Examination of X Chromosome Markers in Rett Syndrome: Exclusion Mapping with a Novel Variation on Multilocus Linkage Analysis

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Summary

Rett syndrome is a neurologic disorder characterized by early normal development followed by regression, acquired deceleration of head growth, autism, ataxia, and stereotypic hand movements. The exclusive occurrence of the syndrome in females and the occurrence of a few familial cases with inheritance through maternal lines suggest that this disorder is most likely secondary to a mutation on the X chromosome. To address this hypothesis and to identify candidate regions for the Rett syndrome gene locus, genotypic analysis was performed in two families with maternally related affected half-sisters by using 63 DNA markers from the X chromosome. Maternal and paternal X chromosomes from the affected sisters were separated in somatic cell hybrids and were examined for concordance/discordance of maternal alleles at the tested loci. Thirty-six markers were informative in at least one of the two families, and 25 markers were informative in both families. Twenty loci were excluded as candidates for the Rett syndrome gene, on the basis of discordance for maternal alleles in the half-sisters. Nineteen of the loci studied were chosen for multipoint linkage analysis because they have been previously genetically mapped using a large number of meioses from reference families. Using the exclusion criterion of a lod score less than -2, we were able to exclude the region between the Duchenne muscular dystrophy locus and the DXS456 locus. This region extends from Xp21.2 to Xq21-q23. The use of the multipoint linkage analysis approach outlined in this study should allow the exclusion of additional regions of the X chromosome as new markers are analyzed. This in turn will result in a defined region of the X chromosome that should be searched for candidate sequences for the Rett syndrome gene in both familial and sporadic cases.

Introduction

Rett syndrome is a progressive neurologic disorder characterized by cessation of normal development at 6–18 mo of age, loss of purposeful hand use, acquired deceleration of head growth, autism, and stereotypic hand movements (Rett 1966; Hagberg et al. 1983). This typical phenotype has been observed exclusively

in females. The disorder has an estimated prevalence of 1/10,000 females, and the majority of the cases are sporadic (Hagberg 1985). A few familial cases with inheritance through maternal lines have been reported (Hagberg et al. 1983; Zoghbi 1988). In addition, all MZ twins (seven pairs) identified to date have been concordant, while all DZ twins (11 pairs) have been discordant (International Rett Syndrome Association, personal communication). These observations have led to the hypothesis that Rett syndrome is a genetic disorder most likely caused by a mutation on the X chromosome. Linkage analysis in familial cases by using numerous X chromosome DNA markers is a valid

approach to test this hypothesis. However, given the small number of familial cases with Rett syndrome, the feasibility of genetic analysis in search of a closely linked marker is very limited. Indeed, given the small number of meioses, it will be extremely difficult to obtain a lod score of 2 or greater in support of linkage to a specific marker on the X chromosome. An alternative linkage approach is that of exclusion mapping, which relies on concordance/discordance analysis for X chromosome DNA markers in the families with affected half-sisters. This approach relies on the hypothesis that the mother is likely a carrier of the Rett syndrome gene because the half-sisters have different fathers. Accordingly, if Rett syndrome is X linked, one would expect both sisters to have concordance for alleles at markers in the putative Rett region. Discordant regions, indicating that the two sisters have inherited different maternal alleles, can be excluded. Typically in this concordance/discordance analysis one can only exclude the specific loci analyzed if one studies a small number of informative meioses. Recently Archidiacono et al. (1991) performed such an analysis using one set of half-sisters and 34 X chromosome markers; 15 markers were informative, and four markers were discordant. Because these markers were widely distributed along the length of the X chromosome, the discordance at the four loci DXS278, DXS92, DXSZ1, and DXYS1 essentially allowed the exclusion of only these specific loci as candidates for the Rett syndrome gene, and it did not permit the exclusion of regions between markers.

In the present study, we performed genetic analysis in two families with affected half-sisters by using more than 60 X chromosome markers which included standard RFLPs and highly informative short tandem repeats. To allow for the exclusion of more than just the discordant loci, we developed a method for multipoint linkage analysis that will allow the exclusion of intermarker regions according to the criterion of lod score less than − 2.

This method will have general applicability for exclusion mapping for any chromosome and can be applied to any genetic disorder in which the number of families or informative meioses is too small for conventional linkage analysis.

Subjects, Material, and Methods

Subjects

Two families with recurrent Rett syndrome were used in the present study (fig 1). Each of the families

had a pair of affected half-sisters, with the mother being the common parent in each case.

Somatic Cell Hybrids

DNA samples from the fathers in both families were not available; therefore somatic cell hybrids were made for each female in the two families, in order to separate and identify the maternal and paternal X chromosomes. Lymphoblastoid cell lines for the females in family 2 have been established elsewhere (Archidiacono et al. 1991). Lymphoblastoid cell lines for each female in family 1 were transformed with Epstein-Barr virus. Transformed cells were then fused to RJK88 cells, a derivation of the Chinese hamster lung cell line V79, which, because of a gene deletion, is deficient in hypoxanthine phosphoribosyltransferase (HPRT) (Fuscoe et al. 1983).

The fusions were performed in monolayers by using modifications of a standard technique (Nussbaum et al. 1983; Zoghbi et al. 1989). Hybrids retaining the active X chromosome were selected for by growth in Dulbecco's modified Eagle medium (Hazelton, Lenexa, KS) containing 10% FCS, 0.1% mM hypoxanthine, and 50 μM azaserine. The retention of either the maternal or the paternal X chromosome for the relevant females was evaluated by Southern analysis. For each of the families, appropriate probes were chosen such that the mothers of the half-sisters were homozygous at these loci while the affected half-sisters were heterozygous, rendering it easy to identify the maternal chromosome of each affected female. In both of these families, the daughter's somatic cell hybrid that did not exhibit maternal alleles was designated as retaining the paternal X chromosome. All somatic cell hybrids were then tested at several other loci to ensure that they had been designated correctly and that the retained X chromosome was intact.

DNA Markers

Sixty-three DNA markers were used in the genetic analysis of familial Rett syndrome cases. A complete listing of these markers along with their regional assignment (Davies et al. 1990; Williamson et al. 1990; Ellison et al. 1991) is provided in figures 3 and 4. Because of the high degree of informativeness at loci containing short tendem repeats (STRs) (Litt and Luty 1989; Weber and May 1989; Boylan et al. 1990), we included in our analysis loci known to have STRs and regional localization on the X chromosome (Luty et al. 1990; Weber et al. 1990; Edwards et al. 1991; Huang et al. 1991; Huang et al., submitted). Eleven

such loci were studied in our families. In addition to standard RFLPs and STR polymorphisms, we studied specific cDNAs and/or genomic sequences which, on the basis of what is known about their pattern of expression and/or function, are potential candidate sequences for the Rett syndrome gene. These include the cDNA for the E1α subunit of pyruvate dehydrogenase (PDHA1) (Ho et al. 1989), the cDNA for the glycine receptor (GLR) (Siddique et al. 1989), the cDNA for ornithine transcarbamoylase (OTC) (Lindgren et al. 1984), the HM11 probe at the monoamine oxidase locus (MAOA) (Ozelius et al. 1988), the STR polymorphisms at the HPRT locus (Edwards et al. 1991; Huang et al. 1991) and at the synapsin 1/A-raf-1 (SYN/ARAF1) genes (Kirchgessner et al. 1991), genomic sequences from the α3 subunit of the γ-aminobutyric acid receptor (GABRA3), and genomic sequences at the L1 adhesion molecule (L1CAM) locus (Moos et al. 1988). Because there were no known polymorphisms at the PDHA1, GABRA3, and L1CAM loci, probes at these loci were used to search for polymorphism in five unrelated females (including the two mothers of affected half-sisters) by using 28 restriction enzymes.

Polymorphism Analysis

DNA was prepared from lymphoblasts and hybrids by proteinase K digestion with subsequent phenol-chloroform extractions and sodium acetate/ethanol precipitations using an automated DNA extractor (Applied Biosystems, Foster City, CA). For Southern analysis, 5–10 μg of high-molecular-weight DNA was digested to completion with the appropriate restriction endonuclease. Gel electrophoresis and Southern transfer were performed according to methods described elsewhere (Zoghbi et al. 1988). DNA probes were labeled by random priming following the protocol of Feinberg and Vogelstein (1984). For probes known to contain repetitive DNA sequences, the labeled probe was prehybridized with sonicated total human placental DNA to block repetitive sequences, according to the method of Sealey et al. (1985).

Genotypes at loci containing STRs were generated by PCR using primers that flank the repeat segments. PCR was carried out in a total volume of 25 μl with 25–50 ng of genomic DNA, 25 pmol of each primer, 1.25 mM MgCl₂, 250 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.01% gelatin, and 0.625 units of Thermus aquaticus DNA polymerase (Perkin Elmer—Cetus, Norwalk, CT). One primer of each pair was labeled at the 5′ end with [γ-^{32}P] dATP.

Temperature and cycling conditions for each STR were according to the published recommendations. Four microliters of each reaction was mixed with 2 μl of formamide loading buffer, was denatured at 85°C for 2 min, and was fractionated by electrophoresis on a 4% polyacrylamide/7.65 M urea sequencing gel for 2–3 h at 1,100–1,200 V. Gels were wrapped in saran wrap and were exposed for 5–24 h at −70°C to Kodak XRP film.

Linkage Analysis

For the two families in the present investigation, multipoint lod scores were computed to determine which regions of the chromosome could be excluded on the basis of whether the two affected family members were concordant or discordant at the loci in question. In this simple situation, with two families, we formulated a more general solution to the problem, obviating the need for further computer analyses as new loci are tested. Because the disease has been assumed to be inherited through the maternal line and because both offspring are affected, the hypothesis being tested is that of one identical maternal disease gene being transmitted to each of the affected half-sibs. This is an approximation—but a reasonable one—as the gene frequency is very small, given that 1/10,000 females are affected and that most are assumed to be sporadic. Hence, the problem was reduced to a simple situation in which the mother was assumed to be heterozygous for the disease allele, with the same disease-inducing allele being transmitted to each affected half-sib. Since inheritance is only through the maternal line and since the gene frequency is extremely small, the fathers were considered to be normal. We looked at all pairs of adjacent informative markers along the chromosome, determined the exclusion regions between each pair of markers on the basis of the three-point lod scores in the intermarker region, and thus created an exclusion map of the chromosome.

For the case in which both sib pairs are informative at two loci, the multipoint lod scores in this intermarker region are dependent only on the genotypes at these two loci and are independent of genotypes at other loci outside this intermarker region. For this reason, we could calculate, on the basis of whether each marker was concordant or discordant in the affected individuals, the exclusion region in an interval of any given length between two adjacent markers. We simply made up a set of marker alleles such that, for each child, it was known which allele came from which parent; gene frequencies are then irrelevant for

the lod score. Each locus can only be either discordant or concordant in a given family, so, for each family, there are four possible outcomes: discordant at both loci, concordant at both loci, discordant at the first locus concordant at the second locus, and concordant at the first locus and discordant at the second locus. Because we have two families in our data set, and because each family must be one of those types, there are 4 × 4, or 16, possible outcomes for two families at two adjacent loci. Then, at a variety of interlocus recombination fractions, multipoint lod scores were calculated for each of these 16 possible outcomes, and, on the basis of these lod scores, the exclusion regions were determined according to the traditional criterion of lod score less than − 2. This value was used in constructing an exclusion region as is the custom in linkage analysis, with a lod score of − 2 corresponding to a likelihood ratio of 100:1 against the disease being located in the region under consideration. The test is conservative in general, especially in cases in which the entire intermarker region can be excluded. Simple power calculations (not shown here) indicate fairly low power for the test, ranging from 47% at a recombination fraction of .001 down to 12% for recombination fractions of .02–.10. However, the probability of falsely excluding the true location of the disease gene is typically less than .001. Once one has evaluated the exclusion region for all possible outcomes at a large number of intermarker recombination fractions, one can simply make a table of these exclusion regions and determine the exclusion region between any two loci. In such a way, new loci can be added to the map, without the need for further redundant calculations. Table 1 shows such exclusion regions for our two families.

In table 1, columns correspond to different intermarker recombination fractions, and the rows correspond to each of the 16 possible outcomes, at any two adjacent loci, for our two families. To find the length of the exclusion region between any two given loci, one would find the row corresponding to the data in the two families and the column corresponding to the intermarker recombination fraction. For example, for two loci separated by a recombination fraction of .07, if at the first locus the first family was concordant and the second family was discordant, while at the second locus they were both discordant, one would locate row CD-DD, and column .07, for which one would see that the entire intermarker region could be excluded. Similarly, one could look up any combination of family data and intermarker recombination fraction to find other intermarker exclusion regions.

The intermarker recombination fractions for informative loci were obtained from published reports (Mandel et al. 1989; Keats et al. 1990; Alitalo et al. 1991; Kirchgessner et al. 1991). Where intermarker recombination frequencies were not available from published studies, we calculated these recombination frequencies by using version 4.0 of the CEPH data base and the LINKAGE package of computer programs. For newly identified markers such as the STRs at the DXS424, DXS425, DXS453, DXS454, DXS458, and HPRT loci, intermarker recombination frequencies were obtained from two recent studies aimed at mapping these markers in the CEPH reference families (Huang et al. 1991, and submitted).

Results

Somatic Cell Hybrid Analysis

Somatic cell hybrids were made from lymphoblasts of each female in the two families, in order to separate their maternal and paternal X chromosomes. Hybrids were generated for all three females in family 1 (fig. 1). Three hybrids retaining one X chromosome and three hybrids retaining the other X chromosome were isolated for individual I-2. Two hybrids retaining the maternal X chromosome and five hybrids retaining the paternal X chromosome were generated for individual II-1. For individual II-2 11 hybrids retaining the maternal X chromosome were identified; however, no hybrids retaining the paternal X chromosome were generated for this individual. Hybrids were also generated for the three females in family 2 (fig. 1). For the mother, individual I-2, eight hybrids retained one X chromosome and two hybrids retained the other X chromosome. One hybrid retaining the maternal X chromosome and 11 hybrids retaining the paternal X chromosome were isolated for individual II-1. Two hybrids retaining the maternal X chromosome and six hybrids retaining the paternal X chromosome were generated for individual II-2.

Polymorphism Data

Sixty-three markers mapping to the X chromosome were genotyped in the two families with recurrent Rett syndrome. A two-allele ScaI polymorphism was identified at the GABRA3 locus by using lambda clone 29 as a probe. This clone contains approximately 15 kb

Table I

Exclusion Regions, on the Basis of Multipoint Linkage Analysis for Different Markers

CONCORDANCE/ DISCORDANCE[a]	EXCLUSION AT INTERMARKER RECOMBINATION FREQUENCY OF[b]													
	.001	.01	.02	.03	.04	.05	.06	.07	.08	.09	.10	.125	.15	.20
CC-CC	There is no exclusion at all for these two loci													
CC-DC	For all recombination frequencies, one can only exclude the exact point of locus 2													
CC-CD	For all recombination frequencies, one can only exclude the exact point of locus 2													
CC-DD	.0006−2	.004−2	.006−2	.008−2	.009−2	.011−2	.013−2	.016−2	Only locus 2					
DC-DC	Exclude entire intermarker region ——————— (up to .125) ———————											.031−2 / 1−.031	(only at the two loci)	
DC-CC	For all recombination frequencies, one can only exclude the exact point of locus 1													
DC-CD	Entire region ——— .006−2 / 1−.006 only at the two loci													
DC-DD	Exclude entire intermarker region ————————— (up to .150) —————————												.039−2 / 1−.030	(only at loci)
CD-CD	Exclude entire intermarker region ——————— (up to .125) ———————											.031−2 / 1−.031	(only at the two loci)	
CD-CC	For all recombination frequencies, one can only exclude the exact point of locus 1													
CD-DC	Entire region ——— .006−2 / 1−.006 only at the two loci													
CD-DD	Exclude entire intermarker region ————————— (up to .150) —————————												.039−2 / 1−.030	(only at loci)
DD-DD	Exclude entire intermarker region													
DD-CD	Exclude entire intermarker region ————————— (up to .150) —————————												.030−2 / 1−.039	(only at loci)
DD-DC	Exclude entire intermarker region ————————— (up to .150) —————————												.030−2 / 1−.039	(only at loci)
DD-CC	1−.0006	1−.004	1−.006	1−.008	1−.009	1−.011	1−.013	1−.016	Only locus 1					

[a] C = concordant; and D = dicordant. The first letter refers to family 1; the second letter refers to family 2; and the data for each locus are separated by a hyphen (-). In other words, CD-CC means that at locus 1, the first family is concordant and the second family is discordant, while at locus 2 both families are concordant.

[b] Length of region between these markers, where multipoint lod score is less than −2. For most cases, either the entire intermarker region or only the loci themselves can be excluded, but in some circumstances the exclusion region is only a portion of the total intermarker region. In these cases the exclusion region is notated as follows: .031−2 means that the region up to a recombination frequency of .031 and to the left of locus 2 can be excluded, while 1−.031 means that the region up to a recombination frequency of .031 and to the right of locus 1 can be excluded.
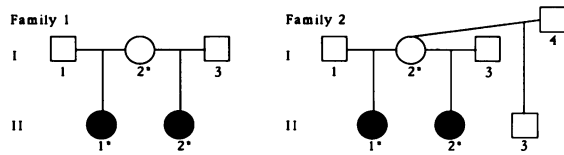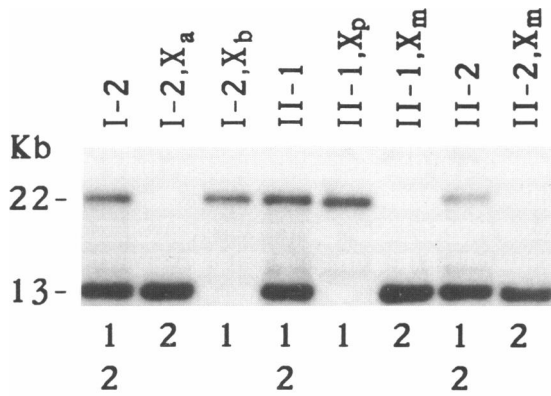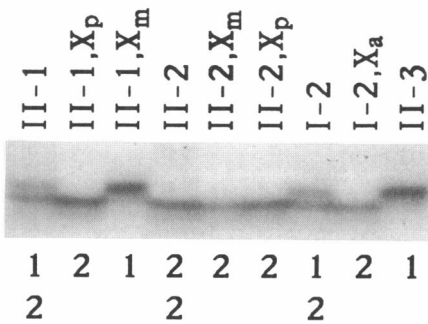
Figure 1   Pedigrees of families with affected half-sisters. The asterisks (*) indicate which individuals have their X chromosomes separated in somatic cell hybrids.

of genomic sequences which include one exon (presumably exon 6) of the α3 subunit of the GABA$_A$ receptor and was provided by Craig Venter (National Institutes of Health). The two alleles were 9.4 and 4.6 kb in size. Both affected sisters in the two families were informative and concordant at this locus. No polymorphisms were identified at the PDHA1 and L1CAM loci. Thirty-six markers were informative in at least one of the affected sib families, and 25 markers were informative in both families. Figure 2a and b illustrates the concordance/discordance analysis in family 1 by using standard RFLPs and in family 2 by using a dinucleotide repeat polymorphism. Figures 3 and 4 include both a listing of all the markers analyzed and the results of the concordance/discordance analy-

Figure 2   a, Southern blotting analysis of PstI-digested DNA from family 1 probed with p99-6. DNAs from somatic cell hybrids retaining either X chromosome are indicated as Xa and Xb for the mother (I-2) or as Xm (maternal) or Xp (paternal) for each affected daughter. The genotypes are shown at the bottom of the gel. b, Analysis of PCR-amplified products containing dinucleotide repeat tracts at DXS453 in family 2. The designations for the source of hybrids is the same as in panel a. The genotypes are shown at the bottom of the gel.

Probe	Locus	Location	Family1	Family2
pDIC56	DXS143	p22.3	○	○
pTAK10B	DXS89	p22.3-p22.2	⊘	⊘
L782	DXS85	p22.3-p22.2	○	○
RC8	DXS9	p22.2	○	○
pSE3.2-L	DXS16	p22.2	○	○
pPA4B	DXS207	p22.2	○	⊘
pD2	DXS43	p22.2	⊘	⊘
p99-6	DXS41	p22.1	○	⊘
pHMA1	DXS208	p22-p21	○	⊘
pTS247	DXS197	p22.2	⊘	⊘
E1αcDNA	PDHA1	p22.1	⊘	⊘
pDP1039	ZFX	p22.1-p21.3	⊘	⊘
HuGlyR	GLR	p22.1-p21.2	⊘	⊘
pB24	DXS67	p21.3	⊘	⊘
C7	DXS28	p21.3	⊘	⊘
K23a	DXS704	p21.3	●	●
J66H1	DXS268	p21.3-p21.2	●	○
p20	DXS269	p21.3-p21.2	⊘	⊘
pERT87-1	DXS164	p21.2	●	○
pERT87-15	DXS164	p21.2	⊘	⊘
pERT87-30	DXS164	p21.2	⊘	⊘
pERT87.8	DXS164	p21.2	⊘	⊘
JBIR	DXS270	p21.2	⊘	⊘
XJ1.1	DXS206	p21.2-p21.1	⊘	○
L754	DXS84	p21.1	⊘	⊘
754-11	DXS84	p21.1	⊘	⊘
pHO731	OTC	p21.1	●	○
pTS264	DXS198	p21-p11	⊘	⊘
pG951-Jd3R	DXS209	p11.4	⊘	⊘
HM11	MAOA	p11.3-p11.23	●	○
L1.28	DXS7	p11.3	●	○
(GT)n	SYN1	p11.2	●	⊘
TIMP-3.9X	TIMP	p11.3-p11.23	⊘	○
XL91B10	DXS426	p21.1-p11.2	●	○
M27β	DXS255	p11.22	●	○
pTAK8B	DXS146	p11.22	●	○
p58.1	DXS14	p11.21	●	○

Figure 3   List of chromosome Xp markers analyzed in families with recurrent Rett syndrome. The last two columns indicate which markers were concordant or discordant in the half-sisters in families 1 and 2. A blackened circle (●) indicates that there was discordance at this locus between the affected half-sisters; an unblackened circle (○) indicates that there was concordance; and a hatched circle (⊘) indicates that the marker was uninformative.

Probe	Locus	Location	Family1	Family2
STRX1/(AGAT)n		p11-q13	⊘	⊘
(AGC)n	AR	q12	●	○
pX22.E	DXS18	cen-q12	⊘	⊘
pSPT/PGK	PGK1	q13	⊘	●
7b	DXYS2X	q13-q22	●	⊘
MFd66/(GT)n	DXS453	q13	●	●
St25-1	DXYS12	q21.31-q21.33	⊘	⊘
pTAK2	DXYS13	q21.31-q21.33	⊘	●
pDP34	DXYS1	q21.3	●	●
XL1E/(GT)n	DXS457	q21.2-q22	⊘	⊘
Mfd72/(GT)n	DXS454	q21.1-q23	●	●
Mdf79/(GT)n	DXS458	q21.1-q23	●	●
XG30B	DXS456	q21-q22	●	○
p22-33	DXS11	q24-q26	⊘	⊘
XL5A/(GT)n	DXS424	q24-q26	⊘	○
pX45d	DXS100	q25	⊘	⊘
pX45h	DXS100	q25	○	⊘
XL90A3/(GT)n	DXS425	q26-q27.1	○	○
(AGAT)n	HPRT	q26	○	○
St1	DXS86	q26	⊘	⊘
pAX-6	DXF1S2	q26-qter	○	⊘
GABA-29	GABRA	q28	○	○
DX13	DXS15	q28	○	○
St14-1	DXS52	q28	⊘	○
L1CAM	L1CAM	q28	⊘	⊘
F8:CB/p114	F8C	q28	⊘	⊘

Figure 4   List of chromosome Xq markers analyzed in familial Rett syndrome cases. The symbols for concordance/discordance at the specific loci are the same as in fig. 3.

sis in the two families. For each of these two families it is indicated whether the two sisters were concordant (unblackened circle) or discordant (blackened circle) or whether the marker was uninformative (hatched circle).

Linkage Data

Direct inspection of the results of the polymorphism analysis as presented in figures 3 and 4 allows the identification of loci that, on the basis of the discordance of alleles at these loci in affected half-sisters, can be excluded as candidate loci for the Rett syndrome gene. In order to exclude intermarker regions, we used table 1 to identify regions that can be excluded using the traditional criterion of lod score less than − 2. Only loci for which genetic data are available, allowing the determination of intermarker recombination frequencies, were used for this analysis. Table 2 shows the data regarding the exclusion of intermarker regions by 19 markers that were informative in the familial Rett cases and that had genetic data in large numbers of meioses such as the CEPH reference families. The only two loci that are included in this table and that do not have CEPH data are GABRA3 and DXS15. These two loci were concordant in both families, hence the region between them cannot be excluded, irrespective of the recombination frequency.

Discussion

We report the results of a novel multilocus linkage analysis in two families with half-sisters affected with Rett syndrome. For this analysis, we used 19 of the 63 markers analyzed. These 19 markers were chosen because they were informative in both families and were genetically mapped on the X chromosome by using reference families. We have focused on analyzing markers from the X chromosome because of the hypothesis that Rett syndrome is most likely due to an X-linked mutation which is lethal in hemizygous males. The occurrence of this syndrome among half-sisters that have the same mother suggests that the mother either is a carrier of the Rett syndrome mutation or has germ-line mosaicism. Previous methylation studies at the phosphoglycerate kinase gene locus in the mother of the affected half-sisters from one of the families analyzed in the present study (family 2) showed a nonrandom pattern of X chromosome inactivation which provides a possible explanation for the normal phenotype of this putative carrier female (Zoghbi et al. 1990b).

In order to investigate further the hypothesis that Rett syndrome is due to a mutation on the X chromosome, we carried out genetic analysis in the form of concordance/discordance analysis to determine which regions are shared by affected half-sisters and hence are candidate regions for the Rett syndrome gene. Because the mother is the common parent in both of the families evaluated in the present study, we reasoned that the X chromosome loci for which the affected sisters have inherited different maternal alleles could be excluded as candidates for the Rett syndrome gene. By analyzing a large number of closely spaced loci, we can perform multipoint linkage analysis in order to exclude intermarker regions in addition to the specific polymorphic markers analyzed. For this analysis, we determined exclusion regions according to the traditional criterion of lod score less than − 2 and generated a table of these exclusion regions. From this analysis, we were able to exclude the region between the DMD locus and the DXS456 locus, a region which extends from Xp21.2 to Xq21-q23. No loci could be excluded either (a) telomeric to and including DXS28 (Xp21.3) on the short arm of the X chromosome or (b) telomeric to and including DXS11 (Xq24-q26) on the long arm of the X chromosome. Among the individual loci excluded in the present study are several sequences which are either expressed in neuronal tissue or known to affect neuronal function. These include the OTC lo-

Table 2

Exclusion Regions for Two Families with Affected Half-Sisters

Loci	Concordance/ Discordance[a]	Recombination Frequency	Exclusion Status
DXS143–DXS85	CC-CC	.056	No exclusion
DXS85–DXS16	CC-CC	.050	No exclusion
DXS85–DXS9	CC-CC	.060	No exclusion
DXS164–OTC	DC-DC	.042	Exclude entire region
OTC–DXS7	DC-DC	.110	Exclude entire region
DXS7–SYN1/ARAF1	DC-DC	.100	Exclude entire region
SYN1/ARAF1–DXS255	DC-DC	.070	Exclude entire region
DXS255–DXS146	DC-DC	.036	Exclude entire region
DXS146–DXS14	DC-DC	.001	Exclude entire region
AR–DXS453	DC-DD	.080	Exclude entire region
DXS255–AR	DC-DC	.050	Exclude entire region
DXS453–DXYS1	DD-DD	.055	Exclude entire region
DXYS1–DXS454	DD-DD	.113	Exclude entire region
DXS454–DXS458	DD-DD	.029	Exclude entire region
DXS458–DXS456	DD-DC	.050	Exclude entire region
DXS456–DXS425	DC-CC	.211	Exclude DXS456
DXS425–HPRT	CC-CC	.100	No exclusion
HPRT–GABRA3	CC-CC		No exclusion
GABRA3–DXS15	CC-CC		No exclusion

[a] Defined as in table 1, footnote a.

cus, the MAOA locus, and the synapsin 1 gene locus. All three show discordance between one or both pairs of the affected sisters. Other loci, such as GLR, L1CAM, and PDHA1, could not be excluded, because of the lack of informativeness. On the other hand, a polymorphism at the α3 subunit of the GABA$_A$ receptor was developed in both families, and it showed concordance in both families, rendering a mutation at this locus a possible candidate for the Rett syndrome mutation.

Recently Archidiacono et al. (1991) reported a concordance/discordance analysis of Rett syndrome by using family 2 and 34 DNA markers. Fifteen of the 34 markers were informative, and four were discordant. The four discordant markers were DXS278, DXS92, DXSZ1, and DXYS1. Because three of the four markers map to different regions on the X chromosome, the finding of discordance allowed the exclusion of only these specific loci as candidates for the Rett syndrome gene. DXS278 and DXS92 lie close together, mapping to Xp22.3 and Xp22.2, respectively. However, because these markers are not genetically mapped in the CEPH families at this point, intermarker regions in Xp22 cannot be excluded. The discordance at DXYS1 is in agreement with data from the present study, which also reveal discordance at this locus in family

1. DXSZ1 maps to the centromere; the discordance shown by Archidiacono et al. (1991) at DXSZ1 in family 2 is in agreement with our finding that the entire region between DXS255 (Xp11.2) and the androgen receptor (AR) (Xq12) can be excluded.

The use of two families in the present study, as well as a large number of X chromosome markers, many of which have been genetically mapped, allowed the exclusion of an extensive portion of the X chromosome. The use of exclusion mapping to define a candidate region for the Rett syndrome gene is a valid and effective approach, given both the small number of families with recurrent Rett syndrome and the lack of other clues as to the molecular basis of this disorder. Other approaches to identify the Rett locus have included pursuing the X chromosomal breakpoints in Rett patients with X;autosome translocations. Two reports have described the occurrence of such translocations in Rett syndrome patients. The report by Zoghbi et al. (1990a) describes a de novo X;3 translocation in a patient with Rett syndrome. The X chromosomal breakpoint maps between DMD and DXS28. Since DXS28 is not informative in either family described in the present study, the region telomeric to DMD and known to contain the translocation breakpoint cannot be excluded at this time. A report

by Journel et al. (1990) describes an X;22 translocation in a patient with Rett syndrome. This translocation is also present in both a sister of the patient presumed to have a forme fruste because of milder clinical features of Rett syndrome (Hagberg and Rasmussen 1986) and in her asymptomatic mother. The X chromosomal breakpoint is in Xp11.22. The data from the present study exclude the region between Xp21.2 and Xq21-q23, which suggests that the X;22 translocation reported by Journel et al. (1990) is less likely to be causative for Rett syndrome in the family they describe. The use of exclusion mapping when new polymorphisms are developed in Xp21.3-Xp22.1 will be very valuable in determining whether the X;3 translocation (Zoghbi et al. 1990a) is a candidate for causing the Rett phenotype in that patient.

Given that a substantial portion of the X chromosome has been excluded as candidate for the location of the Rett syndrome gene, it is compelling to consider other hypotheses for the genetic basis of Rett syndrome. Such hypotheses include the possibility that Rett syndrome is due to an autosomal mutation which has sex-limited expression. It is hard to explain such a hypothesis in light of the normal phenotype in the putative carrier mothers, unless one suggests that Rett syndrome is an autosomal recessive disorder with sex-limited expression. This latter possibility is complicated by the recurrence of the syndrome in half-sisters, rendering the likelihood for the two different fathers to be carriers extremely low. Because of the evidence for maternal inheritance in some familial Rett cases, the possibility that Rett syndrome is due to a mutation in the mitochondrial genome should be considered. The fact that the Rett phenotype is observed exclusively in females whereas mitochondrial disorders are known to affect both sexes equally argues against this hypothesis. Other possible mechanisms may include an autosomal dominant locus which is affected by both genomic imprinting and sex-limited expression. To test any of these hypotheses will be extremely difficult, given the small number of familial cases. To date, the only hypothesis that could be tested effectively is the hypothesis that Rett syndrome may be due to a mutation on the X chromosome. As additional STR polymorphisms are identified and genetically mapped on the X chromosome, these polymorphisms can be analyzed both in the families described in the present study and in other families with affected sisters, to determine whether any additional regions of the X chromosome can be excluded. This can be done easily by using the table presented in the present study

for the multipoint linkage analysis, without the need for any further calculations. This approach for multipoint linkage analysis is simple and can be applied to any disorder in which familial cases and informative meioses are few. If an X chromosomal region which is concordant among all sibship cases is delineated, expressed sequences in this region can be analyzed in both sporadic and familial Rett syndrome cases, to determine whether any mutation can be identified.

Acknowledgments

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