Duchenne muscular dystrophy — a molecular service

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Summary

In 1987 a carrier detection and prenatal diagnostic service for Duchenne muscular dystrophy using molecular technology was instituted at the Department of Human Genetics, University of Cape Town, to serve affected families in southern Africa. DNA samples from 100 affected male subjects and 350 of their relatives from a total of 110 families have been banked. To date restriction fragment length polymorphism (RFLP) analysis and deletion screening has been performed on the DNA of 60 male patients and 116 female relatives at risk of being carriers of the faulty gene. The DNA probes used were pERT 87-1 (MspI polymorphism) pERT 87-15, pXJ1.1, pXJ2.3 (*Taq*I polymorphism), pXJ1.2 (*Bcl*I polymorphism), P20 (*Msp*I and *Eco*RV polymorphism) and the cDNA probes. DNA deletions have been detected in 30 of the 60 affected boys and the carrier risks of 49 women have been determined by RFLP analyses. In those families where the risks were uncertain because the affected males had died, prenatal exclusion testing was offered to potential carriers. Two pregnancies were terminated when male fetuses were shown to be affected, since they had the same deletion as that observed in the proband.

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Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive disorder affecting about 1 in 3 300 newborn boys.[1] There is no cure for this severe disorder and, since male patients do not live to procreate, the most important facet of management is detection of women who are carriers.

Molecular diagnosis has resulted in dramatic progress in the management of affected families. Linkage studies using DNA markers flanking the DMD gene led to the isolation of genomic probes which could detect submicroscopic deletions in the DNA of affected boys.[2,3] The screening of DNA from male patients with the recently cloned and sequenced complementary DNA (cDNA) of the DMD gene has revealed that deletions are present in more than 60% of affected boys.[4–6] These deletions do not occur randomly: there are two regions of the gene that are preferentially deleted in these patients.[7,8]

A high-frequency deletion region occurs near the centre of the gene and a lower frequency occurs in the 5′ end. In the 40% of patients in whom no deletion is found, the current approach is RFLP analysis for carrier detection and antenatal diagnosis.[9,10]

The application of these molecular advances has major implications in the genetic service being offered to families with DMD. Our experience using the intragenic genomic probes and the cDNA subclones is reported.

Patients and methods

Affected male patients were referred to our department by their clinicians or through the State Health Genetic Service. In all patients the diagnosis of DMD was confirmed by clinical appraisal and appropriate ancillary investigations. An allelic disorder, Becker muscular dystrophy (BMD), is differentiated by its later age of onset and its milder clinical course.

Families with BMD have access to our DMD service, but for the sake of clarity, BMD is not considered further in this article.

In families where there was a clear history of the disorder, 30 ml blood specimens were collected in ethylenediaminetetraacetic acid (EDTA) tubes from the proband, his male and female siblings, both his parents and his maternal grandparents (where possible). Blood was also obtained from potentially heterozygous female relatives.

In families where the possibility of a new mutation had to be considered, blood was obtained from the proband, his unaffected brother(s) and his mother. In those instances where the affected male subjects were deceased, blood was collected from the consultant, her unaffected brother(s) and both her parents.

Creatine kinase (CK) levels were not available for all of the potential carrier females. Where possible this information was provided by the referring clinician or State Health nursing sister together with the normal range established by the laboratory performing the CK tests.

Molecular probes

The probes used were the 5′ genomic probes: pERT 87-1; pERT 87-15; pXJ1.1; pXJ1.2; and pXJ2.3; the intronic probe P20, which recognises sequences near the centre of the gene; and the cDNA subclones cDMD 1 - 2a, 2b - 3, 4 - 5a, 5b - 7, 8 and 9 - 14. The probes used in this study are schematically depicted in Fig. 1.

Molecular analysis

DNA isolation from leucocytes, restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting and hybridisations were performed as described by Van den Plas *et al.*[11] Denhardt's solution and salmon sperm were replaced in the hybridisation solution by the skim milk powder 'Blotto'.[12] DNA probes were labelled according to the nick translation procedure of Rigby *et al.*[13] and hybridised to patient DNA digested with *Taq*I, *Msp*I, *Bcl*I or *Hind*III.

Deletion screening and carrier detection

Initially, patient DNA was screened for microdeletions in the DMD gene using the intragenic probes pERT87, XJ, P20 and the cDNA subclones. These deletions were identified by the absence of allelic fragments when the DNA was hybridised to genomic probes or by the absence of one or more exonic fragments when hybridised to the cDNA probes.

Once a deletion was found in the proband, the DNA of the women at risk of carrying the mutation was screened with the probe that detected the deletion. If both of the RFLP fragments were present (heterozygous for the RFLP) it was unlikely that the woman was carrying the mutation. If only a single fragment was present, it was uncertain whether both alleles were present (homozygous for the RFLP) or whether one allele was present and the other deleted (hemizygous). Dosage studies would theoretically be useful in these circumstances. This technique, however, has been found to be unreliable both in our own and other investigators' laboratories.[14] For this reason, we estimate the risks for a potential carrier using a combination of pedigree data, CK level and DNA analysis.[14,15]

All carrier-risk estimates are given with a 5% uncertainty factor due to the chance of crossing over occurring during meiosis.[6,15] For this reason, low-risk estimates are given as 'less than 5%' and high-risk women have a carrier risk of 95 - 100%.

Results

Deletion screening

Molecular investigations were undertaken in 60 DMD patients from different population groups. Details of ethnic background and molecular findings are given in Table I.

Of the deletions found in 30 unrelated affected male patients from all ethnic groups, 8 were detected with the 5′ probes pERT87, XJ and the cDNA 1 - 2a subclone. The intronic probe P20 and the cDNA 8 subclone detected deletions around the central region of the gene in a further 22 patients. Most of the deletions identified with the cDNA involved different exonic fragments and had different end-points. Although the general trend of deletion distribution favoured the central 'hot spot', a reversal of this distribution was observed in indigenous

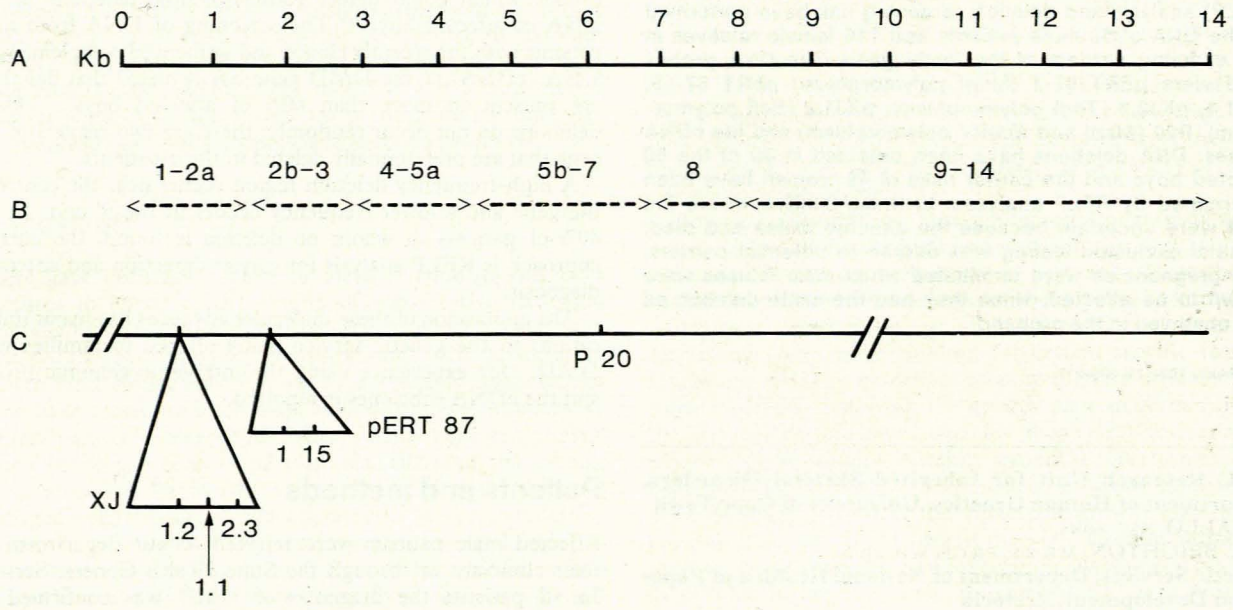

Fig. 1. A schematic representation of the DMD locus and the probes used in this study (A = size in kilobases (Kb); B = cDNA subclones for use in hybridisation; and C = genomic physical map indicating approximate positions of genomic probes).

TABLE I. SUMMARY OF DELETIONS FOUND IN UNRELATED PATIENTS FROM DIFFERENT ETHNIC BACKGROUNDS

Population	Total No. of families studied	Affected boys studied		Total No. of deletions observed	
		Familial cases	Sporadic cases	Central hot spot	5′ hot spot
Indian origin	**16**	**6**	**10**	**8**	**1**
Mixed ancestry	**13**	**9**	**4**	**6**	**2**
Black	**11**	**7**	**4**	**1**	**4**
White	**20**	**14**	**6**	**7**	**1**
Total	**60**	**36**	**24**	**22**	**8**

Statistical comparison of deletion distribution with Yates correction: $\chi^2 = 9,22$, df = 3 ($P = 0,026$).

black patients. A statistical comparison of deletion distribution among the different groups of patients shows $\chi^2 = 9,22$ ($P < 0,5$).

In 30 male patients in whom deletions were not detected, RFLPs of the DNA probes were used in haplotype analysis in order to establish the phase of the faulty gene.

Carrier status

The RFLPs that proved useful for family studies and carrier-risk estimations were the TaqI polymorphism for XJ1.1, XJ2.3, pERT87-15 and the cDNA 8 subclone; the MspI polymorphism of pERT87-1 and P20; and the BclI polymorphism of XJ1.2. The results of investigations for carrier risk estimation are summarised in Table II.

Thus far, our department has banked DNA from 116 female subjects with one or more affected male relatives.

Pedigree data

From pedigree data we could identify 22 obligate carriers by virtue of them having an affected son as well as a family history of DMD. Ninety-four female subjects were classified as potential carriers because of their relationship to affected male patients in their families. Of the potential carriers, 22 had an initial risk of 50% of being heterozygous gene carriers, since their mothers were obligate carriers of the defective gene.

Molecular studies

The analyses of DNA RFLPs reduced the initial risk of 7 of the female subjects at 50% risk to less than 5%, since they inherited the opposite maternal allele to their affected male relative(s). Conversely, 8 had their risks increased to more than 95%.

RFLP analyses increased the initial risk estimates of 8 of the other 72 women to more than 95%. Conversely, 26 had their risk reduced to less than 5% when it was shown that: (*i*) the proband inherited the same maternal allele as his unaffected brother indicating that the proband had a new mutation; and (*ii*) the female subject was heterozygous for an RFLP at a locus where the proband had a deletion.

Carrier-risk estimations remained unchanged in 45 women either because further confirmation required dosage studies, because of a lack of RFLP heterozygosity in key family members or because key family members were not available for DNA studies.

In 4 kindred with a history of DMD, the affected male subject was deceased. In 3 of these families an unaffected male sibling was available for study and carrier risk was estimated on the assumption that the disease-associated allele would be the opposite of that inherited by the unaffected brother. Two potential carriers had their risks thus increased when they inherited the opposite maternal allele and 2 female subjects were declared 'low risk'. In the 4th family the risks remained the same, since a key family member was not available for study.

Prenatal diagnosis was requested by 2 potential carriers of the DMD gene whose risk estimates had been increased after RFLP analyses. In both instances a male fetus was screened for a DNA deletion that had been detected in the proband. Both fetuses inherited the same maternal allele as the proband, as well as the deletion. The pregnancies were terminated at the parents' request.

Discussion

The gene for DMD is one of the largest known, spanning more than 2 000 kb.[16] It is reasonable to assume that the large size predisposes the gene to the high mutation rate that has been established.[17]

DMD therefore poses a major problem in South Africa in view of the population's high annual birth rate. Since no effective treatment is available, the most effective way to assist affected kindreds is to offer prenatal diagnosis to high-risk women through molecular technology.

TABLE II. SUMMARY OF THE MODIFICATION OF CARRIER-RISK ESTIMATES AFTER DNA STUDIES

	Before DNA analysis		After DNA analysis		
	Females at risk	Carrier status	Risk increased	Risk decreased	Not conclusive
	22	**Obligate**	**—**	**—**	**—**
	22	**50% risk**	**8**	**7**	**7**
	72	**Potential**	**8**	**26**	**38**
Total	**116**		**16**	**33**	**45**

DMD is found in all ethnic groups in southern Africa and deletion screening has shown that deletions are generally more frequent in the central region of the gene, as has been reported in various publications.[7,8] There is, however, a difference in deletion distribution frequency in indigenous black patients. The study of a larger number of these patients and detailed analyses of the deletions will be necessary in order to elucidate the biological significance of this situation.

The application of the DMD gene probes in a genetic service in southern Africa has resulted in 33 women being given a low-carrier risk (i.e. a maximum of 5% due to the chance of crossing over during meiosis). A further 16 women have been established as high-risk carriers.

In those cases where a deletion was detected in the proband's DNA, prenatal diagnosis can be offered with an accuracy approaching 100%. In those families where RFLP analyses were used to track the faulty gene, prenatal diagnosis could be offered with 95% accuracy.

Germ-line mosaicism is a complicating factor, which warrants discussion. Recently, Bakker *et al.*[18] have shown that in those instances where new mutants have been assumed because the proband had inherited the same maternal alleles as his unaffected brother, the recurrence risk of DMD in a male pregnancy is in fact about 14%. Prenatal diagnosis based on haplotype information can therefore either exclude the X chromosome or predict a 14% recurrence risk if the male fetus has inherited the same X chromosome as the proband.

Since the inception of this molecular service to families of affected boys, the number of requests for antenatal diagnosis has been relatively small (only 2). Nevertheless, the approach outlined will permit high-risk women to have children without undue anxiety, providing the families are fully investigated before the potential carriers become pregnant. For this logistical reason, it is necessary that family members should be referred for a molecular work-up as soon as the first case of DMD/BMD is diagnosed.

We are most grateful to the physicians who referred patients to us, to the staff of institutions who allowed us access to patients under their care, to the patients and their family members whose blood specimens were used in this study and to the genetic nurses of the Department of National Health and Population Development who willingly assisted with documentation and collection of blood samples. We are most grateful to Mrs G. Schutte for her technical assistance. We also thank Drs L. M. Kunkel, R. G. Worton and P. L. Pearson for their generous provision of genomic probes. We are most appreciative of assistance and co-operation from the Muscular Dystrophy Foundation of South Africa. This study was funded by grants from the South African Medical Research Council, the Mauerberger Foundation, the Harry Crossley Foundation and the University of Cape Town Staff Research Fund.

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