Detection of Heterozygotes for Intragenic Deletions in Families with Recurrence of Duchenne or Becker Muscular Dystrophy

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Abstract

More than 60% of Duchenne/Becker muscular dystrophy (DMD/BMD) cases is due to deletions in the dystrophin gene, therefore the large majority of female carriers is heterozygote for an intragenic deletion. A new protocol is presented here for detection of these heterozygotes, based on multiplex semi-quantitative PCR amplification of genomic DNA. The method is non-radioactive, fast and easy to perform. The technique was successfully applied to a series of 60 females from DMD/BMD families, in which polymorphic DNA markers failed to define the carrier status.

Key words: DMD/BMD, deletions, carrier detection, semi-quantitative multiplex PCR.

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Duchenne muscular dystrophy (DMD), with an incidence of 1/3500 male births, is the most common X-linked lethal disease in man. Both DMD and its milder allelic form Becker muscular dystrophy (BMD) are caused by mutations of the dystrophin gene, that encodes for a 427 Kd cytoskeletal protein in muscle. With its 79 exons spanning over 2.5 megabases of genomic DNA, this is the largest human gene identified so far [13]. Dystrophin is virtually absent in the skeletal muscle of DMD patients, whereas it is present in reduced amount and/or size in BMD subjects. The two allelic forms correlate with the effect of the mutation on the reading frame of dystrophin cDNA: destroyed in DMD and preserved in BMD.

More than 60% of mutations of the dystrophin gene are deletions; duplications and point mutations account for approximately 10% and 30% respectively [4, 6]. As for any other genetic disease, carrier detection and prenatal diagnosis are the only procedures available to reduce the incidence of DMD/BMD. Given the peculiar mutation pattern found in the disease, the assessment of the carrier status in female relatives of DMD/BMD patients bearing an intragenic deletion is of paramount relevance. This kind of study can be done using polymorphic markers within the dystrophin gene, either directly, by testing markers located inside the deleted sequence for loss of heterozygosity, or indirectly, performing linkage analysis with markers lying outside the deleted region. However, these approaches are limited to the cases in which informative markers are present within the family; besides, the dystrophin gene exhibits an unusually high frequency of intragenic recombination [5], therefore affecting the accuracy of the linkage analysis.

An alternative approach to identify carrier individuals bearing an intragenic deletion can be based on semi-quantitative PCR. Protocols based on radioactive or fluorescent labeling of PCR products were previously reported in the literature [1, 2, 7, 10]. In both cases the procedures require the use of either cumbersome techniques or expensive pieces of equipment, hence hindering their usefulness for routine screening.

Here we report a novel procedure based on semi-quantitative PCR coupled with the silver staining method [12]. In our hands this approach turned out to be easy and fast to perform, with a high level of reliability and reproducibility.

Material and Methods

Characterization of patients

All the DMD and BMD patients were diagnosed at the Neurology Department University of Padua. At-risk female relatives were identified after the family tree reconstruction, as a part of the genetic counseling procedure.

DNA was extracted from peripheral blood lymphocytes by a standard salting out protocol [9] and its concentration measured by a Perkin-Elmer Lambda 15 spectrophotometer.

The screening for intragenic deletions in the affected males was performed by 3 sets of primers for multiplex Polymerase Chain Reaction (PCR) involving exons: 3, 6,

8, 45, 48, 50; 4, 12, 13, 17, 19, 60; 43, 44, 47, 49, 51, 52, according to methods described elsewhere [12].

Different DNA polymorphic markers, both inside and outside the deleted region, were analyzed in each family in order to characterize the X-chromosome inheritance. All the markers were amplified by PCR and separated in agarose or polyacrylamide gels, according to the original protocols [3, 9, 11]. Markers utilized in this study were: TG repeats [9], Pert 87.8 TaqI RFLP and Pert 87.15 XmnI RFLP [11], STRs 44, 45, 49 and 50 [3].

Semi-quantitative PCR

100 ng/l dilutions were prepared from the DNA stock solutions obtained as described above. Three µl from the diluted samples was loaded in 0.8% agarose gel, to verify the actual amount of DNA in the diluted samples. In few instances the appropriate adjustments were made to obtain similar DNA concentrations (see below).

Semi-quantitative PCR was performed in a total volume of 12 ul containing: 100 ng genomic DNA, 5 pmol of each primer (6 or 7 primer pairs), 1 mM of each dNTP, 1x buffer (TRIS-HC1 65 mM pH8, $(NH_4)_2SO_4$ 15 mM, 2 mercaptoethanol 10 mM, BSA 0.1 g/l), 1.5 mM $MgCl_2$, 0.7 units of RTB DNA polymerase (AMED, Italy). The PCR reaction was performed in a thermal cycler MJ Res. (USA). The cycling parameters were: 94°C as initial denaturation step for 3 min.; 94°C as denaturation 30 sec., 65°C as annealing and extension for 4 min. (17 cycles); 65°C as final extension for 6 min. In some instances, reducing the number of cycles to 16 improved the detection of the difference between non-deleted and deleted exons.

The total amount of the amplification products was mixed with 6 µl non-denaturing loading buffer (Ficoll 15%, xylene cyanol 0.25%, bromophenol blue 0.25%, 0.1 M EDTA pH8) and run in 8% polyacrylamide gel (29:1 acrylamide:bis-acrylamide) containing 5% glycerol. The electrophoresis was performed at room temperature for 3 hours at 280 volts. The visualization of the products was obtained after silver staining. The gels were dried in a Biorad Gel Dryer (mod. 583) at 65°C for 40 min.

In order to ensure that the amount of template DNA would not affect the ratio between the different bands in the same lane, we performed a template titration with DNA amounts ranging from 50 to 200 ng in a multiplex amplification. In all the samples, the ratio between the bands was maintained (data not shown).

All the samples were analyzed in at least three independent experiments.

Dried gels were scanned in reflection mode with a Microtek E6 scanner using a 400 dpi resolution in 256 gray levels. The resulting images were analyzed using the NIH Image software (developed at the US National Institute of Health and available as freeware at http://rsb.info.nih.gov/nih-image) to measure the relative intensity of the bands. Results were interpreted by comparing in each sample the ratio between non-deleted and deleted exons. In carrier females this value was consistently higher than in controls.

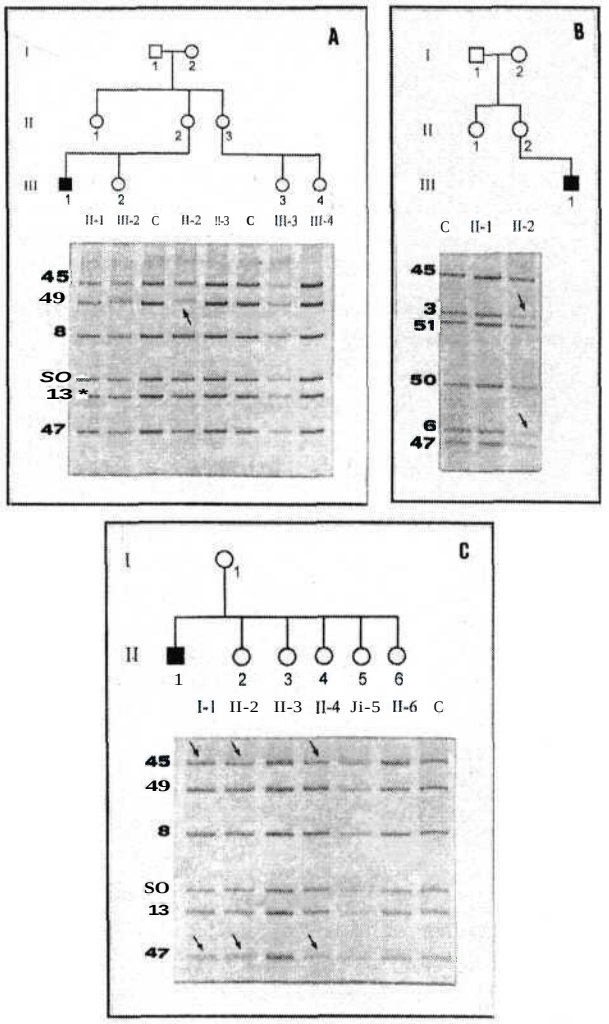

Figure 1. Family trees and gel electrophoresis offamilies no. 1 (A), no. 2 (B), no. 3 (C). Exons are listed on the left side: the bands with decreased intensity, indicating heterozygosity for the deletion, are marked with arrows. Females from non DMD/BMDfamilies were used as controls, "C".

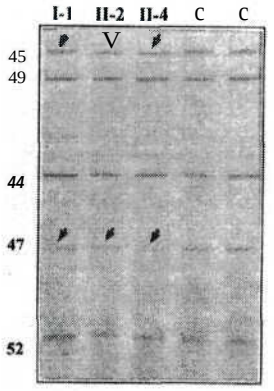

Figure 2. Control gelforfamily no. 3. Numbers and arrows are as in figure 1.

Tables I, 2, 3 and 4. Area values are the numeric output of the Gel Plotting Macro from NIH Image software. Area Ratio = Exon ND/Exon Del. The individuals who resulted to be carrier of the deletion are indicated with an asterisk.

Sample	Exon ND	Exon Del	Area ND	Area Del	Area Ratio	Average	2x St Dev
II-1	8	49	2379	2970	0.80		
III-2	8	49	1942	2861	0.68	0.80	0.02
II-3	8	49	4606	5006	0.92		
Control	8	49	3958	4639	0.85		
Control	8	49	4064	4887	0.83	0.84	0.02
II-2 *	8	49	2794	2282	1.22	1.22	—

Sample	Exon ND	Exon Del	Area ND	Area Del	Area Ratio
II-2 *	47	6	4192	3584	1.17
II-1	47	6	10439	12168	0.86
Control	47	6	5674	6348	0.89
II-2 *	51	3	5255	2540	2.07
II-1	51	3	9511	7680	1.24
Control	51	3	6541	5145	1.27

Sample	Exon ND	Exon Del	Area ND	Area Del	Area Ratio	Average	2x St Dev
I-1 *	49	45	3077	1997	1.54		
II-2 *	49	45	2754	2290	1.20	1.38	0.34
II-4 *	49	45	3051	2201	1.39		
II-3	49	45	3753	4455	0.84		
II-5	49	45	1672	1842	0.91	0.91	0.14
II-6	49	45	2835	2875	0.99		
C	49	45	2237	2336	0.96	0.96	—
I-1 *	13	47	2559	1375	1.86		
II-2 *	13	47	2188	1795	1.22	1.59	0.66
II-4 *	13	47	2923	1733	1.69		
II-3	13	47	3602	2855	1.26		
II-5	13	47	1311	1395	0.94	1.09	0.33
II-6	13	47	2259	2140	1.06		
C	13	47	2245	1813	1.24	1.24	—

Sample	Exon ND	Exon Del	Area ND	Area Del	Area Ratio	Average	2x St Dev
I-1	49	45	1516	965	1.57		
II-2	49	45	1185	870	1.36	1.44	0.24
II-4	49	45	1268	921	1.38		
C	49	45	1665	1734	0.96		
C	49	45	1916	2178	0.88	0.92	0.12
I-1	44	47	1099	744	1.48		
II-2	44	47	984	578	1.70	1.68	0.40
II-4	44	47	1247	668	1.87		
C	44	47	1287	1145	1.12		
C	44	47	1523	1482	1.03	1.08	0.14

Results

In this work we used multiplex PCR to assess the carrier status of females from DMD/BMD families bearing a known deletion by comparing the relative amounts of amplified products from non-deleted and potentially deleted exons.

The experimental conditions were first tested on 16 carriers of known deletions and on a series of 30 normal subjects, to exclude the possibility of false positives. All the deletions were clearly identified and no false positives were found.

The method was then applied to 60 cases in which the heterozygosity for an intragenic deletion was suspected. In 20 cases the carrier status was proved, in 38 it was clearly excluded, whereas in last two (3.2%) the result was still unclear. In these two instances it was not possible to obtain reproducible multiplex amplifications, presumably due to the poor quality of the template DNA.

Figure 1 shows three families in which we succeeded in assessing the carrier status by the new protocol.

In family no. 1 the patient was diagnosed as BMD. The family history of the disease was negative. A deletion of exons 48-49 of the dystrophin gene was detected in the patient's DNA. Analysis by STRs 44, 45 and 49 polymorphic markers showed the inheritance of the grand-father's X-chromosome in the affected male and in the females II-1, II-2, II-3 and III-3 (Figure 1 A). On the contrary, females III-2 and III-4 resulted to carry a different X-chromosome. It was impossible to exclude that a new mutation occurred in the grand-father's spermatogenesis and passed to the male patient through his mother. However, if germinal mosaicism occurred, also the mother's sisters (II-1, II-3) could have received the mutated chromosome. On the contrary, the analysis demonstrated that the deletion was present only in the mother of the affected child.

In family no. 2 the patient was a sporadic case diagnosed as DMD. A deletion of exons 3-6 was identified. Analysis by the polymorphic markers TG repeat, Pert 87.8 TaqI RFLP and Pert 87.15 XmnI RFLP did not produce informative results. The present analysis unequivocally demonstrated that only the mother of the affected child carried the deletion (Figure 1B).

In family no. 3 the deletion screening identified a deletion of exons 45-48 in the BMD patient. The mother was an obligate carrier, because of the positive family history, but she was uninformative for the polymorphic markers STRs 44, 49 and 50. The analysis by our method showed that, among her five daughters, two (II-2 and II-4) were also heterozygotes for the deletion (Figure 1C).

In order to confirm the visual analysis of the gels, we quantified by densitometry the amount of amplified product from non-deleted and potentially deleted exons within each lane. The intensity of each band was represented as the area of the corresponding peak in the densitometry plot. For each individual, we calculated the ratio between the intensity of non-deleted and deleted exons; this value was consistently higher in the individuals scored as hemizygotes for the deletions (Tables 1, 2, 3). In only one instance -exon 47, subject II-2, family no. 3- the result of the densitometric analysis was not resolutive, despite the fact that exon 45 was clearly deleted. We therefore repeated the amplification of subjects I-1, II-2 and II-4 plus two controls, using 16 cycles with exons 44, 45, 47, 49 and 52. Figure 2 and Table 4 report the results of the densitometric analysis, that confirmed the hemizygosity of exons 45 and 47 in all the three subjects.

Discussion

The assessment of the carrier status remains one of the most crucial and difficult task in the analysis of DMD/BMD families in which an intragenic deletion is transmitted, because of the masking effect of the healthy X-chromosome. The use of polymorphic markers along the dystrophin gene and, most importantly, in the deletion hot-spot region marked a step forward in the direct and indirect detection of deletion heterozygotes. However, polymorphic markers are not always informative. Moreover, the existence of intragenic recombinations should always be taken into account.

The method presented here allows a quick and direct carrier assessment in families with known deletions in the dystrophin gene. Densitometric analysis confirmed that the simple visual evaluation of the gels is a reliable way to identify carrier females. As opposed to similar methods previously published in the literature [1, 2, 5, 8], the protocol reported here does not require the handling of radioactive materials or the use of specialized equipment to detect fluorescently labeled PCR products. We therefore think that our method is more suitable for routine analysis. In addition, this technique could find a more general application in all the situations in which detection of heterozygosity for a given deletion or quantification of genomic copy number is required.

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