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CARRIER DETECTION BY DNA ANALYSIS IN DUCHENNE MUSCULAR DYSTROPHY FAMILIES*

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We applied DNA analysis techniques to Turkish families whose members were afflicted with Duchenne / Becker muscular dystrophy. The aim of this study was to establish a prenatal diagnosis of this anomaly and to determine the carrier state.

All of the techniques used in established diagnosis centers are now applied routinely in our laboratory. Both Southern analysis and polymerase chain reaction (PCR) methods were used for deletion detection in patients and restriction enzyme fragment length polymorphism (RFLP) determination for linkage analysis in women at risk. CA repeated sequence length polymorphism, the most recent technique for linkage analysis, was also applied.

About 250 individuals from seventy - nine families were investigated and thirty - six entire families were screened. Twenty - five women were found to be carriers while thirty seven were non - carriers. The carrier state could not be determined in three women. *Key words: Duchenne muscular dystrophy, polymerase chain reaction, restriction enzyme fragment length polymorphism, deletion, Southern analysis.* ·

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease, which affects about one in 3,400 males. Boys afflicted with this X - linked recessive disorder become nonambulatory at around age ten and die at about age twenty. Becker muscular dystrophy (BMD) is the milder and rarer form of the disorder in which nonambulation occurs much later in life. Both disorders are caused by mutations in the dystrophin gene[1,2].

In families with DMD, high levels of lactic dehydrogenase (LDH) and creatine phosphokinase (CPK) enzymes indicate the carrier state in women. However,

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about 30 percent of all carriers have normal levels of these enzymes. Therefore, DNA analysis should be carried out to determine the carrier state in all women at risk. It is then possible to perform prenatal diagnosis using the same techniques. In addition, the use of DNA analysis techniques allows for early diagnosis in about 60 percent of patients.

DNA analysis techniques that have been developed in the last five years are routine in our laboratory and are applied to DMD families. A variety of methods are employed in order to identify carriers related to both familial and sporadic cases. We are currently examining seventy - nine affected families. In this study, the strategies that have been developed for carrier detection and prenatal diagnosis are discussed and examples of interesting cases are presented.

A commonly accepted strategy for DNA analysis of this disorder circumvents problems arising from the unique features of the gene, such as its very large size and great variety of mutations. Dystrophin gene mutations are different in each DMD family and can be observed as large mutations (mostly deletions and some duplications) in 60 - 70 percent of patients via the commonly used DNA analysis techniques. Identification of the deletion reveals the site of the mutation, enabling linkage analysis to be performed more precisely. This is because in such a large gene, the probability of crossing - over in each meiosis is about 5 percent. Moreover, the DNA of a female carrier may be assayed for the presence of a particular deletion in one of her X - chromosomes. For these reasons, deletion detection simplifies carrier determination to some degree. This direct method assures a fast, simple prenatal diagnosis. However, in 30 - 40 percent of DMD families, the mutation is not detectable. Thus for carrier testing and prenatal diagnosis in such families, linkage analysis determines whether or not the DMD allele has been inherited.

The strategy for DNA analysis in DMD families may be summarized as follows:

A) Deletion Detection in Patients:

Either the Southern analysis or the polymerase chain reaction may be used. The first method detects deletions in about 60 - 70 percent of patients[3]. Ninety - eight percent of these deletions are detectable by the latter method which is fast and lower in cost[4,5].

1. Southern Analysis:

This technique detects specific DNA fragments (generated by restriction enzymes) using of complementary DNA (cDNA) probes. The probes themselves are part of the fragment to be detected. The method may be summarized as follows: a patient's DNA sample is cleaved by a restriction enzyme and subjected

to agarose gel electrophoresis to separate the fragments according to size. The fragments are then denatured, transferred onto a membrane and fixed. A radioactively labeled probe specific for the fragment is hybridized to the DNA samples on the membrane and the target fragment (s) is identified after autoradiography on X - ray films. A total of eight cDNA probes (specific to exons of the dystrophin gene) are used to screen the entire gene. Detection of the absence of a fragment in a patient is interpreted as a deletion. The first probe using Southern analysis takes at least eight days and four days are required for each additional probe.

2. Amplification by polymerase chain reaction (PCR):

The multiplex PCR technique was first applied in DMD in 1988 and was shown to detect about 50 percent of the deletions detectable by Southern analysis[4]. Recently, a second assay (multiplex II) has been developed[5]. Both assays together detect about 98 percent of all deletions and require only two days for results. PCR assays can be used in deletion detection in patients and in a fetus at risk because they are simple, fast, do not require radioisotopes, and need only 5 percent as much DNA as the Southern assay. However, all deletions detected by PCR should be confirmed by Southern analysis, which is completely reliable.

In each multiplex assay, nine pairs of primers, members of each pair being complementary to the opposite DNA strands, are used to amplify nine regions of differing lengths, each in a different exon commonly deleted in DMD patients. The fragments are amplified by 10^5 - 10^6 - fold in a single reaction and visualized after gel electrophoresis. Failure of amplification of one or more exons indicates a deletion.

B) Deletion Detection in Carriers:

Whenever a deletion is identified in a patient, DNA samples of women at risk in the family may be analyzed for the presence of a particular deletion by dosage analysis, even though the assay is complicated by the existence of two X - chromosomes. Alternatively, the mutation can be directly identified if a junction fragment is observable.

1. Dosage Analysis:

A female's DNA sample is subjected to Southern analysis using the same probe that detects the deletion in the related patient. A deletion in one of her X - chromosomes results in autoradiographic bands with half the intensity.

2. Junction Fragment Detection:

A junction fragment arises as a result of fusion of the two DNA fragments flanking the deletion. Such a fragment acquires an abnormal size as compared to a standard. It is this feature that renders analysis very simple: the mutation can be

identified by a single analysis in all females at risk in the family. Unfortunately, junction fragments are rarely detectable by cDNA probes.

C) Linkage Studies to Determine Inheritance of DMD Allele:

The most commonly applied technique comprises the use of restriction enzyme length polymorphisms (RFLP) which arise as a result of differences in DNA sequence in the noncoding regions of the gene. An RFLP does not change the sequence of the gene product (protein), but may be used to distinguish between the two alleles of the gene in the mother. Analysis in affected and unaffected boys in a family will reveal which allele is linked to DMD in that particular family. Intergenic RFLPs are detectable by some probes specific to introns and by some of the cDNA probes. In order to minimize the uncertainty in diagnosis due to the probability of crossing - over, the large size of the gene necessitates that at least three RFLPs be used: one intergenic and two which flank the gene. In cases where the site of deletion is known, linkage analysis using only two probes that flank the deletion is sufficient.

For prompt detection, the PCR technique can be used for three RFLP analysis: pERT87 - 8 (TaqI), pERT87 - 15 (BamHI) and pERT87 - 15 (XmnI)[6]. A region containing the restriction enzyme site is amplified, digested by the restriction enzyme and assayed via gel electrophoresis.

Recently the PCR technique has also been applied to another type of polymorphism: CA repeated - sequence length polymorphisms. Two such sequences are defined for the dystrophin gene: one is at the 3' end while the other is at the 5' end[7,8]. Because the lengths of the CA repeated sequences vary in different X - chromosomes, the alleles may be directly analyzed via gel electrophoresis after amplification. These polymorphisms are very useful since few RFLP assays exist in these regions.

Material and Methods

Seventy - nine unrelated patients diagnosed as having DMD or BMD in the Neurology Department of İstanbul University Faculty of Medicine, and 175 of their family members were enrolled in this study. Diagnosis was based on pedigree data, clinical examination, elevated serum creatine kinase values and electromyograms. In some patients, the muscle biopsy histopathology was also determined.

Plasmids containing the probe insert were grown in *E.coli* HB101 strain and isolated by the alkaline lysis method. The insert was cleaved out using the appropriate restriction enzyme and separated from the plasmid vector by agarose gel electrophoresis. The gel piece containing the probe fragment was then subjected to electrophoresis in closed dialysis tubing and the probe was recovered.

DNA was isolated from 10 ml peripheral blood samples. Cleavage of DNA by restriction enzymes and the Southern transfer were carried out essentially as previously described[9]. Radiolabeling was performed by the random - priming technique. 50 ng of probe DNA was labeled using 40 μCi ^{32}P - dCTP in 30 μl of reaction volume. The hexanucleotide primers were supplied by Dr. Linné; ^{32}P - dCTP was supplied by Amersham at a specific activity of 3.000 Ci / μg. The percentage of incorporation was determined after TCA precipitation. The whole reaction mix was used for hybridization without prior separation of unincorporated ^{32}P - dCTP typically in a volume of 20 - 40 ml. Autoradiography took 1 - 4 days at −70 °C with Kodak Xomat XAR film and Dupont lighting plus or Quanta III screens.

When the DNA samples on the membrane were to be reprobed, the previously hybridized probe was removed in 0.4 N NaOH and the membrane was neutralized in 0.2 x SSC and 0.1 % SDS. Labeled probes in hybridization buffer were occasionally reused after denaturation (100 °C for 10 minutes).

Multiplex assays were performed as described by Chamberlain et al[4], except that only two temperatures were employed in the amplification cycles: four minutes at 60 °C for annealing and elongation and one minute at 92 °C for denaturation. After 30 cycles, fragments were separated on a 2 % NuSieve, 1 % agarose gel. Primers were supplied by Dr. P. Ray and Dr. T. Friedman and Taq polymerase by Cetus and Boehringer.

PCR for RFLP analyses, CA repeat polymorphisms and the Y - chromosome sequence were performed in three steps: one minute at 52 °C for annealing, two minutes at 72 °C for elongation and one minute at 92 °C for denaturation. For RFLP analysis, 15 μl of the reaction mix was digested with the appropriate restriction enzyme in 20 μl of digestion buffer containing 10 units of the enzyme. pERT87 - 15 (XmnI) digestion products were assayed on 1 % agarose gel and the 3' CA repeat polymorphisms on 15 % acrylamide gels. All other assays were on 3 % NuSieve, 1 % agarose gel.

For isolation of DNA from the chorionic villi, the sample was first washed in TE and resuspended in 0.45 ml SE. SDS was added to 1 %, proteinase K to 1 mg/ml and the sample was incubated at 56 °C for two hours. DNA was precipitated by the addition of two volumes of ethanol after phenol - chloroform (1:1) extraction (twice) and chloroform extraction (twice). DNA was dissolved in 0.2 ml of TE. 20 - 30 mg villi yielded about 100 μg of DNA.

Results

In our studies with DMD families in Turkey, both deletion detection and RFLP analyses were carried out simultaneously. In cases where a deletion was detected, DNA samples of women at risk were analyzed for linkage in regions

flanking the deletion; some were also analysed for dosage. In cases where no deletion was detectable, RFLP linkage analysis was carried out: three polymorphisms (one intergenic, two flanking) were used for sporadic cases whereas two intergenic polymorphisms were sufficient for familial ones to establish an accurate diagnosis with all certainty. All of the above - mentioned DNA analysis techniques can now be routinely applied in our laboratory, where all the results described in this work were obtained.

A) Mutation Screening:

1. Southern Analysis:

Eight different cDNA probes were employed spanning only the first 9 kilobase pairs of the protein coding region of the gene, since no deletion had been reported at the outset in the remaining 5 kilobase pairs. A DNA sample of each patient was cleaved separately with two different restriction enzymes to obtain accurate results (Fig. 1). Of seventy - nine unrelated patients, forty - seven (59.5 %) were observed to have deletions.

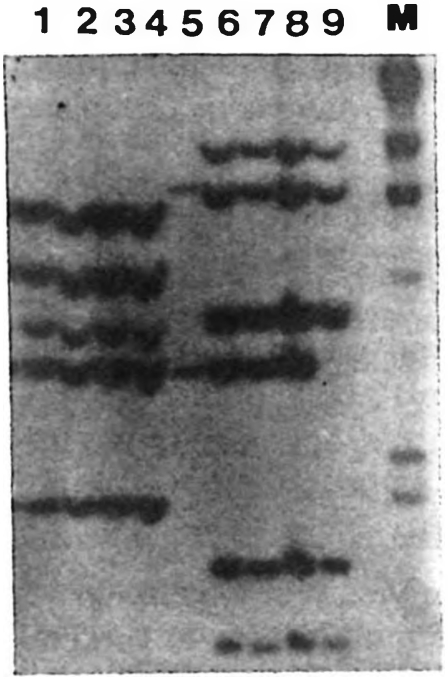

Fig. 1: Southern analysis of different patient DNA samples using the probe cDNA8. Samples 1-5 were digested with the restriction enzyme Taq^{I}, 6-9 with Hind III. There is a large deletion in sample 5, spanning fragment 1, 3, 5 and 6. The last lane is the lambda DNA/Hind III size marker.

Women at risk were screened for the deletion carried by the patient in the family. A decrease in dose in autoradiographic bands as a result of a deletion in one of the X - chromosomes could be detected upon careful observation (Fig. 2). Of the eighteen women included in dosage analysis, ten had a pattern similar to the standard; the others were carriers. In a few samples, band intensities were also determined by a density scanner.

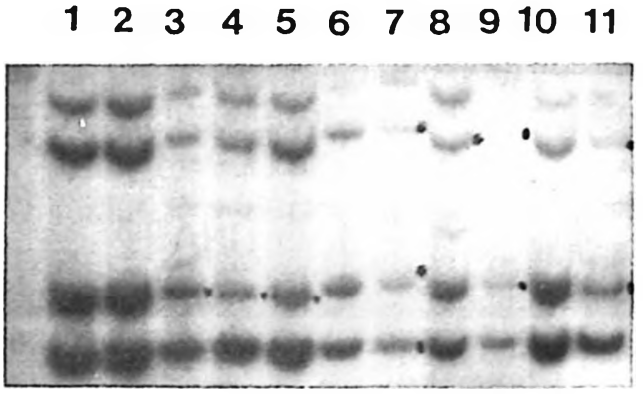

Fig. 2: Dosage analysis for DNA samples of women at risk, using the probe cDNA8 and Hind III. Samples 1 and 5 are standards. A decrease in band intensity relative to the control pattern was observed insamples 3, 4, 5, 7, 8, 9 and 11 as marked to the right of the bands.

2. Polymerase Chain Reaction Analysis:

DNA samples from seventy - nine patients were amplified using multiplex I and multiplex II primers. An example is shown in Fig. 3. Deletions were detected in forty-five patients (57 %) after agarose gel electrophoresis was performed.

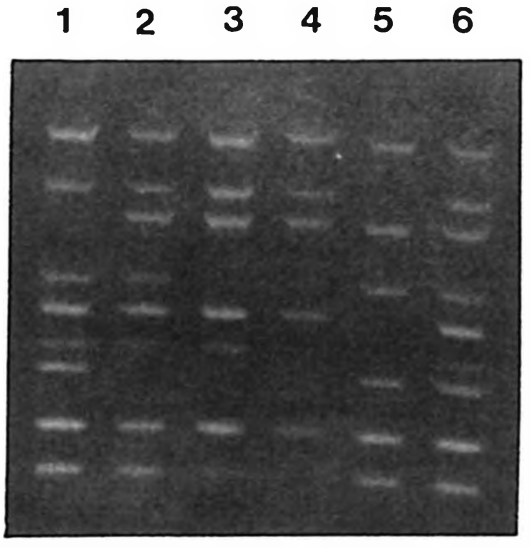

Fig. 3: Multiplex II amplification products of different samples. The last sample is an unaffected male, all others exhibit deletions corresponding to one or more regions in the gene.

B) Junction Fragment Detection:

In deletion screening of patient DNA samples with cDNA probes, some fragments, not of the expected size, were observed. These junction fragments were detected in six patients and were used to perform carrier analysis in women at risk. Out of nineteen women assayed, the junction fragments were present in six, indicating the carrier state; thirteen were non - carriers.

C) Linkage Analysis:

The mothers of DMD boys were screened for RFLP using five intergenic probes

(pERT87 - 1, pERT87 - 8, pERT87 - 15, XJ1.1 and XJ2.3), two flanking (754 and 99.6) and one cDNA probe (cDNA8). Probes pERT87 - 15 and cDNA8 were used to assay two RFLPs each. RFLP sites are shown in Fig. 4 and an example of RFLP detection in Fig. 5.

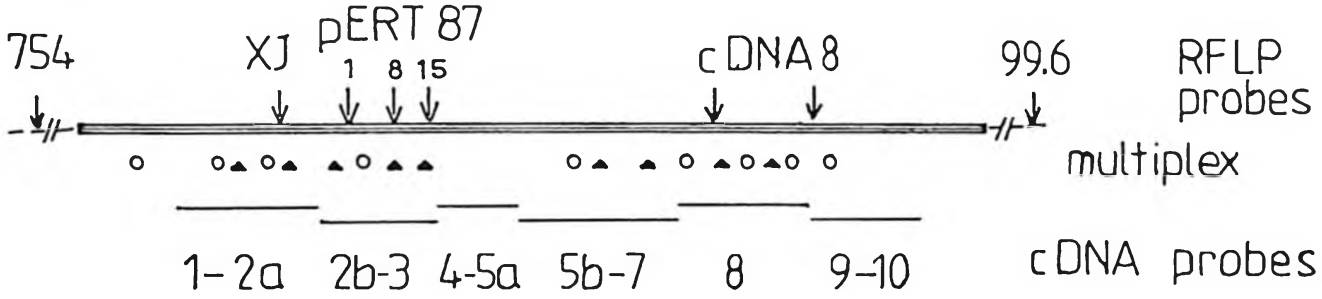

Fig. 4: Map of the dystrophin gene showing the locations of the probes detecting RFLP ( ), the regions spanned by the cDNA probes (—) and regions amplified by multiplex (▲) and multiplex II (o).

Fig. 5: An example of RFLP detection in 18 unrelated females using the probe 754 and the restriction enzyme PstI. Females exhibiting both bands are heterozygous for the RFLP.

Not all the mothers were assayed for all of the ten RFLPs, but the assay was terminated whenever:

1. Three RFLPs (one intergenic, two flanking the gene) were detected.
2. Two RFLPs flanking the mutation were detected.
3. One RFLP close to the mutation was detected in the familial cases.
4. A junction fragment was detected.

Among the RFLPs for which the mother was found to be heterozygous, the most suitable ones were chosen to be used in the analysis of all family members. The allele associated with DMD was determined and the carriers were identified. Interesting families are described individually to demonstrate the analyses.

Family I : The patient was reported to be a sporadic case, and no deletion could be detected either via multiplex PCR assays or by hybridization to cDNA probes. The mother was found to be heterozygous, having one intergenic and two flanking probes. Haplotypes were determined in five members of the family. The maternal grandfather's haplotype had to be deduced since his blood sample was not available (Fig. 6 a). The patient was shown to have inherited one of the grandmother's alleles, while the sister had inherited the grandfather's. Therefore, the sister was not a carrier. The data did not rule out inheritance of DMD from the grandmother.

Family II : The patient was reported to be a sporadic case, and no deletion was detected either via multiplex PCR assays or by hybridization to cDNA probes. The

father's blood sample was not available, thus the two sisters' common allele was assumed to have been inherited from the father (Fig. 6 b). Sister 2 had the same maternal allele as the patient, but sister 1 had the other, and was therefore definitely not a carrier. Sister 2 would be a carrier if the mutation was carried by the mother. Since the mutation was not defined, she should be considered a carrier.

Family III : The patient was reported to be a sporadic case and a deletion was detected with the probe cDNA 1 - 2 a. The mother was found to be heterozygous for two probes that closely flanked the deletion. Analysis of the family revealed that the sisters inherited a different maternal allele than the patient, thus they were definitely not carriers (Fig. 6 c).

Family IV : The patient was reported to be a sporadic case and a deletion was found in the cDNA8 region. Blood samples from only six of eleven members of the family were available. The mother was heterozygous with only the probe cDNA8 (PstI), which is very close to the mutation. The three sisters' common allele was assumed to be the paternal allele (Fig. 6 d). Sisters 2 and 5 inherited the same maternal allele as the healthy brother, so they were definitely not carriers.

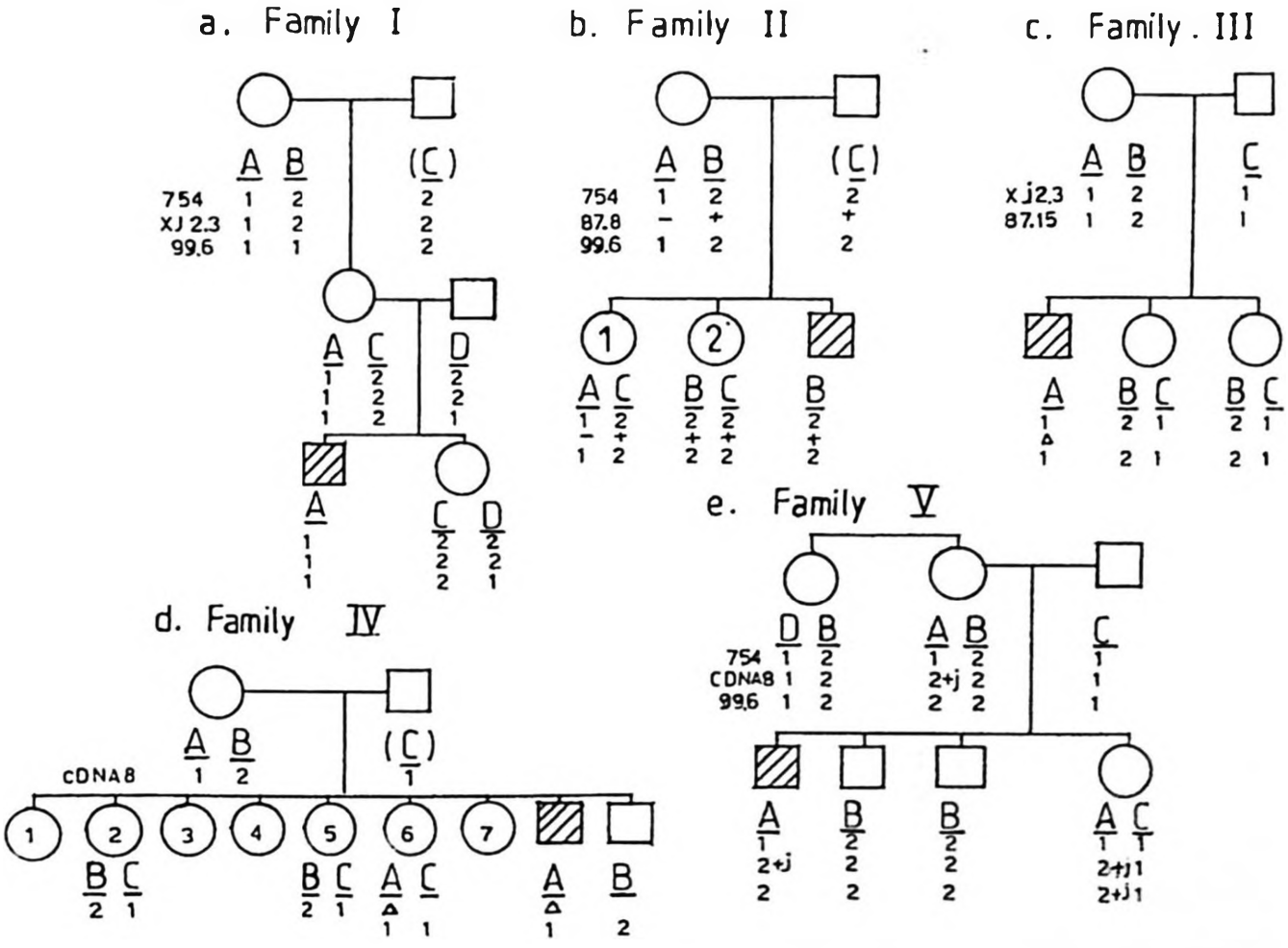

Fig. 6 a, b, c, d, e: Analysis in families I-V using various, probes. Δ indicates a deletion and J a junction fragment. Father's allele if deduced is indicated by parenthesis.

Sister 6 inherited the same maternal allele as the patient. Dosage analysis with the densitometer revealed that she carried the deletion, so did the mother, indicating that the mutation had occurred before the mother's. The densitometric analysis was the determining factor for this sister since the case was known to be sporadic.

Family V : Since the patient's maternal uncle also had DMD, both the sister and the maternal aunt sought carrier analysis. No deletion could be detected in this patient; moreover, he seemed to be heterozygous for the probe cDNA8. However, his DNA sample could be amplified using Y - chromosome specific primers via the PCR technique, indicating that the sample had not been mixed up with a female's Southern analysis after prolonged electrophoresis revealed that one polymorphic fragment was actually not the same size but smaller than the larger polymorphic allele, and so was identified as a duplication junction fragment. Both mother and sister were found to carry the junction fragment, and therefore were carriers, while the aunt did not have it, thus was not a carrier (Fig. 6 e). Probes cDNA8 and 99.6 also confirmed that she had inherited a different maternal allele.

Family VI : The patient was reported to be a sporadic case and the gene was totally deleted in the probe cDNA8 region. The mother was not heterozygous with either of the two RFLP assays in the same region: cDNA8 (PstI) and cDNA8 (Taq I). However, the maternal aunt was heterozygous with probe cDNA8 (PstI), indicating that she carried no deletion (Fig. 7 a). Thus she, and consequently her three daughters, were not carriers. Expectedly, the sister of the patient was "homozygous" for the probe: her alleles were either 3.4 / 3.4 or deletion / 3.4. The family was further analyzed with another probe (pERT87 - 15) for which the mother was heterozygous, and it was shown that the sister had inherited a different maternal allele than the patient's. She was considered to be a carrier with only 5 % probability (probability of crossing-over) since the mutation was rather distant from the pERT87 - 15 region.

Family VII : The two brothers were the only ones known to be afflicted in the family. No deletion could be detected in their DNA samples. The mother was heterozygous for only probes XJ1.1 and cDNA8P. The sisters were assayed and shown to have the same maternal allele with the first probe but different ones with the second, revealing a crossing - over event (Fig. 7 b). The first sister was not a carrier because she had inherited a maternal allele that was different from that of her brothers'. Whether the second sister was a carrier or not was not determined since the relative locations of the crossing - over and the mutation could not be determined.

Family VIII : This family was one of the most difficult to be completely screened. The two brothers were the only ones known to be afflicted in the family and no

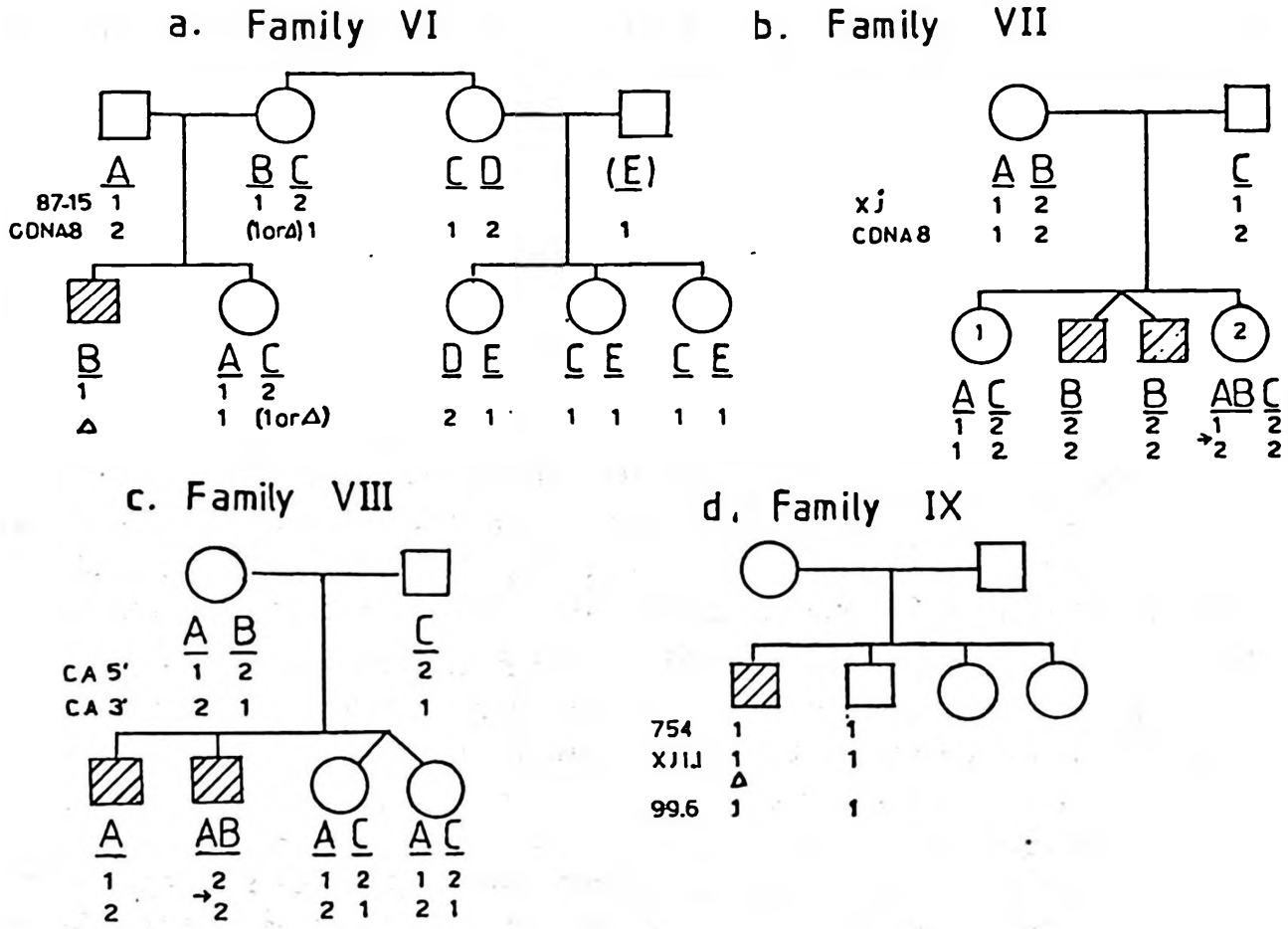

Fig. 7 a, b, c, d: Analysis in families VI-IX using various probes. Δ indicates a deletion. Father's allele if deduced is indicated by parenthesis.

deletion could be detected. The mother was homozygous for all RFLP probes studied. Later, when the CA repeat polymorphisms were applied, she was found to be heterozygous with both of them. Analysis showed that the sisters inherited a common maternal allele and that there was a crossing - over event in one of the patients (Fig. 7 c). Since the first brother had the same allele as the sisters, it was concluded that the crossing - over was in the second brother and that both of the sisters were carriers.

Family IX : The patient was reported to be a sporadic case and a deletion was found in the cDNA8 region. The unaffected brother had inherited the same haplotype, but not the deletion (Fig. 7 d).

Prenatal Diagnosis:

A woman with two affected brothers sought prenatal diagnosis at twelve weeks of pregnancy. The following assays were carried out within six days: blood samples from the family members were obtained and DNA was isolated. The mother of the pregnant woman was assayed for RFLP by PCR analysis and found to be heterozygous for pERT87 - 15 (BamHI). Family analysis revealed that the

pregnant woman had inherited the same maternal allele as the affected brothers and was therefore, a carrier. When the samples of the affected brothers were assayed via multiplex I and multiplex II, three fragments failed to amplify in each assay. There was not enough time to confirm the deletion by Southern analysis, but it was unlikely that failure of six consequent fragments to amplify was due to factors other than a deletion. DNA of the chorionic villi sample (CV DNA) was assayed by multiplex PCR to show that the fetus was not an affected male (all nine fragments amplified in both assays). Moreover, the following additional PCR analyses showed that the fetus was a female:

a) Absence of a Y - chromosome sequence: reaction mix contained 50, 100 or 250 ng of CV DNA with and without 100 ng of a male control DNA. Only the samples containing the male DNA showed a 112 bp product, indicating that the CV DNA contained no inhibitors for the reaction and that the sample itself did not contain the Y - chromosome sequence. Thus the fetus was female.

b) pERT87 - 8 (TaqI) RFLP for which the pregnant woman was a heterozygote: the assay showed that the fetus was a homozygote, indicating that the CV sample was not significantly, if at all, contaminated with the mother's.

Discussion

At present the only way to perform prenatal diagnosis for DMD is via application of DNA analysis techniques which are also used for carrier testing. These techniques will retain their importance in the future since a cure for the disease does not seem feasible at present due to the nature of the defect, and new affected families arise at high frequency unlike other genetic disorders (one third of all DMD cases are sporadic).

DNA analysis techniques that are widely used in genetic diseases were applied to Turkish DMD families in the Biology Department of Boğaziçi University. To our knowledge, this is the largest family study on a genetic disorder ever performed in Turkey. The study covered 254 individuals in 79 families. Thirty-six families were completely screened. In all the families, a total of twenty-five women were found to be carriers, while thirty-seven were non - carriers. In three women, it was impossible to determine the carrier state for various reasons. One subject had a crossing - over within the gene (family VII), while the other two belonged to different families and had inherited a maternal allele common to both the affected and healthy brothers. However, in a significant number of the families, analyses could not be completed due to unavailability of blood samples from some family members. Recently, families have become more interested and cooperative after being informed about the anomaly by the Turkish Myopathy Association.

The greatest difficulty in carrier and prenatal diagnosis of DMD is the strict requirement for haplotype analysis in all family members, unlike in certain other

genetic diseases. Such family studies are employed for several reasons in all established centers studying DMD. Some of these reasons are demonstrated in families described in this work. If a crossing - over has occurred in a patient, a single assay will mislead the analysis (different 5' - CA alleles were associated with the mutation in two brothers in family VIII). If a crossing - over in a sister has occurred, a single analysis will lead to an incorrect conclusion (probe XJ1.1 in family VII). Some cases are sporadic, so inheritance of the same haplotype does not necessarily indicate inheritance of DMD (two brothers in family IX).

Analysis is further complicated by germinal mosaicism in mothers, which is due to a mutation prior to proliferation of the germ cells. Thus the germ cells may contain three alleles, one of which harbors a mutation and is not present in the blood cells. Germinal mosaicism in DMD mothers is estimated to be above 10 percent, therefore, all sporadic cases should be suspected as being germinal mosaic if the mother desires more children.

Familial cases are much easier to analyze since they do not harbor the difficulties inherent in sporadic cases including germinal mosaicism. In general, only about one half of families are believed to be sporadic. However, in our families only 16 percent (16 / 79) were known to be familial for certain, which may simply be due to misinformation, and resulted in an otherwise avoidable extra burden in our analyses. To simplify the analysis, the carrier state of a mother may be determined via dosage analysis. However, the equipment used was not designed for this purpose, therefore, the analysis was very time consuming and cumbersome, although reliable.

This study also resulted in the early diagnosis of some suspected cases. Of fourteen boys who were too young for diagnosis, five had detectable deletions in the dystrophin gene. Considering the fact that deletions were detected in 47 of the total 79 diagnosed patients studied, 60 percent of the patients may be expected to be diagnosed with certainty via deletion detection.

We have shown that the RFLP probes employed in this work may all be used in Turkish families since they detect heterozygotes with reasonable frequencies. CA repeat length polymorphisms are also high enough to be conveniently employed. Application of the 3' - CA repeat polymorphism is more difficult than that of 5' - CA, not only because acrylamide gels are more difficult than NuSieve - agarose, but also because the interpretation is complicated by the hetero duplex band apparent only on acrylamide gels.

The greatest obstacle during this study was financial. Families were not charged a fee for the assays because the study was supported by a research grant. We should like to mention that costs were kept at a minimum by not using kits, and by optimizing the techniques applied.

Our aim is to decrease the number of new cases in families by prenatal diagnosis. Only one prenatal diagnosis established via PCR assays proved to be fast and efficient However, a lot of cases are expected to be more difficult. These cases include families with patients in whom no deletion is detected or in whom the deletion corresponds only to a single multiplex band. Southern analysis is essential in diagnosing these families: in the first group for RFLP analysis and in the latter group for confirmation of the deletion. Thus fresh radioactive material is needed, which has a half - life of fourteen days and is costly. Generally, radioactive material is purchased every four to six weeks.

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REFERENCES

1. Emergy AE. *Duchenne Muscular Dystrophy.* Oxford Monographs on Medical Genetics No: 15. New York: Oxford U Pr, 1987.
2. Darras BT. Molecular genetics of Duchenne and Becker muscular dystrophy. *J Pediatr* 117:1, 1990.
3. Koenig M, Hoffman EP, Bertelson CJ, et al. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 50:509, 1987.
4. Chamberlain JS, Gibbs RA, Ranier JE, et al. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res* 16:11141, 1988.
5. Beggs AH, Koenig M, Boyce FM, Kunkel LM. Detection of 98 % of DMD/BMD gene deletions by polymerase chain reaction. *Hum Genet* 86:45, 1990.
6. Roberts RG, Cole CG, Hart KA, et al. Rapid carrier and prenatal diagnosis of Duchenne and Becker muscular dystrophy. *Nucleic Acids Res* 17:811, 1989.
7. Beggs AH, Kunkel LM. A polymorphic CACA repeat in the 3' untranslated region of dystrophin. *Nucleic Acids Res* 18:1931, 1990.
8. Hugnot JP, Récan D, Jeanpierre M, et al. A highly informative CACA repeat polymorphism upstream of the human dystrophin gene (DMD). *Nucleic Acids Res* 19:3159, 1991.
9. Tolun A, Yüksel-Apak M, Özdemir C, et al. Carrier state determination in families at risk for DMD. *Doğa* 13:173, 1989.