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Evidence for a New Locus for X-Linked Retinitis Pigmentosa (*RP23*)

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Purpose. X-linked retinitis pigmentosa (XLRP) is a degenerative disease of the retina characterized in the early stages of disease by night blindness as a result of rod photoreceptor loss, progressing to severe disease with loss of central vision by the third decade in affected males. XLRP displays exceptional genetic heterogeneity, with five reported loci on the human X-chromosome. To investigate the level of heterogeneity for XLRP in the patient pool in the current study, extensive haplotype analysis, linkage analysis, and mutation screening were performed.

Methods. Haplotype analysis of a family with diagnosed XLRP was scored with more than 34 polymorphic markers spanning the entire X-chromosome, including regions already identified as harboring XLRP genes and retina-specific genes. Two-point and multipoint lod scores were calculated. Affected male DNA was amplified with primers specific for the retinoschisis gene (XLRS1), and the products were screened for nucleic acid alterations by direct automated sequencing.

Results. In this article haplotype and linkage data are presented identifying a new locus for XLRP on the short arm of the X-chromosome, distinct from previously reported gene localizations for XLRP. The phenotype is atypical, in that the onset of vision loss in the male members of this family is unusually early, and female obligate carriers have normal fundi and waveforms. Informative recombination events in this family define a locus for XLRP (*RP23*) on Xp22 between the markers DXS1223 and DXS7161, spanning approximately 15 cM. A maximum lod score of 2.1 was calculated for the locus order DXS7103–8 cM–(*RP23*/DXS1224)–4 cM–DXS999. This new locus (*RP23*) encompasses the retinoschisis disease gene; therefore, *XLRS1* was screened for a mutation. No sequence alteration was identified indicating that mutations in the coding region of the gene responsible for retinoschisis do not cause *RP23*.

Conclusions. The results describe evidence for a new locus for XLRP (*RP23*), adding to the established genetic heterogeneity for this disease and the number of genes expressed in ocular tissue residing on the X-chromosome. (*Invest Ophthalmol Vis Sci.* 2000;41:2080–2086)

The human X-chromosome is home to a large number of genes involved in inherited diseases of the eye, of which retinal diseases comprise a majority (Fig. 1[1–20]). These genetic disorders are a significant cause of visual impairment and blindness and include retinitis pigmentosa (RP), congenital stationary night blindness (CSNB), and progressive cone dystrophy (COD). X-linked retinitis pigmentosa (XLRP) is the most severe form of RP, with male patients showing concentric visual field loss before the 20th year of life, leading to severe visual handicap.[21] Female carriers show variable clinical symptoms of the disease with visual impairment usually beginning in middle age, although absence of ocular abnormalities does not exclude the carrier state.[22] XLRP accounts for 7% to 30% of RP cases with an incidence of approximately 1:20,000.[23,24] The disease is degenerative and characterized by constriction of the visual fields due to peripheral photoreceptor loss resulting in night blindness and pigmentary retinopathy. Loss of central vision occurs in advanced stages of disease when the patient's visual impairment is severe.

Accumulated genetic mapping data from families displaying X-linked forms of all the aforementioned diseases demonstrates remarkable genetic heterogeneity. Of particular note is the recent increased detection of genetic heterogeneity for X-linked CNSB ([CSNBX] *CSNB4*[8] see Fig. 1) and X-linked progressive cone dystrophy ([XLPCD] *COD2*[6] see Fig. 1). Such levels of heterogeneity can confound attempts to identify the causative genes in the absence of cytogenetic abnormalities.

After the first genetic linkage report of an RP gene (*RP2*) to Xp11,[25] subsequent genetic analyses of XLRP families have identified up to four other XLRP loci (see Fig. 1; *RP3*, *RP6*, *RP15*, and *RP24*[3,4,18,26,27]) located more distally on Xp. *RP6* is to date only statistical,[4] and only one family has been reported with *RP24*[18] and one family with *RP15* (with a rare dominant cone-rod phenotype).[3] The majority of XLRP families, therefore, fall into the categories of *RP2*[28] or *RP3*[4,5,26] through

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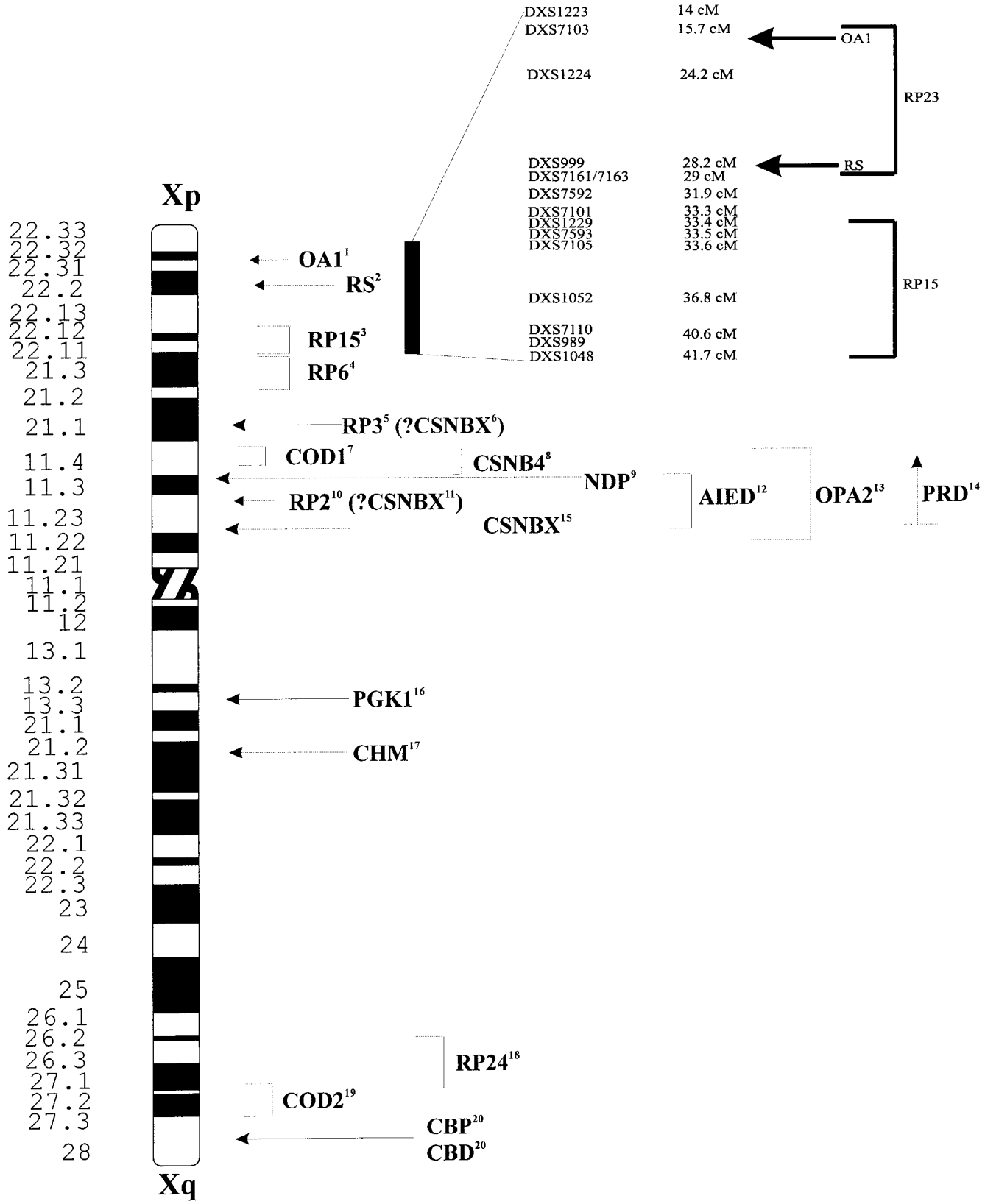

FIGURE 1. Ideogram of the human X-chromosome showing map locations of various eye diseases incorporating a genetic map of Xp22.11–Xp22.32. Genetic distances (Genethon 1996) taken from The Integrated X-Chromosome Database (http://ixdb.mpimg-berlin-dahlem.mpg.de/). Superscript numbers refer to studies identifying the genes.

genetic mapping studies (see Fig. 1); however, mutation detection in the causative genes creates a more complex picture that leaves more than 60% of disease currently unaccounted for.[10,29] In fact, the isolation of the *RPGR* and *RP2* genes has made haplotype analysis leading to crossover detection an even more essential part of XLRP research because of the potential existence of genetically undefined loci on the X-chromosome.

We have extensively haplotyped many XLRP families, and in this article we report a new locus for atypical XLRP in a single family that is not associated with any previously described loci.

Materials and Methods

Clinical Assessment

This study, which involved human subjects, conformed to the tenants of the Declaration of Helsinki. Individual IV-2 was familiar with the clinical histories of three other males in the family, all of whom had the onset of poor vision before the age of 2 years. Thus she sought an ophthalmic evaluation for her son (individual V-2) at age 2 years, when he was noted to have limited central vision and poor night vision and frequently ran into objects.

On examination, the patient fixed and followed with both eyes and had no evidence of tropias, phorias, or nystagmus. His retinoscopic refraction was +3.50 sphere and +1.25 sphere for the right and left eyes, respectively. The anterior segment examination findings were completely normal, and the fundus examination demonstrated normal optic nerves (cup disc ratio [C/D] = 0.3), abnormal grayish macular reflexes, and extensive whitish gray spots (discrete and not flecklike) distributed throughout the midperiphery of the posterior pole. These spots appeared at the level of the pigment epithelium and were not associated with any overlying intraretinal pigment migration. In many areas these white spots were coalescent. When he was examined 4 years later, the patient had no nystagmus, but he clearly demonstrated eccentric fixation with preferential use of the left eye and more symptomatic photophobia. His visual acuities were estimated to be in the 20/800 range in both eyes. Findings in a fundus examination were virtually unchanged, except that retinal arteriole attenuation was now evident. Karyotype evaluation in 1997 demonstrated normal 46XY chromosomes in all cells analyzed.

Individual V-2 was most recently clinically examined in 1998 at age 11. The patient reported no photophobia, and his visual acuity was limited to counting fingers at 2 to 3 feet. His peripheral vision was markedly constricted, and the changes in the pigment epithelium were nearly confluent in the midperiphery, giving rise to an overall grayish appearance (see Fig. 2). Small patches of retinal pigment epithelium (RPE) atrophy were now present in the midperiphery, and a few small patches of intraretinal pigment were noted. There was a circular area of approximately 1.5 disc diameters, centered over the fovea that appeared to have relatively normal retina and RPE. The retinal arterioles were clearly attenuated in all quadrants, but there was no optic nerve pallor. Electroretinograms (ERGs) and formal visual field testing of the child were not performed because of his hyperactive nature, because of the family's reluctance to have him sedated, and because such testing would not alter his diagnosis, prognosis, or treatment.

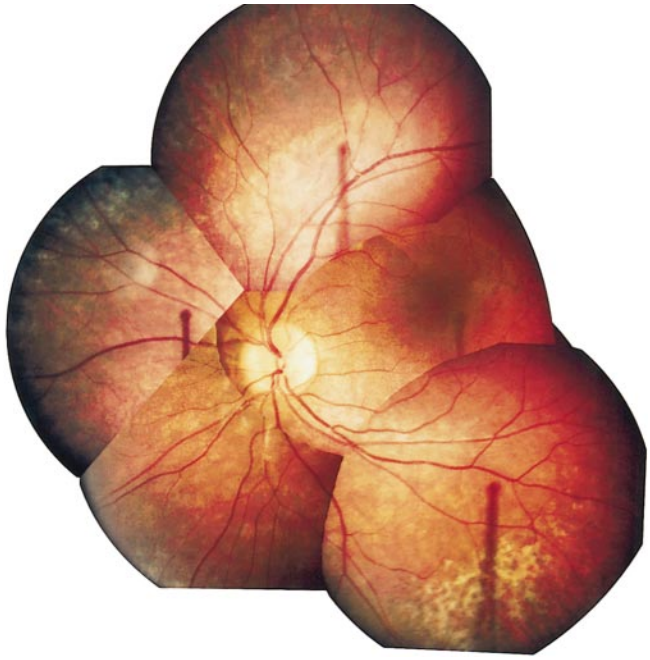

Figure 2. Photomontage of the fundus of affected male V-2 showing attenuated retinal arterioles and near-confluent changes in the pigment epithelium in the midperiphery.

Subject IV-4 was initially evaluated by a genetic retina specialist in Boston at age 21. At that time, extensive electrophysiologic and psychophysical testing demonstrated central visual acuities of 2/400 with mild myopia (-1.00–2.00×180 OU), nondetectable ERG recordings bilaterally, abnormal color vision, and only residual temporal and inferior fields of vision. The clinical examination was remarkable for the absence of foveal reflexes in both eyes, diffuse granularity of the RPE in the central maculae, and moderate intraretinal pigment in the retinal midperipheries. A follow-up examination was conducted when the patient was 35 years of age by one of the authors (MBG). At that time, his visual acuities had deteriorated to hand motion vision in both eyes, and his maculae and retinal midperipheries were relatively unchanged except for more notable areas of RPE atrophy and increased amounts of intraretinal pigment in the retinal peripheries. Posterior subcapsular cataracts, which were not present at age 21, were clearly evident by age 35. Both retina specialists independently concluded that the diagnosis was consistent with RP, with a severe phenotype and macular involvement.

Both the mother (individual IV-2) and the maternal grandmother (individual III-2), who were obligate carriers for the condition based on the pedigree, were examined and underwent ERG testing. The mother's uncorrected Snellen visual acuities were 20/25-2 and 20/15-1 in the right and left eyes, respectively. The visual acuities in the grandmother were 20/25-1 and 20/25-2 in the right and left eyes, respectively. The clinical fundus examinations were completely normal, with no evidence of retinal or RPE changes for either individual. The ERGs were conducted using Jet electrodes with an LKC EP-IC-XL instrument (LKC Technologies, Inc., Gaithersburg, MD) with a Ganzfeld chamber, in compliance with the international protocol standards and compared with age-matched control standards. Both carrier females demonstrated normal wave-

forms, with amplitudes and implicit times that were within the normal range (data not shown). For this study, the participation of family members was approved by the University of Pittsburgh Biomedical Institutional Review Board, in accordance with the guidelines of the Office for the protection from Research Risks, and informed consent was obtained from the patients before their participation.

Haplotype Analysis

The forward primer for each microsatellite was end labeled with ^{32}P-γdATP at 37°C for 45 minutes using T4 polynucleotide kinase (New England Biolabs, Hertfordshire, UK). Polymerase chain reaction (PCR) was performed as previously described.[31] Alleles were detected by electrophoresing the PCR products on 6% denaturing polyacrylamide gels (Promega, Southampton, UK). Details of primer sequences and PCR conditions for all microsatellites used in this study are available from The Genome Database (http://www.hgmp.mrc.ac.uk/gdb/gdbtop.html).

Linkage Analysis

Two-point linkage analysis for XLRP and informative markers on chromosome Xp were scored by computer (Linkage, ver. 5.1 using Mlink; Columbia University, New York, NY). The frequency of the XLRP gene in the general population was taken to be 0.0001. Penetrance values for carriers were set at 0.0000. Alleles at marker loci were assumed to have equal frequency. Multipoint linkage analysis (Linkmap; Columbia University) with loci order DXS7103–DXS1224 (using genetic distance of 8 cM) and DXS999 (using genetic distance of 4 cM) was performed (genetic distances taken from the Integrated X-Chromosome Database; http://ixdb.mpimg-berlin-dahlem.mpg.de/[see Fig. 1]).

Sequence Analysis

All six exon fragments of the *XLRS1* gene were amplified with intronic primers described.[2] PCRs were performed in 25-μl reactions in the presence of 0.5 U *Taq* polymerase (Biotaq from Bioline, London, UK); 200 μM each of dATP, dCTP, dGTP, and dTTP; 200 picomoles of each primer and 1× KCl reaction buffer (from Bioline) including 1.5 mM $MgCl_2$. PCR conditions were as described.[2] Amplification products were purified with centricon concentrators (Amicon, Gloucestershire, UK) according to the manufacturer's instructions. The purified DNA sample (5 μl) was cycle sequenced in both directions (with primers used for amplification) using a termination cycle sequencing kit (ABI prism Ready Reaction Dye FS kit, Perkin–Elmer, Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Reactions were then electrophoresed on a sequencer (model 373A; Perkin–Elmer Applied Biosystems).

Results

Clinical Characteristics

XLRP has been reported to be particularly severe compared with many of the autosomal forms,[21] and macular lesions have also been noted in a higher percentage of X-linked cases; however, the onset of vision loss in the male members of this family was unusually early. There was clear evidence of progressive retinal and RPE changes with both rod and cone involvement (see Fig. 2), and affected male IV-4 had no recordable ERG response at age 21. Both obligate female carriers examined had normal fundi and waveforms.

Haplotype Analysis

The family was analyzed with more than 34 polymorphic marker loci spanning the entire X-chromosome. Figure 3 details haplotype results for the family members. Initial efforts were concentrated on microsatellites surrounding the *RP2* and *RP3* loci on the short arm of the X-chromosome, because these are the reportedly common loci for XLRP. As the haplotype of this portion of the X-chromosome was constructed, it became evident that the disease in this particular pedigree did not segregate with these intervals (see Fig. 3). Other loci (e.g., *CSNB4/COD1*[8,33] and *RP15*[3]) and markers linked to known retina-specific genes (e.g., *X-arrestin*[34] and *RetGC2*[35]) were excluded.

The distal boundary of XLRP in this family was defined by a recombination event observed in individual III-2, an obligate carrier female who had inherited the disease-associated haplotype at DXS7103, placing the disease proximal to DXS1223. This event was also observed in her obligate carrier daughter (IV-2), affected son (IV-4), daughter of unknown status (IV-6), and affected grandson (V-2). The proximal boundary was defined in individual IV-4, an affected male who was recombinant, relative to his carrier mother, between the markers DXS999 and DXS7161. In summary, haplotype data clearly defined a locus for XLRP (*RP23*) between the loci DXS1223 and DXS7161 in Xp22 with markers DXS7103, DXS1224, and DXS999 cosegregating with disease. This analysis would predict that individual IV-6 was a carrier and IV-8 was not at risk.

Linkage Analysis

Table 1 describes two-point linkage analysis results, demonstrating that disease in this family (*RP23*) was not linked to informative markers mapping to previously identified XLRP loci. Significant lod scores were obtained with markers DXS7103 ($Z_{max} = 1.89$; $\theta = 0$), DXS1224 ($Z_{max} = 1.96$; $\theta = 0$), and DXS999 ($Z_{max} = 1.89$; $\theta = 0$). For markers telomeric to the aforementioned loci significant lod scores were not obtained—i.e., DXS996 ($Z_{max} = 0.36$; $\theta = 0.25$) and DXS1223 ($Z_{max} = 1.01$; $\theta = 0.1$). Insignificant lod scores were also obtained with markers DXS989 (linked to the *RP15* locus; $Z_{max} = 0.70$; $\theta = 0.15$), DXS1110 (linked to the *RP3* locus; $Z_{max} = 0$; $\theta = 0.5$) and DXS426 (linked to the *RP2* locus; $Z_{max} = 0$; $\theta = 0.5$). Multipoint analysis was performed to determine the most likely location of XLRP in relation to DXS7103, DXS1224, and DXS999 (see Figs. 1 and 3). A maximum lod score of 2.06 was scored for the locus order DXS7103–8 cM–(*RP23*/DXS1224)–4 cM–DXS999. The family under study originates from the United States; therefore, lod scores were recalculated with European allele frequencies for linked markers DXS7103 (allele 1 at 0.25 and allele 2 at 0.75), DXS1224 (allele 1 at 0.5, allele 2 at 0.2, and allele 3 at 0.3), and DXS999 (allele 1 at 0.6 and allele 2 at 0.4). Resultant lod scores were $Z = 1.97$ at $\theta = 0$ for DXS7103, $Z = 2.01$ at $\theta = 0$ for DXS1224, and $Z = 1.85$ at $\theta = 0$ for DXS999, slightly increasing the statistical significance for linkage at these loci. To confirm the observation of X-linkage in this family, an autosomal dominant model with partial penetrance was compared with a fully penetrant X-

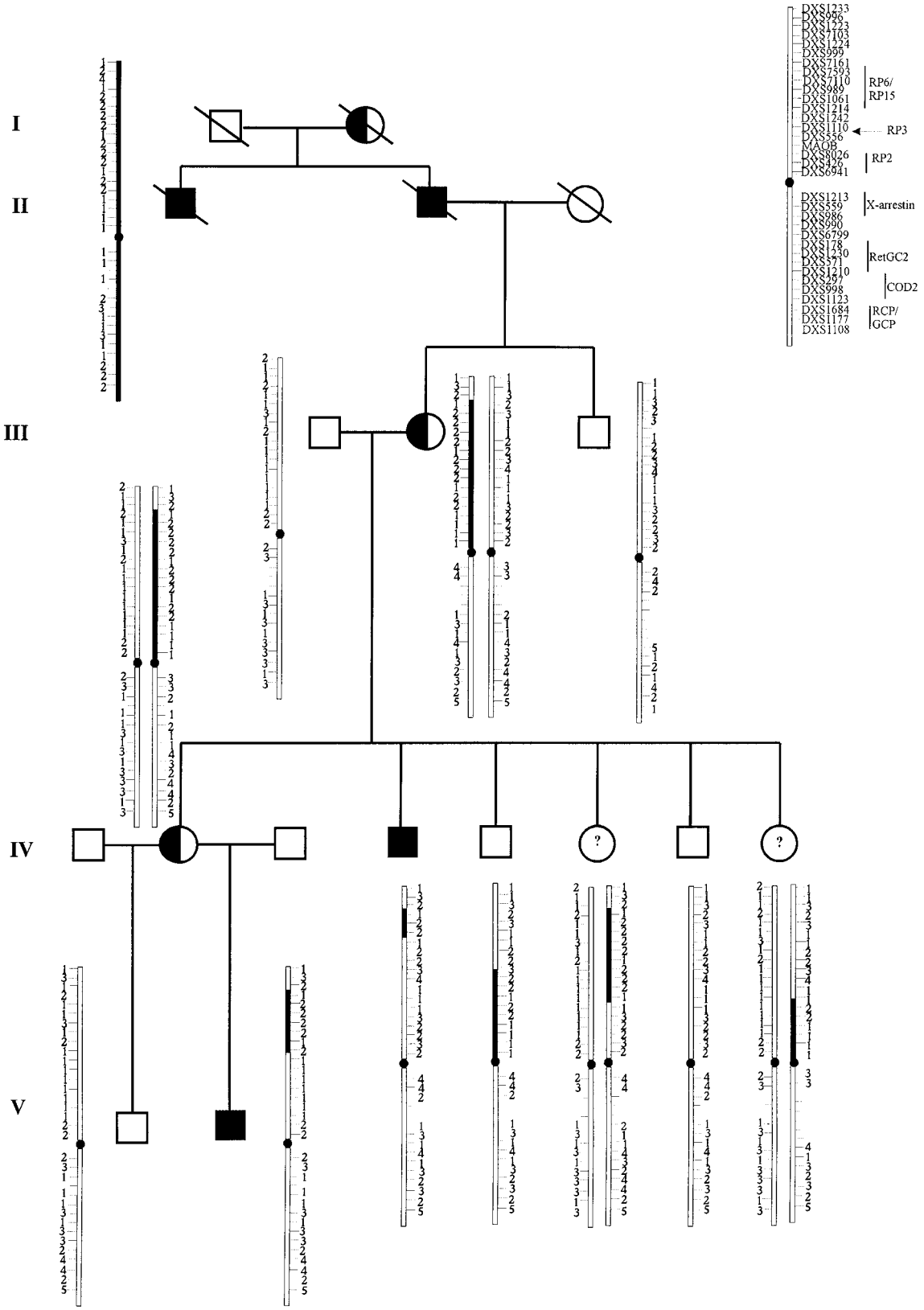

Figure 3. Haplotype analysis of family MGRP-3. Obligate carrier females are represented by *half filled circles*, affected males by *filled boxes*, and females of unknown clinical status by *circles with question marks*. *Open circles* and *squares* denote noncarrier females and unaffected males. The affected haplotype is shown as a *shaded* chromosome. The order of microsatellites on the X-chromosome is shown in the key to the right of the figure. Regions already known to harbor disease genes or retina-specific genes are highlighted in the key (also see Fig. 1). Unmarked alleles were not scored.

TABLE 1. Two-Point Linkage Analysis between XLRP and Informative X-Chromosome Microsatellites on Xp

	Recombination Fraction						
Locus	**0.00**	**0.05**	**0.1**	**0.15**	**0.2**	**0.3**	**0.4**
DXS996	−∞	−0.42	0.04	0.24	0.33	0.34	0.21
DXS1223	−∞	0.87	1.01	1.01	0.95	0.72	0.40
DXS7103	1.89	1.73	1.57	1.40	1.23	0.85	0.44
DXS1224	1.96	1.79	1.63	1.45	1.27	0.88	0.46
DXS999	1.89	1.73	1.57	1.40	1.23	0.85	0.44
DXS989	−∞	0.52	0.62	0.70	0.67	0.52	0.29
DXS1110	−∞	−1.82	−1.24	−0.91	−0.68	−0.37	−0.16
DXS426	−∞	−2.04	−1.23	−0.80	−0.52	−0.20	−0.05

Data are lod scores.

linked model using MLINK with a dummy marker. With a range of assumed penetrances the X-linked model was favored by at least 2 orders of magnitude. The relative likelihood (odds) of observing this segregation pattern if the disease is X-linked versus autosomal dominant is $(1/2)^{10}/(1/2)^{20} = 1 \times 10^3$. These data add significant statistical evidence for X-linked inheritance in this family.

XLRS1 Mutation Screen

All six exons and exon–intron boundaries were sequenced in affected males IV-4 and V-2 and no sequence alterations were detected when compared with the normal gene sequence. Mutations in the coding region of *XLRS1* are therefore excluded as causing disease in this family.

DISCUSSION

An emerging pattern for inherited retinal diseases is the exceptional heterogeneity of these disorders, with growing examples of genetic heterogeneity, allelic heterogeneity, and phenotypic heterogeneity. The complexity of the situation is amply demonstrated on the X-chromosome (Fig. 1). High-resolution mapping by haplotype analysis for X-linked retinal disease has enabled us to define the boundaries for *RP2*[28] and establish a new genetic locus for CSNBX (*CSNB4*[8]), which has potential to be allelic with the gene for *COD1*.[32] The data presented here were obtained in our efforts to establish the number of distinct genes on the X-chromosome involved in eye disease, define their locations, and address the likelihood of allelic heterogeneity.

The family described had an atypical XLRP phenotype, in that the age of onset of disease in affected males is unusually early. However, both obligate carriers tested demonstrated normal waveforms with amplitudes and implicit times that were within the normal range.

Haplotype analysis demonstrated disease segregation with markers on Xp22, excluding all other known locations for XLRP (*RP2*, *RP3*, *RP6*, *RP15*, and *RP24*; Figs. 1 and 3). The location of disease in this family also excluded potential allelism with X-linked progressive cone dystrophy loci (*COD1* and *COD2*) and CSNBX loci (Fig. 1) as well as several candidate genes known to be retina specific that reside on the X-chromosome (*RetGC2*, *X-arrestin*, *RCP*, and *GCP*).

Disease in this family is clinically distinct from the phenotype described by McGuire et al.[3] for *RP15* and segregates with markers distal to this locus (Fig. 1). This novel locus for XLRP has been designated *RP23*, adding to the level of heterogeneity for XLRP loci and RP loci in general.

The critical interval maps to Xp22.32–Xp22.13 spanning approximately 15 cM, cosegregating with the markers DXS7103, DXS1224, and DXS999. Multipoint linkage analysis scores a maximum lod of 2.06 for the locus order DXS7103–8 cM–(*RP23*/DXS1224) –4 cM–DXS999.

The *RP23* disease interval encompasses the retinoschisis locus.[2] *XLRS1* is a neural retina-specific gene potentially involved in cell–cell interactions on membrane surfaces. *XLRS1* was the primary candidate for disease in the family in this study, and the gene was therefore screened for a mutation. All exons, and exon–intron boundaries were analyzed by sequencing an affected male patient. Although this result was negative, indicating that the gene responsible for retinoschisis does not cause *RP23*, mutations outside the coding region cannot be excluded.

The critical interval for *RP23* is large, and international efforts will ultimately result in the entire sequence of this genomic region being publicly available; therefore, we envisage that many genes will be implicated as candidates for this disease. Ongoing analysis of XLRP pedigrees may lead to further refinement of the *RP23* disease interval and facilitate identification of the causative gene.

Acknowledgments

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