MOLECULAR DIAGNOSIS OF DYSTROPHINOPATHIES USING A MULTI-TECHNIQUE ANALYSIS ALGORITHM

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ABSTRACT: Introduction: Dystrophinopathies are X-linked recessive neuromuscular diseases caused by mutations in the dystrophin gene. In this study we aimed to detect mutations within the dystrophin gene in DMD patients, to determine the carrier status of women, and to perform a prenatal diagnosis. Methods: We analyzed 17 individuals from 2 unrelated families with a history of DMD. We used multiplex PCR, multiplex ligation-dependent probe amplification (MLPA), and short tandem-repeat (STR) segregation analysis to accurately detect and characterize the mutations and to identify the at-risk haplotype. Results: The selected methodology allowed for the characterization of 2 single-exon out-of-frame deletions in the affected patients. Nine of 13 women and a fetus were excluded from being carriers. Three recombination events were found and suggested that germline mosaicism had occurred in both families. Conclusions: This methodology proved to be efficient for characterizing the disease-causing mutation in affected individuals and for assessing the carrier status in healthy relatives. These findings helped inform precise genetic counseling and contributed to characterization of the disease in the Argentine population.

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Dystrophinopathies include several muscle diseases caused by mutations in the dystrophin gene (Xp21.2, MIM # 300377), which encodes the dystrophin protein. The type of mutation defines 3 different phenotypes: (1) Duchenne muscular dystrophy (DMD) due to total loss of dystrophin function; (2) Becker muscular dystrophy (BMD) that results from reduced gene expression or a partially functional protein; and (3) X-linked dilated cardiomiopathy (XLDC) caused by a selective loss of the heart-specific dystrophin isoform. The mild end of the spectrum of phenotypes consists of an asymptomatic increase in serum concentration of creatine phosphokinase and muscle cramps with myoglobinuria and isolated quadriceps myopathy.[1] The severe symptoms include progressive muscle diseases that are classified into DMD or BMD when skeletal muscle is affected primarily and XLDC when the heart is affected primarily.[2]

DMD affects 1 in 3500 live male newborns, whereas BMD is less frequent.[3] Intragenic deletions of 1 to several exons are responsible for the DMD/BMD phenotype in two thirds of patients; the remaining cases are caused by genomic duplications or chromosomal micro-rearrangements (Leiden Muscular Dystrophy webpages: http://www.dmd.nl). *De novo* mutations and germline mosaicism are responsible for one third of DMD/BMD cases, and a family history of dystrophinopathies with several affected males accounts for two thirds of cases.[4–6]

Patients with mutations that result in a disruption of the translational reading frame of the dystrophin gene (out-of-frame mutation) show a clinical progression to DMD, which is a severe and fatal disease. On the other hand, patients who bear genetic alterations that do not change the translational reading frame (in-frame mutation) develop BMD, which shows milder symptoms. The correlation between phenotype and type of deletion is in agreement with the "reading frame" theory in 92% of cases and is of diagnostic and prognostic significance.[7–9] Therefore, accurate detection and characterization of the genetic abnormality involved in the pathogenesis of these dystrophinopathies help to predict the disease course, which, in turn, allows for precise genetic counseling and follow-up. In addition, molecular diagnosis helps assess the carrier status of healthy women and the risk of transmitting the dystrophin-mutated chromosome to their offspring.

Currently, there is neither efficient treatment of the progressive muscular dystrophy nor efficient rehabilitation. Genetic counseling and prenatal diagnosis are the only available options that medical genetics can offer. However, different lines of research on DMD therapies are being developed, and among the most promising is a gene therapy based on exon skipping, which leads to the

Abbreviations: BMD, Becker muscular dystrophy; CVS, chorionic villus sample; DMD, Duchenne muscular dystrophy; EDTA, ethylene-diamine tetraacetic acid; MLPA, multiplex ligation-dependent probe amplification; PCR, polymerase chain reaction; STR, short tandem-repeat; XLDC, X-linked dilated cardiomiopathy

Key words: carrier detection; DMD; Duchenne; dystrophin gene; molecular diagnosis

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restoration of the translational reading frame and conversion of DMD to the less severe BMD phenotype.[10,11]

Several methods have been proposed for molecular detection of DMD/BMD carriers. They can be classified into: (1) direct studies that seek and characterize the mutation; and (2) indirect studies that identify the at-risk haplotype but do not characterize the genetic abnormality. One of the most relevant advantages of the indirect studies is that there is no restrictive need to identify the disease-causing mutation and to have access to samples from affected individuals.[12,13]

In this study we have aimed to detect mutations within the dystrophin gene in DMD patients, to determine the carrier status of women, and to perform a prenatal diagnosis. We report the molecular analyses of 2 Argentine families with a history of DMD. We show that combining several methodologies improves the accuracy of diagnosis, which, in turn, provides better genetic counseling.

METHODS

Study Subjects and Biospecimens. Three children who were diagnosed clinically with DMD from 2 independent families were referred to our laboratory to confirm the dystrophinopathy using molecular techniques. In addition, geneticists requested detection of mutation carriers among female relatives, including 1 for prenatal diagnosis for DMD. The protocol was approved by the institutional ethics committee. Informed consent was obtained for all study subjects prior to the molecular studies.

The following clinical criteria were used for the clinical diagnosis of DMD: progressive muscular weakness since childhood; high levels of serum creatine kinase (20 times above normal upper boundary)[1,3]; myopathic changes on electromyography; and a muscle biopsy showing absent or decreased dystrophin levels.[14]

Whole blood was drawn by venipuncture with ethylene-diamine tetraacetic acid (EDTA) as anticoagulant for all study subjects. For the fetus, a chorionic villus sample (CVS) was obtained by trained personnel. Genomic DNA was isolated using the cetyl-trimethyl-ammonium bromide method.[15] DNA concentration and quality were measured by absorbance at 260 nm and by the ratio of A_{260nm}/A_{280nm}, respectively. Some samples were also analyzed by electrophoresis on 1% agarose (Genbiotech SRL, Buenos Aires, Argentina) gels dyed with DNA-gel stain (SYBR Safe; Life Technologies, Foster City, California). All samples were stored at −20°C.

Multiplex Polymerase Chain Reaction Assay. Multiplex polymerase chain reactions (PCRs) were designed to target different regions of the dystrophin gene that are frequently deleted. These PCRs were used to identify deletions in the dystrophin gene in males. The method was performed as previously described elsewhere, with minor modifications.[16–18]

Each multiplex PCR amplified the following regions: *Quadruplex I*—exons 8, 17, 44, and 48; *Quadruplex II*—exons 19, 45, 51, and 52; *Quadruplex III*—muscular promoter, and exons 3, 4, and 13; *Quadruplex IV*—exons 43, 47, 50, and 60; *Pentaplex 1*—muscular promoter, and exons 17, 42, 44, and 45; *Pentaplex 2*—brain promoter, and exons 3, 12, 32, and 47; *Pentaplex 3*—exons 13, 16, 19, 34, and 51; *Pentaplex 4*—exons 6, 8, 41, 48, and 60; and *Pentaplex 5*—exons 4, 43, 49, 50, and 52.

All primer sequences were obtained from the Leiden Muscular Dystrophy site [Leiden Muscular Dystrophy webpages (http://www.dmd.nl)]. All PCR reactions were performed in a thermal cycler (Veriti; Applied Biosystems, Foster City, California). PCR amplicons were analyzed by 2% agarose (Genbiotech SRL) gel electrophoresis in 1× TBE buffer and dyed with SYBR Safe (Life Technologies). Gels were photographed and analyzed with specific software.

Positive controls (wild-type DNA) and negative controls (water) were included in all reactions.

Multiplex Ligation-Dependent Probe Amplification Assay. The commercially available multiplex ligation-dependent probe amplification (MLPA) kit[19–21] for the dystrophin gene was used to determine gene deletion/duplication. The assay conditions and reactions were performed according to the manufacturer's recommendations [MRC Holland, Amsterdam, The Netherlands (www.mlpa.com)]. The reaction products were analyzed using with a DNA analyzer (ABI 3730 XL; Applied Biosystems) and Liz as internal size standard. Data analysis was performed using GeneMapper (Applied Biosystems) or GeneMarker (Softgenetics, State College, Pennsylvania) software for MLPA. Wild-type, deleted, and duplicated DNA controls were included in all reactions.

When the MLPA result suggested a single-exon deletion, the result was confirmed by an alternative molecular technique.[22]

Haplotype Segregation Analysis. Several intronic short tandem-repeat [STR-(CA)n] polymorphisms have been identified within the dystrophin gene, and many are located in deletion/recombination hot-spot regions.[12,23–27] We used a set of 8 highly polymorphic STR markers to perform a segregation analysis in these families. The amplified microsatellites were labeled using a specific ^{32}P-primer [STRs: introns 1 (DYSII), 7, 25, 44, 45, and 63], or a specific 6-FAM-primer [STR: introns 1

(DYSII), 62, and 67]. Haplotypes were determined by comparing intrafamily segregation allele patterns.

Radiolabeled PCRs were performed according to Clemens *et al.* with minor modifications.[12] Briefly, 120 ng of genomic DNA was mixed with 15 pmol of 1 ^{32}P-primer, 15 pmol of 1 unlabeled primer, PCR buffer [10 mM Tris-HC1 (pH 8.3), 50 mM KCI, 1.5 mM $MgCl_2$], 0.2 mM of each dNTP, and 0.75 U of Taq-polymerase (Inbiohighway) in a final volume of 15 μl. Samples were denatured at 94°C for 4 min, followed by 25 cycles of DNA denaturation (94°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 30 s), and a final extension at 72°C for 10 min. Then, 2–4 μl of PCR products were mixed with 1 volume of stop solution (98% formamide, 0.5% EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and heated at 95°C for 3 min. PCR amplicons were analyzed by electrophoresis on a denaturing polyacrylamide sequencing gel. After electrophoresis, the gel was fixed for 20 min with 10% acetic acid and rinsed for 20 min with distilled water. Autoradiography of the dried gel was performed at room temperature for 1–3 days.

PCR reactions using 6-FAM primers were performed using 120 ng of genomic DNA, 0.3 μM of 1 6-FAM-labeled primer, 0.3 μM of 1 unlabeled primer, PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$], 0.2 mM of each dNTP, and 0.5 U of Taq-polymerase (Life Technology, Gaithersburg, Maryland) in a total volume of 25 μl. The samples were denatured at 94°C for 3 min, followed by 33 cycles of DNA denaturation (94°C for 30 s), annealing (58°C for 30 s), and extension (72°C for 30 s), and a final extension at 72°C for 10 min. The PCR products were analyzed using a fragment analyzer sequencer (ABI 3730 XL DNA; Applied Biosystems). Data analysis was performed using PeakScanner software (Applied Biosystems).

RESULTS

Family 1. The family pedigree is shown in Figure 1A. Children II5, III1, and III2 were the first DMD cases reported for this family. The woman I2 had a son (II5) and 2 grandsons (III1 and III2) affected with the disease; therefore she was an obligate carrier. Her daughter (II1) was also an obligate carrier, because she had a brother (II5) and 2 sons (III1 and III2) diagnosed with DMD. The women (II2, II3, II4, III3, and III4) were at risk of being carriers, and the fetus IV1 (unknown gender) was at risk of being either a female carrier or an affected male.

First, we performed 4 multiplex PCR assays (*Quadruplex* I, II, III, and IV) on proband III1 and the CVS corresponding to the fetus IV1. We did not find deletions in the exons included in these PCRs (data not shown).

Then, we performed a segregation analysis using 6 STR(CA)n located within the deletion-prone regions (DYSII, 7, 25, 44, 45, and 63). The segregation analysis (Fig. 1A) showed that the 2 affected children (III1 and III2) inherited the haplotype that cosegregates with the mutation (C/K/O/Q/E/I) from their mother (II1). This haplotype was also carried by their grandmother (I2). Women II2 and II3 inherited the haplotype not linked to the disease (B/−/−/−/E/H) and were excluded from being carriers.

The segregation analysis within this family evidenced a recombination event in II4 between the STR DYSII and STR 7 (Fig. 1A). Based only on this information, the DMD-causing mutation could not be identified; therefore, it was not possible to determine the carrier status of II4, III3, and III4. The segregation analysis for III4 evidenced another recombination event that occurred in III4 between STR44 and STR45.

The study of CVS corresponding to IV1 revealed that the fetus had a single X chromosome, because all STRs analyzed indicated a hemizygote. These results suggested that the fetus was male, and he was excluded from being affected with DMD because he did not inherit the disease-linked X chromosome.

To identify the mutation and determine the carrier status of II4, III3, and III4, we performed an MLPA assay on the affected male III2. We found that exon 63 was deleted (Fig. 1B). To confirm this result, we analyzed STR62 (intron 62) by PCR in III1 and III2. We found that the deletion of exon 63 also spanned the STR on intron 62. A detailed *in silico* analysis of exon 63 deletion showed a shift in the reading frame with a creation of an early stop codon (UAA) in exon 64, 10 bases downstream of the deletion breakpoint (Fig. 1C). Therefore, the molecular analysis confirmed the clinical diagnosis of DMD.

We found that woman II4 was heterozygotic for STR62. In addition, she carried the 3′ end of the gene that was not linked to the disease. Therefore, she was excluded from being a carrier. Then, we analyzed STR62 on I2, who was an obligate carrier, and found that her peripheral blood leukocyte DNA was heterozygotic for the *locus* deleted on the patient. A possible explanation for this observation is the occurrence of germinal mosaicism for a *de novo* deletion of STR62 and exon 63 on I2. Thus, her daughter (II1) and her deceased son (II5) inherited the dystrophin-mutated X chromosome, whereas her other daughters, II2, II3, and II4, inherited the non-mutated dystrophin X chromosome and were excluded from being carriers.

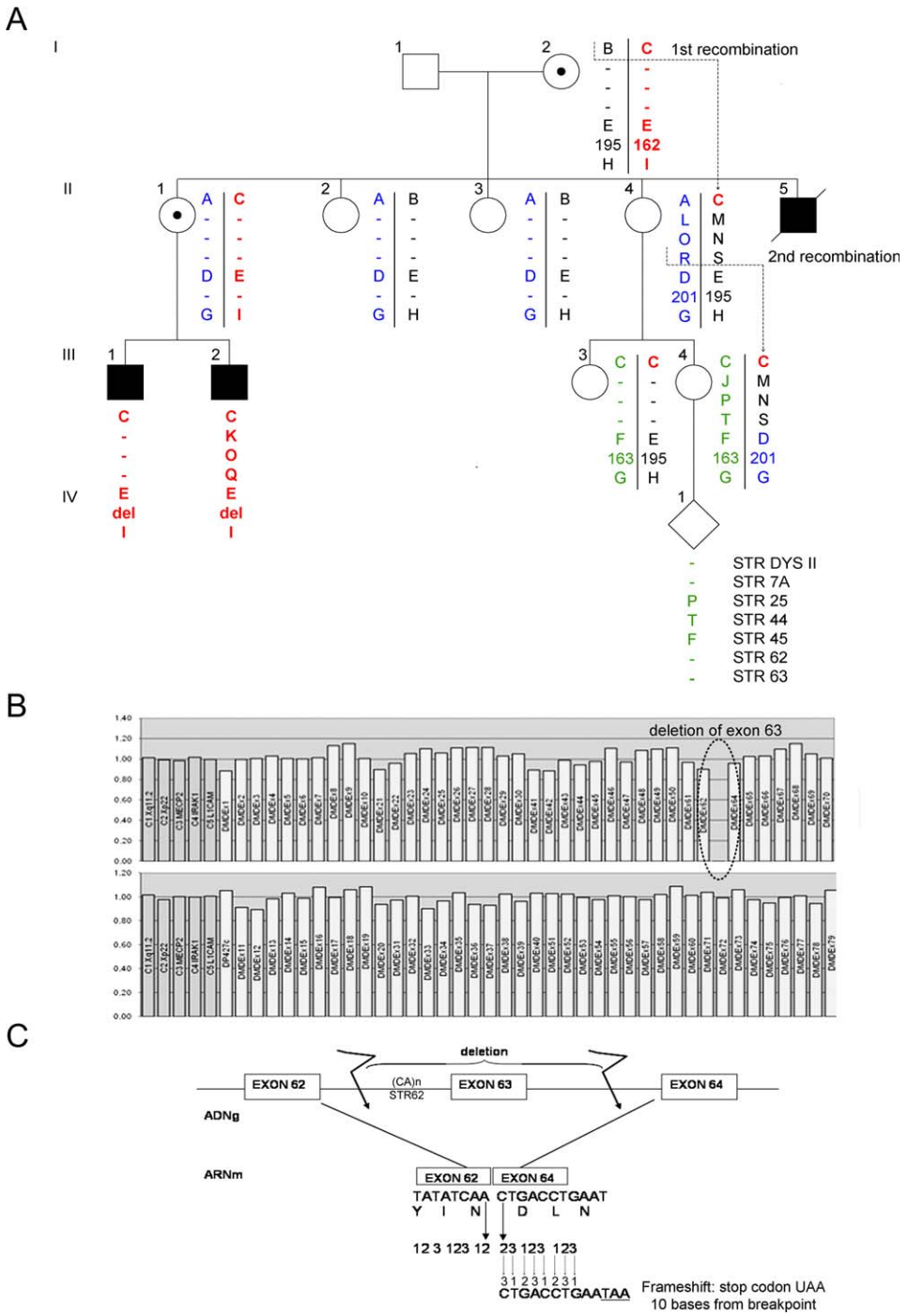

FIGURE 1. Family 1 pedigree and molecular analyses. **(A)** Family pedigree and the haplotype segregation analysis. This family consisted of 4 generations with 3 affected men: 1 in the second generation (deceased) and 2 in the third generation (living). The pedigree analysis showed that 2 women were obligate carriers (I2 and II1). In the haplotype segregation analysis, the letters correspond to the arbitrary names for the ^{32}P-labeled STRs, and numbers correspond to the molecular size of amplicons for 1 6-FAM-labeled STR performed after the MLPA result. Recombination breakpoints are shown by dotted lines, and risk haplotypes for DMD are in bold. **(B)** Deletion of exon 63 for patient III1 detected by MLPA (upper bar graph: Salsa MLPA P034; lower bar graph: Salsa MLPA P035). The results were analyzed using GeneMapper software, and the bar graphs represent the amplification ratio of case:control for each amplicon. **(C)** *In silico* analysis of the deleted dystrophin gene. The prediction shows that this deletion shifts the reading frame and creates an early stop codon in exon 64. Abbreviations: del, deletion; –, STR not analyzed. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In summary, the use of different molecular methodologies allowed us to: (1) identify and characterize the mutation on the affected children; (2) identify 2 obligate carriers; (3) exclude 5 women from being carriers of the mutated gene; (4) exclude a male fetus from being affected with

DMD; and (5) identify a *de novo* mutation and germline mosaicism.

Family 2. The pedigree of Family 2 is shown in Figure 2A. Children II4 and III2 were the first DMD-affected cases in this family. Parent I3 had a son (II4) and a grandson (III2) with DMD; therefore, she was an obligate carrier. Her daughter (II3) was an obligate carrier, because she had a brother (II4) and a son (III2) affected with the disease. The women, I2, II1, II2 and III1, were at risk of being carriers.

First, we sought deletions in the affected patient (III2) using 5 pentaplex PCR reactions. We did not find deletions in the 25 exons studied (data not shown). Therefore, we performed segregation analysis to identify the allele carrying the dystrophin mutation to determine the carrier status of the women (Fig. 2A). We characterized 3 STR(CA)n located in 3 introns (STRs DYSII, STR62, and STR67). This analysis revealed that the haplotype shared by both obligate carriers, I3 and II3 (234-178-270), was the haplotype linked to the mutation. Patient III2 inherited a recombinant chromosome (242-206-270). This recombination event allowed us to predict that the mutation was linked to the 270 allele, because this is the only STR shared by the affected individual and the 2 obligate carriers.

We excluded III1 from being a DMD carrier, because she inherited the haplotype not linked to the mutation (242-206-250). The women, I2, II1, and II2, inherited the at-risk haplotype. However, up to this point of the analysis, their carrier status could not be determined, because a *de novo* mutation may have occurred in I3.

We then performed an MLPA assay on DNAs from III2 and I2. We found a deletion that comprised exon 65 only in III2 (Fig. 2B). We did not find deletions or duplications in I2; therefore, I2, II1, and II2 could be excluded from being carriers. The deletion found in III2 was confirmed by PCR of exon 65 with primers flanking the region where the MLPA probe maps.

In silico characterization of the exon 65 deletion showed a shift in the reading frame with the creation of an early stop codon (UAG) 281 bases downstream of the deletion breakpoint (Fig. 2C). Therefore, the molecular analysis confirmed the clinical diagnosis of DMD.

The molecular study identified a deletion of exon 65 in III2 that was not evidenced in I2, although they shared the same haplotype. We hypothesized that the father of I2 and I3 could have been a germinal mosaic for the deletion, and, whereas I3 inherited the dystrophin-mutated X chromosome, I2 inherited the non-mutated dystrophin X chromosome. Another possible explanation is that a *de novo* deletion occurred in I3, and she transmitted the mutation to her offspring.

In summary, the use of different molecular methodologies allowed us to: (1) identify and characterize the mutation in the affected child; (2) identify 2 obligate carriers; (3) exclude 4 women from being carriers of the mutated gene; and (4) identify a *de novo* mutation and germline mosaicism.

DISCUSSION

Molecular diagnosis of dystrophinopathies is a valuable tool for providing accurate genetic counseling, for detecting the responsible mutation in affected children, and for identifying women at risk of being carriers. In this report, we used different methodologies to achieve these goals in 2 different families with individuals diagnosed with DMD. We provided accurate molecular diagnosis and genetic counseling to all members of both families.

Currently, no "gold standard" technique exists that detects all types of mutations in the dystrophin gene. In addition, there is not a unique molecular diagnostic algorithm suitable for all families with dystrophinopathy-affected individuals. A careful and personalized algorithm must be designed for each particular family, especially in developing countries where economic constraints preclude the use of inexpensive techniques in order to make molecular diagnosis affordable. Each study requires a careful evaluation of the following family and laboratory scenarios: (1) type of molecular diagnosis required (carrier detection, mutation identification, or prenatal diagnosis); (2) sporadic or familial dystrophinopathy; (3) timeframe in which the results are intended (urgency of prenatal diagnoses); (4) family socioeconomic status; (5) availability of DNA samples from affected individuals and their relatives; and (6) laboratory equipment for sample analysis.

Because the ultimate goal of molecular diagnosis is identification of the causative mutation, direct studies are the best choice for molecular analysis (e.g., MLPA, multiplex PCR, and direct sequencing). MLPA allows detection of deletions and duplications, which account for 75% of all dystrophin mutations in men and women and is the method with the highest mutation detection rate.[28] However, this technique is expensive and not affordable for all families and laboratories. An alternative method is the use of multiplex PCR with the caveat that it detects the most frequent gene deletions only in males. When deletions or duplications are not found by MLPA or multiplex

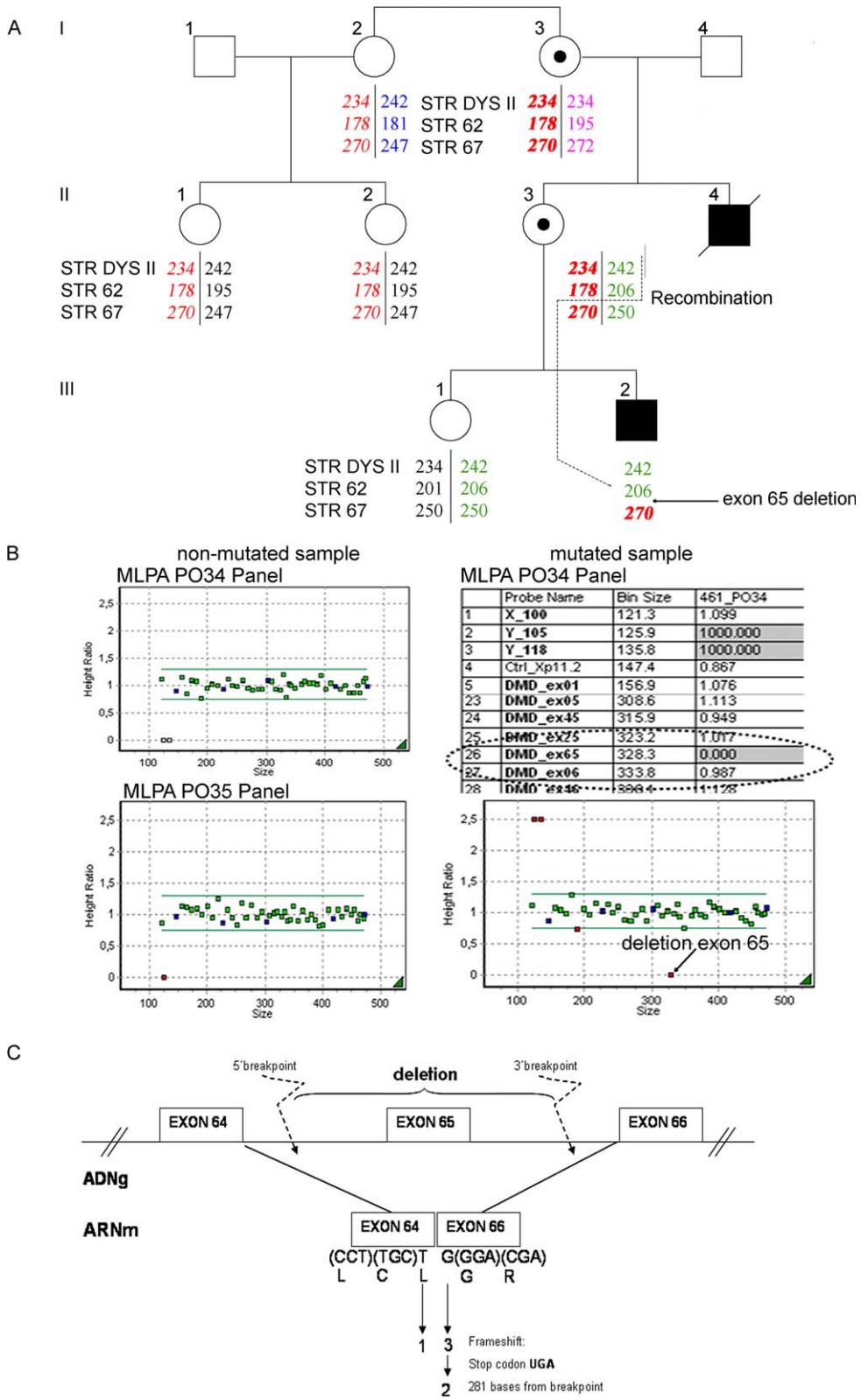

FIGURE 2. Family 2 pedigree and molecular analyses. **(A)** Family pedigree and the haplotype segregation analysis. Family 2 comprised 3 generations with 1 affected man in the second generation (deceased) and 1 in the third generation (living). Pedigree analysis showed that 2 women were obligate carriers (I3 and II3). The numbers correspond to the molecular size of amplicons for 3 6-FAM-labeled STRs. Recombination breakpoints are shown by dotted lines, and risk haplotypes for DMD are in bold. **(B)** MLPA analysis for a non-mutated sample (I2) and the deletion of exon 65 for patient III2. The results were analyzed using GeneMarker software. **(C)** *In silico* analysis of the deleted dystrophin gene. The prediction shows that this deletion changes the reading frame and creates an early stop codon in exon 68. Abbreviations: del, deletion; –, STR not analyzed. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PCR analyses, whole dystrophin gene sequencing is suggested to screen for point mutations and small insertions/deletions.

When direct studies are not conclusive or are unaffordable, indirect studies—haplotype segregation analyses—become additional molecular tools required to perform an accurate molecular diagnosis. The aim of segregation analysis is to identify the at-risk haplotype that cosegregates with the mutation in the affected individuals and in the obligate carriers within the family. One of the most relevant advantages of these studies is that there is no restrictive need to identify the disease-causing mutation and to have access to samples from affected individuals.[12,13] Comparison of haplotypes from relatives and patients (at-risk haplotype) allows for determination of mutation carrier risk. This analysis is informative with approximately 95–100% certainty for most cases. It also allows for detection of molecular events such as recombination, mosaicism, and *de novo* mutations.

Certainty of the study takes into account the probability of a recombination event within the dystrophin gene. Because recombination occurs with an estimated frequency of 10% between both ends of the dystrophin gene, this analysis has a certainty of approximately 90–100% for most cases.[29] The certainty increases when STR *loci* at the 5′ and 3′ ends of the gene are heterozygous. The presence of a deletion at any STR *locus* increases the certainty of the analysis to 100%.[30] Another molecular event that could affect the certainty of the diagnosis is the occurrence of germline mosacism in patients' parents (15–20%).[31,32]

In this study, we have analyzed 3 affected children by multiplex PCR. Although multiplex PCRs were designed to detect 90% of all dystrophin deletions, we were not able to detect deletions in our cohort by this technique. We analyzed 17 individuals by haplotype segregation analysis, and 3 of 17 were analyzed by MLPA as well. Overall results of these different molecular techniques allowed accurate molecular diagnosis in 13 women and 1 prenatal diagnosis. Six women and the fetus were excluded from carrying the dystrophin mutation with almost 100% certainty (Family 1: II4, III3, III4, and IV1; Family 2: I2, II1, and II2). In addition, 3 women were excluded with almost 95% certainty (Family 1: II2 and II3; Family 2: III1). Four women were obligate carriers by pedigree analysis (Family 1: I2 and II1; Family 2: I3 and II3).

We studied the affected patients of both families and found deletions that were predicted to disrupt the translational reading frame generating putative early stop codons that could result in an abnormal truncated protein. The mutations were located within exons that encode for the cysteine-rich domain (exons 62–69) and were predicted to affect all the dystrophin isoforms [Leiden Muscular Dystrophy webpages (http://www.dmd.nl)]. The affected boys had out-of-frame mutations and severe clinical symptoms of DMD. These findings were in agreement with the reading frame rule.[9] Characterization of the mutation permitted: (1) confirmation of the clinical diagnosis of DMD; and (2) potential development of antisense oligonucleotides that target specific exons. This type of therapy would restore the reading frame to allow for synthesis of partially functional dystrophins and to transform the DMD phenotype into a milder dystrophinopathy.[33,34]

In conclusion, the integration of different methodologies that combine direct and indirect studies proved to be useful in characterizing the mutation in affected individuals and identifying relatives at risk of being carriers. The designed algorithms were able to detect mutations in the dystrophin gene, recombination events, and germline mosaicism.

These molecular findings helped in precise genetic counseling and contributed to characterization of the disease in the Argentine population.

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