

Functional implications of the spectrum of mutations found in 234 cases with X-linked juvenile retinoschisis (XLR5)

The Retinoschisis Consortium[†]

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X-linked retinoschisis (XLR5) is the most common cause of juvenile macular degeneration in males, resulting in vision loss early in life. The gene involved in XLR5 was identified recently. It encodes a protein with a disoidin domain, suggested to be involved in cell–cell interactions. We have screened the gene for mutations in 234 familial and sporadic retinoschisis cases and identified 82 different mutations in 214 (91%). Thirty one mutations were found more than once, i.e. 2–10 times, with the exception of the 214G→A mutation

which was found in 34 apparently unrelated cases. The origin of the patients, the linkage data and the site of the mutations (mainly CG dinucleotides) indicate that most recurrent mutations had independent origins and thus suggest the existence of a significant new mutation rate in *XLR5*. The mutations identified cover the entire spectrum, from small intra-genic deletions (7%), to nonsense (6%), missense (75%), small frameshifting insertions/deletions (6%) and splice site mutations (6%). Since, regardless of the mutation type, no females

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with a typical RS phenotype were identified, RS seems to be caused by loss-of-function mutations only. Mutations occurred non-randomly, with hotspots at several CG dinucleotides and a C₆ stretch. Exons 1–3 contained few, mainly translation-truncating mutations, arguing against an important functional role for this segment of the protein. Exons 4–6, encoding the discoidin domain, contained most, mainly missense mutations. An alignment of 32 discoidin domain proteins was constructed to reveal the consensus sequence and to deduce the functional importance of the missense mutations identified. The mutation analysis revealed a high preponderance of mutations involving or creating cysteine residues, pointing to sites important for the tertiary folding and/or protein function, and highlights several amino acids which may be involved in XLRIS1-specific protein–protein interactions. Despite the enormous mutation heterogeneity, patients have relatively uniform clinical manifestations although with great intra-familial variation in age at onset and progression.

INTRODUCTION

X-linked retinoschisis (XLRIS; MIM 312700) is the most common cause of juvenile macular degeneration in males (1,2) and leads to a vitreo-retinal degeneration characterized by cystic spokewheel maculopathy, peripheral retinal lesions, alterations of the vitreous body and a reduced b-wave of the electroretinogram (ERG). Estimations of its prevalence range from 1:5000 to 1:25 000. Affected males have a deterioration in vision which typically presents at between 5 and 10 years of age (3). Visual impairment is usually mild until the fourth decade of life, but thereafter progressive visual deterioration often occurs (3,4). Complications in the later stages of RS include vitreal haemorrhage, choroidal sclerosis, retinal detachment and, in rare cases,

retinal atrophy resulting in blindness. There is a great intra-familial variation in severity; mild, moderate and severe forms of the disease may occur in one family. Female carriers of the disease are unaffected (3).

Little is known about the aetiology of XLRIS. Histopathological findings include splitting within the superficial layers of the retina, degeneration of the photoreceptors, thinning of the ganglion layer and a focally absent or proliferative retinal pigment epithelium (3,5). The underlying cause of XLRIS is thought to be a defect in the Müller cell, the glial cell of the retina which is thought to play a role in the organization of retinal cells during development (5,6).

XLRIS was first mapped to the distal region of Xp in 1969 (7), and subsequent genetic and physical mapping studies eventually localized the XLRIS gene to a 2 cM region between *DXS418* and *DXS999* in Xp22.2 (8–12). Yeast artificial chromosome (YAC) contigs (8,13–15) and a phage P1-derived artificial chromosome (PAC) contig (14) have been constructed across the interval. Based on the PAC contig, the Sanger Centre (Wellcome Trust Genome Campus, Hinxton, UK) has undertaken the sequencing of a 1.3 Mb region containing the RS gene which led to the identification of several new human genes (16,17).

By mapping and expression analysis of expressed sequence tags (ESTs), Sauer *et al.* recently identified a candidate gene for XLRIS, designated *XLRIS1* (18), which maps within the sequenced region. Mutational analysis of *XLRIS1* in nine unrelated XLRIS families revealed a number of distinct mutations co-segregating with the disease, thus providing strong evidence that XLRIS is caused by mutations in this gene (18). The *XLRIS1* gene has six exons and encodes a 224 amino acid protein, processed by N-terminal cleavage into a mature protein with a calculated size of 23 kDa (201 amino acids). The predicted protein sequence contains a highly conserved discoidin domain, shared with a number of other proteins (18,19). The discoidin domain is implicated in cell–cell adhesion and phospholipid binding, a function which is in agreement with the observed splitting of the retina in XLRIS patients, indicating that *XLRIS1* is important during retinal development.

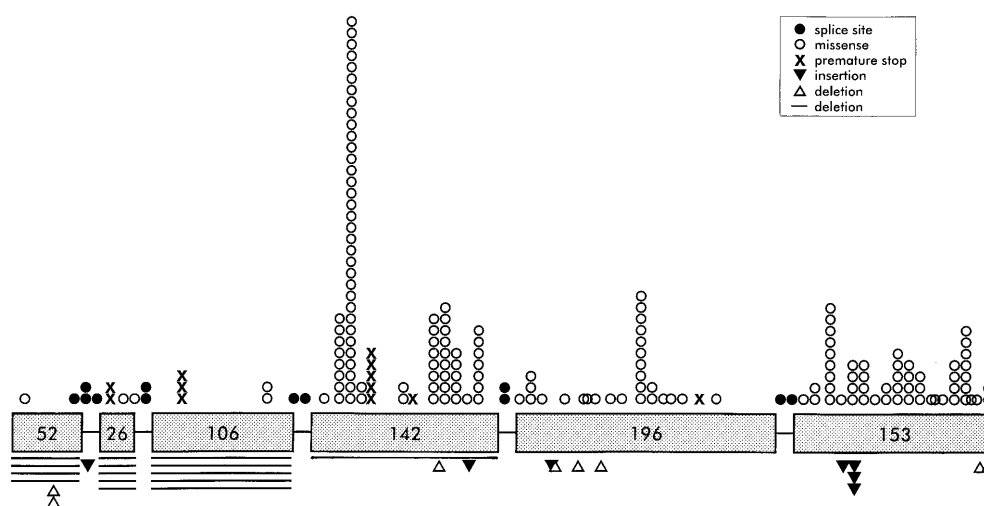


Figure 1. Mutations in the XLRIS protein. The *XLRIS1* gene is drawn schematically; the six exons are represented by bars, numbers indicating their size in nucleotides, and introns by lines. Mutations are indicated by symbols (one for every mutation identified): X, nonsense mutations; open circles, missense mutations; closed circles, splice site mutations; open triangles, small deletions; closed triangles, insertions; lines below exons, larger deletions.

Table 1. Mutations and polymorphisms in the *XLRS1* gene

A. Mutations							
No.	Exon	Mutation	RE site	Protein	Group	Country	Case
4	1	del exon 1		fs	4	4DK	Fam
1	1	2T→C	- <i>MnlI</i> , + <i>MaeII</i>	Met1Thr	5	NL	Fam
2	1	33-36del	-	fs	3, 5	D, US	Fam, Sp?
2	1	52+1g→a	+ <i>MboII</i>	sd?	2, 3	D, US	Fam
1	1	52+1g→t	-	sd?	3	US	Fam
1	1	52+2insT	-	sd?	3	US	Fam
1	1	[52+5g→c; 35T→A]	-	[sd?; Leu12His]	3	US	Fam
5	2	53-78del ^a	-	fs	3	US	Fam
1	2	68C→A	-	Ser23X	4/5	DK	Fam
1	2	76G→T	+ <i>MaeI</i>	Glu26X	4	DK	Fam
2	3	79-2a→g	- <i>PstI</i>	sa?	6, W	D, UK	Fam
5	3	79-184del	-	fs	1, 5	5NL	Fam
3	3	120C→A	-	Cys40X	3, 4, W	D, S, US	Fam
2	3	175T→A	-	Cys59Ser	2, 5	2D	Fam, Sp
1	3	181-184+10del	-	sd?	5	D	Fam
1	3	184+1g→c	+ <i>AluI</i>	sd?	3	US	Fam
1	4	185-1g→c	- <i>BscI</i>	sa?	2	F	Fam
1	4	185-326del		fs	2	D	
1	4	194A→G		Tyr65Cys	5	D	
8	4	208G→A	+ <i>DdeI</i>	Gly70Ser	3, 4	8US	7Fam, 1Sp
34	4	214G→A	- <i>TaqI</i>	Glu72Lys	all	DK, 21NL, 3S, 12D, UK, 5US	33Fam, 1Sp
2	4	216G→C	- <i>PleI</i>	Glu72Asp	2, 4	F, UK	Fam
1	4	221G→T		Gly74Val	3	S	Fam
3	4	223G→T	- <i>MnlI</i>	Glu75X	2, 3	2F, I, S	Fam
2	4	266A→G	+ <i>TspRI</i>	Tyr89Cys	2, 5	2D	Fam
1	4	267T→A	-	Tyr89X	6	UK	Fam
8	4	286T→C	+ <i>AccI</i>	Trp96Arg	3, W	D, 7US	7Fam, Sp
1	4	300delG	- <i>HaeIII</i>	fs	5	D	Fam
10	4	304C→T	+ <i>EcoRII</i>	Arg102Trp	3, 6, W	D, 9UK, US	Fam
5	4	305G→A	+ <i>AluI</i>	Arg102Gln	3, 5, 6	D, 2UK, 2US	Fam
1	4	308T→G	+ <i>HhaI</i> , + <i>CfoI</i>	Leu103Arg	4	US	Fam
1	4	315&316insA	-	fs	W	(D)	Fam
7	4	325G→C	-	Gly109Arg	2-4, 6, W	FI, F, D, 3UK, US	Fam
2	4	326+1g→a	-	sd?	3, 6	UK, US	Fam
1	5	329G→A	- <i>UbaEI</i>	Cys110Tyr	3	US	Fam
1	5	336G→C	- <i>EcoRII</i> , + <i>MnlI</i>	Trp112Cys	2	F	Fam
2	5	336G→T	- <i>EcoRII</i> , + <i>BsmAI</i>	Trp112Cys	1, 2	2I	Fam
1	5	337C→T	+ <i>DrdII</i>	Leu113Phe	3	US	Sp
1	5	350&351insT	+ <i>NcoI</i>	fs	2	F	Fam
1	5	375-378del	- <i>BglII</i>	fs	4	IC	Fam
1	5	380T→C	+ <i>AbaI</i> , - <i>BglII</i>	Leu127Pro	3	US	Fam
1	5	392-393delAA	+ <i>TspRI</i>	fs	5	D	Fam
1	5	404G→T	- <i>XhoII</i> , - <i>BstI</i>	Gly135Val	2	NL	Fam
1	5	407T→C	- <i>BamHI</i>	Ile136Thr	3	US	Fam
1	5	412A→G	- <i>HphI</i>	Thr138Ala	1	NL	Fam
1	5	416delA	+ <i>SmaI</i>	fs	5	NL	Fam
1	5	418G→A	- <i>BsaII</i>	Gly140Arg	3	US	Fam

Continued overleaf

Table 1. Continued

A. Mutations							
No.	Exon	Mutation	RE site	Protein	Group	Country	Case
1	5	419G→A	+Eco47III	Gly140Glu	6	UK	Fam
1	5	421C→G	-HaeII	Arg141Gly	6	UK	Fam
9	5	421C→T	-HaeII	Arg141Cys	2-6	2DK, 2F, D, 3UK, US	8Fam, Sp?
2	5	422G→A	-HaeII	Arg141His	3, 5	D, US	Fam
1	5	428A→T	-MaeIII	Asp143Val	1	NL	Fam
1	5	436G→A	-	Glu146Lys	2	D	Fam
1	5	438G→C	-	Glu146Asp	1	NL	Fam
1	5	460C→T	-FokI	Gln154X	6	UK	Fam
1	5	464A→G	-AfaI, +BspWI	Tyr155Cys	2	I	Fam
1	5	522+1g→t	-NciI, +MseI	sd?	2	F	Fam
1	6	523-2a→g	-BfaI	sa?	3	US	Fam
1	6	533G→A	-	Gly178Asp	3	US	Fam
2	6	544C→T	+BsgI	Arg182Cys	3, 5	D, US	?, Sp
9	6	574C→T	+AvaII, -HaeIII	Pro192Ser	3, 4, 6, W	D, SC, 6UK, US	Fam, 2Sp, 2?
1	6	575C→G	-HaeIII, +CfoI	Pro192Arg	2	F	Fam
1	6	576&577insT	-NlaIV, +MnII	fs	2	A	Fam
4	6	578C→T	+AvaII	Pro193Leu	2, 3, W	A, 2D, US	Fam
3	6	579&580insC	-BbvI	fs	2, 3, 6	A, UK, US	Fam
4	6	589C→T	-AciI	Arg197Cys	1-3, 5	F, D, NL, US	Fam
1	6	590G→A	-MwoI	Arg197His	3	US	?
2	6	596T→C	+HphI	Ile199Thr	3, 6	UK, US	Fam
4	6	598C→T	-FokI	Arg200Cys	1-3, 6	F, NL, UK, US	Fam
4	6	599G→A	-AciI	Arg200His	2, 3, 6	F, UK, 2US	Fam
3	6	608C→T	-MspAII	Pro203Leu	1, 2, 5	F, 2NL	Fam
1	6	618G→A	-FokI, +AflIII	Trp206X	2	F	Fam
1	6	621C→G	-MaeII	His207Gln	4	SC	Fam
1	6	625C→T	-AciI	Arg209Cys	1	NL	Fam
4	6	626G→A	-	Arg209His	1, 5, W	3D, NL	2Fam, Sp, ?
7	6	637C→T	-MspI	Arg213Trp	3, 5, 6	2D, 3UK, 2US	4Fam, 3Sp
1	6	643G→C	-FokI, +PvuII	Glu215Gln	2	E	Fam
1	6	647T→C	-Alu	Leu216Pro	3	US	Fam?
1	6	655-679del	-	fs	5	D	Fam
1	6	655T→C	+HhaI	Cys219Arg	5	D	de novo
1	6	655T→G	+BsaHI	Cys219Gly	6	UK	Fam
1	6	655delT	-BbvI, +MnII	fs	1	NL	Fam
B. Polymorphisms							
Exon	Polymorphism	RE site	Protein	Group	Country	Remark	
5	330T/C		Cys/Cys110	2, 3	US, F		
5	426T/C		Cys/Cys142	3	US	3/98 controls	
5	472G/A		Asp/Asn158	5	D	1/100 controls	
6	606C/T	-FokI	Ile/Ile202	3	US	Sp. case K0193	
6	666G/C		Lys222Asn	-	GER	Sauer <i>et al.</i> (1997)	
6	678C→T	-SfaI	(3'-UTR)	3	US	Fam. case P0597 ^b	

Mutation description follows standard nomenclature (21) using the *XLRS1* cDNA sequence as a reference (GenBank AF014459) with the ATG translation initiation codon counted as +1. Amino acid changes in bold highlight those involving a Cys residue. All exon 6 sequences analysed contained a G at nucleotide 666 (Lys222) instead of an A (and Asn222), and we considered this as the normal sequence. All mutations were analysed at the DNA level only. Indicated are the number of times a mutation was identified (unrelated cases), the mutated exon, the mutation at the DNA level, if a restriction enzyme recognition (RE) site was created (+) or destroyed (-), the consequence of the mutation at the protein level (X, stop codon; fs, frameshift; sd, splice donor site; sa, splice acceptor site; ?, not yet confirmed at the RNA level), group identifying the mutation, the country of origin of the patient/family (A, Austria; D, Germany; DK, Denmark; E, Spain; F, France; I, Italy; IC, Iceland; NL, Netherlands; S, Sweden; SC, Scotland; FI, Finland; UK, United Kingdom; US, United States of America) and if the mutation was found in a familial (Fam) or sporadic (Sp) case.

^aExcept for exon 2, the exon deletions, initially detected by a failure of the exons to amplify, were confirmed by Southern blotting and hybridization with cDNA-derived probes.

^bNext to the 678C/T change in the 3'-UTR, patient P0597 carries a disease-causing 599G→A (Arg200His) mutation.

A more detailed overview of all *XLRS1* mutations identified is available through the Internet at <http://www.dmd.nl/rs.html>

Here we present, through an international collaborative effort, a thorough mutational study of the *XLR51* gene by the analysis of patients from 234 cases with X-linked retinoschisis, revealing mutations in 214 cases (91%). Our analysis includes an extensive alignment of discoidin domain-containing proteins which was used as a guide to understand the potential functional consequences of the missense mutations detected.

RESULTS

The six groups forming the RS consortium screened the entire coding region of the *XLR51* gene in 234 cases for the presence of mutations. The patient set contained both familial (apparently unrelated by family history) and sporadic cases, all presenting typical *XLR51*. Mutation analysis was performed using initial scanning with single strand conformation analysis (SSCA) or denaturing gradient gel electrophoresis (DGGE), or by direct sequence analysis. Ultimately, we identified mutations in 91% (214/234) of the cases (Table 1, Fig. 1). Eighty two different mutations were identified, of which 51 were found only once. Mutations included four different small one-exon deletions (6% of all cases), 11 different splice site mutations (6%), 11 different frameshifting insertions/deletions (indels) (5%, four small insertions/seven small deletion mutations), seven different nonsense (6%) and 49 missense (69%) mutations. Thirty one of the mutations were found more than once, i.e. 2–10 times, with the exception of the 214G→A mutation which was identified in 34 apparently unrelated cases worldwide (14% of all mutations). The mutations were not distributed randomly over the gene. Most mutations, predominantly missense, were found in exons 4–6 encoding the discoidin domain. Of the 33 mutations found upstream (exons 1–3), all but one, i.e. Cys59Ser (see below), cause premature translation termination. The mutational spectrum found in exons 1–3, in particular the absence of missense mutations, argues against an important functional role for this segment of the encoded protein.

To determine the potential effect of the mutations, we have performed a detailed comparative analysis of the *XLR51* protein sequence (Fig. 2). Sauer *et al.* (18) noted that the N-terminus contains a highly hydrophobic putative secretory leader peptide sequence, amino acids 1–23. No obvious protein homologies were found for the N-terminal region, i.e. amino acids 1–60. The remainder of the protein shows a clear homology with the so-called discoidin domain (19). We constructed an alignment of 32 proteins of diverse organisms containing one or two discoidin domains to reveal the consensus sequence and the amino acid variation allowed without affecting its function (Fig. 2). The alignment indicates that the discoidin domain extends from amino acids 63 to 219, both cysteine residues. Consequently, even extremely C-terminally located mutations are expected to destroy the functional properties of the discoidin domain.

The most obvious deleterious mutations are those which delete parts of the gene. The most stringent in this respect are the exon 1 deletions, found in four apparently unrelated Danish families, since these are expected to include the promoter region, thus producing null alleles. The other exon deletions include exons 2 (five US families), 3 (five Dutch families) and 4; the latter was found only once in a German case. The intragenic deletions are expected to produce only severely truncated proteins with no functional significance. The same holds true for the 11 different splice site mutations identified. All but one, i.e. 52+5g→c at the intron 1 splice donor site (see below), hit the 100% conserved GT

splice donor or AG splice acceptor dinucleotides. Normal splicing should thus be abolished, leading either to the skipping of the entire exon or the activation of a cryptic splice site, potentially introducing a (small) deleterious insertion or deletion. Computer analysis, i.e. splice site prediction programs, confirm the deleterious effects of the splice site mutations. However, since either no patient-derived RNA was available or because amplification from blood- or fibroblast-derived RNA failed thus far, the effect on splicing could not be confirmed experimentally. Frequently, splice site mutations leave low levels of normally spliced RNA (10% or less), and thus low levels of normal protein would be produced. As far as we could check, splice site mutations do not coincide with milder phenotypes.

Eighteen different small mutations introduce premature translation termination (11% of the cases), i.e. 11 different small indels and seven different nonsense mutations, again expected to abolish any function of the *XLR51* protein. The most C-terminal of these, 655delT and 655–679del, both destroy the Cys219 residue, the last, but 100% conserved, amino acid of the discoidin domain (Fig. 2).

Most mutations, i.e. 161 out of the 234 cases analysed (69%, 49 different), are missense mutations. Of these, 2T→C, causing a Met1Thr change, is the most dramatic since it abolishes the translation initiation Met codon. Since RS contains only two other Met codons, Met148 and Met214, both not imbedded in a proper Kozak sequence, we expect that this mutation produces no protein at all.

XLR51 contains a high number of cysteine residues, 10 in total. Cys63 and Cys219 are absolutely conserved in all discoidin domain-containing proteins. This indicates that Cys63/Cys219 are of special structural importance, probably involved in forming S–S disulfide bridges. Twelve different missense mutations involve a cysteine residue; in three instances a cysteine is destroyed, while in nine cases a new cysteine appears (Fig. 2). The disease-causing potential of Cys219Arg/Gly is clear since a 100% conserved residue is affected. Twice, however, the mutation seems to affect a non-conserved residue, Cys59 and Cys110. Although the milk fat globule membrane proteins (MFGMs in Fig. 2) contain a cysteine at the Cys59 position, Figure 2 gives the impression that Cys59 lies just upstream of the discoidin motif, clear conservation of which starts at Cys63. However, the motif Cys59/Cys63 is, in a similar amino acid context, conserved at the C-terminal end of *XLR51* (i.e. Cys219/Cys223). Furthermore, not visible from the alignment, most proteins of the discoidin family contain two directly flanking discoidin motifs, placing the last Cys of domain 1 within 4–6 residues of the first Cys of domain 2.

In three cases, the occurrence of a new Cys residue is probably deleterious because it destroys a highly conserved residue (Trp112Cys, Arg197Cys and Arg200Cys). However, in the six other cases, this is not true. In four cases, a new cysteine occurs at a position where it is never found (Tyr65Cys, Tyr89Cys, Tyr155Cys and Arg182Cys). Once a novel cysteine (Arg141Cys) occurs next to one which is normally present (Cys142), and once (Arg209Cys) it appears at a position where other members of the discoidin family do contain a cysteine (Fig. 2). The location of these specific mutations might help to determine the three-dimensional structure of *XLR51*. Either they reside at a position where the additional Cys residue provides a possibility to form new, incorrect, disulfide bridges thus interfering with normal *XLR51* folding, or at a position on the surface of the protein



Figure 2. Discoidin domain alignment. An alignment was made between 29 proteins containing one or two discoidin domains (12 and 17 proteins respectively). If for a specific protein, sequences from more than one organism were known, only the human sequence is presented. Top line: *mis*, amino acids hit by *XLRS1* missense mutations (C if a cysteine was hit or created, asterisk for other changes); *con*, consensus sequence with the amino acid in upper case bold when found in at least 16/18 sequences, upper case when found in 10/18 and lower case when found at least six times. The proteins aligned are (in parentheses known in N number of species, full name and GenBank accession no.): XLRS1 (1* X-linked juvenile retinoschisis precursor protein, AF018963), TRK3 (2* tyrosine receptor kinase, Q16832), EDD1 (3* epithelial discoidin domain receptor 1 precursor, Q08345), AEBP1 (2* adipocyte transcription factor, JC5256), CAP (2* contactin-associated protein, U87223), MFGM (5* milk fat globule membrane protein, Q08431), HEMO (1* silkworm haemocytin, S52093), COAG5 (2* coagulation factor V precursor, M14335), COAG8 (2* coagulation factor VIII precursor, P00451), NRP (4* neuropilin, AF018956), NRP2 (3* neuropilin 2, AF022859) and DISCa (1* slime mold discoidin I chain A, J01282). For proteins containing two discoidin domains, the first and second domain are indicated as -1 and -2 respectively.

where *XLRS1*-specific and functionally crucial interactions with other proteins are disturbed.

Most of the other missense mutations affect highly conserved amino acids in the discoidin domain, changing them to residues which are never found at this position in related proteins (Fig. 2). The exceptions to this rule are: Gly109Arg, a change at a non-conserved position found in several other discoidin proteins; Leu113Phe, a minor change at a non-conserved position found in normal neuropilin; and Arg141Gly/His, where the histidine at this position is found in discoidin itself (Fig. 2). Potentially, these mutations might influence RNA processing like, for example, a silent mutation identified in the fibrillin-1 gene which unexpectedly turned out to affect mRNA splicing (20). Another possibility is that these mutations disrupt the *XLRS1*-specific protein-protein interactions encoded by the discoidin domain. The

possibility that the changes represent rare polymorphisms is unlikely since they were never found in combination with another mutation on the same allele, nor were they found in at least 100 control chromosomes analysed.

In all cases tested so far (37 families), the *XLRS1* mutation identified co-segregated with the disease. An example is shown in Figure 3. Pedigree D0392, analysed at the University of Michigan, shows co-segregation of the 637C→T (Arg213Trp) mutation through nine meioses. The family is of Mexican-Spanish origin with an overall clinical assessment of a mild disease phenotype by examination of four affected males and two carrier females. Clinical signs include foveal and parafoveal cystic changes causing reduced acuity, to 20/50 in a 13-year-old (Fig. 3A, individual no. 5) and to 20/100 in a 22-year-old (Fig. 3A, no. 8). Two older males have acuities of 20/60 at age 54, and

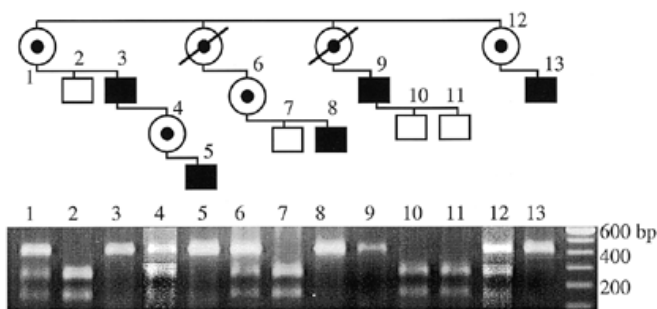


Figure 3. RS in pedigree D0392. (A) Co-segregation of the 637C→T mutation (Arg213Trp) with the RS phenotype. 637C→T in exon 6 removes a restriction site for the enzyme *MspI*. A 413 bp exon 6 fragment was PCR amplified from the genomic DNA, digested with *MspI* and analysed on a 2% agarose gel. In unaffected males, *MspI* generates 216 and 152 bp fragments (lanes 2, 7, 10 and 11). The PCR product of the affected males remains undigested (lanes 3, 5, 8, 9 and 13), while carrier females exhibit a heterozygous state (lanes 1, 4, 6 and 12). Lanes 4 and 12 are photo-enhanced to reveal the faint bands. (B) Electroretinogram (ERG) responses, determined according to Pawar *et al.* (lane 9) of the 54-year-old grandfather (individual no. 3 in A) shows major scotopic b-wave loss but retains a normal scotopic rod photoreceptor a-wave. Photopic flicker time-to-peak is delayed, but photopic single white flash amplitude and waveform remain normal, with normal oscillatory potential development and amplitude. The 13-year-old grandson (individual no. 5) has normal scotopic b-wave amplitude but a partially truncated b-wave to scotopic bright flash (topmost waveform). Photopic 30 Hz flicker, single flash cone responses and the oscillatory potential waveform and amplitudes are normal.

20/80 at age 58 (nos. 3 and 13, Fig. 3A). The ERG for individual no. 5 (Fig. 3B) is normal in amplitude and b-wave development, whereas his grandfather (no. 3) has a major b-wave reduction for the rod-driven scotopic response but a large and normal photopic b-wave with normal amplitude of the oscillatory potentials. By family report, one unexamined male suffered retinal detachment in one eye at age 19.

DISCUSSION

In 20 out of 234 cases (9%), no mutations could be identified despite the fact that the complete gene coding region and immediately flanking intronic regions were entirely sequenced. In at least six families, this concerns clearly X-linked families, amongst which for example, family UMRS-1, which defined the telomeric boundary of the *XLRS* critical region with a recombination between *DXS418* and *DXS257* (9). Furthermore, the typical RS phenotype is very specific, which makes it unlikely that misdiagnosis plays an important role in our failure to detect disease-causing mutations so far. Since we believe that mutations affecting transcription initiation and mRNA processing provide more obvious candidates, we currently are extending our mutation analysis to include the promoter region and larger segments of the introns.

Next to the mutations discussed, we have identified five other sequence changes, probably non-disease-related polymorphisms (Table 1), as well as a deviation from the published *XLRS1* sequence which was present in all our exon 6 sequences analysed (666C→4G and Asn222Lys compared with GenBank AF014459). The 330T/C (Cys/Cys110) change was the only deviation found in one familial *XLRS* case from France. However, since it does not change the protein sequence and since

it was also found in a normal control from the USA, we believe it is highly unlikely that it is disease-causing.

The exon 3 deletion (Table 1, 79–184del) was detected in a recently completed linkage study by a failure of marker *dJ245G19.CA5*, localized 0.6 kb downstream of the exon 3 splice donor site, to amplify in four families (T. Kraayenbrink *et al.*, submitted). Since shared haplotypes (markers *dJ245G19.CA1*, *dJ436M11.CA1*, *DXS999* and *AFM29Iwf5*) were found in four of the five exon 3-deleted Dutch families, they probably have a distant familial relation. Size estimations of the exon 3 deletions failed due to the inability to amplify across this region, both in patients and in healthy individuals, despite the fact that long-range PCR protocols were used and the estimated distance from exon 2 to exon 4 was reported as only 3 kb (18). In controls, amplification across intron 2, i.e. 1.1 kb with the primer pairs we have used, was successful but failed across intron 3 [1.6 kb according to Sauer *et al.* (18)]. Together, these data indicate that intron 3 is much larger than estimated, confirmed by the sequence data from the Sanger Center which are incomplete for intron 3 but already add up to >4.8 kb.

A missense mutation at amino acid position 72 was found in 36 cases. Both substitutions identified, Glu72Lys (found 34 times) and Glu72Asp (found twice), introduce amino acids which are found at this position in other members of the discoidin family (Fig. 2). Since the mutation is found in patients from all populations studied but not in corresponding controls and since it occurs on at least three different haplotypes in the Dutch population [marker *dJ436M11.CA1* (T. Kraayenbrink *et al.*, submitted)], we conclude that it has several independent origins and is very likely to be disease-causing.

Interestingly, 12 of the 26 CpG dinucleotides in the *XLRS1* coding region have been hit by mutations. In five cases, mutations affected both nucleotides, introducing the expected nucleotide change when postulating that the CpG dinucleotide was methylated at a genomic level. Of these, 207CG, 304CG, 421CG and 598CG seem to represent mutational hotspots, with 34, 15, 11 and 9 mutations reported at that position respectively. A remarkable hotspot lies at a stretch of six C residues (positions 574–579) which was hit 18 times yielding five different mutations, of which only one, 579&580insC, is of the type expected for single nucleotide runs.

The 82 different mutations in the relatively small *XLRS1* gene provide direct evidence for multiple origins of *XLRS*. Similarly, although 31 different mutations were identified more than once, varying from two to 34 times, our data indicate that many of the recurrent mutations identified also have multiple, independent origins. Most recurrent mutations were found in populations of different ethnic origin and, where analysed, on different haplotypes, although in some cases distant, unknown familial relationships might exist, as indicated by haplotype sharing (T. Kraayenbrink *et al.*, submitted). Thus, our data indicate that there is a significant new mutation rate in *XLRS1*, thereby providing one plausible cause, i.e. the lack of a family history, which contributes to the observation that many cases of RS remain undiagnosed (2; P.A. Sieving, personal communication). Partly, however, this is also because the ERG is not always abnormal in b-wave amplitude (Fig. 3B).

Based on the mutation spectrum and, regardless of the mutation type, the lack of symptoms in carriers, RS is a disease caused by a loss-of-function rather than a dominant-negative effect. This observation gives some hope for the development of therapeutical

strategies based on the addition of functional XLR51 protein to supplement the existing deficit to prevent the vitreo-retinal degeneration. It remains unclear, however, at which stage of development the damaging effects of this deficit become irreversible.

MATERIALS AND METHODS

Mutation analysis

Blood samples from 234 RS patients (familial and sporadic) were collected by ophthalmologists throughout Europe and the USA and analysed by the six participating groups. Genomic DNA was isolated from the blood samples using standard protocols. The coding sequences of the *XLR51* gene were PCR amplified using the primers described by Sauer *et al.* (18), in some instances modified to improve yields and mutation detection, e.g. by addition of GC clamps for DGGE, or facilitating direct sequencing (for details, see <http://www.dmd.nl/rs.html>). PCR products were sequenced directly or pre-screened using SSCA or DGGE. Fragments with a mobility shift were sequenced to identify the mutation. In cases where no mutation was identified by the SSCA or DGGE scan, all coding regions and splice sites were examined by direct sequencing. To verify that similar changes were not present in healthy individuals, i.e. they represent polymorphic variants, all coding sequences were also analysed in at least 100 additional normal X-chromosomes.

Where possible, segregation of the mutation was checked using the electrophoretic mobility shifts detected or, when the mutation introduced new or removed existing restriction enzyme recognition sites (Table 1), by digestion of the amplification products. The exon deletions, initially detected by a failure of the exons to amplify, were confirmed by Southern blotting and hybridization with cDNA-derived probes.

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