[Frontiers in Bioscience E2, 547-558, January 1, 2010]

A 15-year molecular analysis of DMD/BMD: genetic features in a large cohort

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1. ABSTRACT

Duchenne (DMD) and Becker muscular dystrophies (BMD) are X-linked recessive neuromuscular disorders caused by mutations in the dystrophin gene. In most cohorts, DMD/BMD are due to deletions (60-80%) and duplications (6-10%) involving one or more exons. The remaining cases are caused by different type of point mutations. We analyzed 179 unrelated male patients, 296 women belonging to 137 DMD/BMD families, and 93 independent patients referred for hyperCKemia. We identified 121 deletions and 11 duplications involving one or more exons and one complex rearrangement in the DMD/BMD patients, and 9 deletions in males referred for high levels of serum CK. Carrier status was investigated in 219 female relatives of deleted or duplicated DMD/BMD males, and by linkage analysis in 77 women belonging to families in which the causative mutation was not identified. Four carrier women with clinical manifestations of the disease had unbalanced X inactivation with a degree of X skewing between 70% and 93%. Large cohort studies from different geographic areas may be important for mutation typology comparisons and their appropriate analytical approach.

2. INTRODUCTION

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked recessive neuromuscular disorders caused by mutations in the dystrophin gene. The incidence of DMD and BMD is approximately 1 in 3,500 and 1 in 20,000–30,000 live male births, respectively (1).

Patients affected by DMD have progressive muscular weakness and degeneration of skeletal muscle. The disease is usually diagnosed in affected boys when they are 3–5 years old. In this period, they have difficulty in running and in climbing stairs. At the age of about 11 years, patients are unable to walk and are wheelchair-bound. Shortly thereafter, symptoms become more severe culminating in respiratory and cardiac failure and death at a median age of 20 years. Patients affected by BMD have a broad spectrum of clinical symptoms ranging from mild to severe. With respect to DMD, BMD has a later onset of skeletal muscle weakness and a slower progression. Difficulties in ambulation become evident at the age of 20 years. Dilated cardiomyopathy is common and is the most

frequent cause of death around 40 years. In patients with mild forms of BMD, symptoms appear at the age of 30 and at age 60 patients may be still able to walk (2). Although DMD/BMD are X-linked recessive disorders, carrier females may have clinical symptoms of the disease. This is due to X-chromosome rearrangements involving the dystrophin *locus*, such as balanced X:autosome translocations, or to a complete or partial absence of an X chromosome, or to unbalanced X inactivation (3).

Before the advent of molecular biology tests, dystrophinopathies were diagnosed by measuring serum creatine phosphokinase (CK), skeletal muscle biopsy and electromyography. Serum CK concentrations are more than 10 fold the upper reference limit in DMD and more than 5 fold in BMD patients. The diagnostic sensitivity of the test is 100% in young men and it gradually decreases with advancing age due to the progressive disruption of dystrophic muscle fiber. Elevated CK serum levels are still an indicator for a diagnostic hypothesis of DMD/BMD. Female carriers have serum CK concentrations in the range of 2-10 fold the upper reference limit (1, 4). Skeletal muscle biopsy shows altered fiber size, foci of necrosis and regeneration, hyalinization and deposition of fat and connective tissue only in an advanced stage of the disease. Electromyography is characterized by a short duration and low amplitude, polyphasic rapidly recruited motor unit potentials; however, these findings are not specific and occur in all myogenic disorders (1, 4). On the contrary, the immunohistochemical analysis of muscle biopsies with specific antidystrophin antibodies clearly distinguishes among normal, DMD and BMD samples. There is a positive immunohistochemical signal in normal subjects, absence of signal in DMD patients, and sporadic or interrupted staining in patients affected by BMD and in manifesting female carriers (1).

The dystrophin gene is located at the Xp21 *locus* and, spanning 2.4 Mb, it is one of the largest genes of the human genome. 99.3% of the gene is constituted by introns, whereas the remainder consists of 79 exons that produce a transcript of about 14 kb expressed in skeletal and cardiac muscle, and at low level in the brain (5-9). The gene has 7 distinct promoters linked to unique first exons that give rise to various isoforms of the protein. Three full-length isoforms, localized in brain, in muscle and in Purkinje cerebellar neurons, have the same number of exons but are derived from different promoters (5). Additional isoforms are generated by alternative splicing events, i.e., exclusion of some exons from the primary transcript and/or subversion of the reciprocal order of exons (10, 11).

Mutations not maintaining an open-reading frame (ORF) in the spliced mRNA because of frameshifted triplet codons result in a truncated, nonfunctional dystrophin protein that usually gives rise to the DMD phenotype. Alternatively, mutations not affecting the ORF and predicting the production of a semifunctional protein usually give rise to the less severe BMD phenotype (12). This reading-frame hypothesis, which arises from analysis of genomic DNA, applies to about 90% of cases (13), and recent results obtained in dystrophin cDNA suggest that splicing patterns that convert out-of-frame into in-frame mutations are more frequent than expected (14). The dystrophin *locus* is very unstable. One-third of all DMD/BMD cases are due to new mutations, which occur in patients without a family history of the disease (1).

DMD/BMD are caused by deletions and duplications involving one or more exons or by point mutations (including small insertions or deletions, splicing mutations). About 60-80% of DMD/BMD cases are due to deletions (1, 4, 15, 16), preferentially clustered in two regions of the dystrophin protein: the amino-terminus (exons 2–20, which encode amino acids 12–874) and the central region (exons 45–53, which encode amino acids 2146–2624) (1). About 98% of deletions involving the most frequently deleted exons can be detected in males using multiplex PCR analysis (17-19) or fluorescent *in situ* hybridization (FISH) (20-23). About 6-10% of DMD/BMD cases are due to duplications that can be detected using quantitative PCR analyses (24-28). The latter methods also reveal deletions and duplications in female carriers. Various new techniques have been developed to detect deletions/duplications and are able to screen many target sequences simultaneously: multiple amplifiable probe hybridization (MAPH) (29), multiple ligation-dependent probe amplification (MLPA) (14, 30, 31) and array-comparative genomic hybridization (array-CGH) (32). The remaining DMD/BMD cases are due to point mutations scattered along the entire gene, and pure intronic deletions or exonic insertion of repetitive sequences (5, 13, 33). Testing methods for small mutations are: single condition amplification internal primer sequencing (SCAIP), denaturing gradient gel electrophoresis (DGGE), denaturing high performance liquid chromatography (DHPLC) (13), and detection of virtually all mutations-SSCP (DOVAM-S) (34).

Linkage analysis, based on haplotyping of polymorphic short tandem repeats (STRs) within the dystrophin gene, is used for carrier detection and in the prenatal diagnosis of DMD/BMD families in which the causative mutations cannot be or were not determined in the proband (35-44). However, intragenic recombination in the dystrophin gene occurs with a frequency of 9% to 12%, which is three-four times that expected on the basis of the length of the gene (35, 39, 40, 43). Therefore, determination of the location and frequency of the recombination events improves DMD/BMD carrier detection and prenatal diagnosis in families in which the disease-causing mutation cannot be detected by most conventionally used methods.

Here, we report the outcome of a 15-year study on molecular diagnosis in DMD/BMD-affected males and in female DMD/BMD carriers of a large cohort belonging to independent families with at least one affected male.

3. MATERIALS AND METHODS

3.1. Patients

179 unrelated male patients were diagnosed and classified (either DMD or BMD) from clinical status assessed according to Hoffman (45), and/or from

Table 1. Deletions in the dystrophin gene identified by QF-PCR or MLPA

Deleted regions involving exons	Patients (n)	QF-PCR	MLPA
MP1[1]- PP1[2]	1	yes[3]	
MP1[1]	1	yes[3]	
3-4	1	yes	
3-7	5	yes[3]	
3-13	1	yes[3]	
5-45	1	yes	
6-19	1	yes[3]	
8-16	1	yes[3]	
8-43	1	yes[3]	
10-29	1	yes[3]	
12-13	2	yes[3]	
12-29	1	yes[3]	
12-48	1	yes[3]	
13-44	1	yes	
18-27	1	yes[3]	
19-21	1	yes[3]	
19-50	1	yes[3]	
27	1	no	yes
28-49	1	yes[3]	
30-44	1	yes[3]	
30-51	2	yes[3]	
35-44	1	yes[3]	
41-44	1	yes[3]	
42-43	1	yes	
44	4	yes	
44-47	1	yes	
45	3	yes	
45-46	3	yes	
45-47	12	yes	
45-48	10	yes	
45-49	4	yes	
45-50	2	yes	
45-51	5	yes	
45-52	3	yes	
45-53	1	yes[3]	
45-55	7	yes[3]	
45-63	1	yes[3]	
46-48	3	yes	
46-47	1	yes	
46-49	2	yes	
46-50	1	yes	
46-51	1	yes	
46-55	1	yes[3]	
48	4	yes	
48-49	3	yes	
48-50	7	yes	
48-51	2	yes	
48-53	1	yes[3]	
48-54	1	yes[3]	
49-51	1	yes	
49-52	2	yes	
50	1	yes	
50-51	1	yes	
50-52	2	yes	
51	3	yes	
51-53	1	yes[3]	
52	1	yes	
52-54	1	yes[3]	
53	1	yes[3]	
53-54	1	yes[3]	
55	1	no	yes
56-63	1	no	yes

QF-PCR: quantitative fluorescent multiplex PCR; MLPA: multiple ligation-dependent probe amplification; [1]MP1, muscle promoter exon 1; [2]PP1, Purkinje promoter exon 1; [3]additional PCRs or MLPA were used to define the length of the exonic deletions.

dystrophin immunohistochemistry on muscle biopsy. Ninety-three patients were referred for a high level (>5 fold the upper reference limit) of serum CK, and 296 women belong to 137 families with history of the disease. Four women were referred for clinical manifestations of the disease and positive immunohistochemical analysis with antidystrophin antibodies. Informed consent was obtained for each patient/family according to the procedure established by the local Institutional Bioethics Committee.

3.2. Methods

Genomic DNA was extracted from whole blood, or from amniocytes or chorionic villi for prenatal diagnosis. From 1993 to 2003, male patients were screened for deletions using four multiplex PCRs that amplify 24 exons (17), and carrier status in female subjects was assessed by quantitative PCR carried out as previously described (46). Since 2004, we have used a quantitative fluorescence multiplex-PCR (QF-multiplex PCR) to identify deletions and duplications in male patients and in female carriers as reported elsewhere (24) with minor modifications. Twenty-four dystrophin gene exons were amplified in four multiplex PCR reactions (exons 4, 8, 12, 17, 19, 44, and 45 in multiplex A; muscle promoter and exons 13, 43, 49, 50, and 52 in multiplex B; exons 41, 42, 46–48, 51, and 53 in multiplex C; and exons 2, 3, 5, and 6 in multiplex D). The forward primers were labeled with the fluorochromes VIC, FAM, NED, or PET. Exon 10 of the pyruvate kinase gene (labeled with VIC, FAM, NED or PET in reactions A, B, C and D, respectively) was co-amplified as an internal standard reference for the double-copy gene in each multiplex PCR. PCR products were mixed and separated by a single capillary gel electrophoresis run on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). The length of exonic deletions was defined directly with these multiplex PCR in about 68% of cases, whereas additional PCRs were used in the remaining patients. For patients resulting negative at the above-mentioned tests, we carried out the MLPA analysis using the SALSA P034/P035 DMD kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's recommendations. All single exon deletions detected by QF-multiplex PCR or by MLPA were confirmed by a PCR with different primer pairs.

Haplotype analysis was carried out using the method based on capillary gel electrophoresis of fluorescent-labeled amplified alleles of 15 intragenic STRs spanning the entire dystrophin gene (35).

To analyze X-inactivation in manifesting carriers, we used the PCR-based method for methylation-dependent amplification of the polymorphic triplet repeats at the androgen receptor (HUMARA) *locus* (47, 48) with minor modifications. The HUMARA-1 primer was labeled with the fluorochrome FAM and the amplification products of HpaII-digested and not digested DNA were separated by capillary gel electrophoresis on the ABI Prism 310 Genetic Analyzer.

RESULTS AND DISCUSSION

4.1. Deletions and duplications in male patients

The QF-multiplex PCR of the hot spot exons of the dystrophin gene (24) identified 118 deleted and 7 duplicated DMD/BMD patients (Tables 1 and 2) out of 179 analyzed, and 9 deleted men out of the 93 referred for high levels of serum CK (>5 fold the upper reference limit). One of the deletions identified spans the muscle and the Purkinje promoters, and correlates with a severe muscle phenotype without cardiomyopathy (49). Moreover, the noncontiguous duplication of exons 44-48 and 51-53 was

Table 2. Duplications in the dystrophin gene identified by QF-PCR and/or MLPA

Duplicated regions involving exons	Patients (n)	QF-PCR	MLPA
2	1	yes	
3-7	1	yes[1]	
5-21	1	yes[1]	
10-11	1	no	yes
12-16	1	yes[1]	
13-29	1	yes[1]	
21	1	no	yes
21-29	1	no	yes
45-56	1	yes[1]	
51-62	1	yes[1]	
57-65	1	no	yes
noncontigous: 44-48; 51-59; 64-79	1	yes[1]	

QF-PCR: quantitative fluorescent multiplex PCR; MLPA: multiple ligation-dependent probe amplification; [1] additional PCRs or MLPA were used to define the length of the exonic duplications.

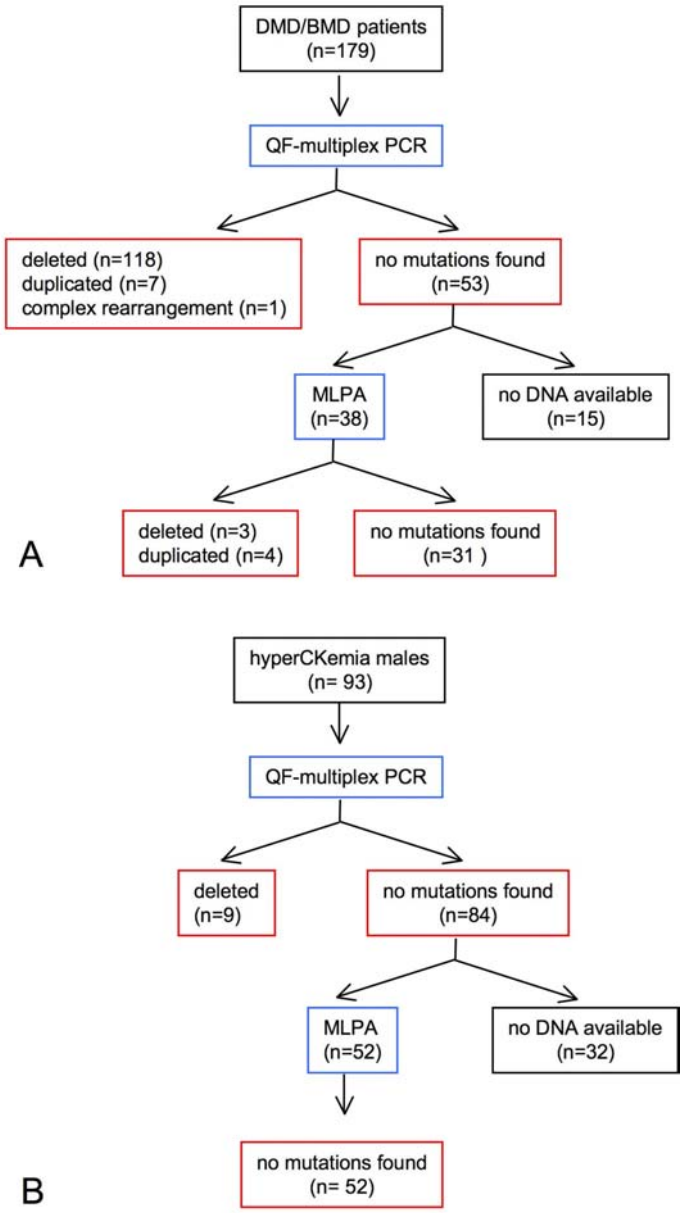

Figure 1. Deletion and duplication screening by QF-multiplex PCR and MLPA in DMD/BMD patients (panel A) and in men referred for hyperCKemia (panel B). The red boxes indicate the results and the blue boxes indicate the methodologies.

Table 3. Deletion and duplication frequencies in different populations published in recent years (2002-2008)

Country of cohort	Unrelated males	Deletions	Duplications	Deletions and Duplications	Methods	References
Italy	164	73.8%	7.3%	81.1%	QF-multiplex PCR/MLPA	this study
Italy	506	74.5%	10.1%	84.6%	log-PCR	(55)
Serbia, Montenegro	123	63.4%	7.3%	70.7%	MLPA	(56)
China	179	66.2%	6.2%	72.4%	MLPA	(57)
China	249	65.0%	5.0%	70.0%	array-MLPA	(58)
Canada	165	60.1%	9.1%	69.2%	QF-multiplex PCR	(59)
Germany	150	42.0%	7.3%	49.3%	multiplex PCR/MLPA	(60)
USA	72	36.0%	26.4%	62.4%	MAPH	(29)
USA	102	40.0%	25.0%	65.0%	Southern/multiplex PCR/ MLPA/array-CGH	(61)
USA	97	17.5%	14.4%	31.9%	Southern/multiplex PCR/ MLPA/ array-CGH	(32)
Taiwan	89	36.0%	24.7%	60.7%	MLPA	(62)

QF-multiplex PCR: quantitative fluorescent multiplex PCR; MLPA: multiple ligation-dependent probe amplification; MAPH: multiplex amplifiable probe hybridization; log-PCR: multiplex PCR products spaced logarithmically on a gel; array-CGH: array-comparative genomic hybridization.

identified in one DMD patient and in his mother, grandmother and aunt. The MLPA, carried out to define the boundaries of this duplication, revealed a more complex rearrangement, involving three noncontiguous duplications of exons 44-48, 51-59 and 64-79 (Table 2). The molecular mechanisms proposed for such nonrecurrent copy-number variations include nonhomologous end joining repair (50-54), homologous recombination (52) and errors of DNA replication (54). Thirty-eight DMD/BMD patients and 52 males with high levels of serum CK, who had no apparent deletions or duplications at the QF-multiplex PCR and for whom DNA was still available, were further analyzed by MLPA. Seven additional mutations were identified, each in a DMD/BMD male, namely deletions of exons 27, 55 and 56-63 and duplications of exons 10-11, 21, 21-29 and 57-65 (Tables 1 and 2). Based on these results and on a cost-benefit analysis, in our laboratory we carry out MLPA only on patients and on their female relatives who are negative at the QF-multiplex PCR, or to define the length of exonic deletions/duplications, whose boundaries are not defined by the QF-multiplex PCR. Deletions or duplications were ruled out in 31 DMD/BMD males (about 19%) investigated by QF-multiplex PCR and MLPA (Figure 1); these patients probably bear point or deep intronic mutations. However, deletions or duplications cannot be ruled out in 15 affected males that were not analyzed by MLPA because their DNA was no longer available (Figure 1). The deletion/duplication rate found in this study is similar to those obtained in populations from Italy (55), Serbia and Montenegro (56), China (57, 58) and Canada (59) (Table 3), whereas, different frequencies were found in other populations (29, 32, 60-62) (Table 3).

The mutation detection rate was much lower in subjects with no clinical symptoms and who underwent molecular diagnosis only because of high CK levels. On the other hand, a similar low incidence (8%) of BMD immunohistochemical patterns has been reported in a retrospective evaluation of muscle biopsies of 40 individuals with "idiopathic hyperCKemia" (63). Interestingly, 3 out of our 9 deleted patients referred only for high CK levels had deletions that included exons 50 and 51, which are mutations predicted to be associated with mild BMD phenotypes. In fact, we previously reported (17) a correlation between genotype and phenotype in two groups of BMD deleted patients selected according to the presence or absence of exons from exon 50 onwards, which marks the region encoding the hinge III region of the protein (exons 50 and 51). On the basis of immunohistochemical and clinical evidence, we previously demonstrated (17) that the in-frame deletion of the hinge III region at the distal rod domain results in a milder phenotype as compared with shorter deletions that do not include the hinge III region. In accordance with our findings, Béroud (64) reported the clinical data of 15 patients with deletion of exons 45-55 and mild BMD phenotypes. Moreover, they developed an algorithm which predicts that the delivery of a micro-dystrophin gene deleted of the large segment spanning exons 45 to 55 could transform the DMD phenotype into the asymptomatic or mild BMD phenotype and rescue up to 63% of DMD patients bearing a deletion. Furthermore, the data of Melis (65) are also in agreement with our results on the correlation between phenotype expression and DNA regions deleted in the dystrophin gene. In fact, they reported two cases of children, investigated because of the incidental finding of elevated serum CK levels, who had deletions involving exons coding for the hinge III region (exons 48-51 and 48-53, respectively).

4.2. Assessment of carrier status and prenatal diagnosis

We assessed carrier/non carrier status by QF-multiplex PCR or by MLPA in 219 women belonging to 102 families with deleted or duplicated males, and carried out 17 prenatal diagnoses on chorionic villi from carrier females. The *de novo* deletion rate (about 35%) was in agreement with data reported for other cohort studies (1).

The carrier risk was assessed by linkage analysis through the segregation of 15 intragenic STRs (35) for females belonging to families in which the causative mutation was not identified. Haplotypes were constructed using the

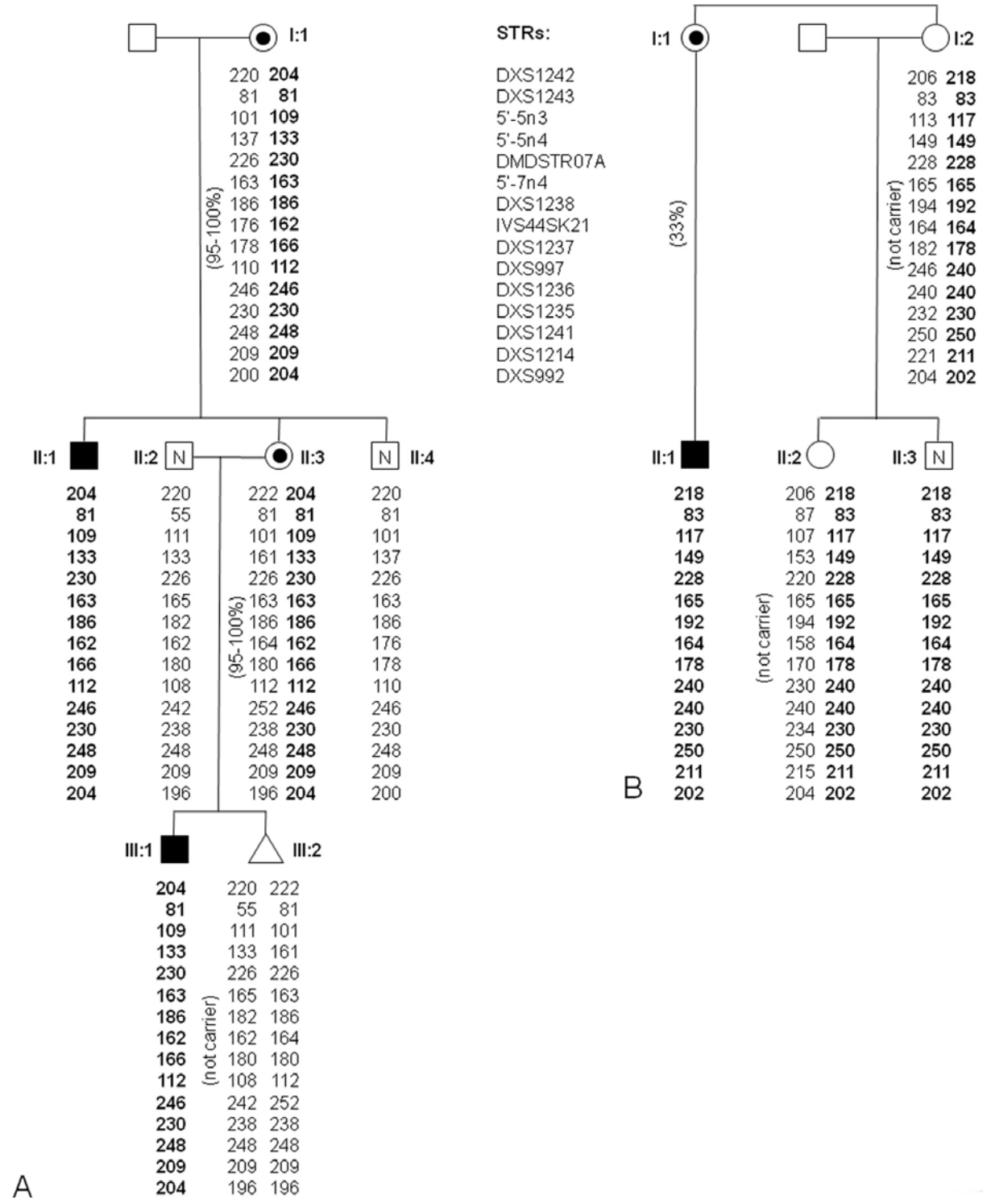

Figure 2. Examples of linkage analysis in pedigrees with familial (A) or sporadic (B) DMD/BMD cases. The percent at each haplotype (in brackets) indicates the likelihood of being a carrier. N: non affected male.

pedigree information in 35 families. Sixteen women were diagnosed, 13 as carriers and 3 as non carriers with a > 95% likelihood, in five families in which sporadic DMD/BMD cases were ruled out because at least two men in the family were affected by the disease (Figure 2A). Haplotyping led to the identification of four families with *de novo* DMD/BMD cases, i.e., one affected and one unaffected male that had the same haplotype (Figure 2B),

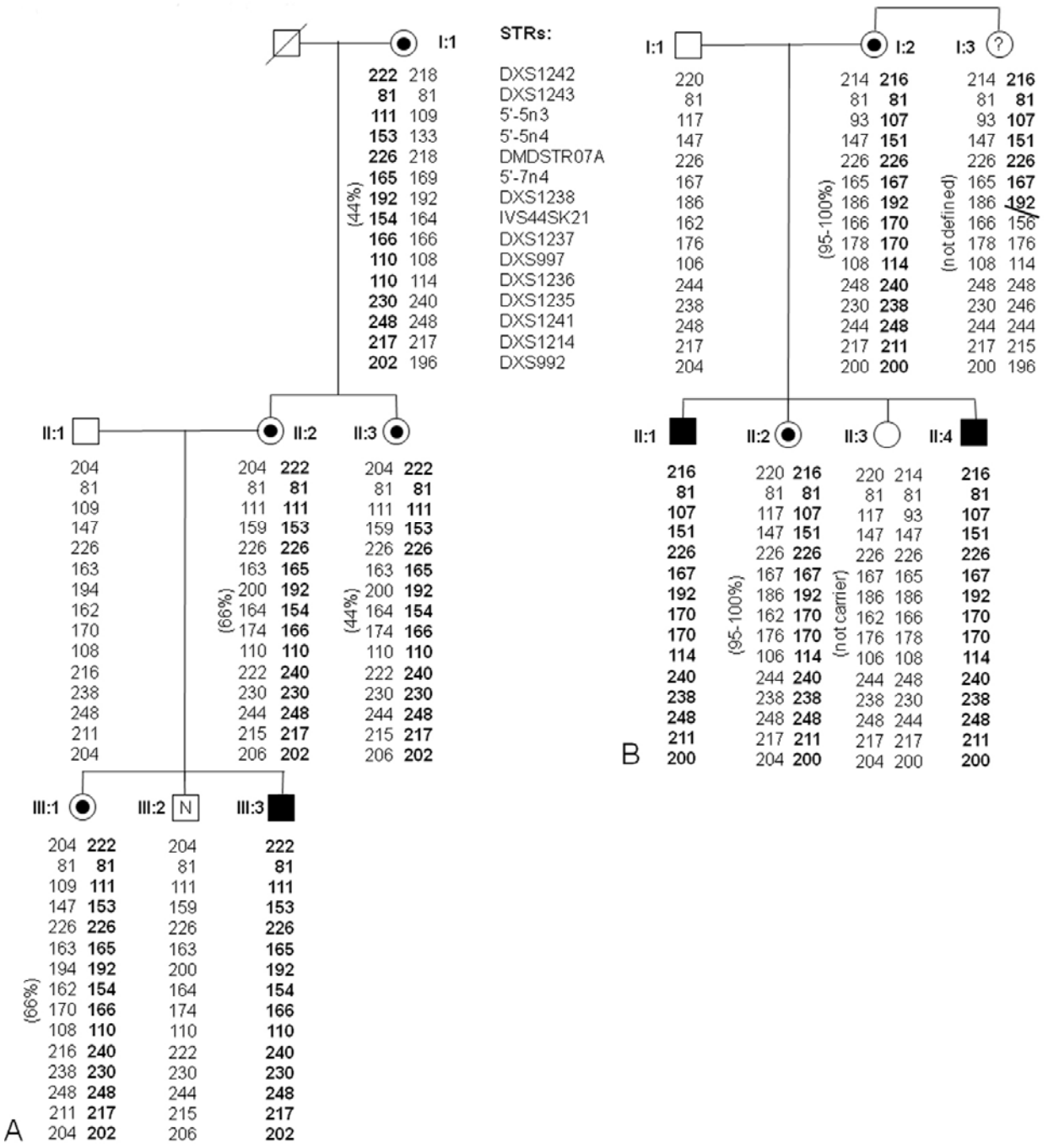

Figure 3. Examples of linkage analysis in pedigrees in which sporadic/non sporadic DMD/BMD cases could not be assigned (A) or in which the carrier status was not defined because of a recombination event (B). The percent at each haplotype (in brackets) indicates the likelihood of being a carrier. N: non affected male. The recombination event in subject I:3 in panel B is marked with a slash.

and 26 families in which sporadic/non sporadic cases could not be assigned (Figure 3A). Because the mutation could originate in the mother or in grandparents of the affected patient, we were able to establish the risk of being carrier in 30 women with a probability that depended on the degree of relationship with the affected males, whereas we were able to establish non carrier status in 22 women with a > 95 likelihood (Figure 2B and Figure 3A). The carrier status was not defined in 2 women because of recombination events in their haplotypes (Figure 3B). Among the 30 prenatal diagnoses performed on the overall case-mix, we carried out 13 prenatal diagnoses by linkage analysis; the

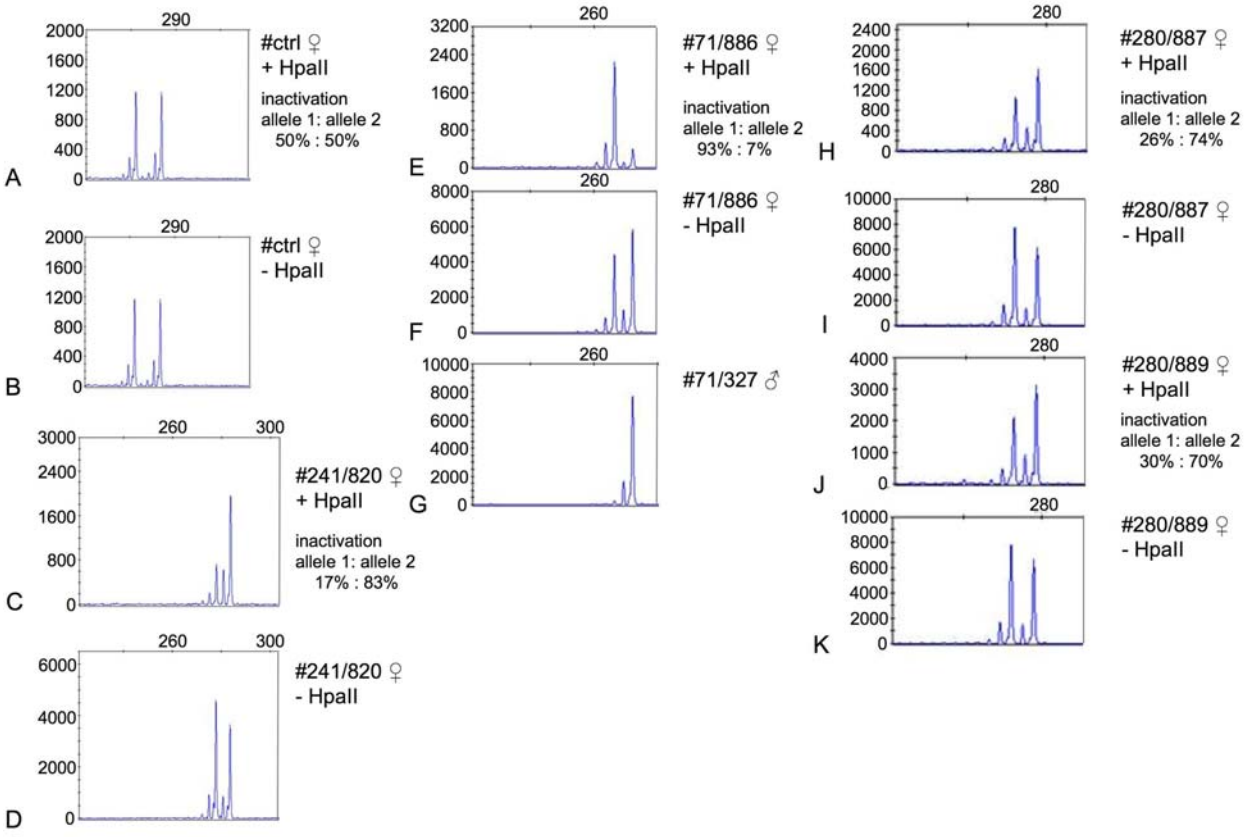

Figure 4. X chromosome inactivation analysis of one control (panels A and B) and four symptomatic carriers (panels C, D; E, F; H, I; J, K). HpaII digested (A, C, E, H, J) and undigested (B, D, F, I, K) amplified HUMARA alleles. Panels A and B show the HUMARA alleles of a woman with random X inactivation (named as control). Panel G shows the HUMARA allele of the affected brother of subject # 71/886; *x-axis*: allele length (bp); *y-axis*: arbitrary units of relative amount (fluorescence).

results were not conclusive for 5 fetuses (2 boys and 3 girls) because of recombinations. Given the high frequency of intragenic recombination in the dystrophin gene, the analysis of a large STR panel is crucial for a faithful characterization of the gene segregation. Since the terminal regions of a gene are notoriously challenging for linkage analysis, because some recombination events could be missed, our STR panel analyzes several polymorphic microsatellites in the 5' and 3' regions of the gene to minimize diagnostic errors (35).

4.3. Unbalanced X inactivation in symptomatic women

We screened four women referred for clinical manifestations of the disease for deletions/duplications by QF-multiplex PCR and MLPA. One woman carried deletion of exons 48-50, a mother/daughter pair carried deletion of exon 1, and one was negative. We investigated the chromosome X inactivation patterns of these women by analyzing the methylation-sensitive digestion of the human androgen receptor gene (47, 48) (Figure 4 A-K). In all symptomatic females we found unbalanced X inactivation with a degree of X skewing ranging from 70% to 93%. One woman (Figure 4E and F) had preferential inactivation of the allele not present in her affected brother (Figure 4G), which indicates that the mutated dystrophin gene is predominantly active. Differently, the mother/daughter pair shared the same alleles (Figure 4H–K), therefore it was impossible to infer the parental origin of the active allele or to hypothesize a recombination event between the dystrophin and the HUMARA *loci*. Familial cases of skewed X inactivation have been described in families (66, 67) in which the Lowe syndrome or the Rett syndrome**,** X-linked diseases, segregate.

4. CONCLUSIVE REMARKS

Deleted/duplicated represent 81.1 % (73.8% and 7.3%, respectively) of our DMD/BMD patient's cohort, as detected by QF-PCR and MLPA. In particular, the latter methodology increased the mutation detection rate by about 4% and is very effective in identifying duplications. In fact, 4 duplications out of 12 identified in this study are located in regions not investigated by the sets of QF-multiplex PCR generally used. Furthermore, a complex rearrangement of three noncontiguous duplications is a rare finding, and its mechanism warrants further studies. The detection rate of deletions/duplications identified in our cohort is one of the highest reported in recent years (see Table 3). Although this high detection rate may depend in part on the diverse procedures used throughout the world, it is highly feasible

that the typology of mutations may differ among populations, and this, in turn, may be partially due to a founder effect of mutations in some geographic regions. This new observation may be particularly relevant in instances of large population studies.

A high mutation detection rate is also important for the direct assessment of carrier/non carrier status and for prenatal diagnosis. The haplotype assessment with a large set of STRs, as reported in this paper, is advisable because of the high rate of recombinations; this seems particularly important when analyzing the 5' and 3' ends of the dystrophin gene. Moreover, our symptomatic women affected by DMD/BMD, an X-linked recessive disease, may be included among those cases due to unbalanced X-chromosome inactivation, whose intimate mechanism is still obscure.

5. ACKNOWLEDGMENTS

This work was supported by grants from Regione Campania (DGRC 2362/07) and from MIUR (PS 35-126/Ind). We are grateful to Jean Ann Gilder for revising and editing the English text**.**

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Key Words: Duchenne/Becker muscular dystrophy, Deletions, Duplications, MLPA, Linkage Analysis, X-inactivation, Noncontiguous Duplications

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