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An Efficient Strategy for Gene Mapping Using Multipoint Linkage Analysis: Exclusion of the Multiple Endocrine Neoplasia 2A (MEN2A) Locus from Chromosome 13

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SUMMARY

Members of four families in which multiple endocrine neoplasia type 2A (MEN-2A) is segregating were typed for seven DNA markers and one red cell enzyme marker on chromosome 13. Close linkage was excluded between the MEN2A locus and each marker locus tested. By means of multipoint analysis and the genetic map of chromosome 13 developed by Leppert et al., MEN2A was excluded from any position between the most proximal marker locus (D13S6) and the most distal marker locus (D13S3) and from within 12 cMorgans outside these two loci, respectively. However, the support of exclusion within an interval was diminished under the assumption of a substantially larger genetic map in females. The strategy of multipoint analysis, which excluded between 1.5 and 2.0 times more chromosome 13 than did two-point analysis, demonstrates the utility of linkage maps in mapping disease genes.

INTRODUCTION

Multiple endocrine neoplasia type 2A (MEN-2A) is a dominantly inherited disorder characterized by medullary carcinoma of the thyroid, pheochromocy-

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toma, and parathyroid adenoma. It is distinguished from MEN-2B by the presence of mucosal neuromas in the latter. It is almost completely penetrant by 40 years of age (Gagel et al. 1982). Although screening tests allow early detection of tumors (Wells et al. 1978), they are both unpleasant and expensive. Identification of a genetic marker very closely linked to the MEN2A locus would improve genetic counseling for family members and make screening unnecessary for the 50% of relatives who do not have the mutation. It would also enable a better understanding of the pathophysiology of the disease and lead to characterization of the gene at the molecular level.

Analysis of classical blood group and serum protein markers (Simpson 1984; Ferrell et al. 1985; Kruger et al. 1986) and a large group of DNA markers defined by restriction-fragment-length polymorphisms (RFLPs) (Goodfellow 1985; Goodfellow et al. 1985; Kidd et al. 1986) have not yielded evidence for linkage. Initially, DNA markers were tested for chromosome 20 (Goodfellow 1985; Goodfellow et al. 1985), because of the suggested deletion of 20p12.2 (Babu et al. 1984; Van Dyke et al. 1984). In all of these studies, one marker at a time was tested for linkage with the MEN2A locus and no statistical use was made of available information on linkage between the markers.

Multipoint linkage analysis permits simultaneous examination of several loci and provides greater precision than two-point analysis for the detection or exclusion of linkage between a disease locus and a cluster of linked marker loci (Lathrop et al. 1985). The precision is gained from the detection of multiple crossovers (Bowcock et al., in press-*a*) and meiotic events that are uninformative in two-point analysis. One can obtain from the linkage data an odds ratio for each possible gene order by comparing the relative likelihoods associated with different gene orders (Ott 1985). This technique has been used to exclude the MEN2A locus from large portions of chromosomes 11p (K. K. Kidd, unpublished data) and 20 (Farrer et al., in press). We report here linkage data between the MEN2A locus and eight loci that have been mapped and ordered on the long arm of chromosome 13 (Leppert et al. 1986). Multipoint analysis using these loci and their map positions enabled us to exclude MEN2A from chromosome 13.

MATERIAL AND METHODS

Subjects and Typing

Members of three kindreds were studied: the New Haven (N) kindred (Kruger et al. 1986), the Kingston R family (Goodfellow et al. 1985), and the Winnipeg C family (Birt et al. 1977). All samples were typed for four polymorphic loci on chromosome 13—namely, ESD, D13S1, D13S2, and D13S3. In addition, the N family was typed for D13S5, D13S6, and D13S7 and the R and C families were typed for D13S4. The red cell enzyme EsD was typed by means of standard techniques (Harris and Hopkinson 1976). DNA isolation procedures are described in Goodfellow et al. (1985). Standard Southern blotting methods were used. Procedures for the interpretation of the RFLP systems are outlined by Cox and Gedde-Dahl (1985) and Leppert et al. (1986).

Linkage Analysis

Lod scores for a set of recombination frequencies from 0 to 0.5 were calculated for MEN2A versus each marker by means of the computer program LIPED (Ott 1974), modified to incorporate correction for age of onset (Hodge et al. 1979). Penetrance (defined here as age-specific probability of detection of the disease) intervals were considered to increase linearly from a value of zero at birth to a maximum of 0.99 at age 35 years and older (Farrer et al., in press). The MEN-2A status was coded as affected, unaffected, or unknown. All analyses assumed a phenocopy frequency of .001 and a gene frequency of .0005 for the MEN2A allele.

A series of joint analyses of MEN2A and two contiguous marker loci was carried out using the program LINKMAP from the LINKAGE package (Lathrop et al. 1984, 1985). In these analyses, age-dependent penetrance of MEN-2A was defined as a step function based on six age intervals (Farrer et al., in press) corresponding as closely as possible to the function used in the LIPED analyses. Distances between markers were fixed according to the genetic map of Leppert et al. (1986) for the long arm of chromosome 13 (fig. 1). D13S5, which was typed in the N kindred only, and D13S4, which was typed in the R and C families only, were considered as a single locus, since no recombinants were found in the Utah data (Leppert et al. 1986). D13S7, which was mapped to the same location as D13S2 by Leppert et al. (1986), was excluded from the multipoint analysis because, in the one family typed for this locus, it was less informative for linkage than D13S2. In all, six distinct marker loci were considered in the multipoint analyses. Higher-order multipoint analyses were not practical because these data consist of complex pedigrees and several multiallelic marker systems—and were not warranted because the pedigrees are reasonably informative for most loci. Each joint analysis of three loci was done twice; in one analysis rates of recombination among males and among females were assumed to be equal, and in the second analysis a constant map ratio of 3.89 was used to allow for higher recombination rates in females (Leppert et al. 1986).

RESULTS

Close linkage was excluded by two-point analysis between the MEN2A locus and each of the chromosome 13 loci tested (table 1). D13S1 is the only locus for which loose linkage to MEN2A was suggested. On the basis of two-point analysis alone, no more than 48 cMorgans of this chromosome can be excluded from consideration (fig. 1).

By means of multipoint analysis under the assumption of equal recombination rates in males and females, the MEN2A locus was excluded from any position between the most proximal marker locus (D13S6) and the most distal marker locus (D13S3) (fig. 1), with relative odds of at least 500 to 1 (fig. 2). The MEN2A locus was also significantly excluded from within 12.3 cMorgans and 11.9 cMorgans outside the distance between D13S6 and D13S3, respectively, assuming that there are $\geq$12.3 cMorgans from D13S6 to 13cen and 11.9

TABLE 1

RESULTS OF PAIRWISE LINKAGE ANALYSIS BETWEEN MEN2A AND CHROMOSOME 13 MARKERS

MARKER AND KINDRED	RECOMBINATION FRACTION ($\theta_m = \theta_f$)						
	.00	.001	.05	.10	.20	.30	.40
ESD:							
N	−2.46	−1.85	−.34	−.11	.04	.06	.04
R	−5.12	−4.65	−1.76	−1.19	−.65	−.35	−.15
C	.00	.00	.00	.00	.00	.00	.00
Total	−7.58	−6.50	−2.10	−1.30	−.61	−.29	−.11
D13S1:							
N	−10.05	−6.74	−2.00	−1.10	−.36	−.09	−.02
R	−2.64	−1.06	.49	.64	.62	.46	.24
C	−3.76	−1.39	.19	.36	.38	.26	.09
Total	−16.86	−9.19	−1.32	−0.10	0.64	0.63	0.31
D13S2:							
N	−6.97	−6.41	−3.20	−2.11	−1.11	−.59	−.24
R	.38	.38	.34	.30	.22	.15	.08
C	−6.55	−4.33	−1.33	−.72	−.21	−.04	−.01
Total	−13.14	−10.36	−4.39	−2.53	−1.10	−.48	−.17
D13S3:							
N	−2.61	−.70	.69	.69	.44	.19	.04
R	−4.33	−2.01	−.41	−.20	−.06	−.01	.00
C	−3.24	−2.80	−1.20	−.82	−.40	−.16	−.04
Total	−10.18	−3.83	−.92	−.33	−.02	.02	.00
D13S4/D13S5:							
N	−2.53	−2.23	−.69	−.32	.00	.07	.02
R	−2.44	−1.98	−.53	−.29	−.11	−.04	−.02
C	−1.21	−1.20	−.92	−.69	−.35	−.14	−.03
Total	−6.18	−5.41	−2.14	−1.30	−.46	−.11	−.03
D13S6							
N	−10.07	−6.06	−1.61	−.64	.09	.24	.15
D13S7							
N	−6.48	−4.93	−1.32	−.64	−.12	.04	.04

cMorgans from D13S3 to 13qter. Sex-specific differences in recombination frequency reduced the support for exclusion of the MEN2A locus from within each interval. However, all lod scores among intervals between D13S6 and D13S3 were < -1.6 (fig. 2).

DISCUSSION

Using multipoint linkage analysis, we have been able to exclude the MEN2A locus from a minimum of 71 cMorgans and a maximum of 95 cMorgans from chromosome 13. The 95-cMorgan estimate of exclusion includes the distances flanking the two markers (D13S6 and D13S3) mapped at the extreme ends. The lower estimate of exclusion (corresponding to the map distance between D13S6 and D13S3) does not take into account subsequent refinements to the genetic map of chromosome 13q—namely, an estimate of 5.5 cMorgans between D13S10 and ESD (Bowcock et al., in press-*b*) and the observation of one recombinant between D13S4 and D13S5 (A. M. Bowcock and L. A. Farrer,

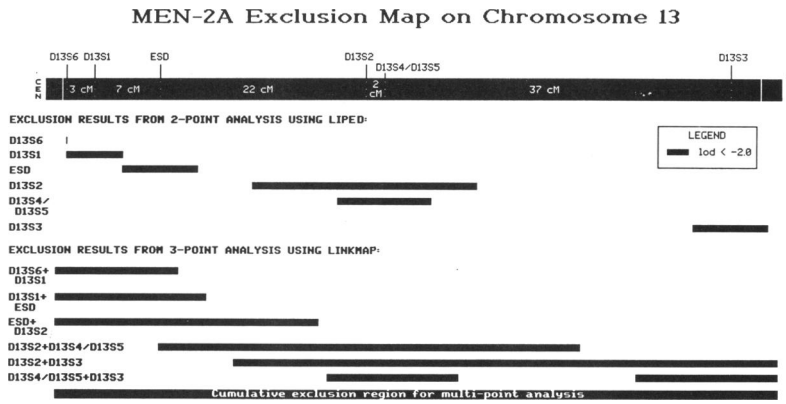

FIG. 1.—Genetic map (from Leppert et al. 1986) of the long arm of chromosome 13. Distances between D13S6 and D13S3 are drawn to scale. Genetic distances (given in cMorgans [cM]) are the map-unit equivalent of small values of recombination (θ) (see Ott [1985] for description of mapping functions). A lod score ≤ -2 is sufficient evidence for exclusion at a particular θ value (Morton 1955). Exclusion regions for the MEN2A locus that extend beyond the scaled map are actually larger than depicted.

unpublished results). There is a remote possibility that MEN2A is located between D13S6 and D13S3 if one assumes large differences between the male and female genetic maps.

Failure to detect linkage because of genetic heterogeneity or variable age of onset of tumors among families may be circumvented by selecting large informative families and applying an appropriate age-correction function (Kidd et al. 1984; Goodfellow et al. 1985). In these analyses, heterogeneity of MEN-2A could not be assessed statistically because no combination of families yielded sufficiently large lod scores. The marginal evidence, when two-point analysis is used, for loose linkage with D13S1 in the K and C kindreds and with D13S3 in the N kindred is negated in the pooled data, although the possibility that the MEN2A locus is located proximal to D13S1 in the K and C families or distal to D13S3 in the N kindred cannot be dismissed absolutely. Multipoint analyses within the K and C families decrease the support for linkage with D13S1 but not for linkage with D13S6, although the peak is shifted distally.

The power of multipoint linkage analysis in demonstrating linkage of a locus for a rare inherited disease to a location on a genetic map has been proven for four X-linked (Drayna and White 1985; Kidd et al. 1985; Baehner et al. 1986; Read et al. 1986) and two autosomal recessive (Tsui et al. 1985; Bowcock et al., in press-*a*) disorders. Although this technique has also been used to exclude disease loci from large chromosomal segments for cystic fibrosis (Farrall et al. 1986; Wainwright et al. 1986), torsion dystonia (Kramer et al., in press) and MEN-2A (Farrer et al., in press), this is one of the first reports of exclusion, by linkage analysis, of almost an entire chromosome as a possible location for a disease gene (Wainwright et al. 1986). The most proximal marker on the long arm of chromosome 13 (D13S6) has been localized to band q13, and the most distal marker (D13S3) has been assigned to q33-34 (Dryja and Morton 1985).

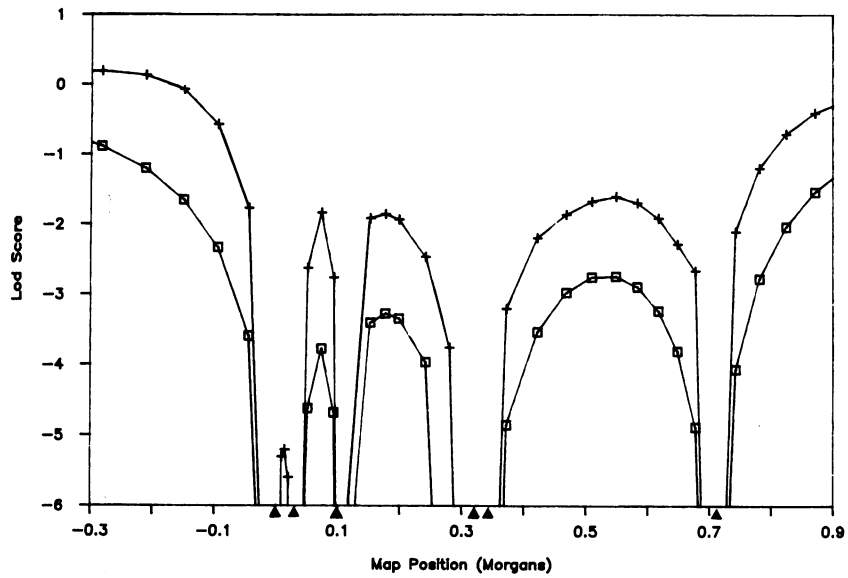

FIG. 2.—Support for exclusion of MEN2A from chromosome 13. The marker D13S6 was arbitrarily set at 0.00, and the other marker loci were positioned from it as described by Leppert et al. (1986): D13S1 at 0.03 Morgans, ESD at 0.10 Morgans, D13S2 at 0.32 Morgans, D13S4/D13S5 at 0.34 Morgans, and D13S3 at 0.71 Morgans. Locations for marker loci are designated by arrowheads on the map-position axis. Two lod-score curves are shown. The bottom curve (denoted by open boxes for computed lod scores at particular map locations) shows support for exclusion under the assumption of equal recombination rates in males and females. Maximum lod scores between D13S6 and D13S1 and between D13S2 and D13S4/D13S5 are -6.90 and -14.70, respectively (data not shown). Lod scores for all map positions < -0.2 and >0.9 are ≤ 0. The top curve (denoted by plus signs [+]) shows support for exclusion when female map distances were fixed as a constant ratio of 3.89 times the male map distances. Maximum lod score between D13S2 and D13S4/D13S5 is -12.43 (data not shown). Lod scores for all map positions < -0.2 and >0.9 are <0.20.

The distance between them represents at least 75% of the physical map and 71 cMorgans of the male genetic map of chromosome 13. Because there is evidence that interference—as judged by the minimum distance separating chiasmata within a chromosome arm—does not act uniformly over equal physical distances throughout the genome (Laurie and Hultén 1985), it is difficult to estimate how much more than 75% of chromosome 13 we have excluded as the site of the MEN2A locus. In any case, the strategy of multipoint analysis excluded between 1.5 and 2.0 times more of chromosome 13 as a site for MEN2A than did two-point analysis.

The present study and others (Farrer et al., in press; Kramer et al., in press; Bowcock et al., in press-*a*) have enabled us to realize some important considerations in and practical limitations to multipoint linkage analysis as a means of mapping disease genes. After relabeling phenotypes to reduce the number of alleles at a genetic locus to no more than three (Braverman 1985; Lathrop et al. 1986), we performed these analyses on a VAX 11/750 mainframe computer using version 3.1 of LINKMAP that was modified to improve I/0 format. Because

of the gain in information from meioses that are otherwise less informative, one should, under ideal circumstances, perform joint analysis of more than three loci if such data are available. (A more mathematical explanation of this point is given in Ott [1985]). A sweep across the genetic map of chromosome 13 by means of a series of three-point analyses, in each of which MEN2A and two marker loci were considered jointly (see fig. 2), required ~45 CPU h; higher-order analyses could not be done in a reasonable (i.e., affordable) amount of time. Our analyses were hindered by a number of factors. The pedigrees ascertained were large and complex. Many key individuals in these kindreds were deceased and could not be typed. Age-of-onset correction is essential for a disease with variable onset. All of these factors contributed to the enormous amount of time required by the computer program. Unfortunately, subsequent versions of LINKMAP, which feature improved computational algorithms, cannot as yet handle complex pedigrees, and programs that can compute likelihoods for joint analysis of a large number of loci (see, e.g., Lander et al. 1986) are still under development. Despite these limitations, however, our study supports the development and use of linkage maps for mapping disease genes (Botstein et al. 1980; Lathrop et al. 1985; White et al. 1985).

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