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Predictive diagnosis of myotonic dystrophy with flanking microsatellite markers

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Abstract

Linkage was shown between the myotonic dystrophy locus (*DM*) and a highly polymorphic AC repeat marker within the kallikrein (*KLK1*) locus ($\hat{Z}$=3·00, $\hat{\theta}$=0·0). Linkage between *KLK1* and the highly polymorphic AC repeat marker within the apolipoprotein C2 (*APOC2*) locus, which had been established in normal families, was confirmed in myotonic dystrophy families ($\hat{Z}$=4·37, $\hat{\theta}$=0·11). These highly polymorphic AC repeat markers flank *DM* on chromosome 19. The gene order is cen-*APOC2* (0·03) *DM* (0·08) *KLK1*-qter with recombination frequencies shown in parentheses. Genotypes for the AC repeat markers can be determined simultaneously by multiplex PCR and separation of the two base pair differences between adjacent alleles on sequencing gels. In informative families, this approach provides rapid diagnosis and is more accurate than methods using markers restricted to the proximal side of the myotonic dystrophy gene.

Myotonic dystrophy (DM) is the commonest muscular dystrophy of adults with a prevalence of about 1 in 20 000.[1] It is inherited in an autosomal dominant fashion and is amenable to presymptomatic and prenatal diagnosis using linked DNA markers.[2] There is no evidence to suggest that DM is genetically heterogeneous.

Asymptomatic members of DM families request presymptomatic testing in order to learn whether they will develop the disorder in the future. The information is of use in planning their lives and, if at high risk, they can choose to have prenatal diagnosis and thus prevent the birth of children who have inherited the DM gene. Prenatal diagnosis is also sought by couples who already have a child with congenital DM.

For a linkage study to be performed on a family, it is first necessary to confirm diagnoses in affected subjects and to assess the status of selected asymptomatic family members. This will allow the correct classification of as many affected family members as possible for establishment of linkage phase. Classification can sometimes be difficult because the clinical features of DM are variably expressed and may appear late in life. In practice, evidence of the disorder is sought by clinical examination, slit lamp examination of the eyes, and electromyography.

It is important to be able to determine which of the asymptomatic parents of an affected subject carries the DM gene if future family studies are to be focused on the relevant side of the family. Sometimes this is not possible, even after full neurological and ophthalmological evaluation. A linkage study using flanking DNA markers can be helpful in this regard. The proportion of cases which represent new mutations is very low.

The DM gene is located at 19q13.2–q13.3.[3] The RFLP markers *APOC2*,[4] *D19S19*,[5] *BCL3*,[6] and *CKMM*[7] are closely linked to *DM* on its proximal side and they have been used extensively for predictive diagnosis. Another closely linked marker, *D19S51*, probably on the distal side, has recently become available.[8] Until marker sequences are isolated from within the DM gene, diagnostic error associated with recombination events close to the gene can be minimised by application of informative flanking markers.

If diagnosis is to be based on informative flanking markers, the efficiency of the testing procedure needs to be optimised. Detection of diallelic RFLPs by Southern analysis is a slow, laborious procedure. The speed of diagnosis has, however, been improved through application of PCR analysis to several RFLP markers on the proximal side of *DM*.[9] Informativeness, as well as speed of diagnosis, has been improved by discovery of a highly polymorphic AC repeat sequence within the *APOC2* locus.[10] This marker has 13 alleles and has been applied to PCR based diagnosis.[11]

We describe the first highly polymorphic AC repeat marker on the distal side of *DM*. This lies within the

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kallikrein locus (*KLK1*) which has been placed onto the CEPH (Centre d'Etude du Polymorphisme Humain) multipoint genetic map as follows: cen-*D19S19* (0·03, 0·04) *APOC2* (0·10, 0·11) *KLK1* (0·05, 0·06) *D19S22*-qter, with male and female recombination frequencies given in parentheses.[12] The two point lod score between *KLK1* and *APOC2* was 28·76 with odds in support of the given order of 6×10^{35}:1. The map deduced for predictive diagnosis is: cen-*APOC2* (0·03) *DM* (0·08) *KLK1*-qter based on the precisely known locations and recombination frequency between *APOC2* and *DM* ($\hat{Z}>90$, $\hat{\theta}=0·02$–0·03).[13] For *KLK1*, nine alleles were observed with allele frequencies, in decreasing size of the AC repeat, of 0·037, 0·037, 0·037, 0·031, 0·025, 0·199, 0·068, 0·559, and 0·007.[12] *KLK1* may be used for diagnosis as a flanking marker in conjunction with any informative marker on the proximal side of *DM*.

Families and methods

Eleven South Australian families were examined. They had been referred for presymptomatic or prenatal diagnosis using linked DNA markers. Family members were assessed before DNA analysis for presence of clinical features of the disorder, characteristic ophthalmological findings on slit lamp examination, or myotonia on electromyography. The diagnosis of myotonic dystrophy was based on established criteria.[2]

KLK1 (AC) and *APOC2* (AC) genotypes were determined simultaneously by multiplex PCR (fig 1) as described previously.[12] Primer sequences for detection of *KLK1* were:

Forward primer:
5′ GGAGCTCTGACTCAGTTCTACTCTATC 3′
Reverse primer:
5′ GACTGAGACATCCTCTCCCACCCT 3′

The *APOC2* primer sequences have been given previously.[10] These are:

Forward primer:
5′ CATAGCGAGACTCCATCTCC 3′
Reverse primer:
5′ GGGAGAGGGCAAAGATCGAT 3′.

The PCR reaction mix (10 µl) was based on Kogan *et al*[13] and consisted of ammonium sulphate (16·6 mmol/l), Tris-HCl, pH 8·8 (67 mmol/l), β-mercaptoethanol (10 mmol/l), EDTA (6·7 µmol/l), bovine serum albumin (170 µg/ml), dimethylsulphoxide (10%), dATP (1·5 mmol/l), dGTP (1·5 mmol/l), dCTP (1·5 mmol/l), dTTP (1·5 mmol/l), magnesium chloride (4 mmol/l), primer (75 ng each), genomic DNA (100 ng), α-^{32}P-dCTP (1 µCi), and 0·5 units AmpliTaq (Cetus-Perkin Elmer). This was overlayed with one drop of paraffin oil and heated to 94°C for three minutes. PCR incubations were carried out in a Perkin-Elmer Cetus thermal cycler for 25 cycles of 55°C, two minutes; 72°C, three minutes; 94°C, two minutes; and completed with 55°C, two minutes; 72°C, 10 minutes. The volume was adjusted to 40 µl with formamide loading buffer (95% formamide, 1 mmol/l EDTA, 0·01% bromophenol blue, 0·01% xylene cyanol). After denaturation at 90°C for three minutes, 3 µl aliquots of each reaction were electrophoresed in 5% 0·4 mm polyacrylamide denaturing (7 mmol/l urea) sequencing gels. The gel was dried and genotypes determined after autoradiography (overnight with intensifying screen at −80°C).

Linkage and risk analyses were carried out using MLINK from the LINKAGE package of computer programs (version 5.04). Age dependent penetrance

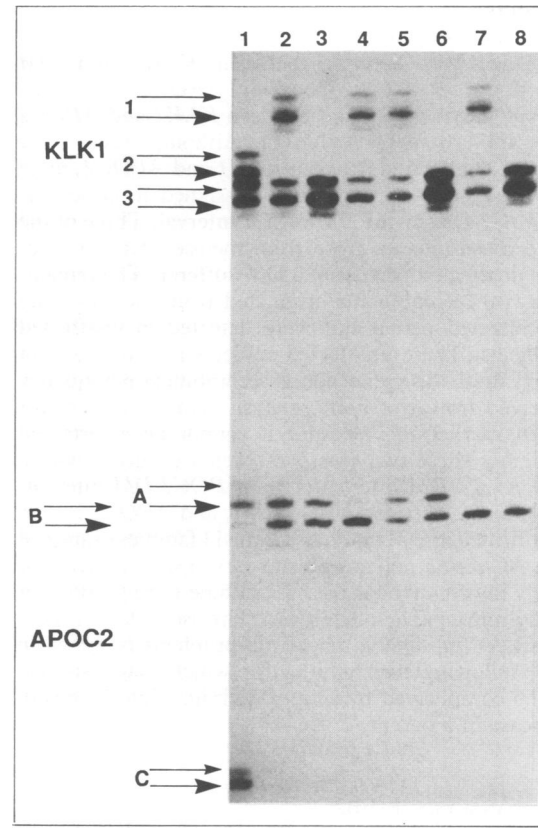

Figure 1 Genotypes determined from family 2, from left to right, are: KLK1: (1) III·1: 2/3; (2) II·2: 1/3; (3) I·1: 3/3; (4) I·2: 1/3; (5) II·3: 1/3; (6) II·4: 3/3; (7) II·6: 1/3; (8) II·5: 3/3. APOC2: (1) III·1: A/C; (2) II·2: A/B; (3) I·1: A/B; (4) I·2: B/B; (5) II·3: A/B; (6) II·4: A/B; (7) II·6: B/B; (8) II·5: B/B. Each allele consists of two primary bands. The lower molecular weight AC strand is more intense than the GT strand, using α-^{32}P-dCTP. Sometimes longer exposure is needed to visualise the GT strand clearly and this is the case for the APOC2 examples shown. In addition to the primary bands, there can be lower molecular weight 'shadow' bands associated with each strand. For simplicity, KLK1 alleles were named 1, 2, 3 and APOC2 alleles were named A, B, C in order of decreasing size of the AC repeat.

was taken into account as previously described.[2] For risk analysis, recombination frequencies with *DM* for *KLK1* and *APOC2* were assumed to be 8% and 3% respectively, with equal recombination frequencies in males and females. Interference was ignored, which for informative flanking markers has the effect of underestimating risk for subjects at high risk, and overestimating the risk to subjects at low risk. The actual chance of double recombination between flanking markers with a recombination frequency of 11% would be less than under the assumption of no interference.

Results

LINKAGE ANALYSIS

Linkage was detected between *KLK1* and *DM* ($\hat{Z}=3{\cdot}00$, $\hat{\theta}=0{\cdot}00$), between *APOC2* and *DM* ($\hat{Z}=8{\cdot}40$, $\hat{\theta}=0{\cdot}00$), and between *KLK1* and *APOC2* ($\hat{Z}=4{\cdot}37$, $\hat{\theta}=0{\cdot}11$) (table 1). Although there were five recombinants between *KLK1* and *APOC2*, none of these could be specifically assigned to either the *APOC2-DM* or the *DM-KLK1* interval. Three of the five recombinants arose from meioses derived from the unaffected partner of a DM sufferer. The remaining two recombinants originated from meioses from an affected parent but were detected in unaffected subjects. These unaffected subjects may or may not carry the disease gene; hence, recombination frequency derived from two point analysis remains at zero for both marker loci because it cannot be ascertained whether these two crossover events occurred within the *KLK1-DM* interval or the *APOC2-DM* interval.

Linkage between *KLK1*, *DM*, and *APOC2* is now confirmed in DM families. Of the 11 families examined for AC repeat polymorphisms, seven were partially or fully informative for *KLK1* and nine were partially or fully informative for *APOC2*. The usefulness of these markers for solving diagnostic problems is shown in the following two families for which diagnosis had been complicated by lack of definite clinical signs of disease in a parent.

RISK ANALYSIS: FAMILY 1

Family 1 (fig 2) was ascertained when II·6 sought genetic counselling during her first pregnancy. She had four sibs (II·1, II·3, II·4, and II·7) with symptomatic DM whose diagnoses had been, or subsequently were, confirmed by neurological examination, electromyogram, and slit lamp examination of the eyes. III·1 had congenital DM. II·7 did not agree to blood collection for DNA studies from herself or her daughter. II·6 had no features of DM on neurological examination or electromyogram, but slit lamp examination of the eyes showed a small number of lens opacities consistent with, but not diagnostic of, DM. II·2 and II·5 have not yet been assessed clinically. I·1 and I·2 had normal neurological examinations and electromyograms and I·2 was completely normal on slit lamp examination of the eyes. I·1,

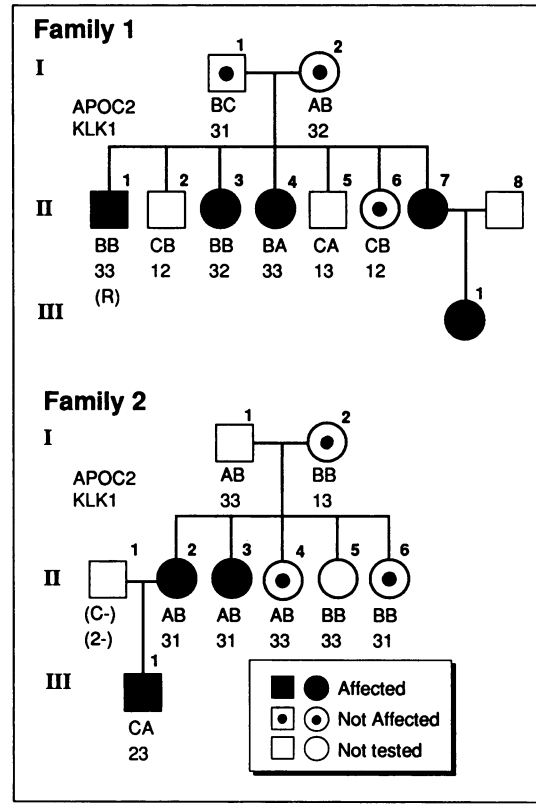

Figure 2 Genotypes for APOC2 (above) and KLK1 (below) in families 1 and 2.

Table 1 Lod scores from 11 DM kindreds.

Linkage comparison	θ						$\hat{\theta}$	$\hat{Z}$
	0·0	0·05	0·10	0·20	0·30	0·40		
DM:KLK1	3·00	2·75	2·48	1·88	1·23	0·55	0·00 (0·00–0·18)	3·00
DM:APOC2	8·40	7·54	6·65	4·79	2·89	1·16	0·00 (0·00–0·06)	8·40
KLK1:APOC2	—	3·83	4·36	3·81	2·53	1·03	0·11 (0·03–0·23)	4·37

however, had early posterior subcapsular lens opacities and numerous cortical dots with polychromatic lustre. These findings were considered consistent with, but not diagnostic of, DM.

Results for family 1 are shown in fig 2. Linkage phase of marker loci was inferred on the basis of the least number of recombinants within the linkage group. There is no recombination between flanking markers except in the maternally derived chromosome of II·1. The two affected subjects II·3 and II·4, who are non-recombinants, share only the B and the 3 alleles. Therefore, the chromosome bearing the B and 3 alleles most likely carries the disease gene and I·1 is the probable transmitter of the DM gene. This is confirmed by computation of risks using MLINK (table 2). Having established the identity and origin of the disease chromosome, II·2, II·5, and II·6 are at very low risk of developing DM (table 2).

RISK ANALYSIS: FAMILY 2

Family 2 (fig 2) was ascertained after the birth of III.1 with severe congenital DM. His mother, II·2, had the typical clinical and electromyographic features of DM. Her sister II·3 was subsequently shown to be mildly affected on clinical and electromyographic criteria although slit lamp examination of the eyes was normal. II·4 and II·6 were normal on clinical assessment, electromyogram, and slit lamp examination of the eyes. I·2 had no abnormalities on neurological examination or electromyogram, but slit lamp examination showed opacities in the anterior and posterior subcapsular regions of the lenses consisting of flakes and flecks and an occasional polychromatic crystal. The features were considered to be consistent with, but not diagnostic of, DM. I·1 did not wish to be examined. He did, however, consent to donate a blood sample for the DNA analysis.

The *KLK1* and *APOC2* results for family 2, with inferred linkage phase, are shown in fig 2. Recombination between flanking markers could not be assessed in generation II because in I·1 *KLK1* was uninformative and in I·2 *APOC2* was uninformative. The affected subjects II·2 and II·3 share the same parental chromosome segments around the region of interest. Thus, the chromosome carrying the DM gene could bear either the A and 3 alleles, or the B and 1 alleles. The affected child III·1 resolved this uncertainty. He received the A and 3 alleles which flank the disease gene from his grandfather, I·1. The disease gene in III·1 could only come from the grandmother, I·2, if there was a double crossover between flanking markers in the meiosis of II·2. The very low likelihood of this event is reflected in the risk analysis (table 2). Affection status is associated with the A and 3 alleles, putting II·4 at high risk (92·7%) and reducing the risk for II·5 and II·6 to 3·1% and 1·3%, respectively.

Table 2 Genetic risks computed from the MLINK program of the LINKAGE package, incorporating age dependent penetrance and DNA results.

Subject	Age examined for clinical signs (y)	Prior risk (%)	Posterior risk (penetrance/DNA) (%)
Family 1			
I·1	68	50	99·85
I·2	62	50	0·15
II·2	Not tested*	50	0·35
II·5	Not tested*	50	0·34
II·6	28	50	0·19
Family 2			
I·1	Not tested*	50	99·98
I·2	51	50	0·02
II·4	26	50	92·72†
II·5	Not tested*	50	3·10
II·6	19	50	1·33

*For risk analysis, untested subjects were coded as affection status unknown. Absence of clinical signs would further reduce the risk for II·2 and II·5 in family 1 and II·5 in family 2.
†Ignoring the absence of clinical signs, the risk based on DNA markers alone is 96·54%.

Discussion

The frequency of recombination between *KLK1* and *APOC2* in the DM families was 11%. This is the same as that determined from normal families from CEPH.[12] Locus order, of *DM* in relation to *KLK1* and *APOC2*, could not be confirmed with absolute certainty from informative three point crosses owing to the absence of any definite crossovers involving *KLK1* and *APOC2* in the DM families. Locus order was inferred from the firmly established background map based on a large number of informative meioses[12] and from the precisely determined recombination frequency between *APOC2* and *DM*.[14] This order agrees with physical mapping which places *KLK1* distal to the human chromosome 19q breakpoints (near the DM locus) in human-rodent somatic cell hybrids.[12] One set of reciprocal hybrids had a breakpoint at *CKMM* and another hybrid had a breakpoint distal to *ERCC1*.

The diagnostic value of highly informative PCR based markers flanking *DM* is exemplified by the two families presented here. Genetic risks based only on informative markers proximal to *DM* can be misleading when an undetected crossover occurs between the marker and the DM locus. Such a crossover is detectable using an informative distal marker, such as *KLK1*, in addition to a proximal marker. The problem in using *KLK1* and *APOC2* as flanking markers for predictive diagnosis is that there will be a recombination event between them in 11% of meioses. This is an issue when an affected subject requests prenatal diagnosis or when an asymptomatic subject requests presymptomatic diagnosis, since in 11% of diagnostic tests a single crossover will be recognised

and no definitive result can be given. This may be resolved in such cases by the subsequent use of two RFLP polymorphisms at *D19S51*. This locus is also distal to *DM*, but much closer to it than *KLK1*.

A diagnostic strategy starting with a PCR reaction, simultaneously detecting hypervariable AC repeats within *KLK1* and *APOC2*, will quickly provide answers to many diagnostic requests. *KLK1* does not map sufficiently close to *DM* to be useful for diagnosis when it alone is informative. It must be applied in conjunction with an informative marker on the proximal side of the disease gene. Given the level of heterozygosity expected from allele frequencies for *KLK1* (64%)[12] and *APOC2* (81%),[10] this marker set represents a powerful combination for predictive diagnosis. In addition, both markers have multiple alleles allowing the detection of non-paternity and identification of mislabelled samples from family members, which further improves the accuracy of the diagnosis.

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