Direct Deletion Analysis in Two Duchenne Muscular Dystrophy Symptomatic Females Using Polymorphic Dinucleotide (CA)$_n$ Loci within the Dystrophin Gene

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Duchenne muscular dystrophy (DMD) is the most common hereditary neuromuscular disease. It is inherited as an X-linked recessive trait in which males show clinical manifestations. In some rare cases, the disease can also be manifested in females. The aim of the present study was to determine the molecular alteration in two cases of non-related DMD symptomatic carriers with no previous history of DMD. Multiplex PCR is commonly used to search for deletion in the DMD gene of affected males. This method could not be used in females because the normal X chromosome masks the deletion of the mutated one. Therefore, we used a set of seven highly polymorphic dinucleotide (CA)$_n$ repeat markers that lie within the human dystrophin gene. The deletions were evidenced by hemizygosity of the loci under study. We localized a deletion in the locus 7A (intron 7) on the maternal X chromosome in one case, and a deletion in the region of introns 49 and 50 on the paternal X chromosome in the other. The use of microsatellite genotyping within the DMD gene enables the detection of the mutant allele in female carriers. It is also a useful method to provide DMD families with more accurate genetic counseling.

Keywords: DMD, DNA deletions, Duchenne muscular dystrophy, STR's analysis, Symptomatic female carriers.

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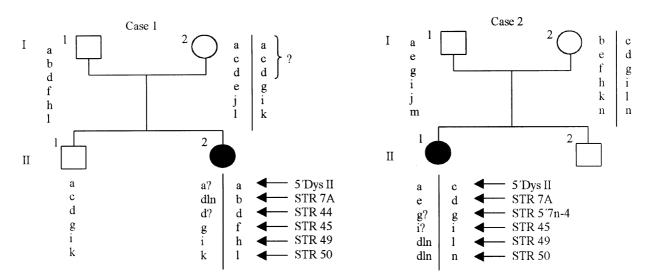[#]Present address: Department of Neurology. University of California, San Francisco.

Introduction

DMD and the milder allelic Becker muscular dystrophy (BMD) are two X-linked recessive diseases that are characterized by the absent, or abnormal dystrophin, in skeletal muscle, which results in early muscle degeneration and death. DMD is a common disease that affects 1 : 3500 newborn males (Emery, 1996), while BMD is less frequent. Both of the diseases are caused by mutations, mostly deletions of one or more exons of the dystrophin gene (Den Dunnen *et al.*, 1989; Koenig *et al.*, 1989; Abbs *et al.*, 1991; Alcantara *et al.*, 2001). It was previously reported that as many as one third of the cases arise from new mutations, while the remaining two thirds are hereditary (Anderson *et al.*, 1986; Edwards, 1986; Barbujani *et al.*, 1990).

Recent biochemical and clinical studies have shown that many females with a primary myopathy have an underlying dystrophinopathy, despite a negative family history for DMD (Nonaka *et al.*, 1991; Pegoraro *et al.*, 1994). Seventy percent of the heterozygous female carriers of DMD show increased-serum-creatine kinase (CPK) levels (Gruemer *et al.*, 1985); this is generally their only physiopathological finding. Although the majority (90%) of these female carriers is asymptomatic (Emery, 1967; Moser and Emery, 1974; Emery, 1993; Baranzini *et al.*, 1997), some of them could reveal proximal muscle weakness. This latter category of heterozygotes has been referred to as "manifesting" or "symptomatic" carriers (Emery, 1967; Moser and Emery, 1974; Baranzini *et al.*, 1997). On the basis of the biochemical findings on muscle biopsy, it has been hypothesized that all female dystrophinopathy patients show a skewed X inactivation, where the X chromosome that carries the normal dystrophin gene is preferentially inactivated (Arikawa *et al.*, 1991; Minetti *et al.*, 1991; Hoffman *et al.*, 1992).

A deletion analysis of affected DMD males can be performed by a multiplex polymerase chain reaction (PCR)

Fig. 1. Pedigrees of 2 families with sporadic DMD manifesting carriers. Haplotypes of segregation analysis are shown next to each analyzed individual. The STR loci order is indicated with arrows at probands haplotypes. dln, deletion; ?, The status of homo-hemizygous was not clearly established.

(Abbs *et al.*, 1991), or Southern blot hybridization (Darras *et al.*, 1988). However, the ascertainment of female carriers by these techniques is often difficult because of the presence of the normal X chromosome that masks the pathogenic deletion in its mutant counterpart. Short tandem-repeat (STR) $(CA)_n$ polymorphisms have been identified in different regions of the dystrophin gene (Beggs and Kunkel, 1990; Oudet *et al.*, 1990; Feener *et al.*, 1991; Miller *et al.*, 1992; King *et al.*, 1994); several of them are located in deletion-prone regions (Clemens *et al.*, 1991). We used a set of seven highly polymorphic STR markers within the human dystrophin gene to perform a segregation analysis in 2 symptomatic females and their relatives in order to search for the hemizygous patterns that reveal the presence of a mutated DMD gene.

Materials and Methods

Patients Both of the females with dystrophinopathy and no history of DMD were referred for a differential diagnosis of DMD by the Department of Neurology, Hospital Frances and Hospital Garrahan.

The studied families were as follows Case 1: The pedigree is shown in Fig. 1. The proband (30 years old) (II2) presented mental retardation with signs of aggressive behavior since childhood. At age 10, she began to suffer frequent falls and fatigue, subsequently her muscle weakness was noted by tripping and difficulty in climbing stairs. The serum-total-creatine kinase (CPK) activity was 462 IU/L (at age 27) and 850 IU/L (at age 28) (normal levels 24-170 IU/L). Electromyography-detected myopathic changes and a muscle biopsy showed the presence of dystrophin at a lower level than normal, which is consistent with that of a symptomatic DMD carrier.

Case 2: The pedigree is shown in Fig. 1. The proband (16 years

old) (II1) presented learning and behavior anomalies at school. At age 7, she showed walking and running difficulties and progressive muscular weakness. The serum-total-CPK activity was 4240 IU/L (at age 8), 6155 IU/L (at age 10), 7818 IU/L (at age 11), 3649 IU/L (at age 12), and 1750 IU/L (at age 14). Electromyography-detected myopathic changes and a muscle biopsy showed the presence of dystrophin at a lower level than normal, which was consistent with data for dystrophinopathy.

DNA analysis DNA was isolated from leukocytes that were obtained from peripheral blood, collected in sodium EDTA, according to the CTAB method (Murray and Thmpson, 1980). The DNA samples of the probands and their parents were used for a segregation analysis.

Amplification of STR (CA)n loci and electrophoresis Seven STR-(CA)n that were located in introns 7, 25-28, 44, 45, 49, 50 and the 5' region of exon 1 were used for the analysis (Table 1). PCR was performed according to Clemens *et al.* (Clemens *et al.*, 1991) with minor modifications; one of the primers was end-labeled with ^{32}P and used instead of the labeled deoxynucleotide in the PCR. Briefly, 120 ng of genomic DNA were mixed with 15 pmol of each primer in a total volume of 15 μl, containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphate, and 0.75 units of Taq polymerase (Life Technologies, Gaithersburg, USA). The samples were heated to 94°C for 4 min, followed by 25 cycles of DNA denaturation (94°C for 30 s), annealing (60°C for 30s), and polymerization (72°C for 30 s). The final 72°C incubation was extended for a further 10 min. Next, 2-4 μl of the PCR products, which were mixed with one volume of a stop solution (98% formamide, 0.5% EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and heated to 95°C for 3 min, were electrophoresed on denaturing-polyacrylamide sequencing gel. Autoradiography of the dried gel was performed at room temperature for 1-3 days.

Table 1. Oligonucleotide primers for PCR amplification and direct DNA sequence analysis of (CA)n loci used for segregation analysis

Probe number	Forward primer	Reverse primer	Location	Reference
5'DYS-II	tcttgatatatagggattatttgtgtttgttatac	attatgaaactataaggaataactcatttagc	5' region of exon 1	(Feener et al., 1991)
07A	ttctggttttctggtctg	gaatcaatctctctgtcaag	intron 7	(www.dmd.nl)
5'-7n4	gtgaagctacaaaaatattagag	caacaatatctcaccatacttg	intron 25-28	(King et al., 1994)
STR 44	tccaacattggaaatcacatttcaa	tcatcacaaatagatgtttcacag	intron 44	(Clemens et al., 1991)
STR 45	gaggctataattctttaactttggc	ctctttccctctttattcatgttac	intron 45	(Clemens et al., 1991)
STR 49	cgtttaccagctcaaaatctcaac	catatgatacgattcgtgtttgc	intron 49	(Clemens et al., 1991)
STR 50	aaggttcctccagtaacagatttgg	tatgctacatagtatgtcctcagac	intron 50	(Clemens et al., 1991)

DNA sequencing Sequencing of the locus STR-7A in family members of Case 1 was performed after amplification of the corresponding DNA fragment using unlabeled primers and PCR conditions, as described. The PCR products were purified by a QIAquick PCR purification Kit. Direct sequencing was performed with a fmol™ kit (Promega, Madison, USA).

Results

We determined the segregation of the STR (CA)n alleles that were located within the deletion-prone regions 5'DYS-II, 07A, 5'-7n4, STR 44, STR 45, STR 49, and STR 50. The PCR products of the alleles were analyzed by superimposing the traces of the proband to that of her relatives. The haplotypes of the allele lengths at these loci were determined in both cases by assigning arbitrary letters to each allelic variation (Fig. 1.)

Case 1: STR haplotypes initially suggested hemizygosity at locus STR 7A for the proband (II2), who should have been heterozygous (allels b and c) (Fig. 2a STR 7A). The hemizygosity was suspected because a similar pattern to that obtained for the father was observed in the proband. Also, a reduction in the signal was seen where the maternal allele (2bp lighter than the paternal one) should have been present (Fig. 2a STR 7A, left arrow). However, the suspected absence of the maternal allele could be attributed to a technical artifact, rather than to a biological finding. In order to further investigate this possibility, we then sequenced STR 7A and its flanking region, where a single nucleotide polymorphism (SNP) (C/T) is located. Because the probands father (I-1) was hemizygous for the T allele (data not shown), and the mother (I-2) was homozygous (or hemizygous) for the C allele, therefore, an heterozygosity (T/C) was expected for the proband (II-2). However, we only detected the paternal allele in this individual (Fig. 2b). As expected, the probands brother (II-1) only displayed the maternal allele (C) (data not shown).

The PCR fragment that amplifies locus STR 7A actually contains 2 polymorphic tandem repeats that lay next to each other; a $(CA)_n$ and $(TC)_n$ (Fig. 2b). By sequencing this fragment, we were able to identify and count the exact number of repeats from each STR in the DNA from the proband and her relatives. While I-2 presented a total of 28 repeats [$(CA)_{16}$ and $(TC)_{12}$], II-2 displayed 29 repeats [$(CA)_{18}$ and $(TC)_{11}$], the same as her father. The lack of the maternal allele that contained a total of 28 repeats was evident. If this allele had been present in the proband, then the double bands in the sequence would have been observed (Fig. 2c). These results confirm that only the paternal allele (2 nucleotides heavier than the maternal) is present in the proband.

Case 2: This case is a clear example of how an indirect analysis (using STRs) becomes a direct one. A deletion of two of the six loci that were studied for this family was identified in proband II-1, revealed by the absence of the paternal allele at STR 49 and STR 50 (Fig. 3). In this case, the lack of an allele was clearly visualized, in contrast to Case 1 where the shadow bands interfered. This result made sequencing unnecessary.

Discussion

The identification of highly polymorphic $(CA)_n$ loci within the dystrophin gene allows researchers to perform a genetic segregation analysis in DMD and BMD families. This is a highly accurate method to establish carrier status and improve counseling. The STR $(CA)_n$ polymorphisms from 5' and the central region of the dystrophin gene proved to be a useful molecular tool, due to their location in the most frequently deleted spots (Den Dunen et al., 1989; Baranzini et al., 1998). One of the most frequent uses of STR segregation analysis in DMD families is to determine the carrier status of females. Also, it can be performed without the need of living-affected relative (Alcantara et al., 2001).

We describe here the molecular analysis of two symptomatic DMD/BMD females by genetic segregation of polymorphic markers. The detection of a heterozygous deletion by this method was successful in both of the cases that were studied. There was evidence of parental origin of the deleted allele in both cases. In Case 1, the deletion was found in the maternal X chromosome. Even though the same haplotype was shared between the proband and her healthy brother, the carrier status of the mother could not be ruled out, due to the probability of a recombination event. In Case 2, the deletion was found in the paternal X chromosome. This suggests the non-carrier status of the probands mother, and
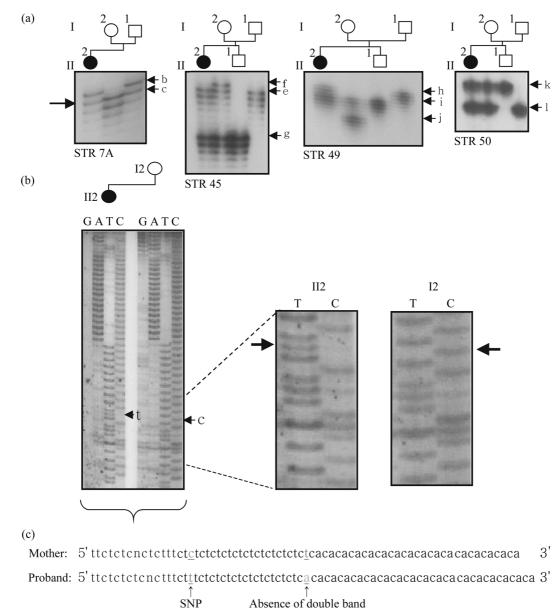
Fig. 2. STR's and sequence analysis in case 1. (a) Allele assignment: Autoradiography of polyacrylamide gel showing the alleles at four of the six analyzed STR loci. In STR 7A, the left arrow indicates the reduction in the signal (in II2) were the maternal allele should have been present. (b) Sequence of STR 7A and flanking region. The arrows indicate the SNP detected, the absence of the C maternal nucleotide variant in II2 is clearly seen. (c) Alignment of the mother and probands sequence at the STR7A locus. This figure shows the sites where the maternal C and T nucleotides at the SNP and at the beginning of the (CA)$_n$ repeat, respectively, should have been present in the proband.

confirms that it was a de-novo mutation. Due to the dystrophin mosaic pattern that was found in the muscle biopsy in both cases, it is highly improbable that the other X chromosome could carry another mutation. This suggests that the skewed X inactivation was the mechanism that resulted in the symptomatic females (Hoffman *et al.*, 1992).

Associated mental retardation is a clinical feature that is present in both Duchenne and Becker muscular dystrophy patients, and its pathogenesis is still unknown (Bardoni *et al.*, 2000; Anderson *et al.*, 2002). A correlation between the cognitive impairment and location and extent of the deletion may exist. Previous works suggest that mutations in sequences, which are located in a distal part of the gene (3' from intron 44) that affects some brain-specific dystrophin isoforms (Dp 140, Dp 71), may be related to the cognitive impairment (Meizard *et al.*, 1998). Since both of our patients showed mental retardation, we sought then to determine whether the deletions that were found could affect any of these isoforms. In Case 2, a link between the mapping of the deletion and the cognitive impairment for II-1 was found,
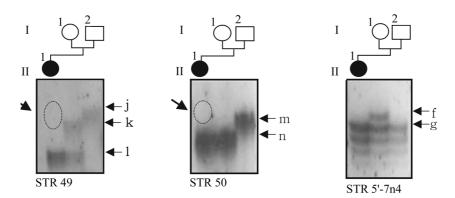
Fig. 3. STR's analysis in Case 2. Autoradiography of polyacrylamide gel showing the alleles at three analyzed STR loci. The arrows indicate the deletion of the paternal allele, which maps at STRs 49 and 50.

since the deletion was detected at STRs 49 and 50. However, this correlation could not be proved, but only presumed in Case 1, since the hemizygous status of STR 44 in II-2 was not clearly seen (Fig. 1. Case 1, STR 44 allele D).

In conclusion, the segregation of STR $(CA)_n$ loci from 5' and the central region of the dystrophin gene resulted in useful information for detecting not only the carrier status, but also the deletion location in female carriers. Our findings dramatically modified the genetic counseling for these families.

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