ABSTRACT: We report a Becker muscular dystrophy (BMD) family with one 5-year-old affected patient and a 69-year-old asymptomatic grandfather. Dystrophin gene multiplex polymerase chain reaction and multiplex ligation-dependant probe amplification analysis showed that both males carried an in-frame deletion of exons 45–55. Segregation analysis revealed two additional asymptomatic boys in this family. Our finding supports previous predictions that exons 45–55 are the optimal multiexon skipping target in antisense gene therapy to transform the severe Duchenne muscular dystrophy into the milder BMD, or even asymptomatic, phenotype.

Muscle Nerve **39:** 239–243, 2009

ASYMPTOMATIC BECKER MUSCULAR DYSTROPHY IN A FAMILY WITH A MULTIEXON DELETION

VERÓNICA FERREIRO, PhD,[1] FLORENCIA GILIBERTO, PhD,[2] GARCÍA M. NOELIA MUÑIZ,[2] LILIANA FRANCIPANE, MD,[1] DIEGO M. MARZESE,[3] ALEJANDRA MAMPEL, MD,[4] MARÍA ROQUÉ, PhD,[3] GUSTAVO D. FRECHTEL, MD PhD,[1] and IRENE SZIJAN, PhD[2]

[1] Genetics Division, Clinical Hospital "José de San Martín," University of Buenos Aires, 2250 Paraguay, 1120 Buenos Aires, Argentina
[2] Department of Genetics and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina
[3] Department of Cellular and Molecular Biology, Faculty of Medical Science, National University of Cuyo, Mendoza, Argentina
[4] Institute of Medical Genetics, Faculty of Medical Science, National University of Cuyo, Mendoza, Argentina

Accepted 9 September 2008

The severe Duchenne muscular dystrophy (DMD, MIM# 310200) and the milder Becker muscular dystrophy (BMD, MIM# 300376) are allelic conditions characterized by progressive muscular degeneration and wasting accompanied by increased serum creatine kinase (CK). They are both caused by mutations in the dystrophin gene (Xp21.2, MIM# 300377). DMD affects ≈1 out of 3,500 live male newborns, while BMD is less frequent. Around two-thirds of patients show intragenic deletions ranging from one to several exons of the dystrophin gene. The remaining cases arise from genomic duplications or microrearrangements. The reading frame rule[8] explains how two different phenotypes result from mutations in the same gene. Clinical progression of the disease can be predicted by whether the mutation maintains (in-frame, BMD) or disrupts (out-of-frame, DMD) the translational reading frame of the dystrophin gene. This hypothesis explains the phenotypic differences observed in ≈92% of DMD/BMD cases.[7] However, several exceptions have been described. First, dystrophin protein has been detected in muscle biopsies of patients with out-of-frame mutations and the BMD phenotype. Second, large in-frame deletions have been found in DMD patients. Lastly, large in-frame deletions have been discovered incidentally in patients with no clinical signs, as is the case of the family described in this work.

The opportunity to transform a DMD into a BMD phenotype is a potential new therapeutic strategy based on development of antisense oligonucleotide technology.[13] The rationale is to induce exon-skipping at the pre-mRNA level in order to restore an open reading frame. Such exon-skipping exists among patients and can explain exceptions to Monaco's rule.[9] The family we report supports previous predictions that exons 45–55 are the optimal exon-skipping target to transform the DMD phenotype into the asymptomatic or mild BMD phenotype among the more than 45% of patients who have out-of-frame deletions in the dystrophin gene.[1]

Abbreviations: BMD, Becker muscular dystrophy; Cys, cysteine; del, deletion; DMD, Duchenne muscular dystrophy; MPm, dystrophin muscular promoter; PCR, polymerase chain reaction; CK, serum creatin kinase; MLPA, multiplex ligation-dependant probe amplification; pre-mRNA, primary transcript RNA; $STR(CA)_n$, short CA tandem repeat

Key words: asymptomatic DMD/BMD; multiple-exon skipping; DMD molecular diagnosis; DMD gene therapy; antisense; DMD

Correspondence to: V. Ferreiro; e-mail: vferreiro@ffyb.uba.ar

Published online 14 November 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mus.21193

FAMILY REPORT

Patient (III1). After an uneventful pregnancy the proband was born normally at term in 1997 to healthy unrelated parents who had no family history of muscle disease. Free walking was possible at the age of 16 months. He was referred to a neurologist because of frequent muscle cramps at the age of five. The physician noted his large calves and found a CK level of 20145 IU/L; thus, a DMD diagnosis was suspected. The patient was referred to a geneticist who noted that he was able to walk, run, and go up and down steps with no difficulty. However, the elevated CK level and the mild muscular symptoms led him to conclude that the patient had oligosymptomatic DMD. Nonetheless, several complementary tests were performed in order to make a definitive differential diagnosis between DMD and BMD. Electromyography showed short-duration and low-amplitude motor unit potentials, compatible with a primary myopathy. Muscle biopsy revealed myopathic changes, such as slight atrophy of type 1 and type 2 fibers, with diameter variations and perimysial/endomysial fibrosis. Immunohistochemistry showed the presence of dystrophin with standard antibodies (Dys1, Dys2, and Dys3), with a limited number of isolated attenuated fibers observed by Dys 2 immunoreactivity (data not shown). The boy remains clinically oligosymptomatic up to the writing of this work.

Molecular Analysis. DNA was extracted from whole blood using standard procedures.[10] Exon analysis by multiplex polymerase chain reaction

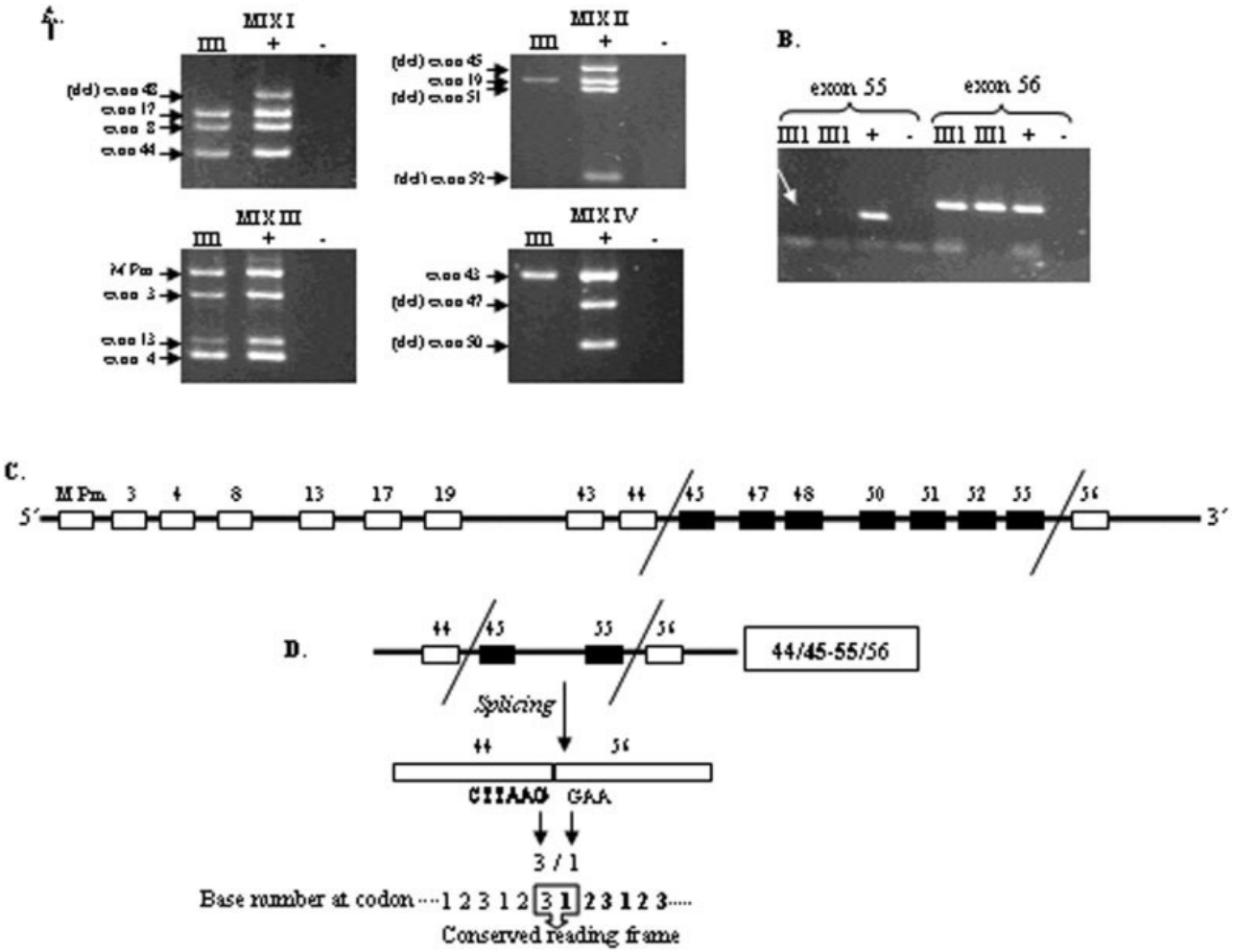


FIGURE 1. Deletion analysis of the proband (III1). **(A)** Multiplex-PCR results. The arrows show the corresponding amplified exon. (del) deletion. (+) positive amplification control. (−) no DNA control. MPm, muscular dystrophin promoter. **(B)** Simplex-PCR results. The white arrow shows the deleted exon 55. **(C)** Diagram of the patient's dystrophin gene. Only the analyzed exons are shown. White boxes represent amplified exons by multiplex and simplex PCR and black boxes deleted ones. **(D)** Reading frame analysis. The mutation found in the patient is an in-frame deletion. (44/**45–55**/56): borders of the deletion found in III1 (last exon amplified / first exon deleted − last exon deleted / first exon amplified).

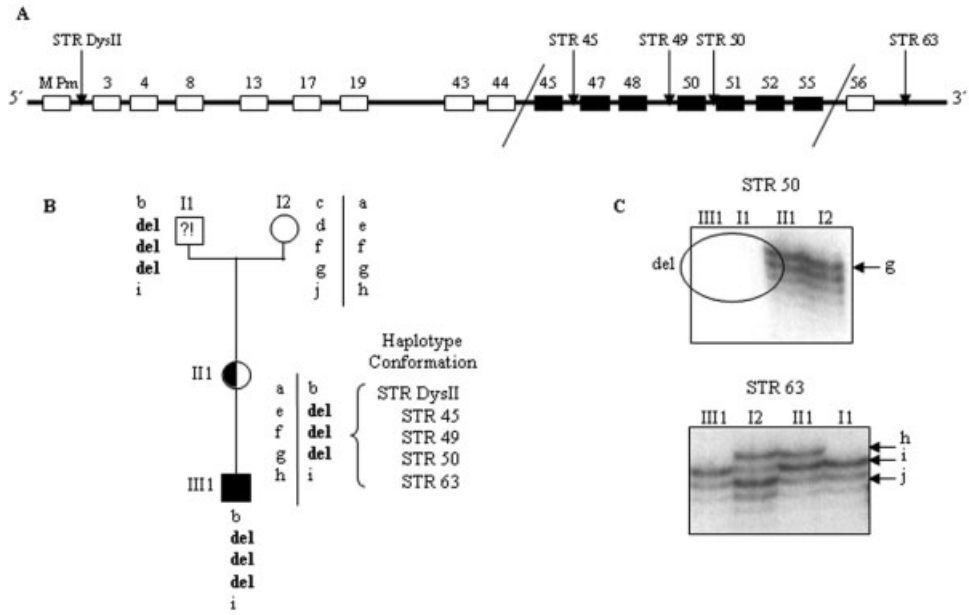


FIGURE 2. Segregation analysis of the proband, his mother and grandparents. **(A)** Location of STRs used in the segregation analysis, within the dystrophin gene. Black boxes denote exons deleted in the proband. **(B)** Pedigree and haplotype analysis. The letters used for naming the alleles were arbitrarily chosen. del, deletion. **(C)** Electrophoretic allele pattern for STRs 50 and 63. The circle indicates the absence of amplification of STR 50 in III1 and I1.

(PCR) of deletion hot spots followed the protocol described in the literature.[4] A large in-frame deletion of exons 45–55 was found (44/**45-55**/56) (Fig. 1). This finding was consistent with the child's clinical profile and allowed us to make a diagnosis of BMD.

Mother and Grandparents. The family returned for further genetic consultation to determine the carrier status of the proband's mother by segregation analysis when the patient was 7 years old. Blood samples were collected from the proband, his mother, and his grandparents. Total DNA was extracted from leukocytes by standard procedures.[10]

Segregation Analysis. Five intragenic short tandem repeat polymorphisms, STR-$(CA)_n$, were analyzed. Three of them mapped within the deletion (STRs 45, 49, and 50), and the remaining two (STR-DYSII and 63) mapped at the 5′ and 3′ ends, respectively (Fig. 2A). PCR amplification was performed as previously published.[5] All five loci were informative. STR segregation analysis showed that the mother (II1) carried the deletion in her paternal allele; surprisingly, such deletion was also found in the grandfather's constitutive DNA (I1) (Fig. 2B). This rare finding is clearly observed in STR50, where no amplification products were detected in III1 and I1. Results from STR63 are shown as an amplification control for both males (Fig. 2C).

Multiplex and simplex PCR analysis confirmed the deletion in the 69-year-old grandfather. Nevertheless, he had no clinical muscle symptoms, and cardiac involvement was ruled out by echocardiography; however, serum CK levels were increased (854 IU/L). This finding, together with the fact that the gene deletion was found in tissues of different embryonic origin (gametes, epithelial, and blood cells), ruled out the possibility of germline mosaicism in the grandfather. These data are consistent with an asymptomatic male who harbored a large deletion in the dystrophin gene.

In order to confirm the presence of the deletion in the mother, a multiplex ligation-dependant probe amplification (MLPA) analysis was performed.

MLPA Analysis. The MLPA technique is a gene-dosage method that can be used to detect deletions, duplications, and certain point mutations.[12] The MLPA was performed in a thermal cycler with a heated lid, using the SALSA DMD probemix PO34 and PO35, from MRC-Holland (Netherlands). Taken together, both kits contain a probe mixture for all 79 DMD exons. Several control fragments on different chromosomes are included as well as the standard MLPA control probes used to determine ligation efficiency and DNA concentration. All reactions were carried out according to the manufacturer's recommendations (www.mlpa.com). The MLPA

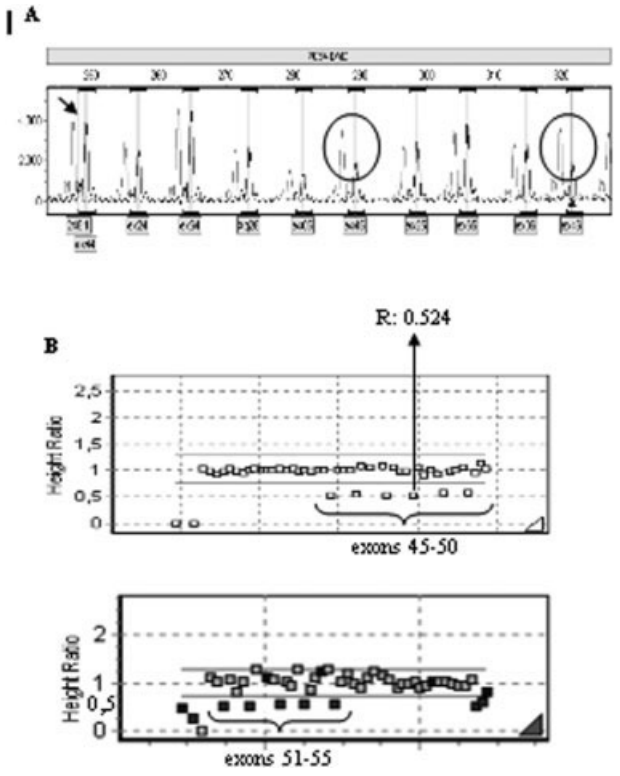


FIGURE 3. MLPA analysis. **(A)** MLPA partial electropherogram for II1. The gray electropherogram corresponds to the control sample and the black one to the II1 sample. The arrow indicates the last exon amplified before the deletion (exon 44). The circles highlight hemizygosity of exons 45 and 46. **(B)** MLPA ratio analysis for II1. PO34 DMD kit (upper panel) and PO35 DMD kit (lower panel). The boxes at 0.5 height ratio represent exons in hemizygosis for II1. R: 0.524, dose-ratio between control and mother samples for exon 48.

products were analyzed using an ABI 3100 genetic analyzer from Applied Biosystems (Foster City, California), with LIZ as the internal size standard. Data analysis was performed using GeneMarker software for MLPA. This assay confirmed the deletion in III1 and I1 (data not shown) and showed hemizygosity of exons 45–55 in the mother (Fig. 3).

Family. As this case was initially thought to be sporadic but turned out to be hereditary, six additional family members without overt clinical symptoms were analyzed (Fig. 4). The three sisters (II1, II2, and II3) were obligate carriers. All of them showed normal serum CK levels (30 IU/L, 143 IU/L, and 105 IU/L, respectively), underscoring the limitation of using serum CK to diagnose female DMD carrier status. Haplotype analysis showed one carrier girl (III3), and two additional 19- and 21-year-old boys with the deletion (III4 and III5) (Fig. 4). They both showed elevated CK levels (849 IU/L and 978 IU/L, respectively) but had no clinical symptoms.

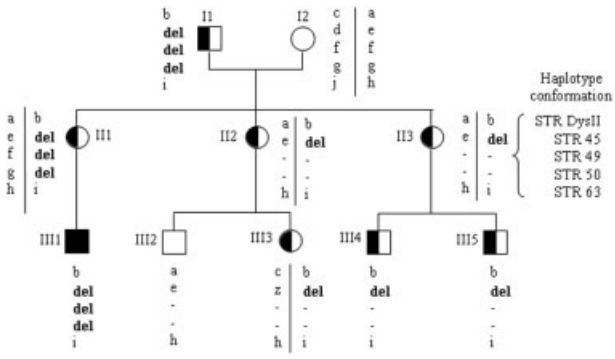
DISCUSSION

It is known that, whereas in-frame deletions result in a BMD phenotype, out-of-frame deletions generally result in DMD. However, it is also important to locate the deletion within the dystrophin gene in order to predict its effects on the resulting protein function. The dystrophin protein contains four domains: amino-terminal, rod, cysteine-rich, and carboxyl-terminal. It has been reported that in-frame deletions in the amino-terminal, cysteine-rich, and carboxyl-terminal domains result in the severe DMD phenotype, while large in-frame deletions in the rod domain result in a mild BMD phenotype. Nevertheless, the latter is rarely related to elevated serum CK levels as the only manifestation of the disease.

Here, we describe the molecular diagnostic strategy used in a family with a sporadic case of BMD and three asymptomatic males who harbor a large deletion. The molecular results showed a "benign" mutation (44/**45–55**/56) inherited from the proband's grandfather. This deletion has been found in other populations, but it is always related to the BMD phenotype (http://www.dmd.nl). Only one asymptomatic case has been reported previously.[1]

Retrospective evaluation of individuals with idiopathic persistent elevated serum CK showed abnormal dystrophin staining in only 8% of the analyzed patients.[3] Thus, our patients are not only rare within the DMD group but also within the idiopathic hyperCKemia one.

The finding of asymptomatic subjects was fortuitous, suggesting that many subclinical individuals are probably not aware of carrying a very mild BMD. The number of ascertained patients could be the tip of the iceberg, and the current prevalence for BMD (1:18,000) could be underestimated.



FIGIRE 4. Pedigree and haplotype analysis for the complete family. The letters used for naming the alleles were arbitrarily chosen. Three asymptomatic males were detected (I1, III4 and III5-). del, deletion. (−) locus not analyzed.

The lack of genotype–phenotype correlation in DMD/BMD cases is a matter of intense study. Some investigators analyzed the contribution of genetic modifiers in the resulting phenotype. The activation of DMD gene transcription by a mutation in its promoter region or by a mutated transcriptional factor may be related to a change in the phenotype severity. A previous study reported a mutation in the MYF6 gene of a patient with a dystrophin in-frame deletion which resulted in a severe BMD phenotype.[6] Similarly, undetermined genetic modifiers may play a role in the phenotypic expression of the disease in our family. Other investigators speculate that overexpression of utrophin, an analog of dystrophin, turns a severe DMD into a mild BMD phenotype without affecting dystrophin expression.[2] This compensatory effect could explain the transformation of the expected BMD phenotype into an asymptomatic one in our patients. Unfortunately, the family's reluctance to consent to muscle biopsy did not allow us to measure their utrophin expression to confirm this hypothesis. Consequently, the molecular mechanism for the absence of symptoms in our patients remains unknown.

The finding of a "benign" deletion is extremely important in the antisense gene therapy field. Most mutations in the dystrophin gene either generate a stop codon in the mRNA or are out-of-frame mutations which translate into a truncated protein and lead to a severe DMD phenotype. Exon skipping during splicing, which normally occurs with a very low frequency, restores the reading frame of the gene leading to the production of a shorter yet functional protein, transforming the DMD into a milder BMD phenotype.[8,11] This strategy would help those patients with out-of-frame deletions in their dystrophin rod domain. For this reason, the finding of a large 11 exon-deletion (44/**45–55**/56), resulting in mild BMD or even an asymptomatic phenotype, is extremely important to design a strategy for restoring the reading frame of all patients with out-of-frame deletions within this region. A DMD patient with a deletion of exon 45, the most frequently deleted exon, or any other out-of-frame deletion that maps within exons 45–55, would be equally benefited by an antisense RNA gene therapy strategy for exons 45–55. Approximately 45% (76 out of 170) of the patients with out-of-frame deletions in the dystrophin gene diagnosed in our lab could be treated with this strategy. This finding supports the design of an optimal multiexon skipping target that encompasses exons 45–55 to transform DMD into BMD phenotype, as was theoretically predicted earlier.[1]

We thank all the members of the families for participating in this study and Dr. Ezequiel Surace for language supervision. We also thank Dr. Hugo Arroyo, Dr. María Soledad Monges, and Dr. Fabiana Lubieniecki for technical assistance. This work was supported by grants from CONICET PIP No. 05196, and was approved by the Bioethical Committee of the Clinical Hospital of Buenos Aires, Argentina. The authors have no conflicts of interest directly relevant to the content of this study.

REFERENCES

1. Béroud Ch, Tuffery-Giraud S, Matsuo M, Hamzolen D, Humbertclaude V, Monnier N, et al. Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. Hum Mutat 2007;28:196–202.
2. Blake DJ, Weir A, Newey SE, Davies KE. Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev 2002;82:291–329.
3. Dabby R, Sadeh M, Herman O, Berger E, Watemberg N, Fayek S, et al. Asymptomatic or minimally symptomatic hyperCKemia: histopathologic correlates. Isr Med Assoc J 2006;8:110–113.
4. Ferreiro V, Szijan I, Giliberto F. Detection of germline mosaicism in two Duchenne muscular dystrophy families using polimorphic dinucleotide $(CA)_n$ repeat loci within the dystrophin gene. Mol Diagn 2004;8:115–121.
5. Ferreiro V, Giliberto F, Francipane L, Szijan I. The role of polymorphic short tandem $(CA)_n$ repeat loci segregation analysis in the detection of Duchenne muscular dystrophy carriers and prenatal diagnosis. Mol Diagn 2005;9:67–80.
6. Kerst B, Mennerich D, Schuelke M, Stoltenburg-Didinger G, Von Moer A, Gossrau R, et al. Heterozygous myogenic factor 6 mutation associated with myopathy and severe course of Becker muscular dystrophy. Neuromuscul Disord 2000;10:572–577.
7. Koenig M, Beggs AH, Moyer M, Scherpf S, Heindrich K, Bettedem T, et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. Am J Hum Genet 1989;45:498–506.
8. McClorey G, Fletcher S, Wilton S. Splicing intervention for Duchenne muscular dystrophy. Curr Opin Pharmacol 2005;5:529–534.
9. Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988;2:90–95.
10. Murray MG, Thompson WF. Rapid isolation of high molecular-weight plant DNA. Nucleic Acid Res 1980;8:4321–4325.
11. Nowak KJ, Davies KE. Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. EMBO Rep 2004;5:872–876.
12. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 2002;30:e57.
13. Van Deutekom JC, van Ommen GJ. Advances in Duchenne muscular dystrophy gene therapy. Nat Rev Genet 2003;4:774–783.