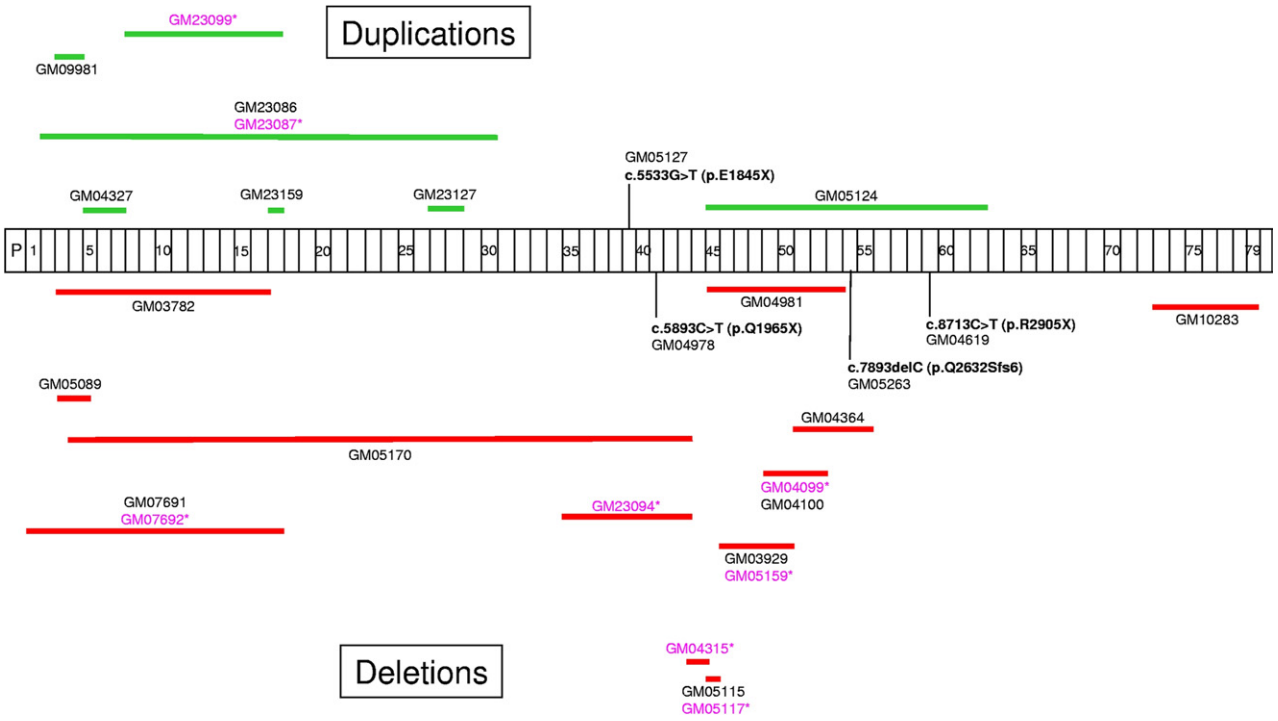

Figure 1. Composition of DMD reference material panel. Schema of the promoter (P) and 79 exons of the DMD gene are indicated by **numbered black rectangles**. The locations of deletions or duplications of DMD exons in each cell line are indicated by **horizontal bars**. Duplications are indicated above the exon map, and deletions are shown below. The exonic locations of point mutations are indicated by **vertical lines**. Coriell numbers (GMXXXXX) are shown for each cell line, and DNA from female donors is indicated with an **asterisk**.

patient population to create and validate new assays, perform quality control, and provide quality assurance through proficiency testing. Currently, there are no commercial sources of reference materials for *DMD* genetic testing. Laboratories that perform this test use nonrenewable and possibly unconfirmed sources of *DMD* genomic DNA including residual patient specimens to fulfill the international, federal, and state regulations, and professional guidelines requiring inclusion of reference or quality control materials representing polymorphisms or mutations detected by the assay during analysis of patient samples[12–16] (Washington State Legislature, *http://www.doh.wa.gov/hsqa/fsl/lqa_home.htm*, accessed August 30, 2010; New York State Clinical Laboratory Evaluation Program, *http://www.wadsworth.org/labcert/clep/clep.html*, accessed July 15, 2010; College of American Pathologists, *http://www.cap.org/apps/docs/laboratory_accreditation/checklists/molecular_pathology_sep07.pdf*, accessed July 30, 2010; and American College of Medical Genetics, *http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm*, accessed July 15, 2010). Similar concerns exist for proficiency test providers to ensure that their challenges reflect the diversity of variation that is included in clinical assays. For *DMD*, this includes not only deletions but also duplications and point mutations, which are present in 35% of patients. Reference materials from female carriers with all three types of mutations should also be available because the presence of an extra X chromosome in the female carrier may make interpretation of a duplication or deletion more complicated than in male patients.

The present study describes the development and characterization of a comprehensive genomic DNA reference material panel for *DMD* genetic testing. In addition, for the first time, to our knowledge, cell lines were identified in the Coriell collection with duplications and point mutations in the *DMD* gene. DNA from each of the 31 selected cell lines was characterized in volunteer laboratories using a variety of assay methods. Deletions, duplications, and point mutations were identified throughout *DMD*. In addition, the *DMD* panel includes samples with both large and small deletions and duplications, samples from female duplication or deletion carriers, and samples with point mutations. Several of the samples have mutations that are targeted by experimental DMD therapies.

The results obtained using the various assays are generally in good agreement. There were a few discrepancies in the data between methods, which were resolved on close examination of the assay design and limitations of detection. While the goal of the study was not to highlight differences among assays, characterized reference materials can greatly facilitate identification of previously undefined limitations of an assay. For example, the Affymetrix Human SNP Array 6.0, although not designed as a *DMD* assay, has the capacity to detect small deletions and duplications throughout the genome. During the study, it was discovered that the probe coverage in *DMD* exons 28 and 72 was not sufficient to detect copy number variation in these exons. Similarly, use of characterized reference materials as validation samples during assay development or as proficiency testing samples can assist laboratories in identification of unknown characteristics of their assays.

Results from the present study also highlight the need for extensive characterization of genomic DNA, either from cell lines or residual patient samples, using a variety of methods before their use as reference materials in the laboratory. Many of the existing Coriell cell lines used in the study had been previously characterized using PCR or Southern blot analysis. In some cases, deletions, duplications, and point mutations were identified using the methods used in this study, whereas previous methods had not identified a mutation in the cell line (labeled "Present study" in Table 1). Similarly, mutations in reference materials derived from patient specimens may be incorrectly identified, depending on the method used to characterize it initially. Thus, CLSI document MM-17[11] recommends characterization of reference materials using a method different from that used to identify the mutation initially.

The present study also highlights the important role that patient groups and registries, such as DuchenneConnect, can have in improvement of laboratory services. Resources, such as DNA, for rare disorders are, by definition, limited, and may be difficult to obtain. Patient groups are becoming aware of their role in the development of new tests and treatments for their rare disorders and are increasingly becoming active partners with the research community. The willingness of patients with DMD or BMD and their families to share their resources with the genetics community through the Coriell Cell Repositories will benefit not only the quality and availability of genetic testing for DMD but also may contribute to the development of treatments for this disorder.

The genomic DNA reference materials characterized in this study will be useful for quality assurance, proficiency testing, test development and validation, and research. DNA samples purified from these cell lines, and other DNA samples characterized by the GeT-RM, are publicly available from the National Institute of General Medical Science Repository at the Coriell Cell Repositories (*http://ccr.coriell.org/Sections/Collections/NIGMS/?Ssid=8,* accessed May 14, 2010). More information about the GeT-RM program and available reference materials are available on the GeT-RM website (*http://wwwn.cdc.gov/dls/genetics/rmmaterials/default.aspx,* accessed May 14, 2010).

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