Clinical use of DNA markers linked to the gene for Duchenne muscular dystrophy

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SUMMARY Seventy families with Duchenne muscular dystrophy (DMD) known to the Institute of Child Health fall into three categories with respect to potential linkage analysis with the X chromosome DNA markers RC8 and L1.28 that bridge the DMD gene.

Families in which there is at least one obligatory female heterozygote (n=13). Here 'prediction' and 'exclusion' of DMD gene transmission may be possible, the accuracy being dependent on the closeness of the linkage of the DNA marker(s) to the DMD gene; an illustrative case is reported. **Families in which there is a single affected boy, who also has one or more healthy brothers (n=26).** Given an informative restriction fragment length polymorphism (RFLP), the probability that the boy represents a new mutation can be reassessed; it is also possible to 'exclude' the DMD gene in a sister.

Families with a single affected boy with no brother (n=30). Here 'exclusion' of the DMD gene in a sister may be possible.

Only in one family was there no possibility of useful linkage analysis. The linkage analysis required is described, and the need to check DMD families for informative RFLPs is stressed.

Duchenne muscular dystrophy (DMD) is an X linked recessive condition in which the basic defect is unknown. Until now, advice to female relatives on the risk of being a carrier has depended on pedigree analysis and measurement of the plasma creatine phosphokinase value. The latter is often higher in obligate carriers of the DMD gene than in normal women, but the overlap is such that creatine phosphokinase measurement often fails to alter significantly the risk based on pedigree analysis. There is, therefore, a need for a more direct way of tracing the inheritance of the DMD gene within families.

This approach has become possible with the isolation of cloned DNA fragments from the human X chromosome which can be used as probes for DNA sequences that are linked to the DMD gene locus.^{1 2} We have analysed the 70 families known to the genetics unit at the Institute of Child Health and assess the proportion of DMD families who could benefit from their use in conjunction with genetic counselling. We report the use of one probe, RC8, to assist in a difficult clinical analysis during pregnancy.

Materials and methods

Probe RC8. The RC8 recombinant was isolated from an X chromosome-specific genomic DNA library cloned in lambda phage.³ It contains a human DNA sequence located at Xp21-Xp223, within the region of the short arm of the X chromosome where the gene for Duchenne muscular dystrophy had been mapped tentatively by the study of X:autosome translocations in girls manifesting the symptoms of DMD. RC8 shows a restriction fragment length polymorphism (RFLP) in DNA digested with the restriction enzyme TaqI, reflecting normal variation in the locations of the DNA sequences recognised as cleavage sites by the TaqI enzyme around the X chromosome sequence identified by the RC8 probe. Approximately 21% of British women are heterozygous for the most common TaqI RFLP which manifests as two bands of 5.3 kilobases (kb) and 3.2 kb respectively after Southern blotting.¹

Consider a family in which a woman has inherited the DMD mutation from her mother and is a known carrier. If the restriction pattern of her DNA shows

she is also heterozygous for the TaqI RFLP, then a similar analysis on her father (from whom she must have inherited the X chromosome that does *not* carry the DMD gene) will identify which of the two restriction patterns is associated with the X chromosome bearing the DMD mutation. This assumes that the RC8 probe sequence and the DMD gene locus are closely linked, and thus that the DMD gene and the particular restriction pattern with which it is in phase are co-inherited from generation to generation. Linkage studies have shown that the sequence hybridising to RC8 is co-inherited with DMD approximately 85% of the time.²

Probe LI.28. A second X chromosome probe, LI.28, also shows a TaqI polymorphism; approximately 41% of the British women are heterozygous.² In addition, a small number (less than 10%) of women are heterozygous for a BamHI polymorphism. The genomic sequence hybridising to LI.28 is located between the centromere and the DMD mutation, on the other side of the DMD locus from RC8. Therefore, RC8 and LI.28 bridge the DMD mutation. Davies *et al.*² have shown that one allele of the RFLP is co-inherited with DMD in an informative family 85% of the time; the genetic distance between LI.28 and DMD is approximately 15 cM.

Restriction analysis. DNA was prepared from 10 ml of whole blood using the phenol extraction method of Kunkel *et al.*⁴ Restriction enzyme TaqI was obtained from Bethesda Research Laboratories (Cambridge), and used according to their instructions. The restricted DNA was subjected to electrophoresis on 1% agarose gels and blotted on to nitrocellulose as outlined by Southern.⁵

Pedigree analysis in family S. A formal Bayesian analysis was used, that combined the *prior* probability of the disease gene (d) being in phase with one restriction pattern with the *conditional* probability of obtaining the restriction patterns observed in the family (given a recombination frequency of 0.15) to give a final absolute fractional risk of the propositus being a carrier (Appendix).

Results

Families with DMD. A total of 70 families are known to the genetics unit at the Institute of Child Health in which at least one male had a definite diagnosis of DMD. These families had been referred over the past 15 years for genetic counselling. Inspection of the pedigrees of these DMD families showed three main categories with regard to the type of information that could be obtained:

(a) Families in which there is at least one obligatory female heterozygote

In these families, linkage analysis (given an informative RFLP) could either predict the presence of the DMD gene or exclude it from certain subjects with varying degrees of accuracy. Family S (described below) is such a family and the results with this family illustrate the way linkage analysis can be used.

(b) Families in which there is a single affected boy who also has one or more healthy brothers

Until now, pedigree analysis in these families assumes a prior risk of 1/3 that the boy represents a new mutation, or (put the other way) a 2/3 chance of his mother being a carrier.⁶ This prior risk is then modified by taking account of any healthy boys the mother may have produced; the more normal boys, the less likely it is that she is a carrier, and the more likely it is that the affected boy represents a new mutation with a low risk of recurrence. If it were possible to show that the affected boy *and* his healthy brother inherited the same region of the X chromosome surrounding the DMD gene locus, this would establish that a new mutation was responsible. This approach depends on the mother's DNA having an appropriate RFLP; the accuracy of the analysis depends on how closely the probe and the DMD gene locus are linked. Fig. 1 shows the result obtained using the existing RC8 and LI.28 probes. In this category of family, linkage analysis can allow both reassessment of the probability of the boy representing a new mutation and exclusion of certain relatives from carrying the gene.

(c) Families with a single affected boy with no brother

Even if one cannot determine whether the mother is a carrier or the affected son represents a new mutation, it is possible to exclude the presence of the DMD mutation in a male fetus or daughter that has inherited the normal restriction pattern surrounding the DMD locus (Fig. 2). The reliability of this exclusion will depend upon how closely linked the probes are to the DMD locus, and also whether the maternal grandfather is available for study, although the latter makes little difference to the reliability in this group. This approach again depends upon the mother being heterozygous for an RFLP with either or both the bridging probes. Even if the affected boy is dead, a healthy brother can provide equivalent information. It is only impossible to obtain linkage information when both the affected boy and the maternal grandfather are dead, and there are no brothers.

Proportion of families that could be helped. The 70 families on our register have been classified accord-

Without linked DNA markers		With use of one, or both, bridging DNA markers

Probability of mother being carrier	$2/3$	$1/3$ ($\theta_A = 0.15$)	$1/6$ ($\theta_A = 0.15$) ($\theta_B = 0.15$)

Fig. 1 Reassessment of the probability of mother being a carrier versus affected boy representing a new mutation. The best possible result for the mother using RC8 and LI.28 (see Appendix for calculations using one marker locus).

Without linked DNA marker		With use of one, or both, bridging DNA markers

Probability of sister being carrier	$1/3$	$1/6$ ($\theta_A = 0.15$)	$1/15$ ($\theta_A = 0.15$) ($\theta_B = 0.15$)

Fig. 2 Reassessment of the probability of a sister of an isolated affected boy being a carrier. The best possible result for the sister using RC8 and LI.28 (see Appendix for calculations using one marker locus).

ing to the above criteria (Table). In more than half, one or more sisters of the boy with DMD would be seeking advice as to carrier status, as well as the mother of the proband. There was only a single

family for whom no information could be obtained; all other families gave some useful data if the mother was heterozygous for RC8 or LI.28, or both. Since 21% and 41% can be expected to be heterozy-

Table Seventy Duchenne muscular dystrophy (DMD) families known to the clinical genetics unit

Type of family	Type of information	With sister(s) of DMD boy	With just maternal aunt(s) of DMD boy	Others	Total (%)
2 or more DMD boys	Carrier prediction and 'exclusion'	11	2	—	13 (18.5)
Single DMD boy with healthy brother(s)	Reassessment of prob of new mutation and carrier 'exclusion'	8	8	10	26 (37)
Single DMD boy or healthy brother if DMD boy dead	Carrier 'exclusion'	22	4	4	30 (43)
Single DMD boy dead	None	1	—	—	1 (1.5)
Total		42	14	14	70 (100)

gous for RC8 and LI.28 respectively, approximately 10% of women will be informative for both probes, and 50% for one probe.

Case history family S. The proband (IV-4) presented at the genetic clinic 12 weeks into a planned pregnancy, seeking confirmation of her belief that she was definitely not a carrier for DMD. A family pedigree (Fig. 3) showed that her mother (III-6) was an obligate carrier, having an affected son (IV-3) and two affected cousins (III-1,3). The prior risk that the proband was a carrier was 1 in 2. A single creatine phosphokinase estimation had been carried out on the proband, her sister (IV-1), and her mother 13 years earlier, and on the strength of these results (none of which was very high) the family were led to believe that the proband was probably not a carrier but that her sister was. At that time the limitations of a single routine creatine phosphokinase estimation in a prepubertal girl as a method for carrier detection were not fully appreciated.

The situation was further complicated by the fact that creatine phosphokinase estimates during pregnancy are uninterpretable unless extremely high.⁷ With no reliable information on which to modify her 1 in 2 prior genetic risk, the proband was left with an almost impossible decision of whether to trust to luck, or contemplate fetal sexing with termination of the pregnancy if the fetus was male, knowing that there was a 75% chance that it would be a normal boy. A decision was made with the proband and her family to check whether linkage analysis could

provide more information on carrier status for the DMD gene, and whether fetal DNA analysis would be informative.

Restriction fragment analysis

Fig. 4 shows the TaqI Southern blot probed with RC8 for the proband (G), her mother and father (D, B), her normal brother (F), her affected brother (C), and her sister (E) together with a control (H), showing the common RFLP. The family does not show the common RFLP with bands at 5.3 kb/3.2 kb, but showed the usual band at 3.2 kb and (for some family members) an additional band at 2.9 kb. The mother had both bands and was heterozygous, and her son with DMD had received the 3.2 kb band while her normal son inherited the 2.9 kb band. Father had a 3.2 kb band and must have transmitted this to the proband and to her sister. Since the proband had only a 3.2 kb band, she must have inherited the 3.2 kb band from her mother, while her sister had inherited the 2.9 kb band from her mother. Fig. 5 gives the most likely genotypes in this family, but because of the possibility of crossing over between the DMD locus and the RC8 sequence, we cannot say that the proband was definitely a carrier. Accepting a recombination frequency of 0.15, the probability of the proband being a carrier can be reassessed as 0.83. With a recombination frequency of 0.10, the probability of being a carrier would have been 0.89; with a recombination fraction of 0.20, the probability of her being a carrier would have been 0.76 (Appendix).

Discussion

This paper analyses the potential value in genetic counselling of the existing DNA probes linked to the DMD locus, and illustrates the principles and problems of this type of analysis with a case report. Clearly, it is far from ideal to assess a family at a time when a woman at risk is already pregnant, but nevertheless important additional information was available to the proband by the time a decision on fetal sexing had to be made. Women facing a high risk of giving birth to a boy with DMD adopt a wide range of views, from those who consider 1 in 50 too high a risk to those who would not contemplate selective abortion of males unless reliable prenatal diagnosis of DMD could be offered.⁸ There are certainly some women who will alter their decision as a result of the changes in estimate of risk made possible by the use of these gene probes, especially if both show informative RFLPs in the family.

In the event, the mother of the proband was heterozygous for a rare RFLP with the RC8 probe, but not with the probe LI.28. This allowed a

Fig. 3 Abbreviated pedigree of Family S.

Fig. 4 Southern blot on DNA from the Family S using TaqI and RC8 probe, plus explanatory pedigree.

Lane G is the propositus showing a single 3.2 kb band (and artefact vertical line).
Lane H is control DNA showing the common RFLP found with RC8 and TaqI with a 5.3 kb band.

reassessment of the risk of the propositus being a carrier by 16 weeks' gestation, but since the propositus herself was not heterozygous for the RFLP, prenatal analysis of a male fetus would have been uninformative. Without an opportunity to identify a male fetus with a greater than 50% chance of being affected, and with considerable reservations about mid-trimester abortion, the propositus opted to continue the pregnancy without intervention of any kind.

This decision was fully informed. The propositus understood the severity of DMD, having lived with her 18 year old affected brother until recently. She was not denying the risk and requested a neonatal test if the baby was a boy. It is relevant that she said

she would probably take a different view if fetal sexing were available in the first trimester, a facility that is likely to become available as a service in the future with karyotyping and DNA analysis on trophoblast material obtained at from 6 to 10 weeks' gestation.⁹⁻¹²

The restriction patterns in the family show that it may be necessary to know the DNA polymorphism pattern of the father to assess the genotype in a sister, contrary to the usual assumptions for X linked inheritance. Were the propositus not available, the genotype of her sister (IV-1) could not be deduced without analysis of paternal DNA.

The family showed a rare RFLP not previously encountered in this laboratory, although it had been

seen before by a collaborator using RC8. It was therefore essential to check that the pattern was consistent with an RFLP showing X linked Mendelian inheritance within the family, and was not due to partial digestion of the DNA samples. In searching for RFLPs with a particular probe and restriction enzyme for use in gene mapping, some investigators¹³ recommend the use of a small panel of 9 normal controls so as to pick out just the common RFLPs in the population. Clearly, the more common the RFLP in the population, the more useful it will be in gene mapping and clinical practice, but as the present family shows, it is still important for those working in the clinical service to be able to recognise the rarer RFLPs and distinguish them from laboratory artefact.

As more pedigree data accumulate, the confidence limits for the estimated distance between the RC8 or LI.28 sequences and the DMD locus will become narrower. Currently, the distances are estimated as 15 cM for RC8 and 15 cM for LI.28.² At the time of advising the propositus, the confidence limits were wider with an estimated distance of 20 cM from RC8, and so she was given a risk of being a carrier of about 75 to 80%. The information available to the propositus was limited for two reasons; her mother was heterozygous for an RFLP with just one of the two probes that bridge the DMD gene locus, and the propositus herself was not heterozygous for any RFLP. If the mother had had an informative RFLP with both RC8 and LI.28, then there would have been a good chance of establishing carrier status, or excluding it, in her daughters, with 97% accuracy. The reason for this substantial improvement in the prediction is because if the two alleles identified as being in phase with the disease gene are transmitted unchanged, then the only way the disease gene could not also be transmitted on that X chromosome would be if there had been a double cross over during female meiosis (both between RC8 and DMD, and DMD and LI.28). The probability of one cross over is 0.15, but for a double cross over it is $0.15 \times 0.15 = 0.0225$.

If the propositus had been heterozygous for an RFLP like her sister, then the risk of any male fetus being affected could in principle have been determined antenatally. It is also notable (Fig. 5 and Appendix) that the sister had only a 0.23 probability of being a carrier, and although this in itself only reduces the risk that a boy will be affected to 1 in 9, if it could be shown that her male fetus had inherited the 3.2 kb band (which came to her from her father), the risk would have fallen to about 1 in 25.

Even these first generation probes, with fairly loose linkage to the DMD gene, can therefore provide useful information for genetic counselling.

Fig. 5 The most likely genotype in Family S, showing a schematic representation of the X chromosome carrying the DMD gene and the restriction fragment length polymorphism, and the Southern blot pattern for each member. This interpretation is not certain because RC8 is 15cM distance from the DMD locus (see text).

'Exclusion' of carrier status can be improved further by combining linkage data with creatine phosphokinase estimates.¹⁴ This is a legitimate procedure since they are clearly independent parameters. That a high proportion of DMD families could benefit from such studies if they had informative RFLPs indicates the urgency in establishing which families do indeed have the appropriate RFLPs, and although at the present time only a proportion can be expected to achieve a useful assessment of their risk, it is the authors' view that they should not be denied this benefit while awaiting a more generally applicable test. While emphasising that this approach is not the solution to the detection of the DMD gene, it would be inappropriate to exclude a family from potential benefit by failing to store a DNA sample on an affected boy or a maternal grandfather before he dies. Knowing how long it can take to do family studies, it may well turn out that the molecular geneticist will beat the clinicians to it, and there will be more closely linked probes available with even better predictions by the time the family DNA samples are obtained.

Appendix*

Linkage analysis in family 'S' (Fig. 4)

This assumes a prior probability that either linkage phase is equally likely.

Linkage phase in mother.	B1—D B2—dis	B1—dis B2—D
With recombination fraction θ	θ^2	$(1 - \theta)^2$
Normalising	$\frac{\theta^2}{\theta^2 + (1 - \theta)^2}$	$\frac{(1 - \theta)^2}{\theta^2 + (1 - \theta)^2}$ [Let $\theta^2 + (1 - \theta)^2 = x$]
Considering chance of LS <i>being</i> a carrier	θ $\frac{\theta^3}{x}$	$1 - \theta$ $\frac{(1 - \theta)^3}{x}$
Absolute probability	$\frac{\theta^3 + (1 - \theta)^3}{x}$	
Considering chance of LS <i>not</i> being a carrier	$1 - \theta$ $\frac{\theta^2 (1 - \theta)}{x}$	θ $\frac{\theta (1 - \theta)^2}{x}$
Absolute probability	$\frac{\theta^2 (1 - \theta) + \theta (1 - \theta)^2}{x}$	
FINAL PROBABILITY OF LS BEING A CARRIER	$\frac{\theta^3 + (1 - \theta)^3}{\theta^3 + (1 - \theta)^3 + \theta^2 (1 - \theta) + (1 - \theta)^2 \theta}$	

Linkage calculation for Fig. 1.

The calculation for flanking loci follows similar principles but is too complex to present here.

* θ = recombination fraction between A and disease gene locus.
 μ = mutation rate for disease.

Appendix contd.

Linkage phase in mother.

	Carrier		Non-carrier
	A1—D A2—dis 2μ	A1—dis A2—D 2μ	
Prior probability			$1 - 4\mu = \sim 1$
Conditional probability given restriction patterns in family	$\theta (1 - \theta)$	$(1 - \theta)\theta$	μ
Joint probability	$2\theta (1 - \theta)\mu$	$2\theta (1 - \theta)\mu$	μ

FINAL PROBABILITY OF MOTHER BEING A CARRIER

$$\frac{4\theta (1 - \theta)}{4\theta (1 - \theta) + 1}$$

$$= 0.337$$

(for $\theta = 0.15$)

Linkage calculation for Fig. 2.

The calculation for flanking loci follows similar principles but is too complex to present here.

	Carrier		Non-carrier
	A1—D A2—dis 2μ	A1—dis A2—D 2μ	
Linkage phase in mother.			$1 - 4\mu = \sim 1$
Prior probability			
Conditional probability	θ	$(1 - \theta)$	μ
Joint probability	$2\theta\mu$	$2 (1 - \theta)\mu$	μ

For daughter.

Conditional probability she is a carrier given her restriction pattern	$1 - \theta$	θ	0
Conditional probability she is not a carrier	θ	$1 - \theta$	1

FINAL PROBABILITY DAUGHTER IS A CARRIER

$$\frac{4\theta (1 - \theta)}{4\theta (1 - \theta) + 2\theta^2 + 2 (1 - \theta)^2 + 1}$$

$$= 0.17$$

(for $\theta = 0.15$)

If the maternal grandfather was A2 then the final probability of the daughter being a carrier is decreased to 0.14 (for $\theta = 0.15$).

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