A Larger Spectrum of Intragenic Short Tandem Repeats Improves Linkage Analysis and Localization of Intragenic Recombination Detection in the Dystrophin Gene

An Analysis of 93 Families from Southern Italy

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Duchenne/Becker muscular dystrophies (D/BMD) are X-linked recessive disorders resulting from dystrophin gene mutations. Intragenic recombination in the dystrophin gene occurs with a high frequency. Therefore, determination of the location and frequency of recombination improves D/BMD carrier detection and prenatal diagnosis in families in which the disease-causing mutation cannot be detected by most conventional methods. We describe herein a linkage analysis performed using a fast method based on capillary gel electrophoresis of fluorescent-labeled amplified alleles of 15 intragenic short tandem repeats spanning the entire dystrophin gene. On characterization of recombination events in 93 unrelated D/BMD families from southern Italy, we mapped 25 intragenic recombinations out of 273 informative meioses analyzed. The terminal regions of a gene are notoriously challenging for linkage analysis because some recombination events could be missed in case of lack of informativeness of the outermost markers. Many recombination events (10/25) identified in this study were located at the terminal regions of the dystrophin gene, and some were found by typing of several informative short tandem repeats located in these regions. Moreover, about 24% of the recombination events found in this study mapped to the 3' region of the gene, in contrast with the low frequency (4 to 15%) reported by others. (*J Mol Diagn* 2007, 9:64–69; DOI: 10.2353/jmoldx.2007.060056)

Duchenne/Becker muscular dystrophies (D/BMD) are X-linked recessive genetic disorders resulting from mutations in the dystrophin gene located at the Xp21 region. The locus is very unstable: one-third of all D/BMD cases are due to new mutations, which occur in patients without a family history of the disease.¹ Intragenic deletions and

duplications together account for over two-thirds of the mutations that cause D/BMD; the remaining cases are due to point mutations or small insertions/deletions scattered along the entire gene (Leiden Muscular Dystrophy, <http://www.dmd.nl>, accessed January 2006). Several methods have been devised to identify deletions, duplications, and point mutations in affected males and in female carriers.^{2–7} Linkage analysis, based on short tandem repeats (STRs) at polymorphic loci in the dystrophin gene, is widely used for carrier detection and in the prenatal diagnosis of D/BMD families in which the causative mutations cannot be or were not determined in the proband.^{8–11} In recent years, many novel STRs have been identified and mapped in the dystrophin gene (Leiden Muscular Dystrophy, <http://www.dmd.nl>, accessed January 2006). However, intragenic recombination in the dystrophin gene has been shown to occur with a frequency of 10 to 12%,^{12–14} which is about four times that expected on the basis of the length of the gene. This high recombination rate is not restricted to families segregating for D/BMD but occurs also in healthy families.^{12,13} Therefore, determination of the location and frequency of recombination improves D/BMD carrier detection and prenatal diagnosis in families in which the disease-causing mutation cannot be detected by most conventional methods.

Here, we report a linkage analysis and the characterization of recombination events in 93 unrelated D/BMD families from southern Italy. The analysis is based on a fast method based on the separation by capillary gel electrophoresis of the fluorescent-labeled amplified alleles of 15 intragenic STRs that span the entire dystrophin gene.

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Figure 1. Electropherogram of the 15 intragenic STRs labeled with NED (channel 1), PET (channel 2), VIC (channel 3), or FAM (channel 4) fluorochromes.

Materials and Methods

The D/BMD families studied in this article were two- or three-generation families that were previously characterized for the presence of exon deletions or duplications.^{5,15} Deletions were detected in 62 families (67% of the families). Informed consent was obtained for each patient/family according to the procedure established by the local Institutional Bioethics Committee.

Genomic DNA was extracted from peripheral blood samples with the Nucleon procedure (Amersham, Buckinghamshire, UK). Five fluorescent-labeled multiplex PCR reactions were designed to amplify 15 dystrophin intragenic STRs (ie, DXS1242, 5'-5n3, IVS44SK21, in multiplex A; DXS997, DXS1214, DXS992 in multiplex B; 5'-5n4, DXS1238, DXS1235 in multiplex C; DXS1237 and DXS1236 in multiplex D; DXS1243, STR07A, 5'-7n4, and DXS1241 in multiplex E) using previously reported primer oligonucleotides (Leiden Muscular Dystrophy, <http://www.dmd.nl>, accessed January 2006). The forward primers were labeled with 5-carboxyfluorescein (FAM), PET, NED, or VIC fluorochromes (Figure 1). Multiplex polymerase chain reaction (PCR) mixtures (25 μ l) contained 200 ng of genomic DNA, 0.25 mmol/L deoxynucleoside triphosphate mixture, 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1 nM/gelatin, 2.5 mmol/L MgCl₂ (2 mmol/L MgCl₂ in multiplex reaction B), 30 nM/gelatin dimethyl sulfoxide (in multiplex reactions B, C, and E), and 0.5 U of *Taq* polymerase. Amplification reactions were performed using a touch-down protocol (denaturation at 95°C for 3 minutes; 39 cycles with denaturation at 95°C for 20 seconds, annealing at 62°C for 40 seconds, -0.5°C

per cycle for 14 cycles and at 55°C for 40 seconds for 25 cycles, and polymerization at 72°C for 45 seconds; final extension: 72°C for 7 minutes) and a thermal cycler PCR System 9600 (Applied Biosystems, Foster City, CA). These PCR conditions were designed to provide a robust amplification of the 15 STRs under the same thermal profile. PCR products (0.5 μ l from each multiplex reaction) were mixed with 0.5 μ l of GeneScan-500 LIZ size standard (Applied Biosystems) and were separated by capillary gel electrophoresis (15 kV at 60°C for 30 minutes) on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) using the POP-4 polymer. We used the Genotyper 3.7 (Applied Biosystems) software for data analysis and created a macro that allowed us to label the peaks and identify the alleles of each marker automatically.

Results

A typical electropherogram of the 15 STRs is shown in Figure 1. The automated analysis is highly reproducible: the interindividual variability, calculated as the experimental CV for each allele of each STR, was between 0.6 and 5.5% ($n \geq 15$), and the between-run variability ranged between $3 \times 10^{-3}\%$ and $4 \times 10^{-3}\%$ ($n = 5$). The method we used for linkage analysis in D/BMD families is fast (all of the multiplex PCRs are amplified in ~2.5 hours under the same thermal conditions, and the capillary gel electrophoretic run takes ~30 minutes), accurate, and easy to perform. For several STRs, we identified a higher number of alleles (Table 1) than reported earlier (Leiden Muscular Dystrophy, <http://www.dmd.nl>, accessed Janu-

Table 1. Heterozygosity (HT) Values and Allele Numbers of the 15 Intragenic STRs in the Dystrophin Gene

Markers (STRs)	HT found in this study	HT reported by others	Allele number identified in this study	Allele number reported by others*
DXS1242	0.79	0.63*	15	8
DXS1243	0.43	0.57*	7	10
5'-5n3	0.80	0.76 ^{16,17}	13	9
5'-5n4	0.81	Not reported (PIC 0.64) ¹⁷	13	8
STR07A	0.62	0.68*	8	12
5'-7n4	0.54	Not reported (PIC 0.52) ¹⁷	5	4
DXS1238	0.84	0.85*	15	12
IVS44SK21	0.80	0.84 ¹⁸	12	10
DXS1237	0.84	0.87*	15	13
DXS997	0.75	0.65*	7	4
DXS1236	0.83	0.91*	19	19
DXS1235	0.70	0.70*	14	6
DXS1241	0.40	0.51*	6	5
DXS1214	0.83	0.79*	10	7
DXS992	0.71	0.87*	9	11

*Leiden Muscular Dystrophy page (<http://www.dmd.nl>).
PIC, polymorphism information content.

ary 2006). However, the observed heterozygosity values did not substantially differ from those previously reported (Leiden Muscular Dystrophy, <http://www.dmd.nl>, accessed January 2006).¹⁶⁻¹⁸

Twelve STRs had high heterozygosity values (0.6 to 0.83) (Table 1). We included in the panel the DXS1243, 5'-7n4, and DXS1241 markers, which have heterozygosity values of about 0.4 to 0.5 (Table 1), so that the STR loci

investigated were evenly spread along the dystrophin gene (Figure 2 A). All of the subjects were typed with the 15 STRs. We were thus able to locate some recombination events more accurately: the DXS1243 STR marks the start of recombination in family FD# 73 and the 5'-7n4 STR marks the end or the start of recombination in family FD# 228 and in families FD# 134 and FD# 40, respectively (Figure 2).

Figure 2. A: Simplified map of the dystrophin gene showing the location of the 15 STRs (below) and of exons (above). **B:** Intragenic recombination events detected in this study and exon deletions present in some families¹⁵; the black bars indicate the intervals where recombination events occurred; the red bars indicate the deletion extent and position. The family identification numbers are indicated.

Table 2. Informative Meioses and Recombination Events between the Intragenic STRs in the Dystrophin Gene

Marker (STRs) pairs	Informative meioses for STR pairs (n)	Recombination events (n)	Distance (kb)
DXS1242/DXS1243	81	0	144
DXS1242/5'-5n3	191	2	223
DXS1242/5'-5n4	191	1	508
DXS1243/5'-5n3	105	1	78
5'-5n3/5'-5n4	197	2	285
5'-5n3/DXS1238	209	1	912
5'-5n4/STR07A	167	1	44
5'-5n4/IVS44SK21	182	1	675
STR07A/5'-7n4	142	1	331
STR07A/DXS1238	175	2	582
5'-7n4/DXS1238	165	2	251
DXS1238/IVS44SK21	198	1	49
IVS44SK21/DXS1237	200	3	188
DXS1237/DXS997	171	0	105
DXS997/DXS1236	200	1	39
DXS1236/DXS1235	193	0	43
DXS1235/DXS1241	87	0	302
DXS1235/DXS1214	178	1	537
DXS1241/DXS1214	95	1	235
DXS1214/DXS992	174	4	321

See also Figure 2.

The unequivocal haplotype reconstruction in 93 D/BMD families allowed us to map 25 intragenic recombinations (Figure 2B) out of 273 informative meioses analyzed. Thus, the recombination frequency was about 9%. Table 2 lists the recombination events found in our population, the informative meioses analyzed for each STR pair and the distances between the marker pairs. All recombinants were checked by retyping individuals. No double recombinants were identified, whereas in one family (FD# 73), we found two different recombinants in one generation, and in another family (FD# 241), we found two different recombinants in two generations. A deletion was detected in 13 of the 23 recombinant families. In three deleted families, one relative has a recombination event mapped in a region corresponding to one of the deletion breakpoints segregating in the pedigree (FD# 89: deletion of exons 3 to 7, recombination STR07A/DXS1238; FD# 145: deletion of exons 45 to 55, recombination IVS44SK21/DXS1237; FD# 245: deletion of exons 45 to 47, recombination IVS44SK21/DXS1237) (Figure 2A). In the remaining 10 deleted families, there was no correlation between the deletion site and the recombination site (Figure 2A). Nineteen of the 25 recombinations (76%) mapped in the 1.492-mb region between the STRs DXS1242 (located 1.2 kb from the 5' of the exon 1 B) and DXS1236 (at intron 49), six (24%) in the region of at least 1 mb between the STRs DXS1235 (at intron 50) and DXS992 (>20 kb from the 3' region of exon 79) (Figure 2). Figure 3 shows the haplotypes of two pedigrees in which no deletions or duplications segregated; the use of several markers located at the 5' and 3' regions of the gene allowed us to identify recombination events also in case of lack of informativeness of some STRs at

the terminal regions of the gene. In pedigree FD# 73, the DXS1242 marker is not informative, but two independent recombinations were mapped (Figure 3A) subsequent to segregation of the DXS1243, 5'-5n3 and 5'-5n4 marker alleles, which are not included in the STR panels reported by other groups.^{11,19,20} A similar picture is depicted for the recombination event mapped at the 3' region of the gene between the DXS1214 and DXS992 markers in pedigree FD# 8 (Figure 3B). Although subject II:2 carried the maternal not-at-risk haplotype from the DXS1242 to the DXS1214 markers, the identification of the recombination between DXS1214 and DXS992 precluded a conclusive diagnosis about the carrier/noncarrier status of subject II:2 because this region could contain a mutation.

Discussion

The estimate of the recombination frequency in a population and precise mapping of the recombination events are critical for genetic counseling and prenatal diagnosis. Given the high intragenic recombination frequency of the dystrophin gene, the analysis of a large STR panel is crucial for a faithful characterization of the gene segregation. This analysis can be easily performed thanks to an automated, fast, and accurate method based on the separation by capillary gel electrophoresis of the fluorescent-labeled amplified alleles of STRs that span the entire dystrophin gene. The terminal regions of a gene are notoriously challenging for linkage analysis because some recombination events could be missed in case of lack of informativeness of the outermost markers. We identified many recombination events by typing several informative markers located at these regions of the dystrophin gene. In fact, the use of several STRs in the 5' region of the gene (from DXS1242 to DMDSTR07A) allowed us to identify six recombination events that could be missed in case of lack of informativeness of the DXS1242 marker using smaller STR panels, which include only few markers, as reported by others.^{11,19,20} Furthermore, the use of several STRs with high heterozygosity in the 3' region (from intron 50) of the dystrophin gene enabled us to identify about 24% of all the recombinations found in our population. In contrast, a low number of recombinants (4 to 15% of total recombinants) was identified in this region in other populations,^{13,14} probably because of the typing of a smaller STR panel.

In conclusion, by detecting and mapping recombination events along the entire dystrophin gene, STR haplotyping with 15 intragenic markers could minimize diagnostic errors in linkage analysis of D/BMD families. Moreover, an accurate linkage analysis using several heterozygous STRs spanning the whole dystrophin gene could also help to detect hidden germline mosaicisms,²¹ thereby improving genetic counseling.

Figure 3. Pedigree and haplotype analysis of two D/BMD families showing recombination events located at the 5' (A) or at the 3' (B) region of the dystrophin gene. The thin black bar in FD# 73 (II:1 subject) indicates the region that cannot unambiguously be attributed to one haplotype. The **arrows** indicate the proband of each family.

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