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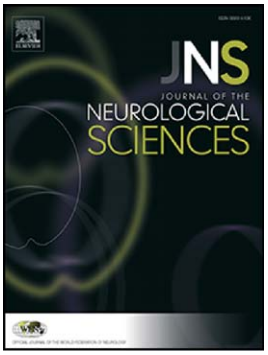

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Symptomatic female carriers of Duchenne Muscular Dystrophy (DMD): genetic and clinical characterization.

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ABSTRACT

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused by mutations in the dystrophin gene and is characterized by muscle degeneration and death. DMD affects males; females being asymptomatic carriers of mutations. However, some of them manifest symptoms due to a translocation between X chromosome and an autosome or to a heterozygous mutation leading to inactivation of most of their normal X chromosome. Six symptomatic female carriers and two asymptomatic were analyzed by: I) Segregation of STRs-(CA)n and MLPA assays to detect a hemizygous alteration, and II) X chromosome inactivation pattern to uncover the reason for symptoms in these females. The symptomatic females shared mild but progressive muscular weakness and increased serum creatin kinase (CK) levels. Levels of dystrophin protein were below normal or absent in many fibers. Segregation of STRs-(CA)n revealed hemizygous patterns in three patients, which were confirmed by MLPA. In addition, this analysis showed a duplication in another patient. X chromosome inactivation assay revealed a skewed X inactivation pattern in the symptomatic females and a random inactivation pattern in the asymptomatic ones. Our results support the hypothesis that the DMD phenotype in female carriers of a dystrophin mutation has a direct correlation with a skewed X-chromosome inactivation pattern.

INTRODUCTION

Duchenne muscular dystrophy (DMD) and the milder allelic Becker muscular dystrophy (BMD) are X-linked recessive diseases caused by absent or abnormal dystrophin, respectively, in skeletal muscle, which results in early muscle degeneration. DMD is a fatal disease that affects 1:3500 newborn males, while BMD is less severe and less frequent [1]. Both dystrophies are caused by mutations, mostly deletions, in the dystrophin gene [2,3]. One third of the cases arise from new mutations, while the remaining two thirds are inherited [4,5]. DMD and BMD usually affect males, with the majority of females being asymptomatic carriers. However, some of these females can reveal symptoms that vary from mild muscle weakness to a more severe clinical course and are classified as manifesting or symptomatic carriers [6,7,8,9,10]. Studies on muscle biopsy suggest that female dystrophinopathy patients show a skewed X inactivation, where the X chromosome that carries the normal dystrophin gene is preferentially inactivated [11,12]. Many of these symptomatic carriers have gross chromosomal rearrangements due to translocation between the X chromosome and an autosome, with one breakpoint involving the DMD gene [13]. In these cases the normal X chromosomes appear to be preferentially inactivated since the dystrophin levels are below normal, leading to DMD or to a milder variant [14]. On the other hand, a small number of females heterozygous for dystrophin mutations, have most of their normal X chromosome randomly inactivated, and manifest mild DMD symptoms [15]. Furthermore, a different X inactivation pattern was seen in pairs of monozygotic twins heterozygous for mutation in the dystrophin gene, which resulted in clinical manifestation in only one of them [16].

It is more difficult to ascertain the DMD mutations in female carriers than those in males because the presence of the normal X chromosome masks the alteration in its mutant counterpart [17]. Thus, in order to detect mutations (mostly deletions) in females clinically diagnosed with muscle disease, more laborious and expensive methods need to be used than for analysis of male patients. These methods include segregation analysis of short tandem-repeat (STR) (CA)n polymorphisms and MLPA assay, which are able to detect hemizigous patterns and thereby reveal

the mutated DMD gene in female-carriers. The use of (STR) (CA)n polymorphisms, located throughout the dystrophin gene including the deletion-prone regions [18], is not as informative as MLPA analysis, however, both methods are essential for validation of the identified mutations.

The determination of maternal and paternal X chromosome activation status is useful for the analysis of non-random X inactivation patterns. One of the methods to test the X inactivation is based on the methylation-sensitivity of the restriction enzymes HpaII and HhaI [19]. It was shown that the methylation of HpaII and HhaI sites in the first exon of the human androgen-receptor locus correlates with X inactivation, moreover, the presence of an STR close to this site allows the use of a PCR assay to identify the methylation pattern of the maternally and paternally derived X chromosomes.

This study aimed to analyze six symptomatic and two asymptomatic female carriers using a strategy that includes: i) identification of the hemizygous patterns in the dystrophin gene by segregation of STRs-(CA)n markers and MLPA assay; and ii) determination of the X chromosome inactivation pattern. Comparing these results with clinical and histopathologic data provides a correlation between genotype and phenotype and thus unable us to uncover the reason for the manifesting DMD status in these females.

MATERIALS AND METHOD

Patients

Six females with muscular dystrophy and no DMD history and two females without these symptoms were referred for a differential diagnosis of DMD by several hospitals in Argentina. The *a priori* diagnosis was established by current clinical, biochemical and histological criteria. After approval of the study protocol by the Bioethical Committee of Hospital de Clinicas "Jose de San Martin", informed consent for genetic analyses was signed by the patients. The clinical features are depicted in Table 1. The six symptomatic females presented with muscle alterations compatible with dystrophinopaty and several of them, in addition, with learning and behavioural anomalies. One of the females had a DMD associated with an autism syndrome. The two asymptomatic females showed minor myopathic signs such as muscle cramps, myalgia and one of them (#60), a mild cardiac dysfunction. Both females presented with high CK levels.

Mutation analysis

Mutation screening was performed by segregation analysis of Short Tandem Repeats of (CA)n (STRs-(CA)n) and Multiplex Ligation-dependent Probe Amplification (MLPA). The DNA from peripheral blood leukocytes of probands and their parents was isolated by the CTAB method [20]. Nine STR-(CA)n located in the following introns: 7, 25, 44, 45, 49, 50, 62,79 and the 5´ region of exon 1 were assayed as previously described [17]. Briefly, 120 ng of genomic DNA were mixed with 15 pmol of each primer, of them end-labelled with ^{32}P, in a total volume of 15 µl containing 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM each deoxyribonucleotide triphosphate, and 0.75 units of Taq polymerase (Inbio Highway, Argentina). The samples were heated to 94ºC for 4 min, followed by 25 cycles of DNA denaturation (94ºC for 30s), annealing (60ºC for 30s) and polymerization (72ºC for 30s) and a final incubation at 72ºC for 10min. Next, 2-4 µl of the PCR products were electrophoresed on denaturing-polyacrylamide sequencing gel. Autoradiography of the dried gel was performed at room temperature for 1-3 days. MLPA was carried out with two SALSA probemixes, 034 and 035, according to the manufacturer´s

recommendations (MRC Holland, Amsterdam, Netherlands) in order to screen all the dystrophin exons. PCR products were analyzed on an AB1 3130 automated sequencer using Genescan software (Applied Biosystem). The dosage quotient was calculated using Gene Marker software.

X-chromosome inactivation pattern analysis (HUMARA assay)

Genomic DNA samples were isolated from peripheral blood leukocytes (PBL) by salting out procedure [21] to detect X-chromosome inactivation by the *AR* (human androgen receptor gene) assay as was originally described with minor modifications [19]. Briefly, for each patient's DNA sample, two reactions were prepared: two μg of DNA were either treated (T) with 20 U Hpa II (methylation sensitive restriction enzyme), or non-treated (N), i.e., incubated with the enzyme digestion buffer with not enzyme. Both reactions were carried out in 20 μl of final volume at 37°C overnight and stopped by heating the mixture at 95°C for 10 min. Two μl of treated and non-treated DNA were amplified by touchdown-PCR, spanning a highly polymorphic (CAG)n repeat in the first exon of the AR gene (Xq11). T and N PCR products were separated on a polyacrylamide gel on an ABI3130XL automated sequencer. The amount of gene products from the two X chromosomes was determined by the use of SoftGenetics GeneMarker. XIP (X-inactivation pattern) was calculated as follows XIP = 100 – 50 (A'/A) (A+B) / (A'+B'), where A and B signals correspond to non-treated, and A' and B' correspond to Hpa II-treated. Statistical analysis was performed using Prism 5.0 GraphPad Software (USA). X-inactivation pattern was considered as random for values ≤ 80% (ratios ≤ 80:20), moderately skewed for values >80% and ≤ 90% and highly skewed for values > 90%, according to Amos-Landgraf et al. [22].

RESULTS

Six female patients manifesting different grades of DMD plus two females not manifesting DMD but with some features suggesting a DMD carrier status, were studied (Table 1). The females with DMD symptoms represent 6% of the total DMD carrier females studied in our laboratory, which is consistent with previously reported data [6]. The age of disease onset in five of the symptomatic females was between 7 and 10. One of these patients (#499) had an onset of autism at the age of 3 with symptoms that included hyperactivity, impulsiveness and loss of previously acquired functions plus an onset of DMD at the age of 10. All these females shared myopathic changes on electromyograms and a progressive muscular weakness, which was less severe than in DMD males. Most of them are still able to walk, but with some problems such as frequent falls, fatigue, lameness and an inability to run. One of these patients (#482) became wheel-chair bound at the age of 19. The sixth female (#514), who was born from consanguineous parents, had an earlier onset, before the age of 6, and presented with more severe symptoms. This female became wheel-chair bound at the age of 6 and died at the age of 13 from heart failure. Four of the females (#287, 334, 433 and 499) showed behavioural and mild learning anomalies, however, all of them finished elementary school and three were attending high school. The remaining two females, the autistic patient (#499) and the one with more severe DMD symptoms (#514), did not attend elementary school. The patient who became wheel-chair bound at the age of 19 (#482) had a difficulty in using her hands, however, she had skills for painting.

The biochemical and genetic data of the patients indicate that the CK levels varied between 462 and 5070 U/L in the six symptomatic females (Table 1). The muscle dystrophin protein levels, analyzed by immunohistochemistry, were lower than normal (#287, 334, 433), or there was an absence of dystrophin in many of the fibers (#499). These results are consistent with the dystrophinopathy syndrome. The two females not expressing DMD (#60 and 645) had had mild myopathic signs, however, they presented with biochemical or histological data that suggest a DMD

carrier status. They showed high CK levels and one of them (#645) also showed a decreased dystrophin in all muscle fibers.

Detection of deletions in the dystrophin gene was previously arrived at by segregation analysis of STRs-(CA)n in families with affected females. Figure 1 shows the pedigrees and haplotypes of six families. Three of the females (#287, 334 and 433) showed a hemizygous pattern with an absence of maternal or paternal allele at the loci of 7A, 49/50 and 45 respectively, which revealed a deletion in these regions of the dystrophin gene (STRs results of #287 and #334 were already reported [17]). There was no absence of any parental allele in the haplotypes of the other three affected females. One of these patients, #514, displayed a homozygosity for 6 of the 7 STRs studied with the exception of STR 49. Her mother was also homozygous for 6 of the STRs and shared these alleles with the father. These results revealed the parents' consanguinity. In addition, the paternal allele at the 49 STR locus changed to a smaller one (d→e) during transmition to his daughter.

MLPA analysis confirmed the results of STRs segregation assay showing deletions of the exons 7-20 (#287), 45-52 (#334) and 45 (#433). In addition, this analysis revealed a duplication of exons 18-28 in patient #499. No alterations were detected in the dystrophin gene by MLPA in two affected females (#482, #514) nor in the two females not expressing DMD.

The pattern of X-chromosome inactivation (XIP) in the six symptomatic and the two asymptomatic females is depicted in Figure 2. All the females were found to be heterozygous at the *AR* locus. The symptomatic females showed an extremely skewed X-chromosome inactivation pattern: XIP>90%, whereas, the two females not expressing DMD (#60 and 645) showed a random pattern of inactivation: XIP 52% and 71% respectively (Figure 2 and Table 1).

DISCUSSION

In order to clarify the mechanism responsible for clinical symptoms in female DMD mutation-carriers we studied six females with a clinical diagnosis of dystrophinopathy and two females without dystrophinopathy, but with biochemical/histological data suggesting a DMD carrier status. Furthermore, their genotypic and phenotypic profiles were compared. The clinical presentation of 5 of the females, who are currently aged between 17 and 41, was similar to mild dystrophinopathy, characterized by a later onset and less severe muscle problems than in DMD males. Of note is that one of the females (#499) presented clinical features of two syndromes: autism and dystrophinopathy. Behavioral and mild learning anomalies were apparent in most females but there was a severe anomaly in the autistic patient. In contrast to these 5 female patients the sixth female (#514) showed severe DMD symptoms, like those displayed in DMD males. She had serious walking and respiratory problems and died at the early age of 13. The two females without dystrophinopathy (#60 and 645) did not share the clinical features of the symptomatic patients.

The immunostaining of muscle biopsy with antidystrophin antibody highlighted a reduced dystrophin labelling of the fibers and a mosaic distribution of dystrophin-positive (or attenuated) and dystrophin-negative fibers. However, there was no correlation between the percentage of negative fibers and the clinical presentation since one of the females (#645) without dystrophinopathy and a mildly increased CK level, showed a marked decrease of dystrophin, indicating a DMD carrier status. These results are in full agreement with other reports [23].

Segregation of STRs and MLPA analyses allowed for the detection of deletions in three females and a duplication in one of the patients. Two of these mutations were clustered in the central hot spot region and two other mutations, one deletion and one duplication were located at the 5´ region of the gene, which is a common site for duplications [9]. All the mutations were out of frame. No gross deletions/duplications were found in two of the female patients (#482 and 514) nor were they found in the two females not expressing DMD (#60 and 645), suggesting an absence of gross rearrangements and the presence of point mutations as the cause of dystrophinopathy or carrier

status. Interestingly, one of the patients (#514) who was homozygous for most STR loci due to her parents´ consanguinity (cousins), additionally showed a retraction in the paternal 49 STR-allele. This patient was the one that had a severe muscular disease and the only one who died at an early age. The absence of gross rearrangements revealed by STRs and MLPA analyses suggests that there was no chromosomal translocation between X and an autosome. Thus, another type of mutation would be responsible for the dystrophinopathy, and the severe presentation may be due to additional alterations in a glycoprotein complex or in other factors resulting from parental consanguinity.

Skewed X chromosome inactivation has been proposed as a possible explanation for the presence of DMD-symptoms in female carriers of dystrophin gene mutations. Such females should have an active X chromosome with the mutation and an inactive normal X chromosome. This hypothesis has been proved in females with an X-autosome translocation, in whom the X-autosome can not be inactivated and thus, the normal X-chromosome became inactivated in all the muscle cells, leading to severe DMD-like symptoms [9]. However, in cases with a skewed X-inactivation in the majority of the fibers (for example 80%) some muscle cells should have dystrophin-positive fibers. In these cases the results concerning the correlation between the patient phenotype and an X-inactivation pattern are controversial [7,8,22]. All the symptomatic females studied in our laboratory presented a skewed X-inactivation pattern (XIP), showing a relationship between the clinical phenotype and the pattern of X-inactivation. No translocations have been revealed by the methods used for detection of gross rearrangements. These data suggest a different reason for a skewed X-inactivation than the presence of an autosomic fragment joined to X chromosome with a mutated dystrophin gene. The lack of translocation in the only patient with a severe form of dystrophinopathy (#514) correlates with the partially skewed pattern of X-inactivation, unlike to that observed in patients with an X-autosome translocation [9].

In summary, different findings concerning the correlation between DMD symptoms and a skewed X chromosome inactivation in females have been reported previously. Our results support

the hypothesis that the DMD phenotype in female carriers of dystrophin mutations is connected to a skewed X chromosome inactivation pattern, leading to less expression of the wild type dystrophin.

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CONFLICT OF INTERESTS

The authors declare that they have no competing or other interests that might be perceived to influence the results and discussion reported in this paper.

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LEGEND TO FIGURES

Figure 1: Pedigrees of six families with DMD symptomatic female carriers. The haplotypes resulted from segregation analysis are shown next to each analyzed individual. They are denoted by assigning arbitrary letters to each variation. dln: deletion.

Figure 2: X-chromosome inactivation pattern in six symptomatic carriers of Duchenne Muscular Dystrophy and in two asymptomatic females. N indicates non-treated and T treated with *Hpa* II. XIP indicates X-chromosome inactivation pattern which was considered as random for values ≤ 80% (ratios ≤ 80:20), moderately skewed for values >80% and ≤ 90% and highly skewed for values > 90%. Each single signal corresponds to a PCR amplified allele of the (CAG)n repeat within the first exon of the *AR* gene, on an ABI3130XL automated sequencer. To quantify the relative inactivation, the AUC (area under the curve) of both alleles (non-treated and treated DNA) was measured using the GeneMarker software. The HUMARA assay shows an extremely skewed X-chromosome inactivation pattern, XIP>90%, in all the six symptomatic cases (in the upper part of the figure: #287, 334, 433, 482, 499 and 514) and a random pattern of X-inactivation, XIP 52% and 71%, in the two asymptomatic cases (in the bottom of the figure: #60 and 645).

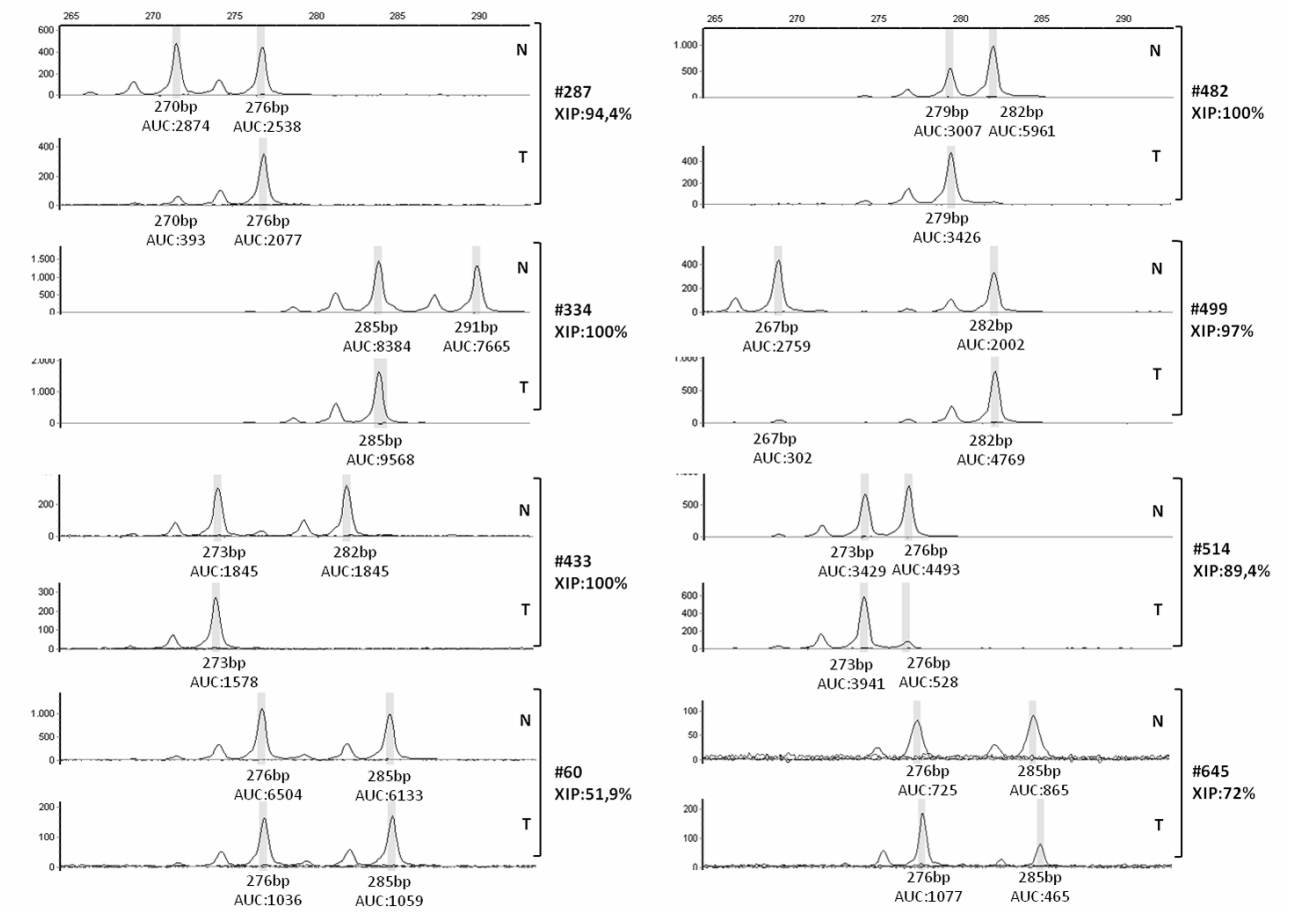

Figure 1

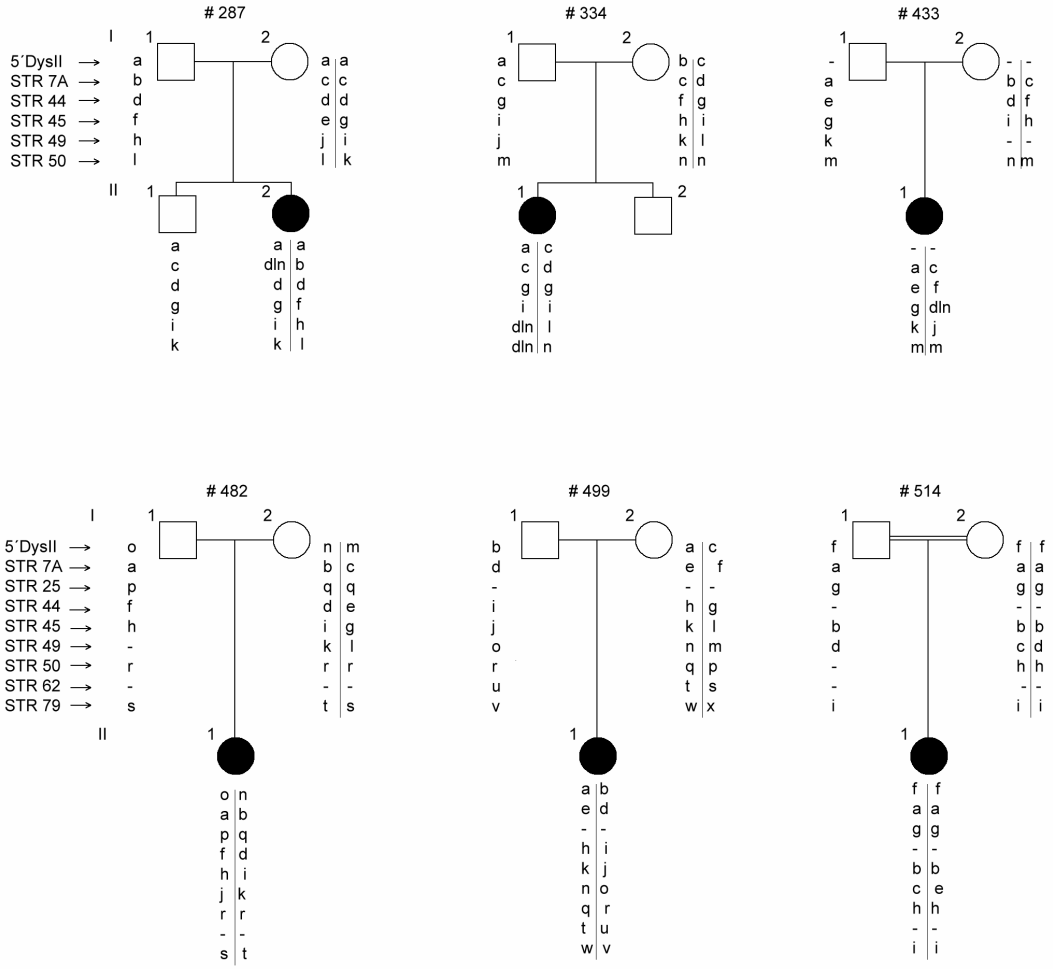

Figure 2

Table 1: Clinical, biochemical and molecular data of symptomatic female carriers. X-inactivation pattern

Patient ID*	Age of Onset (years)	Current Age (years)	EMG[†] data & muscular weakness	Other signs	Learning & behaviour	CK[**] Levels (U/L)[‡]	Muscle Biopsy	Mutations STRs[§] assay	Mutations MLPA[α] assay	XIP[β]
287	10	41	Myopathic changes Progressive weakness	Frequent falls Fatigue Lameness (at present)	Aggressivenes s Mild MR[γ] Elementary school finished Brain NMR[δ]: alteration	462:27 y[ε] 850:28 y	Dys[λ] 1,2,3 Lower level than normal	Hemizygosity at 7A *locus* Absence of maternal allele	Deletion: Exons 7-29	94:6

334	7	25	Myopathic changes Progressive weakness	Walking problems No running	Behavioural & mild learning anomalies at High school	4240:8y 1750:14y	Dys 1,2,3 Lower level than normal	Hemizygosity at 49/50 *loci* Absence of paternal allele	Deletion: Exons 45-52	100:0
433	8	17	Myopathic changes Progressive weakness	Walking problems No running	Behavioural & mild learning anomalies at High school	1630:8y	Dys 2,3: Negative fibers, Isolated positive Altered fiber structure Increase of endomysial fibrosis and of internal nuclei.	Hemizygosity at 45 *locus* Absence of maternal allele	Deletion: Exon 45	100:0
482	8	21	Myopathic changes Progressive	Wheel-chair bound at 19 years Diffic	Normal learning: Elementary school	5070:12y	Not determined	Not detected	No gross Rearrang m.	100:0

			weakness	ulty in the use of hands	finished Painting Skills					
499	3: Autism ~10: DMD	24	Myopathic changes Progressive weakness	Autism Walking problems Inability to hold objects Convulsions	Aggressiveness No speaking. Special care needs	5000:15y	Dys 1,2,3: Partial deficiency: 15-30% of fibers. Mosaic distribution	Not detected	Duplication: Exons 18-28	97:3
514	Before 6	Dead:13 (Cardiac strike)	Progressive weakness	Wheel chair bound: 6 years	Breathing diffic.[v] Incomplete school	Not reported	Not determined	Not detected	No gross Rearrangem.	90:10

645	9	17	No Myopathic changes	9 years: Frequent falls 17 years: Normal walking/running	Normal learning University study	315:9y	Dys 1,2,3: Decrease: all muscle fibers	Not detected	No gross Rearrangem.	72:28
60	4	17	No Myopathic Changes	4 years: Cramps Decreased strength Mild cardiac dysfunction	Behavioural & learning anomalies	3642:17y	Dys 1,2,3: Normal	Not detected	No gross Rearrangem.	52:48

*ID: patient´s identification number; [†]EMG: electromyography; [**]CK: creatine kinase; [‡]U/L: units per liter; [§]STRs: short tandem repeats; [α]MLPA: multiplex ligation-dependent probe amplification; [β]XIP: chromosome X inactivation pattern; [γ]MR: mental retardation; [δ]NMR: nuclear magnetic resonance; [ε]y: years; [λ]DYS: dystrophin; [ν]diffic.: difficult.