Use of DNA Probes in Detecting Carriers of Duchenne Muscular Dystrophy: Selected Case Studies

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Detection of Duchenne muscular dystrophy carriers by genetic analysis is illustrated by four case studies. The technique is most useful in obligate families, in excluding carrier status in isolated cases, and in families in which the affected child demonstrates a molecular deletion. A major limitation of this technique is that the accuracy of carrier status in isolated (i.e., no family history) cases is limited by the probability that the affected child may represent a new mutation. To improve the carrier risk estimate generated by the DNA data, particularly in isolated cases, we suggest that measuring creatine kinase activities in the serum and performing the genetic analysis on the nonaffected males may be helpful.

Additional Keyphrases: heritable disorders · genetic analysis

Duchenne muscular dystrophy (DMD) is the most common X-linked recessive disease, with an estimated incidence of 1 in 3500–5000 newborn males (1). Because DMD is a lethal disorder for which there is no effective treatment, the major emphasis has been given to prevention by means of carrier detection. Carrier detection has depended on pedigree information along with biochemical tests, of which the assay of creatine kinase (CK; EC 2.7.3.2) in serum is the most frequently used. However, this approach has not been successful for detecting carriers in many DMD families, mainly because some definite carriers (30–60%) show CK activities (2–4) that are within normal limits.

There are three classifications of carriers, based on genetic considerations (2, 5). Definite (obligate) carriers are mothers of an affected son who also have a relative with DMD in the female line of inheritance. Probable carriers are mothers with two or more affected sons but with no other affected relatives. Possible carriers are mothers of an isolated case. The sisters and other female relatives of any type of carrier mother are also possible carriers. In practice, both definite and probable carriers are considered to be obligate carriers. In DMD, it is absolutely essential to distinguish between obligate and nonobligate carriers, because the accuracy of the risk estimate is influenced by the type of DMD family, as will be shown in the subsequent cases.

The use of DNA probes is a new and an important addition to existing laboratory methods for the detection of carriers of DMD. DNA probes from the short arm of the X chromosome, which recognize restriction fragment lengths polymorphisms (RFLPs) linked to the DMD gene, are available. The RFLPs can be used within affected families to trace the X chromosome carrying the mutant gene (6–9). However, unlike other genetic disorders for which the RFLP technique is currently being used, sporadic mutations are thought to play a contributing role in the overall incidence of DMD. This presents a unique situation, particularly in the isolated cases of the disease, when one is using DNA probes linked to the disease locus. Our purpose in this case study is to illustrate some of the benefits and limitations of the genetic technique when specifically applied to detection of DMD carriers.

Materials and Methods

Blood specimens were collected from patients with DMD and from their family members at the muscular dystrophy clinics in Richmond, Charlottesville, and Norfolk, VA. About 20 to 30 mL of blood was drawn by venipuncture into evacuated tubes containing EDTA. DNA was isolated from the leukocytes by conventional treatment with protease K (EC 3.4.21.14) and phenol/chloroform extraction techniques (10). Ten micrograms of DNA was digested with the appropriate restriction enzyme and separated by electrophoresis in a 8.0 g/L horizontal agarose gel. After electrophoresis, the DNA was transferred in 0.4 mol/L NaOH reagent to a nylon membrane (Bio-Rad Labs., Richmond, CA) (11, 12).

The X chromosome pERT probes (provided by Dr. L. M. Kunkel) (13, 14) were radiolabeled with 32P by nick translation (Amersham Corp., Amersham, Bucks., U.K.) (15). The nylon filter had been prehybridized at 37 °C in equal volumes of formamide and the following mixture: 5× SSC (1× SSC = 0.15 mol of NaCl and 150 mmol of Na citrate per liter), sodium phosphate buffer (50 mmol/L, pH 6.5), 5× Denhardt’s solution (1.0 g of ficoll, 1.0 g of polyvinylpyrrolidone, and 1.0 g of bovine serum albumin per liter), 1 g of sodium dodecyl sulfate per liter, and 250 mg of yeast RNA per liter. Hybridization was performed in the same solution to which the radiolabeled probe had been added to give 1 × 106 counts/min per milliliter. The filter was washed in 2.0 g/L sodium dodecyl sulfate and 2× SSC twice at room temperature and then in 2.0 g/L sodium dodecyl sulfate and 0.5× SSC at 60 °C for 60 min. Samples were autoradiographed with an intensifying screen at −70 °C for one to five days. A description of the DNA probes and their approximate orientation on the X chromosome are shown in Table 1 and Figure 1 (16).

In each family, the relevant females were tested with the pERT probes. The probes that differentiated the two X chromosomes (informative probes) were then used to test the other family members. Approximately 10% of the females studied were homozygous for all pERT87 probes.

Results

Family A

Family A comprises four subjects in two generations, and the son (II-2) has DMD (Figure 2). The family attended the
clinic to obtain information about the carrier status of their
daughter (II-1). Pedigree analysis indicates that the mother
(I-1) is an obligate carrier, owing to the presence of a
previous case of the disease on her side of the family.

Probe pERT87-8 revealed a polymorphism in the mother's
DNA, exhibiting the 2.2-kb and 4.4-kb alleles. This renders
her "informative" and permits testing of the other family
members with this probe. The mother transmitted the DMD
gene with the 2.2-kb allele, the allele her affected son
inherited. The daughter was deemed a carrier because she
must have inherited a normal 2.2-kb allele from her father
and the defective 2.2-kb allele from her mother. The carrier
prediction would be accurate with a probability of approxi-
mately 92–96%, given an estimated crossover frequency of
2% to 4% between the DMD gene and the pERT87 loci (17).
Recombinational events may occur between either the
mother and the proband or the mother and the daughter.
The daughter's genetic risk of being a carrier, 50% as based
on pedigree data alone, was increased to 92–96% by the
restriction analysis.

Family B

Family B visited the clinic because the carrier status of the
daughters (II-1 and II-2) was unknown (Figure 3). The
proband represented the first known occurrence of DMD in
this kindred. The mother (I-1) was informative for probe
pERT87-15, exhibiting the 2.8-kb and 1.6/1.2-kb combina-
tion. One daughter (II-1) demonstrated a double dose of
the opposite allele (1.6/1.2-kb) from that of her affected brother
(2.8-kb), suggesting that she was not a carrier unless a
crossover had occurred (probability 2–4%). Her sister (II-2)
was heterozygous for pERT87-15, displaying the 2.8-kb and
1.6/1.2-kb combination. Because the 1.6/1.2-kb allele was
derived from her father (I-2), she must have received the
defective allele (2.8-kb) from her mother. However, daugh-
ter II-2 in this family does not have a 92–96% genetic risk of
being a carrier like daughter II-1 in family A because it is
not known for certain whether her mother is a carrier. If one
assumes the mother is a carrier, then the daughter's risk of
also carrying the defective gene is 92–96%. On the other
hand, if the mother is not a carrier, then the daughter's risk
is minimal. Thus the carrier state cannot be resolved with
the same degree of accuracy in nonobligate families.

Even if RFLP linkage data indicate that a female relative of
an isolated case possesses the same X chromosome as the
affected male, she does not necessarily carry the defect,
because the affected male may be the result of an isolated
sporadic mutation. The presence of sporadic mutations is the
major limitation of the DNA probe analysis in DMD carrier
detection. In isolated cases of DMD, one cannot predict
carrier status with the same high confidence with which one
can exclude it. If the prevailing assumption is correct that a
third of all DMD alleles represent new mutations (18, 19),

Table 1. Characteristics of pERT87 Probes

<table>
<thead>
<tr>
<th>Allele size, kb</th>
<th>Enzyme</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>q</td>
</tr>
<tr>
<td>pERT87-1</td>
<td>XmnI</td>
<td>8.7</td>
</tr>
<tr>
<td>pERT87-8</td>
<td>BstXI</td>
<td>4.4</td>
</tr>
<tr>
<td>pERT87-15</td>
<td>TaqI</td>
<td>3.1</td>
</tr>
<tr>
<td>pERT87-30</td>
<td>XmnI</td>
<td>1.6/1.2</td>
</tr>
<tr>
<td>pERT87-J-bir</td>
<td>BamHI</td>
<td>30</td>
</tr>
</tbody>
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Fig. 1. Orientation of the pERT87 loci to the DMD region (Xp21)
The order is based on deletion cases. Distance from probes 754 and c7 are given
in centimorgan (cM)

Fig. 2. Family A, pERT87-8 hybridization on BstXI digest
© denotes obligate carrier status

Fig. 3. Family B, pERT87-15 hybridization on a XmnI digest
then the probability of daughter II-2 in family B being a carrier would be approximately 67%. Although the prior carrier risk of 33% (derived from the pedigree) was increased by the addition of the RFLP data, this daughter could still be provided with a risk estimate only in the intermediate range.

To improve the accuracy of the risk prediction, additional information derived from other carrier tests may be beneficial. In this case the daughter II-2 had a serum CK activity of 478 U/L (upper limit for healthy women, 180 U/L), which reclassified her into a high-risk category and made carrier status virtually certain for her and her mother as well.

Family C

Mother (II-3), having had two children with DMD, is an obligate carrier (Figure 4). DNA analysis was performed to determine whether her sister (II-2) and the sister's daughter (III-1) were carrying the DMD gene. Both mothers were informative for the pERT87-15 TaqI RFLP, making it possible to test all other family members with this probe. The mother (II-3) transmitted the DMD loci with the 3.3-kb allele, because both affected children (III-5 and III-6) inherited this fragment, whereas the healthy son III-4 has the 3.1-kb allele. The sister II-2 appears to be a DMD carrier for the following two reasons: she shares the same pERT87 alleles (3.1/3.3-kb) with her obligate carrier sister and she had a slightly increased value for serum CK (220 U/L). However, without being able to establish the RFLP pattern of the grandparents (I-1 and I-2), who were not available, we cannot with certainty assign the carrier status of II-2.

On the basis of these data alone, there are three possibilities regarding the carrier status of II-2. First, by demonstrating the same RFLP pattern, she may have inherited the same X chromosome as II-3 and consequently be a DMD carrier. This possibility would be further supported by her slightly increased serum CK activity. Secondly, although she exhibits the 3.3-kb allele, it may actually be an entirely different 3.3-kb fragment from that of her obligate sister. If the grandmother was heterozygous at the 3.3-kb allele, she may have transmitted genotypically different 3.3-kb fragments to each of her daughters. In this case, the sister would not be a carrier. Lastly, an isolated sporadic mutation may have occurred between either the grandmother or grandfather and the obligate mother. In this situation, regardless of which X-chromosome II-2 has inherited, she would not be a carrier.

With this information, it is not possible to determine the carrier status of the sister. This family illustrates a general limitation of the DNA technology. If certain key family members are unavailable to donate DNA, reliable answers regarding carrier state cannot always be provided. Without being able to determine the pERT87 alleles of the grandparents, a risk estimate utilizing the DNA probes for the sister could not be produced. However, restriction analysis was also performed on the sister's two healthy sons, II-2 and III-3. The sons both exhibited the 3.3-kb allele, which was the same fragment the affected boys have. Thus, the sister does not appear to be carrying the DMD gene on her 3.3-kb fragment and therefore would not be a carrier unless a crossover had occurred (probability 2–4%). Although DNA testing was not done on the sister's daughter (III-1), she also would have a low risk estimate of being a carrier. Despite the uncertainty about the origin of the mutation, DNA analysis was able to reduce the probability of carrier status of the two females (II-2 and III-1) at risk in family C.

Family D

Family D also contains an isolated case of the disease (Figure 5). Restriction analysis was performed to assess the carrier status of the daughter II-1. Mother I-2 was informative for probe pERT87-8; therefore, this probe was used to test the other family members. As shown in Figure 5, the DNA from the affected son failed to hybridize with pERT87-8. Therefore, this individual has a deletion within the Xp21 region. The recognition that DNA from 7–8% of patients with DMD fails to hybridize with the pERT probes was not only a fundamental advance in the understanding of the...
molecular pathology of the disorder but also had a major impact on the determination of DMD carrier risk (13, 14, 17, 20). Carrier predictions in families demonstrating a deletion are highly accurate, because the deletion is considered to be the primary genetic defect in the case. The probe is no longer binding a sequence of DNA linked to the defect but is actually identifying the causative mutation. Thus, in families with DMD-associated molecular deletions, the prediction is presumably almost 100% accurate. As is the case with family D, because the DNA from daughter II-2 does not exhibit the deletion she will have almost no risk of carrying the DMD gene. The recognition of a deletion also elucidates the origin of the defect in this family. It indicates that the level of occurrence of the DMD mutation was between the mother and affected son, because the mother's DNA displays the normal pERT87-8 alleles. However, germline mosaicism in DMD, whereby a deletion was transmitted to more than one offspring by a woman who showed no evidence of the mutation in her somatic cells, has recently been reported (21), so the mother does have an uncertain risk of still being a carrier. This finding shows that carrier testing should be performed on all sisters of such males.

Discussion

Accurate determination of DMD carrier status is of utmost importance. The benefits include relief of the psychological burden, more accurate family planning, and the knowledge of the possible need to perform prenatal diagnosis. In this study, the relative value of DNA analysis for DMD carrier assessment was illustrated through the use of four case studies. Each of the cases (and the corresponding family history) demonstrates different important aspects of DMD carrier detection by DNA analysis. The strengths and limitations of this technique must be well recognized if one is to convey meaningful and reliable information to the affected families.

It is apparent that DNA analysis is most helpful when the family is of the obligate type, as in family A. In this situation, linkage analysis can predict or exclude carrier status with the same high degree of accuracy. After having determined the defective allele in family A, the probability of the daughter being a carrier was 92–96%. The prediction of carrier status is straightforward in obligate families, but unfortunately less than a third of the families that attended the clinics were obligate families.

Family B highlights the major pitfall of the molecular DNA approach in DMD. The accuracy of linkage analysis depends on the proximity of the marker to the disease loci, and requires knowledge of which allele for a given informative marker is segregating with the disease gene (linkage phase). The linkage phase cannot be determined with certainty in isolated case families because of the possibility of a sporadic mutation. This limitation appears to be peculiar to DMD, because sporadic mutations do not seem to play a significant role in other genetic diseases to which the RFLP analysis is currently being applied. Although daughter II-2 in family B has inherited the same allele as the affected brother, she still has a 59% chance of not being a carrier. The additional information provided by her increased serum CK activity greatly alters this and places her at a very high risk. In this situation, an increased serum CK value for the sister increased the likelihood that the isolated DMD case did not arise from a new mutation, and that the sister had therefore inherited the DMD gene. In our experience, half of DMD carriers (determined by DNA analysis) have increased serum CK activities. Even though the proportion of carriers with increased serum CK values is low, the finding of an increased value provides strong evidence for the carrier state, which is particularly valuable in cases for which family-history information is sparse or lacking. Although the inclusion of carrier status cannot always be made with great certainty in isolated cases, linkage studies will be extremely beneficial for sisters: one can estimate much smaller carrier risks if the RFLP results indicate a sister has inherited an X chromosome different from that of her affected brother. These studies should, therefore, be performed in families of isolated cases, especially for women who would otherwise be forced to make decisions based on serum CK data alone.

Family C illustrates the importance of obtaining blood samples from nonaffected family members. Exclusion of carrier status of mother II-2 and daughter III-1 was possible only because DNA analysis was carried out on the nonaffected cousins (II-2 and III-3). Once it was established that these boys had the same RFLP pattern as the affected patients, the risk of the mother and daughter being carriers was reduced to about 2%. In isolated cases it is also absolutely essential to collect samples from the healthy brothers of the proband. If the healthy brother has inherited the same pERT allele as his affected brother, this finding would indicate that they both inherited the same X chromosome (within the limits of a possible recombination). Thus the likelihood that the affected male resulted from a new mutation would therefore be great and the mother's carrier risk would be very low (i.e., 2%.

Family D also consisted of an isolated case of DMD, but a highly accurate risk estimate was readily provided because the affected son exhibited a molecular deletion. The sister did not show the deletion, so the probability was almost 100% that she was not a carrier. Unfortunately, a low incidence of pERT87 deletions, less than 5% of the families, has been detected during this investigation. However, it has recently been reported that, with the use of field-inversion gel electrophoresis, a higher deletion rate is detected in DMD (22). Because of the high accuracy that can be achieved in carrier prediction when a deletion can be ascertained, all DMD patients should be initially screened for the presence of molecular deletions.

Carrier detection in DMD has been significantly improved by the addition of DNA probes that recognize RFLPs that are linked to the defective gene. As was shown in the present case studies, the value of RFLP linkage data may be limited, particularly in families with only a single affected male, because the test does not directly identify the defect. With the recent discovery of the dystrophin protein (23), the product of the DMD gene, more direct methods of detecting carriers may soon become possible. This approach would have the potential to further improve the accuracy of carrier detection in all affected families.

References