

## DNA ANALYSIS OF BRAZILIAN DUCHENNE MUSCULAR DYSTROPHY FAMILIES USING (CA)<sub>n</sub> MICROSATELLITE MARKERS

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### ABSTRACT

We investigated nine Brazilian Duchenne Muscular Dystrophy (DMD) families with patients without a deletion or duplication, by haplotype analysis using microsatellite markers of the (CA)<sub>n</sub> type. Our objective was to detect carriers of the anomalous dystrophin gene among women at risk. We assumed that the locus Xp21 is involved in the MD condition in all the families. Looking for informativeness, we used two (CA)<sub>n</sub> microsatellite markers of the 5' end of the dystrophin gene, i.e. 5'DYSI and 5'DYSIII (Feener *et al.*, *Am. J. Hum. Genet.* 48: 621-627, 1991) and, in view of the high intragenic recombination rate, we also used a microsatellite marker of the 49 intron, i.e., STR 49 (Clemens *et al.*, *Am. J. Hum. Genet.* 49: 951-960, 1991). In only one of the nine families the information was insufficient to indict the X-chromosome at risk. There was absolute coherence between increased creatine-kinase (CK) levels of some obligatory carrier women and their genetic condition as evaluated by haplotype analysis. In three of seven families with isolated cases it was possible to define new mutations in which the X-chromosome was originated from the maternal grandfather. It was possible to detect a crossing over at the 5' end-intron 49 interval in one of the nine mothers of patients.

We conclude that (CA)<sub>n</sub> loci are excellent markers in face of their high informativeness. Sensitivity and rapidity provided by the polymerase chain reaction (PCR) assay make them unique when compared to other markers assayed by standard Southern blotting and hybridization. For detection of carriers of the anomalous dystrophin gene, it is absolutely necessary to use markers both from 5' or 3' end of the gene and from the central/distal (3') regions in face of the 12% intragenic recombination rate (Abbs *et al.*, *Genomics* 7: 602-606, 1990).

### INTRODUCTION

Duchenne and Becker muscular dystrophies (DMD and BMD) are variants of a progressive muscular disease caused by alterations in the 2.4-Mb dystrophin gene located at Xp21. Approximately 65% of DMD cases are due to intragenic deletions or duplications (Forrest *et al.*, 1988; Den Dunnen *et al.*, 1989; Koenig *et al.*, 1989) and 98% of these can be detected by polymerase chain reaction (PCR) analysis of exons (Chamberlain *et al.*, 1988; Gibbs *et al.*, 1989; Beggs *et al.*, 1990).

The detection of women carriers of the DMD gene in families of patients with a deletion or duplication is

viable by Southern blotting, either using cDNA probes and determining the intensity of the band, or using intragenic genomic probes which recognize RFLP's, or by using eventual junction fragments. In all these cases detection is not always easy. The evaluation of band intensity requires blots of excellent quality and rigorous controls. For known RFLP's, probes XJ1.1, pERT 87, JBir, P20, and J66 only recognize "diallelic" fragments and their use is hampered when the family is small or when the patient is dead. The occurrence of junction fragments which may indicate the carrier condition is rare. Even so, haplotype analysis using intragenic or extragenic markers has been one of the methods most often utilized for carrier detection, especially among the 35% of cases in which the patient has no deletion or duplication.

Over the last ten years, (CA)<sub>n</sub> loci have been identified in numerous sequences of the human genome. These CA repeats are amplifiable by polymerase chain reaction (PCR) and represent a powerful new class of genetic markers. There are approximately 35,000 to

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130,000 (CA)<sub>n</sub> loci in the human genome. The *n* value roughly ranges from 15 to 30 (Hamada and Kakunaga, 1982; Hamada *et al.*, 1982; Tautz and Renz, 1984; Litt and Luty, 1989). The function of the (CA)<sub>n</sub> blocks is unknown but they are assumed to serve as hot spots for recombination (Slightom *et al.*, 1987) or to participate in gene regulation (Hamada *et al.*, 1984).

Weber and May (1989) proposed that blocks of human (CA)<sub>n</sub> repeats may exhibit length polymorphism based on other examples in which the number of repeats within a tandem repeated DNA block varies among the individuals of a species, as is the case for vertebrate minisatellites (Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987), for human alpha-satellite DNA (Willard *et al.*, 1986; Jabs *et al.*, 1989), for color vision genes in man (Motulsky, 1988), and for tandem repeats within malaria parasite antigen genes (Kemp *et al.*, 1987; Weber, 1988).

With the introduction of PCR, analysis of variations in length in simple tandem repeated DNA sequences became viable (Weber, 1990). The use of PCR to detect DNA polymorphisms provides increased sensitivity and rapidity when compared to standard Southern blotting and hybridization.

A marker at the 3' end of the dystrophin gene has been independently described by Beggs and Kunkel (1990) and Oudet *et al.* (1990). Four other CA repeats were later described by Feener *et al.* (1991) in the 5' region around the brain promoter. More recently, the Houston group (Clemens *et al.*, 1991) described four of the six loci identified by them, also of the short tandem repeat (STR) type, characterized by polymorphisms of the CA repeats within introns 44, 45, 49 and 50 of the human dystrophin gene. Since this region is subject to deletion, the new PCR markers have become valuable for the detection of such deletions (Clemens *et al.*, 1991). Furthermore, like the remaining (CA)<sub>n</sub> loci previously identified in the dystrophin gene, they could improve the accuracy of linkage studies and, consequently, of carrier detection and prenatal diagnosis of DMD/B.

## MATERIAL AND METHODS

Nine DMD families ascertained through our University Hospital were studied in order to detect carriers of the abnormal dystrophin gene. We assumed that the locus Xp21 was involved in the MD condition in all the families, although in some cases, for example in family 48, we cannot exclude the minor risk that the MD condition was due to the autosomal recessive form (Duchenne-like). In all but two of these families (194 and 197), the patient was an isolated case, i.e., even though he may have had an affected brother, there was no indication of other generations being affected. In all but one family (family

48) the patients had been studied by PCR and Southern blotting, followed by hybridization, presenting no deletion or duplication (Falcão-Conceição *et al.*, 1992a,b). In two of these families (48 and 164), the patient died but there was a normal brother whose study has permitted us to infer the genetic constitution of the patients in relation to the markers used.

The levels of creatine kinase (CK), pyruvate kinase (PK) and hemopexin (H) of the women at risk to be carriers had been previously determined in our laboratory (Gonçalves-Pimentel *et al.*, 1988; Falcão-Conceição *et al.*, 1988). We looked for a possible coherence between the elevated CK data obtained for some obligatory carrier women and the haplotypes that could indicate the anomalous chromosome.

Carrier detection was based on linkage analysis, using PCR markers of the (CA)<sub>n</sub> type. There is considerable distance between markers of the 5' and 3' end of the dystrophin gene (recombination rate is on the order of 12%). On this basis, we utilized two markers of the 5' end -5'DYSI and 5'DYSIII (Feener *et al.*, 1991), and one of the central gene region, i.e., STR 49 (Clemens *et al.*, 1991).

The informativeness for each marker, as determined by the Authors, is:

	Number of Alleles	% Heterozygous
5'DYSI	5	78.6
5'DYSIII	4	51
STR 49	19	93.3

The six primers used were synthesized and the kits prepared in Leiden. Markers 5'DYSI and 5'DYSIII were used in the same reaction.

PCR was performed essentially according to the method of Saiki *et al.* (1988). One half µl of genomic DNA (100-200 ng/µl) was mixed with 15 pmol of each PCR primer in a total volume of 15 µl, containing 10X PCR buffer, 10X dNTP stock (2 mM dGTP, 2 mM dTTP, 2 mM dATP, 25 µM dCTP) and X µl of sterile water. Radioactive PCR was performed with 1 µl 1 in 10 diluted 32 P α dCTP (3000 Ci/mml Amersham). We used 0.06 Units of Super Taq polymerase (HT Biotechnology Ltd.). Finally, we sealed the mixture with two drops of mineral oil (Sigma). The reaction was carried out in a Perkin Elmer thermocycler as follows: after denaturation at 94°C for 5 minutes, 23 cycles of DNA denaturation (94°C for 45 seconds), annealing (1 minute 53°C) and polymerization (65°C for 2 minutes) are performed. The final 65°C incubation was extended to 5 minutes. Aliquots of the PCR products were mixed with one volume of formamide dye solution (95% formamide, 20 mM EDTA, 0.05%

bromophenolblue and 0.05% xylenecianol). Electrophoresis was performed on a 8% denaturing polyacrylamide gel for 3 hours at 65 watts. The gel was fixed in 5% methanol and 5% acetic acid. After drying the gel, autoradiography was performed for 3-12 hours.

## RESULTS AND DISCUSSION

With 5'DYSI and 5'DYSIII, as expected, five and four alleles were detected, respectively. With respect to 5'DYSI, five of the nine unrelated patients exhibited the 179-bp allele (allele D in our nomenclature), which indeed was the most frequent in the population studied by Feener *et al.* (1991). With respect to 5'DYSIII, the 223-bp allele (B), the most frequent in the population studied by Feener *et al.*, was detected in three of the nine unrelated patients studied.

The length (in bp) of (CA)<sub>n</sub> alleles of the markers used, as related by Feener *et al.*, 1991, (see our Figures 1-5) are:

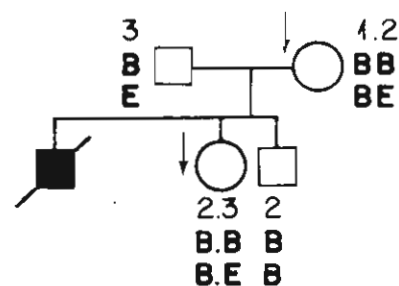
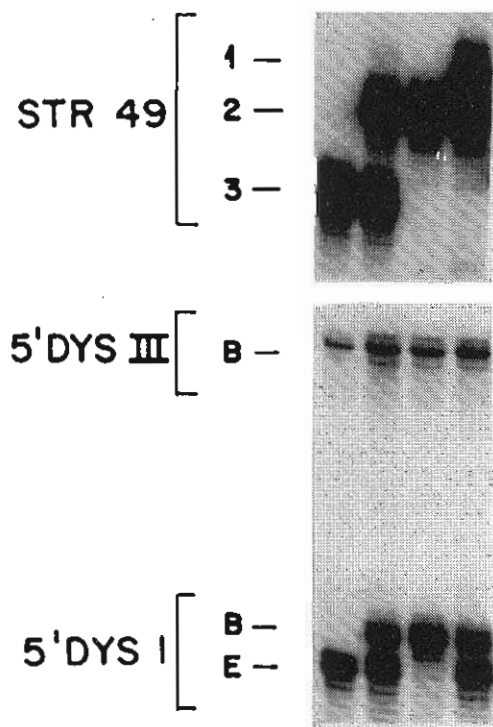
5'DYSI		5'DYSIII	
Alleles	bp	Alleles	bp
A	185	A	225
B	183	B	223
C	181	C	221
D	179	D	219
E	177		

Since some of the STR 49 amplifications were not successful we had to use a different nomenclature for the alleles found, without identifying their length in bp, as made by Clemens *et al.* (1991).

In view of the singularity in the structure of each family, the families will be discussed one by one.

*Family 48 had a sporadic case who died without a DNA study (Figure 1)*

In addition to the patient there was a normal brother. The mother and an adult sister had normal CK levels (mean = 4.5 Sigma Units (SU) in both; normal, mean + s = 3.68 + 2.23 SU). The mother was informative for two of three microsatellites (5'DYSI:BE and STR 49:1,2). If we assume the mother to be a carrier of the X-linked DMD gene and we consider the two informative microsatellites, the patient's sister is not a carrier because she inherited the same X-chromosome as the normal brother. The chance of a double cross over between 5'DYSI and STR 49 is less than 1%. However, if we consider the mother not to be a



*Family 48*

Figure 1 - Autoradiograph showing alleles at three (CA)<sub>n</sub> loci in the human dystrophin gene for members of a DMD family in which the patient is deceased. ↓ Normal CK levels.

carrier, there still will be a 7% risk for the sister to be a carrier because the mother could be a germinal mosaic (Bakker *et al.*, 1989).

We cannot exclude the minor risk that the MD in this patient is caused by the autosomal recessive gene. Because he died many years ago there was no muscular biopsy material available. However, the risk for the sister of patients to be carriers is also 50%, though the risk for affected sons is negligible relative to the risk in the case of the X-linked form. This latter risk was therefore investigated.

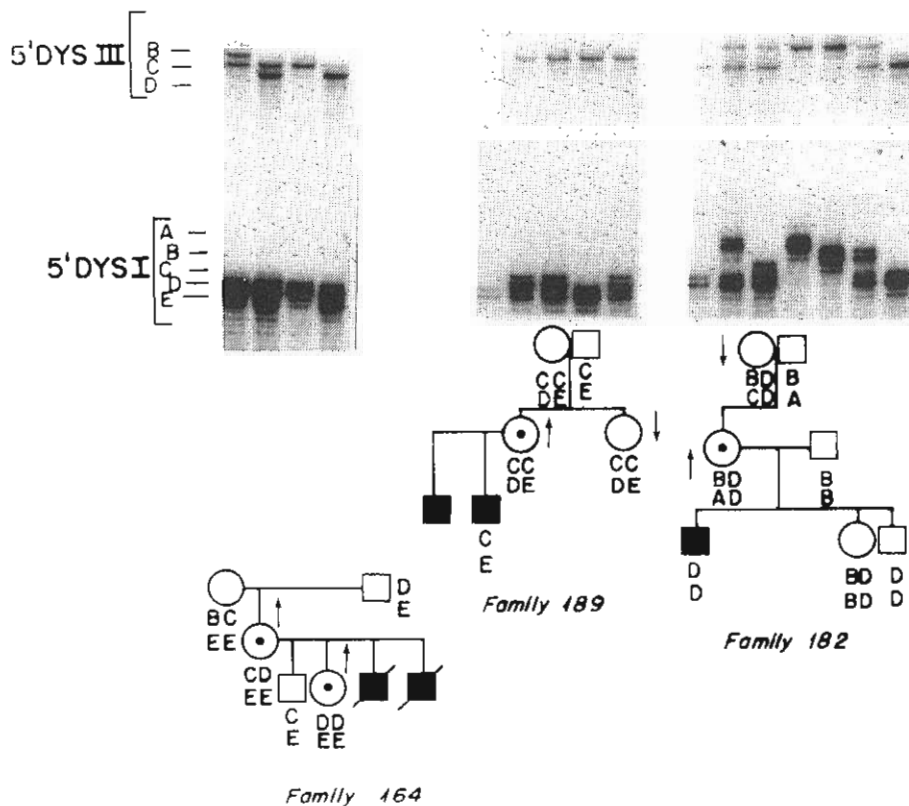


Figure 2 - Autoradiograph showing alleles at two (CA)<sub>n</sub> loci in a human dystrophin gene for members of three families in which the mothers are probable (164, 189) or possible carriers (182). O, Obligatory carrier; ↑, CK levels at the upper part or outside the normal range; ↓, normal CK levels.

### New mutations

In three families (164, 189 and 182, Figure 2), the patients were isolated cases, i.e., there was no indication of other affected generations. In all three families, the mutation in the mother's X-chromosome seems to be a new mutation, within one of her X-chromosome's or within the X-chromosome of the healthy grandfather, as was concluded on the basis of the haplotypes of the two maternal grandparents which were determined, or inferred, by use of the microsatellites. The carrier condition of the mothers in families 164 and 189 was strengthened by the existence of more than one affected son and by CK levels at the upper part of the normal range (mean = 7.3 and 7.0 SU, respectively) or by increased CK levels (mean = 12.0 SU) in family 182.

In family 164, whose patients are deceased, study with the STR 49 marker (data not shown) confirmed the hypothesis that neither X-chromosome received by the patient's sister, a supposed carrier (haplotype differing from that of the normal brother and CK levels at the upper part of the normal range, mean = 6.9 SU), originated from the maternal grandmother.

In family 189, the mother and a maternal aunt have the same genetic constitution with respect to 5'DYSIII and 5'DYSI (CCDE), but only the mother is likely to be a carrier. The CK levels of the maternal aunt were normal (mean = 2.0 SU). The mother, in addition to having CK levels at the upper part of the normal range (mean = 7.0 SU), had two affected sons. It is possible that the anomalous chromosome X with haplotype C;E came from the maternal grandfather. This was also indicated by the study with STR 49 (data not shown).

In family 182, the patient, the normal brother and the sister inherited the same haplotype for the markers 5'DYSIII and 5'DYSI (D;D). However, the study with the STR 49 marker (data not shown) demonstrated that the patient's allele differed from that of the two siblings. These, however, have the same allele, which points to the occurrence of crossing over between the 5' end and the region of the STR 49 marker in the mother. Since the mother had increased CK levels (mean = 11.0 SU) she must be a carrier, possibly due to a mutation in the grandfather. We assumed that the patient's X was the recombinant. Thus, the segment containing the mutation probably originated from the maternal grandfather who had normal

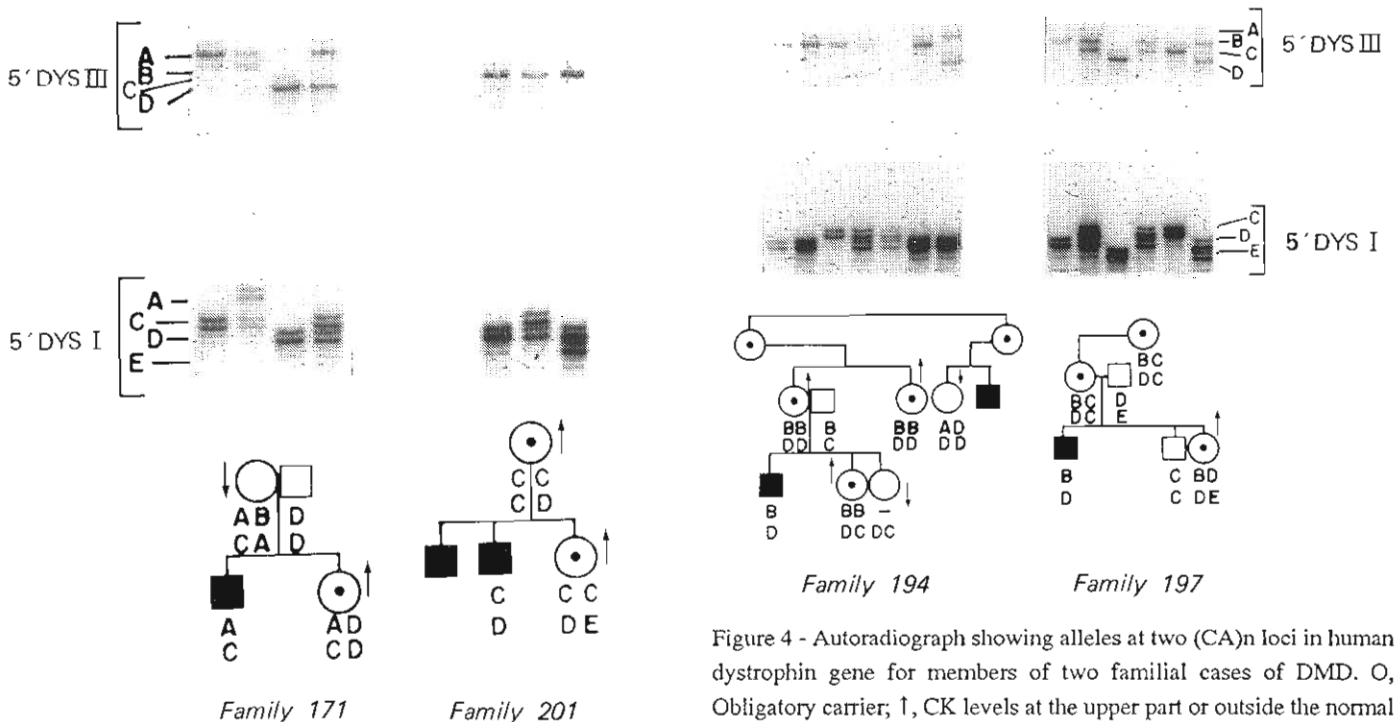


Figure 3 - Autoradiograph showing alleles at two (CA)<sub>n</sub> loci in human dystrophin gene for members of DMD families. O, Obligatory carrier; ↑, increased CK levels; ↓, normal CK levels.

CK levels (mean = 2.0 SU, normal mean + s = 10.08 + 4.10 for adult men) and showed no indications of being a mosaic. The maternal grandmother had normal CK levels (mean = 4.25 SU).

#### Mosaic or non-mosaic?

The patient in family 171 (Figure 3) was an isolated case. His adult sister had very high CK levels (mean = 32.0 SU) and therefore was a carrier. The mother was informative for the markers 5'DYSIII and 5'DYSI (AB and CA). The carrier status of the sister was coherent with the fact that she had the same A;C haplotype as the patient. The same applied to the STR 49 marker (data not shown). Since the mother had normal CK levels (mean = 2.0 SU), there are two hypotheses: either she was a carrier in which the lyonization phenomenon is of the non-random type, or she was a germinal mosaic. Since the patient had no deletion, it would be impossible at this time to clarify whether she was a germinal mosaic.

In contrast, the situation of family 201 was quite clear. The patient was an isolated case because there were no other affected generations. There was an affected brother and the patient's mother and adult sister had high

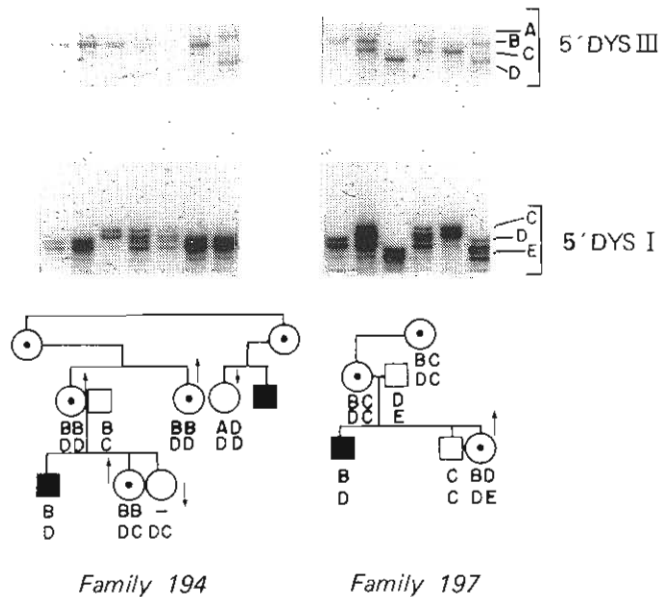


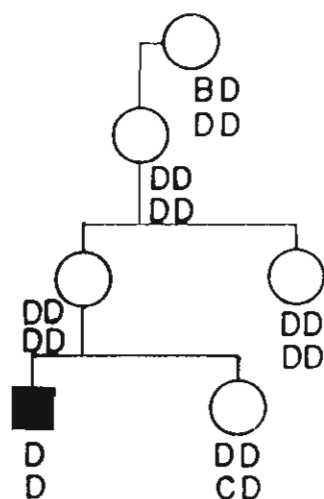
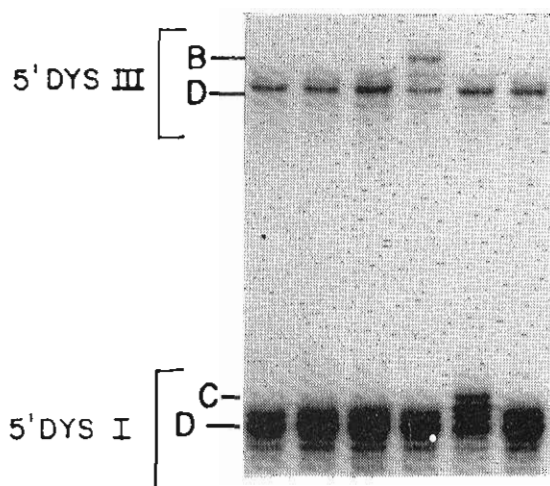
Figure 4 - Autoradiograph showing alleles at two (CA)<sub>n</sub> loci in human dystrophin gene for members of two familial cases of DMD. O, Obligatory carrier; ↑, CK levels at the upper part or outside the normal range; ↓, normal CK levels.

CK levels (mean = 37.0 and 19.0 SU, respectively). The mother was informative for 5'DYSI (CD). The C;D haplotype present in the patient, his mother and his sister reflected an X chromosome with the mutation. The information provided by the STR 49 marker (data not shown) was coherent.

#### Familial cases (families 194 and 197, Figure 4)

In family 194, the mother, an obligatory carrier, was not informative for either marker at the 5' end. CK levels were increased not only in the mother (20.5 SU) but also in the maternal aunt (10.5 SU) and in one of the young sisters (182.0 SU); premenarchal normal: mean + s = 10.87 + 3.49 SU. All of them inherited the same B;D haplotype which was present in the patient. A female cousin had normal CK levels and a different haplotype (A;D).

In family 197, the patient's mother and grandmother were informative for both markers 5'DYSIII and 5'DYSI. The B;D haplotype, which was present in the patient, was also present in the mother, the grandmother and a sister. The CK levels were elevated: mean = 19.0 SU in the premenarchal sister, and at the upper part of the normal range: mean = 8.5 SU in the mother and 9.5 SU in the grandmother. The normal brother had the haplotype C;C. A study of STR 49 (data not shown) was coherent and did not indicate the occurrence of crossing over.



Family 198

Figure 5 - Autoradiograph showing alleles at two (CA)n loci in human dystrophin gene for members of a DMD family.

### No possibility to clarify

In family 198 (Figure 5), the patient was an isolated case. The mother and grandmother were not informative with respect to the two markers and both had CK levels at the upper part and outside the normal range (8.5 and 10.0 SU, respectively). The maternal aunt had the same genetic constitution as the mother in terms of the 5'DYSI and 5'DYSIII markers. Study with the STR 49 marker (data not shown) revealed that the patient's maternal aunt and sister were heterozygous and had inherited the same allele as the patient. Both had CK levels at the upper part of the normal range (6.5 SU). It was impossible to clarify this because the mother was not

informative with respect to the two markers and her CK levels were at the upper part of the normal range.

We conclude that (CA)n loci are excellent markers in face of their high informativeness, sensitivity and rapidity of assay. For detection of carriers of the anomalous dystrophin gene, it is absolutely necessary to use intragenic markers both from the 5' and the 3' end of the gene in face of the 12% intragenic recombination rate (Abbs *et al.*, 1990).

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## RESUMO

Investigamos nove famílias DMD brasileiras com pacientes sem deleção ou duplicação, por análise de haplótipos usando marcadores microsatélites do tipo (CA)n. Nosso objetivo foi detectar portadoras do gene anormal da distrofina entre mulheres sob risco. Assim, admitimos que o locus Xp21 esteja envolvido no condicionamento da DMD em todas as famílias aqui estudadas. Buscando informatividade, usamos dois marcadores (CA)n da extremidade 5' do gene da distrofina, i.e., 5'DYSI e 5'DYSIII (Feener *et al.*, *Am. J. Hum. Genet.* 48: 621-627, 1991) e, em vista da alta taxa de recombinação intragênica, usamos, paralelamente, um marcador (CA)n do intron 49, i.e., STR 49 (Clemens *et al.*, *Am. J. Hum. Genet.* 49: 951-960, 1991). Em apenas uma das nove famílias estudadas não houve informatividade suficiente para indiciar o cromossomo X sob risco. Houve coerência absoluta entre os níveis de creatino-cinase (CK) aumentados e a condição genética de algumas mulheres avaliadas como portadoras. Em três de sete famílias de casos isolados foi possível definir novas mutações originadas no avô materno. Foi possível detectar um crossing over no intervalo 5'-intron 49 em uma das nove mães de pacientes.

Concluimos que os loci (CA)n são marcadores excelentes, dada sua alta informatividade. A sensibilidade e a rapidez de ensaio pela PCR fazem-nos únicos quando comparados com outros marcadores ensaiados por Southern blotting, seguido de hibridização. Com relação à detecção de portadoras do gene anormal da distrofina é absolutamente necessário usar marcadores tanto das extremidades 5' e 3' do gene como das regiões central/distal (3'), em face da taxa de 12% de recombinação intragênica (Abbs *et al.*, *Genomics* 7: 602-606, 1990).

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