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X-linked α-Thalassemia/Mental Retardation (ATR-X) Syndrome: Localization to Xq12-q21.31 by X Inactivation and Linkage Analysis

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Summary

We have examined seven pedigrees that include individuals with a recently described X-linked form of severe mental retardation associated with α-thalassemia (ATR-X syndrome). Using hematologic and molecular approaches, we have shown that intellectually normal female carriers of this syndrome may be identified by the presence of rare cells containing HbH inclusions in their peripheral blood and by an extremely skewed pattern of X inactivation seen in cells from a variety of tissues. Linkage analysis has localized the ATR-X locus to an interval of approximately 11 cM between the loci DXS106 and DXYS1X (Xq12-q21.31), with a peak LOD score of 5.4 (recombination fraction of 0) at DXS72. These findings provide the basis for genetic counseling, assessment of carrier risk, and prenatal diagnosis of the ATR-X syndrome. Furthermore, they represent an important step in developing strategies to understand how the mutant ATR-X allele causes mental handicap, dysmorphism, and down-regulation of the α-globin genes.

Introduction

The rare association of α-thalassemia and mental retardation (ATR) has been recognized with increasing frequency over the past 10 years (Weatherall et al. 1981; Wilkie et al. 1990*a*, 1990*b*). Since the molecular basis of α-thalassemia is amenable to analysis, this situation offers a potentially useful approach to the study of the associated mental handicap.

The human α-globin cluster is located in the terminal band of chromosome 16p. It comprises an embryonic gene (ζ2) and two α genes (α2 and α1) arranged in the following order: telomere–ζ2–α2–α1–centromere. α-Thalassemia is a common inherited disorder in which various well-characterized deletions and point mutations of the α genes result in underproduction of the α-globin chains of hemoglobin A (HbA, $\alpha_2\beta_2$). A greater than 50% reduction in α-globin synthesis results in moderately severe anemia associated with a significant accumulation of excess β chains that form tetramers (β_4, called HbH), a condition referred to as "HbH disease." HbH disease is frequently seen in Mediterranean and Oriental racial groups, in which it is always inherited in a Mendelian fashion, and is not known to be associated with an increased frequency of mental retardation (reviewed in Higgs et al. 1989).

Investigation of patients with the ATR association has demonstrated two unusual features: (1) it often occurs in racial groups in which α-thalassemia is otherwise rare, and (2) the pattern of inheritance is different than that usually seen in α-thalassemia. Molecular analysis has revealed two groups of affected individuals. Some patients have large (1–2-Mb) deletions of the tip of chromosome 16p; the clinical features of this so-called ATR-16 syndrome are quite variable, in part because some patients have additional aneuploidy (Wilkie et al. 1990*a*). By contrast, the second group of patients (15 boys and one 46,XY female), in whom no deletion or other abnormalities of the α-globin complex can be detected, have a remarkably uniform phenotype that is quite different from that of ATR-16

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and that comprises severe mental retardation, characteristic dysmorphic facies, genital abnormalities, and an unusual, mild form of HbH disease (reviewed in Gibbons et al. 1991). The locus encoding this syndrome segregates independently of the α-globin cluster (Donnai et al. 1991), and analysis of several affected pedigrees has shown that the pattern of inheritance is consistent with an X-linked recessive disorder (Harvey et al. 1990; Cole et al. 1991; Donnai et al. 1991; Wilkie et al. 1991*a*; 1991*b*; R. J. Gibbons, unpublished observations). At present the molecular basis of this condition, referred to as the "ATR-X syndrome," is unknown.

In this study we have set out to localize the X-chromosome region encoding the ATR-X syndrome. First, using hematologic and molecular approaches, we have examined the phenotype of female carriers of this disorder and have defined criteria for the diagnosis of the carrier state. Second, using these criteria, we have performed a linkage analysis in five pedigrees including 12 affected individuals, to localize the region containing the ATR-X locus. Finally, we have demonstrated the application of these results to risk analysis in a further two pedigrees.

Subjects and Methods

Families Studied

The selection criteria for including families in the linkage study were (*a*) the presence of at least one severely mentally retarded individual with the ATR-X syndrome and (*b*) the presence of at least one female carrier, diagnosed either on the basis of pedigree anal-

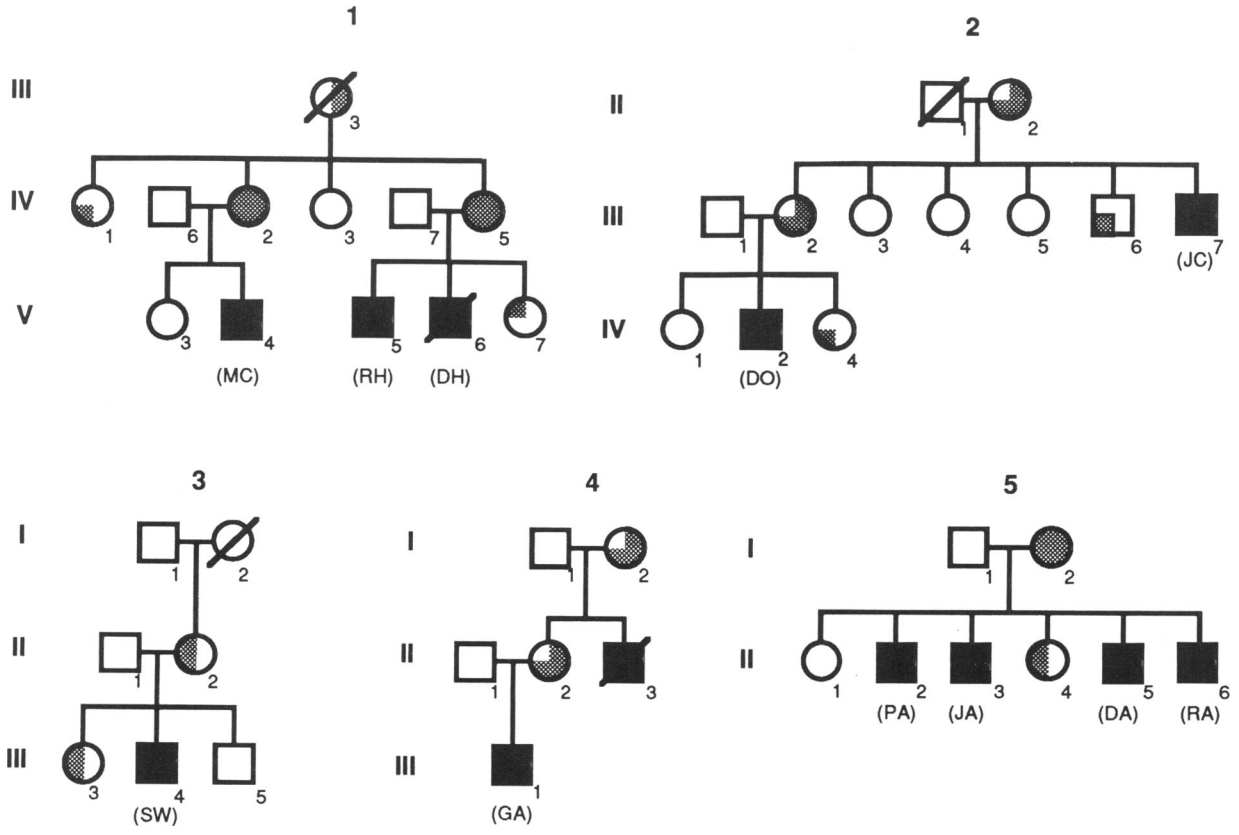

Figure 1 Pedigrees studied for linkage analysis. Pedigrees are numbered as in previous publications, and annotation of probands (in parentheses) follows the nomenclature of Gibbons et al. (1991). In family 3 (Wilkie et al. 1990*b*) further inspection of BCB preparations revealed HbH inclusions in case II-2 that were not previously noted. ○ = Female; □ = male; ■ = affected male; ◍ = obligate carrier deduced from pedigree; ◉ = HbH inclusion positive; ◐ = nonrandom X inactivation; and ◪ = male with 47XXY, HbH inclusion negative and nonrandom X inactivation. The extra X is paternally derived (data not shown). A slash through a symbol denotes that the individual is deceased.

ysis (obligate carrier) or by the detection of HbH inclusions in the peripheral blood, as outlined below. The pedigrees of the five families (family 1 [Wilkie et al. 1991*b*], family 2 [Cole et al. 1991], family 3 [Wilkie et al. 1990*b*], family 4 [Porteous and Burn 1990], and family 5 [Donnai et al. 1991]) that fulfilled these criteria and that were available for study are shown in figure 1. Further genetic analysis and risk assessment was performed in two additional families (family 6 and family 7 [Wilkie et al. 1990*b*]). All of these families are thought to be of northern European origin. Controls for X-inactivation studies (see below) were DNA samples obtained from 24 unrelated phenotypically normal female individuals from a variety of racial groups.

Hematologic Analysis

Routine hematologic analyses were carried out according to a method described elsewhere (Wilkie et al. 1990*a*). To detect HbH inclusions, which are diagnostic of α-thalassemia, blood smears were made after incubation of equal volumes of peripheral blood and a 1% solution of brilliant cresyl blue (BCB) in 0.9% NaCl, at room temperature for 4–24 h (Gibbons et

Table 1

Summary of Probes Used in This Investigation, with Their Location and Associated Polymorphism

Locus	Probe	Region of Enzyme	RFLP	Alleles (kb)	Allele Frequency	Source[a]
DXS143	dic56	Xp22.3	*Bcl*I	7.4/8.9	.56/.44	K. E. Davies[b]
DXS16	pXUT23	Xp22.2	*Bgl*II	17.5/12.5	.84/.16	K. E. Davies[b]
			*Bcl*I	2.5/2.2	.51/.49	
DXS268	J66	X021.3-p21.2	*Pst*I	1.5/1.4/1.35	.6/.3/.1	G. J. B. van Ommen
DMD	Cf56a	Xp21.3-p21.1	*Pst*I	10.8/3.4	.09/.91	K. E. Davies[b]
OTC	pOTC	Xp21.1	*Bam*HI	16/5.4	.75/.25	K. E. Davies[b]
DXS7	L1.28	Xp11.4-p11.3	*Taq*I	12/9	.77/.23	K. E. Davies[b]
DXS255	M27β	Xp11.22	*Pst*I		Multiallelic system	I. Craig
DXS1	p8	Xq11.2-q12	*Taq*I	15/9	.84/.16	ATCC
			*Hin*dIII	7/7.5	.78/.22	
DXS159	cpX289	Xq12	*Pst*I	5.5/1.6	.67/.33	ATCC
DXS106	cpX203	Xq12	*Bgl*II	1/5.8	.64/.36	ATCC
PGK1	pSPT-PGK	Xq13.3	*Pst*I/*Bst*XI	1.05/.9	.79/.21	A. D. Riggs
DXS72	pX65H7	Xq21.1	*Hin*dIII	7.2/.7	.45/.55	K. E. Davies[b]
DXYS1X	pDP34	Xq21.31	*Taq*I	10.6/11.8	.6/.4	K. E. Davies[b]
CA repeat ...			PCR	.174/.172/.170	.54/.42/.04	
DXYS5S	47z	Xq21	*Taq*I/*Hin*dIII	3.2/2.4	.76/.24	D. C. Page
	p31		*Taq*I	4.8/2.8	.86/.14[c]	D. C. Page
DXS3	p19-2	Xq21.3	*Msp*I	4.4/12	.75/.25	G. A. Bruns
			*Taq*I	3,2/5	.62/.38	
DXS87	pA13.R1	Xq21.33-q22	*Bgl*II	4.5/1.1	.58/.42	K. E. Davies[b]
DXS94	pXG-12	Xq22	*Pst*I	6.5/7.2	.52/.48	K. E. Davies[b]
DXS287	pYNH3	Xq22-q24	*Rsa*I	3.2/2.8	.38/.62	ATCC
DXS42	p43-15	Xq24-q25	*Bgl*II	6/9.5	.81/.19	K. E. Davies[b]
DXS11	p22-33	Xq24-q25	*Taq*I	11/20	.84/.16	K. E. Davies[b]
F9	F9probeVIII	Xq26.3-q27.1	*Taq*I	1.8/1.3	.75/.25	G. Brownlee
DXS105	55E	Xq27.1-q27.2	*Pst*I	16/10	.6/.4	K. E. Davies[b]
DXS369	pRNIA	Xq27.3	*Xmn*I	1.25/1.1	.59/.41	K. E. Davies[b]
DXS296	VK21A	Xq27.3-q28	*Taq*I	10.9/9.9	.84/.16	G. Suthers[b]
	VK21C		*Msp*I	12.7/9.9	.89/.11	G. Suthers[b]
DXS374	p1A1.1	Xq28	*Pst*I	5.2/2.9,2.3	.58/.42	K. E. Davies[b]
DXS52	St14-1	Xq28	*Taq*I		Multiallelic system	J. L. Mandel

[a] For the original references for each of the probes, see Kidd et al. (1989). ATCC = American Type Culture Collection.
[b] Probe also available through ATCC.
[c] Authors' unpublished data.

al. 1991). Blood films were scanned at high power (approximately 200 cells/field) for a minimum of 20 min (usually scanning approximately 100,000 cells).

DNA Analysis

Genomic DNA was prepared from venous blood, buccal cells in saliva, or hair roots (Old and Higgs 1983; Hopkins et al. 1989). Restriction-enzyme digestion, preparation of Southern blots, and hybridization with radiolabeled probes were performed by standard methods (Feinberg and Vogelstein 1983; Old and Higgs 1983; Church and Gilbert 1984). The probes used in the present study, as well as their sources, are summarized in table 1 and figure 2. The polymorphic CA repeat at DXYS1X was examined by PCR using the primers and conditions described by Browne et al. (1991). PCR products were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel and were visualized on a UV-light transilluminator after being stained with ethidium bromide.

Methylation studies were performed as described by Vogelstein et al. (1987) for pSPT-PGK and as described by Abrahamson et al. (1990) for M27β. The hypervariable locus DXS255 contains a VNTR detected by the probe M27β (Fraser et al. 1989). At the 5′ end of the VNTR, methylation differences at the sequence CCGG may be detected using the restriction endonuclease *Msp*I and its methylation-sensitive isoschizomer *Hpa*II. On the active X chromosome the sequence is methylated and therefore resistant to cleavage by *Hpa*II; on the inactive X chromosome the sequence is unmethylated or partially methylated (Abrahamson et al. 1990; Hendriks et al. 1991). The methylation pattern at the 5′ end of the PGK1 gene as revealed by the probe pSPT-PGK is less complicated; the eight *Hpa*II sites contained within the *Pst*I/*Bst*XI limit digest are unmethylated on the active X chromosome and are methylated on the inactive X chromosome (Vogelstein et al. 1987).

Linkage Analysis

Linkage analysis was performed using the LINKAGE package, version 5 (Lathrop et al. 1985). The ATR-X locus was coded as a diallelic (normal or mutant), monogenic, X-linked disorder that was assumed to be fully penetrant in males. As discussed below, female carriers were defined by the ability to demonstrate HbH inclusions in the peripheral red blood cells or by the presence of nonrandom X inactivation (or by both criteria); the frequencies of HbH inclusions and nonrandom X inactivation among normal females

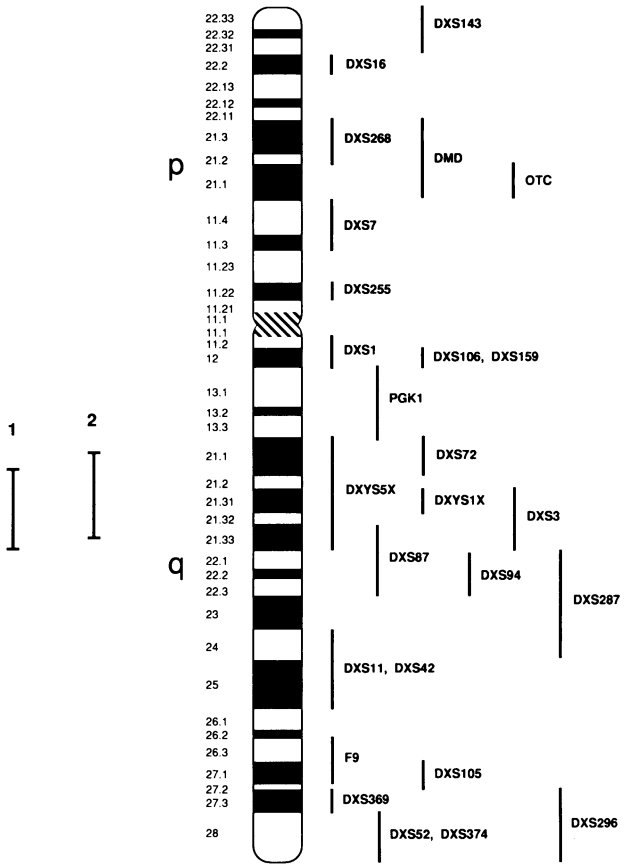

Figure 2 Diagram of the human X chromosome, showing physical locations of probes used as described by Kidd et al. (1989). Vertical bars on the left of the diagram represent the approximate extent of deletion, from the X chromosome, seen in two previously described patients with choroideremia and developmental abnormalities (patient 1 [Hodgson et al. 1987], known as MBU [Merry et al. 1989], and patient 2 [Wells et al. 1991]).

were taken to be .0 and .1, respectively (see below). Since the two components of the mutant phenotype have different penetrances, the degree of certainty with which we were able to diagnose individuals as carriers also differed. To accommodate this when the LINKAGE programs were used, all individuals were coded as "affected" and were assigned to one of three mutually exclusive liability classes, on the basis of their phenotype. The three classes were defined as follows: class I included (*a*) all individuals who had HbH inclusions and (*b*) all females who were obligate carriers on the basis of pedigree information; class II included all females who lacked HbH inclusions but who had nonrandom X inactivation; and class III included (*a*)

all normal males and (*b*) females who both lacked HbH inclusions and had random X inactivation.

The penetrance for each sex in these classes is summarized in table 2. The figures for females are based on a relatively small number of observations (see below) and must be regarded as tentative. The probabilities that a heterozygote would have HbH inclusions or demonstrate nonrandom X inactivation were *not* regarded as independent; it was assumed that all heterozygotes would have either HbH inclusions or nonrandom X inactivation or both (see below). There were only two women in class II (IV-1 in family 1 and IV-4 in family 2). For the purpose of mapping the ATR-X locus, because their carrier status was uncertain these women were coded as being of unknown status in class II. The penetrance figures in class II were, however, used in the subsequent risk analysis (see below). One male with a 47,XXY karyotype (III-6 in family 2) fulfilled the criteria for class II. As the distribution of X-inactivation ratios is not known for such individuals, he was coded as unknown in class II, for all analyses.

Details of the polymorphic loci used in the linkage analysis are shown in table 1 and in the report by Kidd et al. (1989). Two-point linkage analysis was performed using the programs LODSCORE and MLINK. For the purpose of linkage analysis in families 1–5, the mutation rate (μ) was defined as 0, and we assigned an arbitrary mutant-allele frequency of .0001. For multipoint linkage analysis the program LINKMAP was used to place the ATR-X locus in relation to the loci DXS106, PGK1, DXS72, and DXYS1X. The genetic distances between these loci have been previously estimated to be 6.7 cM, 4.2 cM, and 0.4 cM, respectively (Keats et al. 1989; Mahtani et al. 1991). For the multipoint analysis, Haldane's mapping function (Ott 1985) was used to convert recombination fractions (θ's) to genetic distances.

Risk Analysis

When estimating the risk of an individual being a carrier, we assumed that the fitness of ATR-X hemizygotes and heterozygotes was 0 and 1.0, respectively. The value of μ was assumed to be equal in males and females. For a lethal X-linked recessive disorder with equal mutation rates in males and females, the prior probability of a woman being a carrier is equal to 4μ (Young 1991). When a Bayesian approach was used to estimate carrier risks on the basis of a woman's phenotype, it was not necessary to specify μ, as it is canceled out in the calculation. To incorporate genotype data into the carrier risk assessment, we used the program MLINK, which requires that the value of μ in males and females be stated. The value of μ used for risk estimation was .00005 (subsequently, we confirmed that risk figures were consistent over a 10^4 range of mutant-allele frequencies).

Results

Identification of Female Carriers

Obligate carriers and other female relatives of patients with the ATR-X syndrome appear to be of nor-

Table 2

Penetrance Figures Used for Linkage Analysis

Group and Allele(s)[a]	Penetrance		
	Class I: HbH Inclusion Positive and/or Obligate Carrier	Class II: HbH Inclusion Negative and Nonrandom X Inactivation	Class III: HbH Inclusion Negative and Random X Inactivation—Normal Males
Males:			
X	.0	. . .	1.0
X^m	1.0	. . .	.0
Females			
XX	.0	.1	.9
XX^m	.5	.5	.0
X^mX^m	1.0	.0	.0

Note.—Only a proportion of heterozygotes exhibit HbH inclusions; for the purpose of this linkage analysis, this proportion has been assumed to be 50% (see text). All individuals were coded as being "affected" with the phenotype defined for each class (see text). Note that the phenotype defined for class III is the normal phenotype. These figures represent the probability that an individual with the specified genotype will have the phenotype defined for the class.

[a] X and X^m = normal allele and mutant allele, respectively, for the disease locus.

mal intelligence, and, with one exception (see below), their red cell indices are grossly normal. However, it has previously been noted that occasional HbH inclusions can be demonstrated in the peripheral blood of some phenotypically normal female relatives of such patients (Wilkie et al. 1990*b*, 1991*b*; Donnai et al. 1991). The affected red cells are usually rare, representing only 1 in every 2,000–10,000 cells, which is equivalent to approximately 1% of the frequency seen in the affected boys.

To explain this consistently low proportion of cells containing HbH inclusions, we hypothesized that X inactivation might be occurring nonrandomly. If X inactivation were random, then one would expect that in carrier females the number of cells containing HbH inclusions would be half that seen in affected males, whereas, if X inactivation were markedly skewed, such that the X chromosome carrying the mutant ATR-X allele had been inactivated in the majority of red cell precursors, then one would predict that cells with HbH inclusions would be correspondingly rare.

In the absence of distinguishable alleles for the enzyme glucose 6-phosphate dehydrogenase, the pattern of X inactivation cannot easily be determined in red cell precursors. However, it can be assessed in peripheral blood mononuclear cells by analyzing differences in the pattern of methylation between active and inactive X chromosomes at loci where the paternal and maternal X chromosomes can be distinguished by RFLPs. Two such loci are DXS255 (Abrahamson et al. 1990) and PGK1 (Vogelstein et al. 1987).

In a population of normal females, the proportion of active X chromosomes derived from each parent is distributed around a mean of 50:50 (Nance 1964; Migeon 1971; Fialkow 1973). A skewed pattern of X inactivation is observed in some normal females. Vogelstein et al. (1987) observed that 5% females had X-inactivation ratios in excess of 80:20, as determined by the methylation pattern at the PGK1 locus. When the methylation pattern is analyzed at DXS255, up to 10% of the normal female population appear to have a ratio in excess of 90:10, as judged visually, with the unmethylated component of one allele being absent or virtually absent. For example, we analyzed 24 normal females and found only 1 individual with such a skewed ratio (data not shown). In another study, 5 of 50 individuals were shown to have a skewed pattern of X inactivation (Y. Boyd, personal communication). In this study we have taken a value of 10% as the proportion of normal females with a skewed pattern of X inactivation.

To document the X inactivation patterns in known or potential carriers of ATR-X, we initially studied females from families in which there was at least one female carrier diagnosed either on the basis of pedigree analysis or by the detection of HbH inclusions (families 1–5; fig. 1). All (eight of eight) mothers of affected boys have a skewed pattern of X inactivation, with the ratio visually assessed as greater than 90:10. This pattern was seen with M27β (fig. 3) and, where informative, with pSPT-PGK also (fig. 4). In three of the mothers who were studied further, the same pattern of skewed X inactivation observed in DNA from peripheral blood was also seen in DNA from buccal cells and hair roots (data not shown). A significant proportion (4 of 12) of maternal sibs or female offspring also had a similarly skewed pattern of X inactivation. These observations strongly implicate an X-linked defect as the cause of the ATR-X syndrome.

In the light of these findings it is now possible to examine the relationship between the pattern of X inactivation and the presence of HbH inclusions (fig. 1). HbH inclusions were demonstrated in three of seven obligate carriers of the ATR-X syndrome. In addition, HbH inclusions were seen in one other mother of an affected boy (II-2 in family 3) and in three female sibs. With one exception, cells with HbH inclusions were rare (0.01%–0.001% red cells) and

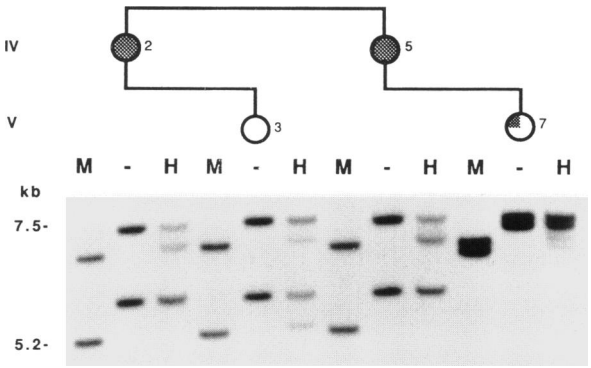

Figure 3 Methylation analysis at DXS255, by using M27β: hybridization of M27β to DNA prepared from the venous blood of females from family 1 (see fig. 1). Shown are fragments generated by *PstI* + *MspI* (lanes M), *PstI* (lanes -), and *PstI* + *HpaII* (lanes H) digests of a single sample. Both mothers (IV-2 and IV-5), who are obligate carriers and exhibit rare HbH inclusions, have nonrandom X inactivation as indicated by the digestion of only one allele by *HpaII*. One daughter (V-3), in whom no HbH inclusions were found, has random X inactivation (both alleles show digestion with *HpaII*), and her cousin (V-7), who has very frequent HbH inclusions, also has random X inactivation.

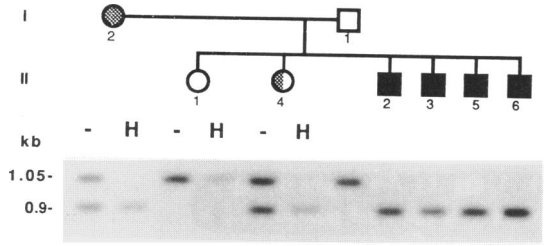

Figure 4 Methylation analysis at PGK1, by using pSPT-PGK: hybridization of pSPT-PGK to DNA from venous blood of individuals from family 5 (see fig. 1). In each case, DNA was digested with *Pst*I and *Bst*XI; for female members of the family, the sample was then divided into two equal parts; one half was not digested further (lanes -), and the other half was digested with *Hpa*II (lanes H). The mother (I-2) and one daughter (II-4), both of whom exhibit rare HbH inclusions, have nonrandom X inactivation. The other daughter (II-1), in whom no inclusions were found, is uninformative for this probe. All four affected boys share the same PGK1 allele, which is the same allele that is resistant to digestion by *Hpa*II (and that hence is inactive) in both their mother (I-2) and the informative sister (II-4).

always associated with a skewed pattern of X inactivation. In the one important exception (V-7 in family 1), a girl of normal intelligence and development, an abnormal blood film was observed, and 0.9% of the red cells contained HbH inclusions, a proportion similar to that observed in affected boys. DNA obtained from the peripheral blood and buccal cells of this girl showed a random (50:50) pattern of X inactivation (fig. 3). These results are consistent with the hypothesis that in female carriers of the ATR-X syndrome the number of cells in which HbH inclusions can be demonstrated reflects the proportion of hemopoietic stem cells in which the abnormal X chromosome is active. The inability to detect inclusions in some obligate carriers is best explained by postulating that the preferential inactivation of the X chromosome bearing the mutant X allele is so extreme in these subjects that production of HbH-containing cells falls below the threshold for detection.

On the basis of these findings we conclude that female carriers of ATR-X may be identified in three ways: (1) they may be obligate carriers, (2) they may exhibit HbH inclusions in their red cells, or (3) they may have a markedly skewed pattern of X inactivation. For the purpose of risk analysis, we estimated that approximately 50% of heterozygotes demonstrate HbH inclusions and that the remainder demonstrate nonrandom X inactivation in the absence of HbH inclusions (table 2). Since up to 10% of normal females show a similarly skewed pattern of X inactivation, this must be taken into account in estimating carrier risk.

Linkage Analysis

Two-point LOD scores were calculated for θ's between the ATR-X locus and all the loci listed in table 1, in the five families that fulfilled the criteria for inclusion in the linkage study (families 1–5 in fig. 1). The results for a selection of these loci are summarized in table 3. A peak LOD score of 5.42 at $\theta = 0$ was seen at the locus DXS72. DXS106 and DXYS1X were the

Table 3

Two-Point Linkage Analysis

	LOD Score at θ of									
ATR-X vs.	.00	.001	.01	.05	.10	.20	.30	.40	θ_{max}	LOD_{max}
DXS143	−∞	−8.20	−4.24	−1.65	−.71	−.06	.07	.03	.30	.07
DMD	−∞	−9.08	−5.11	−2.42	−1.35	−.45	−.08	.04	.42	.04
DXS255	−∞	−5.69	−1.77	.67	1.41	1.60	1.20	.55	.17	1.63
DXS159	−∞	.31	1.26	1.74	1.76	1.46	.97	.40	.08	1.78
DXS106	−∞	−1.50	−.51	.12	.32	.42	.36	.22	.20	.42
PGK1	3.46	3.45	3.40	3.17	2.87	2.21	1.46	.66	.00	3.46
DXS72	5.42	5.41	5.33	4.95	4.46	3.39	2.21	.96	.00	5.42
DXYS1X ...	−∞	3.01	3.92	4.23	4.02	3.20	2.16	1.00	.04	4.23
DXYS5X ...	1.51	1.50	1.48	1.37	1.23	.92	.58	.21	.00	1.51
DXS3	3.34	3.33	3.28	3.05	2.74	2.06	1.30	.52	.00	3.34
DXS87	−∞	−4.49	−1.54	.32	.92	1.16	.95	.52	.19	1.16
DXS94	−∞	−4.20	−2.22	−.91	−.42	−.07	.00	−.02	.31	.00
F9	−∞	−14.69	−8.71	−4.63	−2.96	−1.44	−.68	−.25	.50	.00
DXS52	−∞	−8.69	−4.72	−2.07	−1.05	−.23	.05	.10	.38	.11

closest flanking loci that demonstrated recombination. In family 2, containing the individual (III-5) who demonstrated recombination between DXS72 and DXYS1X, probes at the loci DXYS5X and DXS3 were uninformative. Consequently, we obtained positive LOD scores ($\theta = 0$) at these loci. However, haplotype analysis in this family (data not shown) indicated that there was only a single recombination in III-5. Therefore, this places the locus encoding ATR-X between DXS106 and DXYS1X, corresponding to the interval Xq12-Xq21.31; the flanking markers lie approximately 11 cM apart (Keats et al. 1989; Mahtani et al. 1991). Multipoint linkage analysis (fig. 5) did not refine this genetic localization.

Genetic Risk Analysis

Twelve women (one deceased) in these pedigrees were identified as carriers of the ATR-X mutation on the basis of pedigree information or by the demonstration of HbH inclusions in a proportion of their red cells. Under the assumption that all carriers have either demonstrable HbH inclusions or nonrandom X inactivation or both (as detailed above), seven female relatives could be defined as not being carriers of the ATR-X syndrome. In two female relatives (IV-1 in family 1 and IV-4 in family in family 2) who had nonrandom X inactivation but in whom no HbH inclusions could be demonstrated, the carrier status remained undetermined.

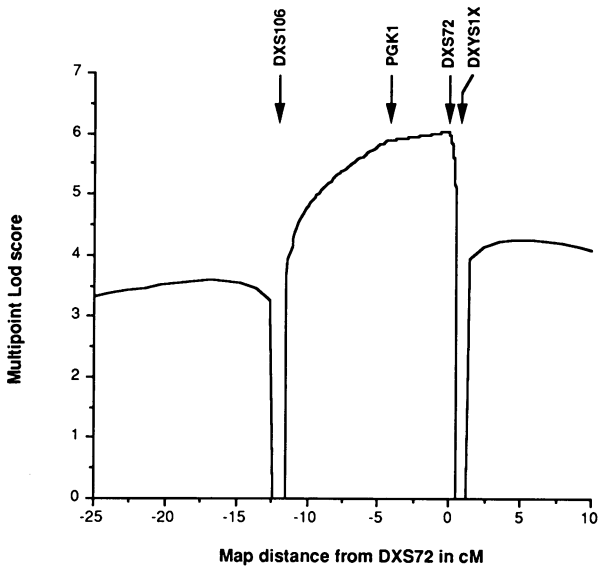

Figure 5 LINKMAP analysis of location of ATR-X locus in proximal Xq. The LINKMAP program was used to calculate multipoint LOD scores for different ATR-X locus locations relative to a fixed map of four markers. The locus DXS72 was arbitrarily placed at the origin, and the other loci were positioned relative to it according to the genetic distances as described in Material and Methods.

For each of these women the prior risk of being a carrier was 50%. Under a Bayesian approach, the presence of nonrandom X inactivation increased their posterior probability of being a carrier to 83% (table 4). The program MLINK was then used to modify the risk assigned to these two women, according to the genotypes at loci flanking the ATR-X locus. As the precise position of this locus within the DXS106-DXYS1X interval is not known, risks were calculated with the ATR-X locus placed at four locations within the interval. If the consultand was recombinant between DXS106 and DXYS1X, then the carrier risk could vary very widely according to the exact position of the ATR-X locus. The calculation of carrier risks at a number of ATR-X locations within this DXS106–DXYS1X interval defines a range of risks (or a risk interval) for the consultand. In this way the lack of precision in the genetic localization of the ATR-X locus could be reflected in the risk assessment (Lange 1986; Suthers and Wilson 1990). If a consultand was not recombinant in the DXS106–DXYS1X interval, then the risk interval would be very narrow. IV-1 in family 1 had inherited the same haplotype as her affected nephews, and the risk interval for her being a carrier was calculated to be 98%–100%. IV-4 in family 2 had not inherited the same haplotype as her affected brother, and her risk interval was reduced to 1%–2%. In the latter case, therefore, the skewed pattern of X inactivation is probably an example of the extreme pattern seen in approximately 10% of the normal population.

Two further families were identified, each containing one affected male but no obligate female carriers (families 6 and 7; fig. 6). Although these two families were not included in the linkage analysis, they are presented here to illustrate the logic that can be applied to the risk assessment of carrier status in the ATR-X syndrome. In family 6, there was insufficient information to indicate whether the mother (I-2) was an obligate carrier. Bayesian analysis of the phenotypes in this family gave her a 56% risk of being a carrier (table 4). Multipoint analysis indicated that her risk interval for being a carrier was 87%–91%, because all three daughters had inherited, in the proximal Xq region, the maternal haplotype that was the opposite of that inherited by the affected child.

Table 4

Risk Analysis

A. Family 1—Consultand Is IV-1 (Same Calculation for IV-4 in Family 2)		
	Risk that IV-1 Is a	
	Carrier	Noncarrier
Prior probability	.5	.5
Conditional probability of IV-1 being in class II	.5	.1
Joint probability	.25	.05
Posterior probability (IV-1)	.83	.17
Incorporating multipoint linkage analysis:		
Family 1, IV-1	.98–1.00	.00–.02
Family 2, IV-4	.01–.02	.98–.99

B. Family 6—Consultand Is I-2		
	Risk that I-2 Is a	
	Carrier	Noncarrier
Prior probability	4μ	$1\text{-}4\mu \sim 1$
Conditional probability:		
Of I-2 being in class II	.5	.1
Of II-2 being in class I	.5	μ
Of II-1, II-3, and II-4 being in class III	.09 [$=(.5\times.9)^3$]	.73 [$=(.9)^3$]
Joint probability	$.09\mu$	$.07\mu$
Posterior probability (I-2)	.56	.44
Incorporating multipoint analysis	.87–.91	.09–.13

C. Family 7—Consultand Is III-1		
	Risk that II-2 Is a	
	Carrier	Noncarrier
Risk probability	2μ[a]	$1\text{-}2\mu \sim 1$
Conditional probability:		
Of II-2 being in class II	.5	.1
Of III-2 being in class I	.5	μ
Of III-3 being in class II	.3 [$=(.5\times.5)+(.5\times.1)$]	.1
Joint probability	$.15\mu$	$.01\mu$
Posterior probability	.94	.06
	Risk That III-1 Is a	
	Carrier	Noncarrier
Prior probability	.47	.53
Conditional probability of III-1 being in class II	.5	.1
Joint probability	.23	.05
Posterior probability (III-1)	.82	.18
Incorporating multipoint analysis	.98–.99	.01–.02

[a] 4μ would be incorrect here, as the grandmother (I-2) is not a carrier; however, either chromosome could carry a new mutation.

In the second of these families (family 7) II-2 had an affected son, and analysis of her DNA revealed a nonrandom pattern of X inactivation. However, both of her parents had entirely normal phenotypes. It was not clear whether she carried a new mutation or whether a new mutation had occurred in her son; analysis of the haplotype revealed that the affected X chromosome is derived from the maternal grandfather (I-

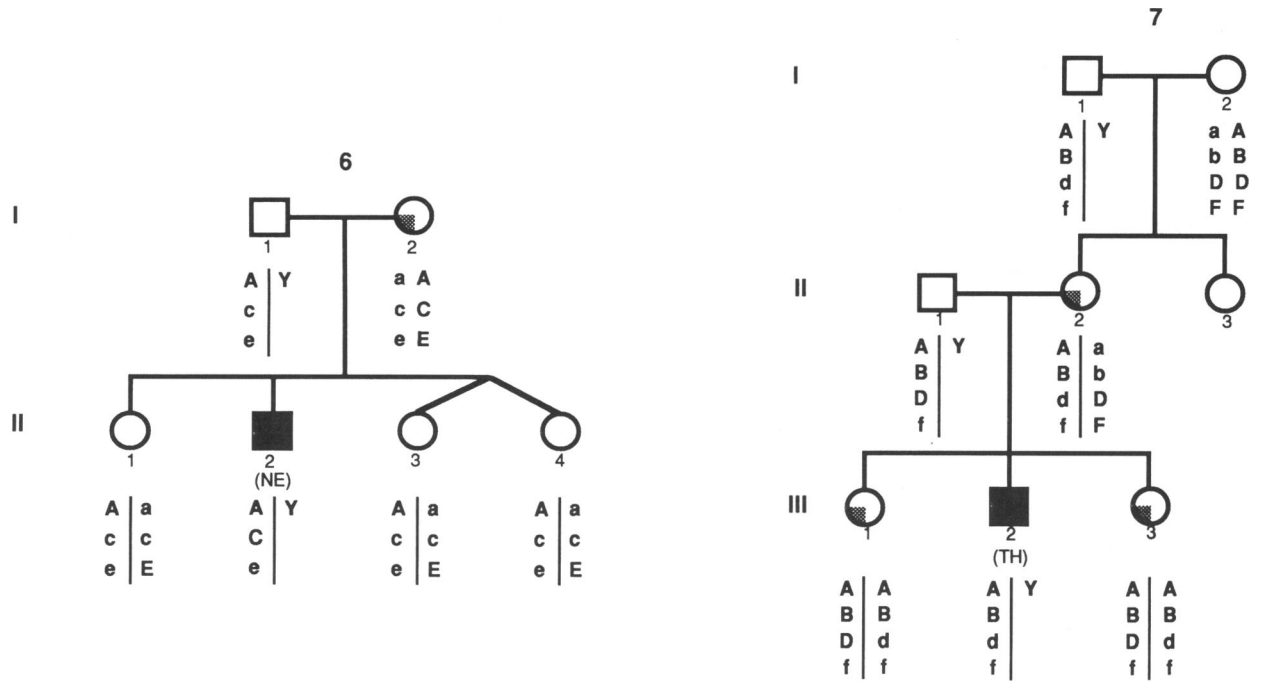

Figure 6 Examples of pedigrees in which the only evidence for the carrier state was a skewed pattern of X inactivation. Symbols are as used in fig. 2. Haplotypes, where available, are given below each individual. Alleles for the informative loci in each pedigree are represented as follows: A and a = alleles at DXS1 that are detected by p8 in a *Hin*dIII digest; B and b = alleles at PGK1 that are detected by pSPT-PGK in a *Pst*I/*Bst*XI digest; C and c = alleles at DXS72 that are detected by pX65H7 in a *Hin*dIII digest; D and d = alleles at DXYS5X that are detected by p31 in a *Taq*I digest; E and e = alleles at DXS3 that are detected by p19-2 in a *Taq*I digest; and F and f = alleles at DXS94 that are detected by pXG-12 in a *Pst*I digest (for further details, see table 1). Previously reported probands whose phenotypes have been reviewed by Gibbons et al. (1991) are shown in parentheses. In family 6, the proband II-2 (NE) is phenotypically female but has an XY karyotype (Wilkie et al. 1990*b*).

1). Her daughter (III-1) wished to know her own risk of being a carrier. A Bayesian analysis of the phenotypes in this family indicated that the risk of the consultand (III-1) being a carrier was 82% (table 4). Multipoint risk analysis (as described above) indicated that the consultand's risk interval was 98%–99%.

Discussion

Approximately 1 in 500 newborn males suffers from some form of X-linked mental retardation; many of these have the fragile-X syndrome, but in others the cause of mental handicap often remains unknown and difficult to categorize further (Glass 1991). The ATR-X syndrome represents a novel form of X-linked mental handicap, one that can be distinguished by its characteristic dysmorphic facial appearance and associated congenital abnormalities; the diagnosis can be confirmed by demonstrating the features of HbH disease (α-thalassemia). The linkage analysis presented here has localized the disease to the interval Xq12-q21.31. The proximal (centromeric) boundary of this localization is defined by one recombination observed between the disease and locus DXS106, in Xq12. The distal (telomeric) boundary is defined by one recombination between the disease and locus DXYS1X, in Xq21.31. High-resolution cytogenetic analysis has not revealed any abnormalities in seven of the cases presented here. Although numerous other loci for X-linked mental retardation map within this interval (Chowcheck et al. 1985; Wieacker et al. 1987; Arveiler et al. 1988; Samanns et al. 1988; Sutherland et al. 1988; Suthers et al. 1988; Cremers et al. 1989; Goldblatt et al. 1989; Schwartz et al. 1990; Miles and Carpenter 1991; Wilson et al. 1991), the ATR-X syndrome appears distinct from all those previously reported.

As more families including individuals with the ATR-X syndrome are identified, it becomes increasingly important to be able to recognize female carriers

of this disorder, to provide appropriate genetic counseling. We have shown that in female carriers of the ATR-X mutation it is sometimes possible to demonstrate HbH inclusions in a small proportion of their red cells, a feature that is diagnostic for the presence of α-thalassemia when the BCB incubation is performed at room temperature with a negative control sample. Possible carriers should therefore be carefully screened for the presence of HbH inclusions, and, if these are detected, it may be concluded that the woman is a carrier. Care must be taken to exclude the coincidental inheritance of other α-thalassemia determinants, especially in populations where this is common.

It appears that the frequency of HbH inclusions in blood smears from carriers of the ATR-X mutation reflects the pattern of X inactivation observed in DNA from leukocytes; usually such carriers have a markedly skewed pattern of X inactivation, and, consequently, very few cells contain HbH inclusions; but, in the one exception (V-7 in family 1) having random X inactivation, the inclusions are much more frequent. Quite often no HbH inclusions can be detected in putative carriers, and in such cases the X-inactivation status of a possible heterozygote may be informative. When nonrandom X inactivation is detected, a simple Bayesian calculation may be used to determine the posterior probability of the consultand being a carrier. Where a carrier is informative for PGK1, phase can be deduced by identifying the preferentially inactivated X chromosome (fig. 4). As a corollary of this, the likelihood of a female being a carrier is increased if the preferentially inactivated X chromosome is the one inherited by the affected male. We consider women who lack HbH inclusions and have random X inactivation to be at very low risk (less than 1%) of being carriers.

Since the ATR-X locus has been localized, it is now possible to incorporate classical genetic linkage analysis in the modification of carrier risk (see table 4). In families with informative markers in the ATR-X interval (e.g., DXS72) and flanking the locus (e.g., DXYS1X and DXS106), prenatal diagnosis may be feasible. However, two cautions should be noted. First, the peak two-point LOD score is only 5.42 (table 3), and the observation of just one further recombinant could alter the gene location relative to the polymorphic loci on Xq. However, it is reassuring that the peak multipoint LOD score of 6.03 in the interval DXS106-DXYS1X was almost two units higher than the highest scores in the flanking regions (fig. 5). Second, the precise location of the ATR-X locus within this interval is not known, and, since this genetic distance (11 cM) is relatively large, there is a significant possibility that a recombination would occur and widen the calculated risk interval. In this situation it is preferable to use multipoint risk analysis, because genotype data from loci flanking the disease gene can be incorporated simultaneously into the risk calculation.

Linkage data are also useful in the characterization of new mutations affecting the ATR-X locus. We detected a new mutation in one of the seven pedigrees examined (family 7): analysis of the haplotypes in the region of the ATR-X locus revealed that the affected X chromosome is derived from the maternal grandfather.

The nature of the underlying abnormality in the ATR-X syndrome is unknown. We have recently investigated two male patients with naturally occurring deletions encompassing most of the Xq21 region (fig. 2). Both of these patients have developmental delay and choroideremia. However, hematologic studies have excluded α-thalassemia (R. J. Gibbons, unpublished data; D. Robins, personal communication). This indicates that nullisomy for loci in the segment removed by these deletions is not responsible for the ATR-X phenotype; investigation of other deletions from this region would be of interest. Another approach to understanding the molecular basis of this disorder lies in elucidating the mechanism by which the ATR-X mutation down-regulates expression of the α-globin genes. Critical *cis*-acting sequences involved in the regulation of α-globin gene expression have been defined (Higgs et al. 1990). Some of the proteins that interact with these sequences have been identified (Jarman et al. 1991), but none of the genes encoding them have been shown to map to this region of the X chromosome. Finally, the observation that in female carriers the chromosome bearing the ATR-X mutation is preferentially inactivated might also help in understanding the pathophysiology of this syndrome.

The pattern of X inactivation is subject to numerous influences during development. For example, a nonrandom pattern of X inactivation is frequently observed in females with structural abnormalities of the X chromosome: in deletions (Wells et al. 1991) and duplications (Thode et al. 1988) the abnormal chromosome is usually inactive, whereas in balanced X; autosome translocations the normal X is usually inactive (Mattei et al. 1982). In these circumstances the skewed pattern of X inactivation probably results

from selection against those situations in which the expressed karyotype is unbalanced. As yet, we have not detected any structural abnormality of the X chromosome in patients with the ATR-X syndrome (Gibbons et al. 1991). A skewed pattern of X inactivation may also result from selection for the normal allele in female carriers of a deleterious mutation (e.g., X-linked immunodeficiency syndromes [reviewed in Schwaber and Rosen 1990; Winkelstein and Fearon 1990] and hypoxanthine guanine phosphoribosyl transferase deficiency [Nyhan et al. 1970]). In these cases the selection that gives rise to the skewed pattern of X inactivation is restricted to certain critical cell types. In the ATR-X syndrome, cell types representing mesoderm (blood cells), endoderm (buccal cells), and ectoderm (hair roots) are equally affected. Therefore, if the skewed pattern of X inactivation observed in carriers of the ATR-X syndrome is due to selection, this must precede tissue differentiation or act in a coordinate manner. Alternatively, it is possible that the ATR-X mutation in some way alters the probability that the chromosome carrying this allele will be inactivated. Indeed, the X-inactivation center maps within the ATR-X disease interval and might be subject to a *cis* effect (Brown et al. 1991). A better understanding of the normal mechanism by which the X chromosome is inactivated may enable this hypothesis to be tested in detail.

An important, unanswered question is why female heterozygotes are phenotypically normal. Skewed inactivation silencing the mutant allele might be an explanation in those in whom it is observed; however, one intellectually normal female carrier (V-7 in family 1) has a random pattern of X inactivation. Although the hematological manifestations in female carriers can be explained, the relationship between genotype and phenotype for the other features of the syndrome is unclear. Further analysis of all these problems requires the identification of more families to study. Nevertheless, this syndrome promises to provide insights into a number of biological mechanisms, including the regulation of α-globin genes, causes of dysmorphism and mental retardation, and the establishment of the pattern of X-chromosome inactivation.

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