A model system for the analysis of gene exclusion: cystic fibrosis and chromosome 19

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SUMMARY We have used multilocus analysis to exclude the cystic fibrosis locus from six polymorphic DNA markers covering most of chromosome 19. A substantial increase in the confidence for exclusion was obtained using the computer programme LINKAGE compared to analysis of pairwise lod scores. A structured approach to the analysis of linkage to autosomal recessive inherited diseases where the biochemical defect is not known is described.

Cystic fibrosis (CF) is the most common genetic disease in North European populations, with an incidence of 1 in 2000 live births in the United Kingdom. The biochemical basis of the mutation in CF is not known, although membrane transport phenomena associated with the disease have recently been described.^{1,2} Evidence from segregation analyses has confirmed that the inheritance of CF is consistent with its being a recessive defect caused by a mutation at a single autosomal locus.³

The use of DNA probes for the analysis of genetic disease allows the determination of linkage between a restriction fragment length polymorphism (RFLP) and a disease locus even when the biochemical defect is unknown. This approach has already been used successfully by us to analyse sex linked genetic diseases such as Duchenne muscular dystrophy,⁴ as well as to study previously unassigned autosomal dominant diseases such as Huntington's disease.⁵

There were, until recently, few clues as to the chromosomal localisation of the CF locus (CF). Mayo *et al*⁶ suggested that CF might be located on chromosome 4, based on data from cell hybrids expressing a ciliary dyskinesia factor. We have shown that the mutation causing CF is unlikely to be on chromosome 4 by exclusion mapping with several DNA and protein markers.⁷ Two different families have been reported showing chromosomal anomalies, one of which suggested that the CF mutation mapped to chromosome 5p,⁸ and the other suggested

chromosome 13q34.⁹ We investigated the latter family initially by mapping the gene for factor X to 13q34 by gene dosage experiments and then excluded CF from linkage to factor X.^{10,11} We have also demonstrated that certain candidate genes, such as complement component 3, which had been proposed as the locus of the CF mutation, do not segregate with the disease.¹²

Since this research was completed, Eiberg *et al*¹³ have reported linkage between CF and PON (para-oxonase) and several groups, including our own, have reported that the mutation causing cystic fibrosis is located on the long arm of chromosome 7 and shows linkage to 3·11 and TCRB,¹⁴ to COL1A2,¹⁵ to MET,¹⁶ and to DOCR1-917.¹⁷

Although linkage between markers and CF has now been shown, our data for chromosome 19 illustrate the use of multipoint computer analyses which analyse multiple linked markers along a single chromosome to maximise data from the few informative families that can be obtained. It is therefore a paradigm for any single gene autosomal recessive disease where the biochemical defect is unknown. Multipoint linkage is of particular value as it usefully extends the exclusion regions between the markers to exclude most of the chromosome.

Materials and methods

GENE PROBES LOCATED ON CHROMOSOME 19
The cloned DNA sequences used in this study are listed in table 1 together with regional assignments of the markers and details of the RFLP defined by

TABLE 1 Polymorphisms used in this study.

Marker	Regional assignment	RFLP enzyme	No of alleles	Frequency of rare allele	References
LDLR	p13-2→p13-1	<i>PvuII</i>	2	0.27	26
C3	p13-3→p13-2	<i>SstI</i>	2	0.39	12
APOC2	cen→q13-2	<i>TaqI</i>	2	0.40	27
D19S9	p13-2→p13-2	<i>EcoRI</i>	2	0.19	21
CYP1	q13-1→q13-3	<i>SstI</i>	2	0.47	28
D19S6	q13-3→qter	<i>ApaI</i>	2	0.48	29

each clone. A cDNA encoding the LDL receptor gene was kindly provided by Dr D Russell. The gene for complement 3 was obtained from Dr G Fey,¹⁸ the cDNA clone for apolipoprotein C2 (APOC2) was isolated in this laboratory,¹⁹ and the cytochrome p450 cDNA recombinant was provided by Dr E Shepherd.²⁰ Random probes D19S9 and D19S6 were provided by Drs D Shaw²¹ and R Bartlett respectively.

CYSTIC FIBROSIS FAMILIES

Twelve European families with at least three affected children and up to four unaffected sibs and two families with two affected children and two normal sibs were used in this study. Blood was collected from all children and parents and DNA prepared by standard methods.²²

HYBRIDISATION ANALYSIS

DNA 5 µg from each family member was digested with the appropriate restriction enzyme according to the manufacturer's instructions (BRL Ltd, Irvine, Scotland), fractionated by electrophoresis on 0.8% agarose, and transferred to Zetapore membranes (AMF Cuno) as described by Maniatis *et al.*²³ Hybridisation and autoradiography were carried out as described previously.²⁴

LINKAGE ANALYSIS

Pairwise lod scores were calculated using the LIPED computer programme adapted by Ott²⁵ for use with microcomputers. The allele frequencies used are given in table 1. The multipoint linkage computer package LINKAGE³⁰ was used to test for linkage to cystic fibrosis at locations within a fixed map of known linked markers, as discussed by Farrall *et al.*³¹ The order of loci *LDLR-C3-APOC2-CYP1-D19S6* was deduced from physical assignments, two point lod scores, and multipoint calculations.^{29,32} There were insufficient data to place *D19S9* accurately within the other markers. Estimates of recombination fractions between markers were taken from HGM8 and our own multipoint estimates. Each map distance is calculated from the upper limit (95% confidence interval) of the recombination fraction

estimate by using Haldane's mapping function.³³ The use of this mapping function effectively disregards the influence of interference in subsequent analyses. By using the upper limits of the recombination fraction estimates and ignoring interference, the estimates of exclusion are cautious. Multipoint likelihoods may be conveniently presented as a location map, in the manner of Lathrop *et al.*³⁰ For pairwise analyses, a lod score of -2.0 calculated for combined sexes was considered to exclude linkage significantly between *CF* and each marker at the relevant recombination fraction. For multipoint calculations, a location score of -9.2 (lod equivalent = -2.0) for *CF* within the fixed map of markers was similarly considered to exclude linkage.

Results

Table 2 shows the pairwise lod scores obtained for each probe and *CF*. Fig 1 shows the physical location of the markers used in this study. Fig 2 shows the multipoint likelihood map for *CF* within the linked markers *LDLR*, *C3*, *APOC2*, and *CYP1*. From the pairwise results we can exclude *CF* from moderate linkage to *LDLR* and *C3*, and tight linkage to *APOC2*, *D19S9*, *CYP1*, and *D19S6*. Multipoint analysis shows that the exclusion regions may be extended between *LDLR*, *C3*, *APOC2*, and *CYP1*. The total excluded genetic length for multipoint analysis is 88 centiMorgans (cM) (*LDLR-C3-APOC2-CYP1-D19S6*). An estimate of the total

TABLE 2 Lod scores at selected recombination fractions between *CF* and six chromosome 19 markers.

Marker	No of meioses	Recombination fraction					
		0.05	0.10	0.20	0.30	0.40	
LDLR	30	-3.70	-2.31	-0.95	-0.35	-0.07	m
	20	0.05	0.40	0.48	0.31	0.11	f
C3		-4.40	-2.21	-0.68	-0.06	-0.004	c
	44	-1.83	-1.02	-0.32	-0.06	0.08	m
	37	-2.20	-1.20	-0.42	-0.11	-0.03	f
		5.62	-2.91	-0.84	-0.13	-0.03	c
APOC2	53	0.12	0.28	0.30	0.19	0.04	m
	26	-1.40	-0.78	-0.27	-0.07	-0.02	f
D19S9		-3.22	-1.40	-0.24	0.07	0.02	c
	21	-0.66	-0.15	0.18	0.15	0.06	m
	12	-1.52	-0.95	-0.42	-0.17	-0.04	f
		-2.18	-1.10	-0.24	0.02	0.02	c
CYP1	32	0.31	0.56	0.55	0.28	0.03	m
	24	-2.40	-1.62	-0.69	-0.23	-0.08	f
D19S6		-3.10	-1.31	-0.11	0.16	0.06	c
	19	-0.72	-0.44	-0.19	-0.07	-0.01	m
	24	-0.55	-0.28	-0.08	0.00	0.00	f
		-1.60	-0.39	-0.11	-0.01	0.00	c

m = male, f = female, c = combined.

Lod scores are calculated for:

Males only χ^2_{m1} { $\theta_{CF,m1}$, $\theta_{CF,m1} = 0.5$ }

Females only χ^2_{f1} { $\theta_{CF,f1}$, $\theta_{CF,f1} = 0.5$, $\theta_{CF,f1}$ }

Combined sexes χ^2_{c1} { $\theta_{CF,c1}$, $\theta_{CF,c1} = \theta_{CF,m1}$, $\theta_{CF,c1}$ }

FIG 1 A physical map of chromosome 19 showing the relative positions of the markers used in this study.

FIG 2 A likelihood map showing the location score for CF at various map positions with respect to four fixed marker loci: LDLR, C3, APOC2, and CYP1. Genetic location is calculated with 3 at the arbitrary origin, that is, $w=0$ Morgans. Location scores $[1(w_d)]$ were calculated assuming equal male and female distances and the LDLR-C3 distance = 30 cM, the C3-APOC2 distance = 15 cM, and the APOC2-CYP1 distance = 10 cM.^{29,32} The broken line indicates a lod -2 exclusion limit.

excluded genetic length derived from pairwise analyses is 102 cM. This is calculated by doubling the exclusion region around each locus and summing the individual regions.

Discussion

By analysing the segregation of cystic fibrosis with informative polymorphic DNA markers we have excluded CF from a major proportion of chromosome 19. This has been achieved by the use of a fixed genetic map and multipoint analysis. The total exclusion limits achieved by multilocus analysis may be compared to that calculated by adding the exclusion limits derived from pairwise scores at each locus. For these data pairwise methods appear to exclude a larger area. However, adding pairwise scores is only valid if the scores for each pair of loci are independent. The data presented here are not independent; the same meioses contribute to several lod tables. Hence, multipoint analysis, which allows correctly for the interdependence of data at several loci, is the only valid method for estimating the total exclusion region for this data set. We note that the confidence for exclusion between markers using multipoint analysis is substantial, an example of the increase in information derived from each family when multipoint methods are used.

In the case of many autosomal recessive disorders of unknown biochemical aetiology, the search for a linkage is made more difficult by the absence of a reliable heterozygote detection system, thereby reducing the statistical power of each disease family; in general, only the parents and affected sibs contribute significantly to the lod score. In such circumstances a 'random' probe approach is less powerful than a structured approach, which involves the construction of genetic maps followed by the systematic exclusion of a disease locus from all points between a set of markers. This becomes particularly important if there is reason to suspect genetic heterogeneity.

The chromosomal location of CF is now known and therefore this paper is not relevant to exclusion mapping for the CF locus. However, it illustrates the power of LINKAGE in increasing the exclusion length using ordered markers on a single chromosome, and provides a model for this approach to an autosomal recessive disease. This model will be useful for the analysis of rare recessive diseases where the maximal information for linkage may be derived from the few families available for testing.

We wish to thank the many clinicians and families who have made this work possible by their enthusiasm and cooperation, Drs Bartlett, Fey, Humphries,

Russell, Shaw, and Shepherd for the use of their cloned gene probes, and Dr Peter Scambler for helpful discussions. This work was supported generously by the UK Cystic Fibrosis Research Trust and the Medical Research Council. MF is a Foulkes Foundation fellow.

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