Autosomal recessive retinitis pigmentosa in Spain: Evaluation of 4 genes and 2 loci involved in the disease

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Short title: ARRP genes and loci in Spanish families

1

Abstract

Autosomal recessive retinitis pigmentosa (ARRP) is a genetically heterogeneous form of retinal degeneration. The genes for the β-subunit of rod phosphodiesterase (PDEB), rhodopsin (RHO), peripherin/RDS (RDS) and the rod outer segment membrane protein 1 (ROM1), as well as loci at 6p and 1q, have been previously reported as cause of ARRP. In order to determine whether they are responsible for the disease in Spanish pedigrees, linkage and homozygosity studies using markers at these loci were carried out on 47 Spanish ARRP families. SSCP analysis was performed to search for mutations in the genes cosegregating with the disease in particular pedigrees. Three homozygous mutations in the PDEB gene were found, thus accounting for 6% of the cases. No other disease-causing mutation was observed in the other genes analysed, nor was significant evidence found for the involvement of the loci at 6p or 1q. Based on these data, it is unlikely that these genes and loci account for a considerable proportion of ARRP cases.

Keywords

autosomal recessive retinitis pigmentosa, β-subunit of rod phosphodiesterase, candidate genes, linkage analysis, Spanish population

Introduction

Retinitis Pigmentosa (RP) is a group of inherited eye disorders that are characterised by a progressive degeneration of photoreceptors. Prominent clinical features include constriction of visual fields, night blindness and, eventually, severe visual loss (Heckenlively et al. 1988). RP is clinically and genetically heterogeneous, comprising X-linked, autosomal dominant and autosomal recessive forms (Boughman et al. 1980, Heckenlively et al. 1988, Humphries et al. 1990). In Spain, RP occurs in about one in 3000-7000 individuals and is inherited in an autosomal dominant manner (ADRP) in 12% of the cases, autosomal recessive (ARRP) in 39% and X-linked (XLRP) in 4%. This leaves 41% of RP cases with a simplex form and 4% in which transmission pattern is unclear (Ayuso et al. 1995).

Over fifteen loci responsible for RP have been mapped so far (reviewed by Dryja & Li 1995). Mutations that cosegregate with ARRP have been found in the β-subunit of rod phosphodiesterase (PDEB) (McLaughlin et al. 1993, Bayés et al. 1995a, McLaughlin et al. 1995, Valverde et al. in press, Valverde et al. 1996), rhodopsin (RHO) (Rosenfeld et al. 1992, Kumaramanickavel et al. 1994), the rod cGMP-gated channel (CNCG) (Dryja et al. 1995) and, very recently, in the α-subunit of the rod phosphodiesterase (PDEA) gene (Huang et al. 1995). In addition, one family in which RP appears recessive was in fact a case of digenic RP, due to the presence of two heterozygous mutations at the peripherin/RDS and ROM1 genes (Kajiwara et al. 1994).

Linkage studies in two large ARRP pedigrees have implicated two additional loci on 6p and 1q (Knowles et al. 1994, van Soest et al. 1994, Leutelt et al. 1995). Nevertheless, the proportion of ARRP families linked to these loci has not been assessed.

To evaluate the involvement in ARRP of most of the genes and regions that have been implicated in the disease so far, we have combined linkage, homozygosity and mutation analyses in 47 Spanish ARRP families.

Materials and methods

Families

Forty-seven Spanish ARRP pedigrees, comprising 30 consanguineous and 17 non-consanguineous families, were used in this study (Table 1). Autosomal recessive inheritance was supported by the absence of the disease in both parents and the presence

of at least two affected sibs or evidence for paternal consanguinity. Pedigree drawings of most of the families have been published elsewhere (Bayés et al. 1995b).

Control samples were obtained from volunteers working at the Department of Genetics, University of Barcelona.

This research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all the subjects after the nature and possible consequences of the study had been explained.

Clinical and electrophysiological studies

Most of the families were examined at the Hospital de la Santa Creu i Sant Pau in Barcelona or at the Fundación Jiménez Díaz in Madrid. The clinical diagnosis was based on complete ophthalmological examination, including measurements of visual acuity, ophthalmoscopy, dark adaptation, perimetry and electroretinogram (ERG) amplitudes (unpublished data).

Analyses of DNA polymorphisms

Genomic DNA was prepared from peripheral blood as described (Miller et al. 1988).

Markers at the PDEB (Taylor et al. 1992) and RHO (Weber & May 1989) loci, as well as anonymous markers D6S291, D6S439 and F13B, were from the MapPairs set (Research Genetics, Huntsville, AL) and were analysed according to the manufacturer's instructions.

Primers and conditions for examining the poly(T) polymorphism at the peripherin/RDS locus were as described by Kumar-Singh et al. (1991).

Linkage and Homozygosity Analyses

Two-point linkage analysis was performed using the MLINK program from the LINKAGE package, version 5.1 (Lathrop & Lalouel 1984). Because all the DNA polymorphisms used in this study were intragenic, lod scores were calculated at a recombination fraction (θ) of 0 in each family. As accepted, lod scores below -2 were assumed to be evidence for excluding linkage.

The detection of heterozygosity in consanguineous patients was also considered an exclusion criterion (Lander & Botstein 1987).

Homogeneity testing was carried out with the HOMOG program (Terwilliger & Ott 1994).

PCR and SSCP analyses

Pairs of oligonucleotide primers surrounding exonic regions of the human peripherin/RDS (Travis et al. 1991), ROM1 (Bascom et al. 1993b) and guanylate cyclase-activating protein (Subbaraya et al. 1994) genes were synthesised to give PCR products in the range of 200-300 bp. Some exons were analysed in two or three overlapping PCR fragments because of their size. Primer sequences, their nucleotide positions within the corresponding gene and the size of each PCR product are listed on Table 2.

PCR conditions: amplification was carried out in a total volume of 50 μl. Each reaction contained 300 ng of genomic DNA, 20 pmol of each primer, 1 unit of Dynazyme DNA polymerase (Finzymes Oy, Espoo, Finland) in the recommended buffer and 200 μM dNTPs. Reactions were generally subjected to 35 cycles of 94º for 40 seconds and 55º for 30 seconds in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). Primers and PCR conditions for the amplification of the coding regions of the PDEB and the rhodopsin genes have been described elsewhere (Keen et al. 1991, Riess et al. 1992a).

SSCP analyses were performed as described previously (Bayés et al. 1995b). Each PCR amplified fragment was assayed under three different conditions, combining acrylamide and glycerol concentrations and running temperatures.

DNA sequencing

PCR products which gave aberrant SSCP pattern were subsequently purified by Wizard™ PCR Preps (Promega, Madison, WI) and cloned into pUC18 using the SureClone™ Ligation Kit (Pharmacia Biotech., Uppsala, Sweden). PCR amplification and SSCP analysis of the clones allowed selection of those carrying the mutation/polymorphism. In each case, three to five clones were sequenced by the dideoxy chain-termination method using the T7 sequencing™ Kit (Pharmacia). In addition, direct sequencing from the purified PCR product was performed, using the Sequenase™ Version 2.0 DNA Sequencing Kit (United States Biochemical Co., Cleveland, OH).

Single-base changes present in the recognition sequence of restriction enzymes were confirmed by digestion with the corresponding enzyme.

6

Results

β-subunit of rod phosphodiesterase (PDEB)

A dinucleotide polymorphism located at the PDEB locus (Weber et al. 1993) was used to assay linkage to ARRP and to analyse homozygosity in the affected members of consanguineous families. Nine families showed an inheritance pattern compatible with PDEB involvement in the disease (see Table 1). SSCP screening of the 22 exons of the PDEB gene in these pedigrees revealed disease-causing mutations present in homozygosity in the affected offspring of three consanguineous families.

PCR-amplification and sequencing of exon 1 of the proband of family B-4 revealed that the individual was homozygous for a 71 bp tandem duplication leading to a premature stop codon (Bayés et al. 1995a). The second mutation was detected in the two affected members of family B-27, which were found to be homozygous for a T-to-G transversion at position 2117 in exon 17, causing a Leu699Arg substitution (Valverde et al. 1996). Finally, the two affected individuals of family M-9 showed a band shift after SSCP analysis of exon 13, caused by a G-to-A transition resulting in an amino acid substitution of Arg by Gln at residue 552 (Valverde et al. in press). None of these mutations was detected in 50 control individuals analysed, suggesting that they are responsible for RP in these families.

Two polymorphisms were found, a G-to-C substitution in intron 6 (Riess et al. 1992b) and G2598A in exon 22 (Collins et al. 1992) (Table 3).

Rhodopsin

Segregation analysis using the poly(CA) polymorphism (Weber & May 1989) within the first intron of the rhodopsin gene revealed lack of cosegregation with the disease locus and/or lack of homozygosity in 24 pedigrees (see Table 1). In the affected individuals of the remaining 23 families, a search for point mutations within the coding region of the gene was undertaken.

In most instances, SSCP of the seven rhodopsin gene PCR products revealed the same pattern as the controls, but new bands were observed in some samples. DNA sequencing showed that all were due to previously reported polymorphisms (Dryja et al. 1991, Sung et al. 1991). The two most common variants, A269G and C3982T, occurred at non-coding regions. The other, C2557A, produced a silent third position change and was found only once (see Table 3).

7

Peripherin/RDS

The poly(T) polymorphism present in the 3' non-coding region of the peripherin/RDS gene (Kumar-Singh et al. 1991) allowed us to rule out this gene as the cause of ARRP in 20 out of 30 consanguineous families (see Table 1).

In the affected individuals of the remaining 10 consanguineous families and of all the non-consanguineous families, the three exons of the gene were thoroughly examined by SSCP. Sequencing of the variant bands identified five polymorphisms, four of which have been already described. One of these, C558T, is a single nucleotide substitution in exon 1 that does not lead to an amino acid change (Farrar et al. 1991). The other three, G1150C, A1169G and G1253A, result in amino acid substitutions in exon 3, Glu304Gln, Lys310Arg and Gly338Asp, respectively (Jordan et al. 1992). We also identified a novel polymorphism, C1294T, in the 3' non-coding region, with an allele frequency of 0.22 (see Table 3). This polymorphism was also analysed in a random sample of 35 individuals and the allele frequency was quite similar (0.24). Numerous polymorphisms were found in exon 3 and all of them alter a restriction site. Digestions with the corresponding enzymes allowed pooling the samples according to their haplotypes, thus facilitating SSCP analyses. Using the appropriate controls we failed to detect any mutation in the ARRP samples by SSCP.

Only one affected individual of family M-9 presented a variant SSCP band in exon 1. DNA sequencing revealed a point mutation at position 780, changing codon 180 from ATC (Ile) to ATG (Met). Examination of the family members showed that the variant allele does not segregate with the disease although it was not detected in controls. Therefore, we deemed this to be a rare variant and not a disease-causing mutation.

Rod outer segment membrane protein 1 (ROM1)

Because of the absence of highly informative polymorphisms within the ROM1 gene, we screened the affected members of all the ARRP families for the presence of mutations.

PCR-SSCP analysis of the three exons of the gene was performed. We detected a previously described polymorphism in exon 2, G1071C (Bascom et al. 1993a), which does not lead to an amino acid substitution (see Table 3). No other variant band was observed.

6p21.1 region and guanylate cyclase-activating protein (GCAP)

All families were typed for D6S291, a marker reported to be linked to an ARRP locus in one pedigree from the Dominican Republic (Knowles et al. 1994). Negative linkage and/or homozygosity results were obtained in 33 families. Non-informative families were tested with marker D6S439, located 1 cM distal to D6S291 (Gyapay et al. 1994). By the same exclusion criterion, 4 more families were ruled out (see Table 1).

The formal test of heterogeneity was performed to assess the hypothesis of linkage to D6S291 within a subgroup of families. Results obtained were not statistically significant.

Since the guanylate cyclase-activating protein (GCAP) gene has recently been mapped to the vicinity of D6S291 on 6p21.1 (Subbaraya et al. 1994), and it is involved in visual transduction, we evaluated its candidacy as being the ARRP-causing gene in the families that could not be excluded by linkage or homozygosity studies. In the SSCP analysis of the four exons of the gene, no mobility shift indicative of the presence of base mutations was observed in the affected members of these families.

1q31-q32.1 region

A polymorphism at the β subunit of the fibrin stabilising factor, F13B, has been reported to be tightly linked to the gene causing ARRP in an inbred family from The Netherlands (van Soest et al. 1994). In some branches of this pedigree, a special form of RP, preserved para-arteriolar retinal pigment epithelium (PPRPE), was diagnosed. Recently, a Pakistani family with similar characteristics has been reported (Leutelt et al. 1995).

We have examined linkage to 1q in our panel of ARRP families and 30 out of 46 pedigrees were excluded (see Table 1). The test of heterogeneity using the F13B marker was not significant. Moreover, ophthalmological data clearly excluded PPRPE in families M-52, M-68, M-71 and M-141. Assessment of PPRPE was not possible in the other cases in which cosegregation with markers on 1q had been observed.

Discussion

In order to investigate the genetic basis of ARRP, we combined linkage, homozygosity and SSCP analyses in 47 Spanish families.

Linkage and homozygosity studies allowed us to rule out the genes analysed in most of the families (see Table 1). A high proportion of the ARRP pedigrees are consanguineous, and so, homozygosity analysis was a particularly useful tool. Because the DNA polymorphisms used were intragenic, the risk of recombination between these polymorphic sites and the putative mutations was very low. An alternative approach, based on the typing of two flanking markers, has been recently reported for the rhodopsin gene (Tarttelin et al. 1996).

The validity of the PCR-SSCP technique for mutation analysis has been widely substantiated (Prosser 1993, Sheffield et al. 1993). In the present study each PCR fragment was tested under three different running conditions in order to optimise sensitivity. The high number of polymorphisms detected justifies this approach. However, the possibility that some mutations remained undetected could not be ruled out.

Our data suggest that none of the genes or regions selected can be considered a major ARRP gene, although mutations or linkage to these loci have been previously reported. Only three disease-causing mutations in the gene encoding the β-subunit of rod phosphodiesterase were found in 47 ARRP Spanish pedigrees. A similar frequency was reported by McLaughlin et al., who found mutations in the PDEB gene in 7 out of 92 ARRP patients (McLaughlin et al. 1995). We failed to detect any mutation clearly involved in ARRP in the rhodopsin, peripherin/RDS and ROM1 genes. Previous analyses of these genes could only detect two ARRP-causing mutations in the rhodopsin gene (Rosenfeld et al. 1992, Kumaramanickavel et al. 1994) and one case of apparently recessive but digenic RP due to simultaneous heterozygous mutations at the peripherin/RDS and ROM1 genes (Kajiwara et al. 1994). In fact, assuming the possibility of this particular type of digenic RP, we performed direct SSCP analysis of the peripherin/RDS gene in all the non-consanguineous pedigrees. However, digenic inheritance was considered to be very unlikely in the consanguineous families, where affected individuals are supposed to be homozygous by descent.

For most of the families studied negative linkage results were obtained with markers on 6p and 1q. SSCP analysis of the candidate gene encoding the guanylate cyclase-activating protein located on 6p was also performed but it failed to detect any mutation in the non-excluded families. In the linkage report of ARRP to 1q, van Soest et al. (1994) suggested the presence of non allelic heterogeneity within a complex inbred family. This would hardly apply to our case since all the families, with a single exception, were nuclear.

Overall, our data strongly support previous evidence showing that genes other than PDEB, RHO, RDS, ROM1 and the 6p and 1q loci may be involved in a large proportion of ARRP, thus emphasising the genetic heterogeneity of the disease. So far, not enough

linkage data are available to demonstrate the existence of a putative major gene. However, if one considers the more extensive linkage data on ADRP (see Dryja & Li 1995 for a review and references therein), it appears improbable that a putative major gene other than RHO would have escaped detection. Moreover, the retinal degeneration that characterises RP is a final outcome that may blur very diverse molecular defects underlying it. According to these arguments, new data would be expected to follow the trend in which several genes may account individually for a small number of cases. Even PDEB, which explains most of ARRP cases ascertained so far, contributes to no more than 5-10% of all ARRP.

Putative candidates may be found among the genes encoding members of the phototransduction cascade (Dryja 1990). In particular, these families have been analysed for the presence of mutations at the recoverin gene, but none was found (Bayés et al. 1995b). To unveil the genetic causes of the disease in the families studied here, analysis of other candidate genes are currently being undertaken.

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12

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Table 1 Linkage analyses in ARRP families

Consanguineous families

Pedigree	ARRP/ non-ARRP offspring *	PDEB	RHO	RDS	6p (D6S291)	1q (F13B)
B-2	2 / 3	ECH	ECH	NI	EH	ECH
B-3	1 / 2	EH	**H**	EH	EH	ECH
B-4	1 / 3	**Mut**	EH	EH	-	-
B-5	1 / 2	EH	**H**	EH	EH	EH
B-14	1 / 1	EH	NI	NI	EH	EH
B-15	1 / 3	EH	NI	EH	ECH	EH
B-22	1 / 2	EH	EH	EH	EH	NI
B-27	2 / 3	**Mut**	EH	ECH	**CH**	ECH
B-31	1 / 4	**H**	**H**	EH	**H**	EH
B-48	1 / 2	**H**	**H**	**H**	EH	EH
E-1	3 / 6	ECH	NI	NI	EH	**H**
E-2	2 / 3	ECH	NI	ECH	ECH	ECH
E-3	2 / 2	ECH	NI	NI	EH	ECH
M-9	2 / 2	**Mut**	ECH	NI	ECH	ECH
M-24	3 / 6	ECH	NI	EH	ECH	ECH
M-33	6 / 7	ECH	NI	ECH	ECH	ECH
M-35	2 / 4	ECH	ECH	EH	ECH	ECH
M-42	2 / 4	ECH	**CH**	ECH	ECH	ECH
M-43	2 / 2	ECH	ECH	**CH**	ECH	ECH
M-52	2 / 3	ECH	ECH	EH	EH	**CH**
M-161	3 / 7	ECH	ECH	ECH	ECH	EH
M-180	1 / 4	ECH	ECH	**H**	EH	ECH
M-201	2 / 5	EH	NI	ECH	ECH	NI
I-1	2 / 2	ECH	ECH	ECH	ECH	NI
P-1	1 / 4	ECH	ECH	EH	EH	**CH**
V-1	1 / 3	EH	**H**	EH	ECH	**CH**
V-2	3 / 6	ECH	ECH	ECH	ECH	ECH
V-5	3 / 3	ECH	ECH	EH	ECH	ECH
V-7	1 / 2	**H**	NI	NI	EH	ECH
V-9	3 / 5	EH	EH	**CH**	ECH	NI

Non-consanguineous families

Pedigree	ARRP/ non-ARRP offspring	PDEB	RHO	RDS	6p (D6S291)	1q (F13B)
B-6	2 / 2	EC	EC	EC	EC	EC
B-26	2 / 3	EC	EC	**C**	**C**	EC
B-30	2 / 2	EC	EC	EC	EC	EC
M-11	2 / 3	EC	EC	NI	EC	NI
M-12	2 / 3	EC	EC	NI	EC	EC
M-40	3 / 6	EC	EC	EC	EC	EC
M-56	2 / 3	EC	NI	**C**	**C**	EC
M-68	2 / 5	EC	**C**	**C**	EC	**C**
M-71	2 / 3	**C**	**C**	NI	EC	**C**
M-131	2 / 3	EC	**C**	EC	EC	EC
M-141	2 / 3	EC	EC	NI	**C**	**C**
M-150	2 / 5	EC	NI	NI	EC	NI
M-231	2 / 3	EC	**C**	EC	**C**	EC
M-235	2 / 3	EC	EC	EC	NI	EC
M-266	2 / 2	**C**	NI	**C**	**C**	NI
V-3	2 / 2	**C**	EC	**C**	**C**	NI
V-4	2 / 5	EC	NI	NI	EC	**C**

Table 2 Primers for mutation analysis of the peripherin/RDS, GCAP and ROM1 genes

Gene	Exon	Primer sequence (5'-3')	Size of the amplified fragment			SSCP conditions*
			5'intronic/ 5' UTR	exonic	3'intronic/ 3' UTR	
RDS	1	gggAAgCAACCCggACTACA CTTCCCAgCCAgCgAgTTgA	0	230	0	12A G rT/12A rT/12A 4°C
RDS	1	CTCATTgATAgggATggggg CATgAAACACCTgCCAgggg	0	230	0	12A G rT/12A G 4°C/12A rT
RDS	1	CgggCATgAAgTACTACCgg CCCCAATATATTCATAgCTCTgA	0	254	23	12A G rT/12A rT/12A 4°C
RDS	2	ggAAgCCCATCTCCAgCTgT TCTCCTTACCCTCTACCCCC	15	247	17	10A rT/10A G 4°C/10A 4°C
RDS	3	AgATTgCCTCTAAATCTC ggAgTgCACTATTTCTCAgTg	15	213	27	12A rT/12A G 4°C/12A 4°C
GCAP	1	gCgAAAAgggCCTgTCCATC CCAgCACTTCCCCTCCCTg	0	231	19	12A G rT/12A rT/12A G 4°C
GCAP	2	AgCCTTgggTTATgATgggC TAACCCTgggCTCTCAgTTC	63	150	47	12A rT/12A G 4°C/12A 4°C
GCAP	3	gAgATAggATAAggATgggC CTCACCCTCTCCTTTAgTgA	32	94	70	12A rT/12A G 4°C/12A 4°C
GCAP	4	ggACTgCAgAAATgAACACC CCCACCCgTAgTgCAgAA	34	171	0	12A G rT/12A rT/12A 4°C
ROM1	1	TTCCATCCCTgACACCTCTg AggACAgggAACTgACAggA	29	200	0	8A rT/8A G rT/12A rT
ROM1	1	AAggCACCTTggCACCTTCC CATCCAgACTCCCAggCAAA	0	254	0	8A 4°C/10A G 4°C/12A G 4°C
ROM1	1	AATgCAgCTCTATACCCTCC gTACCTCAgTTgCAgCTCAT	0	234	0	8A rT/12A rT/12A G rT
ROM1	1	CTACAAggACACAgAggTgC gAgCgAggTggggATTCAAg	0	162	12	8A rT/8A G rT/12A G 4°C
ROM1	2	ACCCCTCTgTCCCTCCCTTT gggAggAggTgTCAgATgCT	24	247	32	8A G rT/12A rT/12A G 4°C
ROM1	3	ACTCTCCCTgACTCTTTCCC CTTCCTCACCCCAAgCTCCA	36	244	0	10A rT/12A rT/12A G 4°C

* SSCP conditions: A: %Acrylamide:Bisacrylamide (29:1); G: 5% of Glycerol; rT: run at room temperature; 4°C: run at 4°C.

Table 3 Sequence variants detected by SSCP analysis in ARRP patients

Gene	Nucleotide change	Amino Acid change	Predicted restriction site change	Allele frequency
	Polymorphisms			
PDEB	1. G to C (intron 6)	-	-	0.11 (2/18)
	2. G2598A	-	*+NcoI*	0.44 (8/18)
RHO	1. A269G	-	*+SacII*	0.23 (11/48)
	2. C3982T	-	*-RsaI*	0.15 (7/48)
RDS	1. C558T	Val106Val	*- SinI* *	0.44 (24/54)
	2. G1150C	Glu304Gln	*+MvaI*	0.28 (15/54)
	3. A1169G	Lys310Arg	*-MboII*	0.22 (12/54)
	4. G1253A	Gly338Asp	*-CfoI*	0.28 (15/54)
	5. C1294T	-	*-HaeIII*	0.22 (12/54)
ROM1	1. G1071C	Arg223Arg	*-MspI*	0.40 (38/94)
	Rare variants			
RHO	1. C2557A	Thr160Thr	*-MvaI*	0.02 (1/48)
RDS	1. C780G	Ile180Met	*-Sau3AI*	0.02 (1/54)

* Detected with a mismatched primer