2.OBJECTIUS

D'acord amb l'apartat anterior, la Retinosi pigmentària que es transmet seguint un patró d'herència autosòmic recessiu (RPAR) és una patologia caracteritzada per presentar una elevada heterogeneïtat tan a nivell genètic com pel què fa a la seva expressió clínica. Les bases moleculars de la RPAR es coneixen en un percentatge baix de casos, i per aquest motiu la RP es defineix com una malaltia complexa alhora d'estudiar a nivell molecular.

Els objectius d'aquest treball, enfocats en l'estudi de les anomalies genètiques responsables d'aquesta patologia, es basen en una estratègia d'anàlisi de gens candidats agrupats en:

a) Gens expressats en l'EPR i caracteritzats per la seva implicació en el cicle visual del procés de la visió. S'inclouen els gens RLBP1, CRBP1 i RGR.

b) Gens expressats en la part neural de la retina amb funció desconeguda. S'inclouen el gen CRB1 (expressat en els fotoreceptors) i el gen USH2A (expressat en les membranes basals de la retina i coclea).

Els objectius concrets d'aquest treball són:

La selecció de pacients afectats de Retinosi pigmentària (RP) en els que, d'acord amb l'estudi familiar, es demostri un patró d'herència autosòmic recessiu.

La classificació d'aquests afectats segons presentin RP no sindrómica o bé síndrome d'Usher tipus II (RP associada amb una moderada a severa hipoacúsia neurosensorial i resposta vestibular normal).

L'anàlisi mutacional dels gens assenyalats anteriorment: i) en pacients amb RP no sindrómica, es planteja l'estudi dels cinc gens; ii) en pacients amb síndrome d'Usher tipus II, es proposa l'estudi del gen USH2A.

L' establiment de la freqüència amb la que les mutacions dels gens motiu d'estudi es presenten en malalts amb RPAR.

L'estudi de la relació entre l'expressió fenotípica de la malaltia dels pacients amb les mutacions que afectin als gens motiu d'anàlisi.

3. FAMÍLIES ESTUDIADES

S'inclouen en aquest apartat els arbres familiars dels pacients analitzats en aquesta tesi. En el context d'un estudi multicèntric espanyol de les distròfies de la retina s'ha disposat de material biològic i informació clínica procedent dels següents centres:

- Fundación Jiménez Díaz de Madrid
- Hospital Virgen del Rocio de Sevilla
- Hospital La Fe de València
- Hospital de la Santa Creu i Sant Pau de Barcelona

La RP es defineix com un grup de malalties hereditàries amb afectació difusa i progressiva de la funció dels fotoreceptors i de l'epiteli pigmentari (Marmor i col., 1983). El diagnòstic clínic d'aquesta malaltia és molt complex, de forma que els criteris clínics definits per la RP i establerts en aquest estudi han estat:

- Afectació visual bilateral no sindròmica
- Mala adaptació a la foscor (per la pèrdua progressiva de la funció dels fotoreceptors, majoritariament bastons en els estadis inicials de la patologia)
- Disminució o abscència de resposta en l'electroretinograma (inicialment dels bastons, encara que quant la malaltia progressa també hi ha afectació dels cons)
- Disminució concèntrica del camp visual des de la perifèria a la part central
- Presència de dipòsits de pigment en el fons d'ull (coincidint amb estadis avançats de la malaltia)

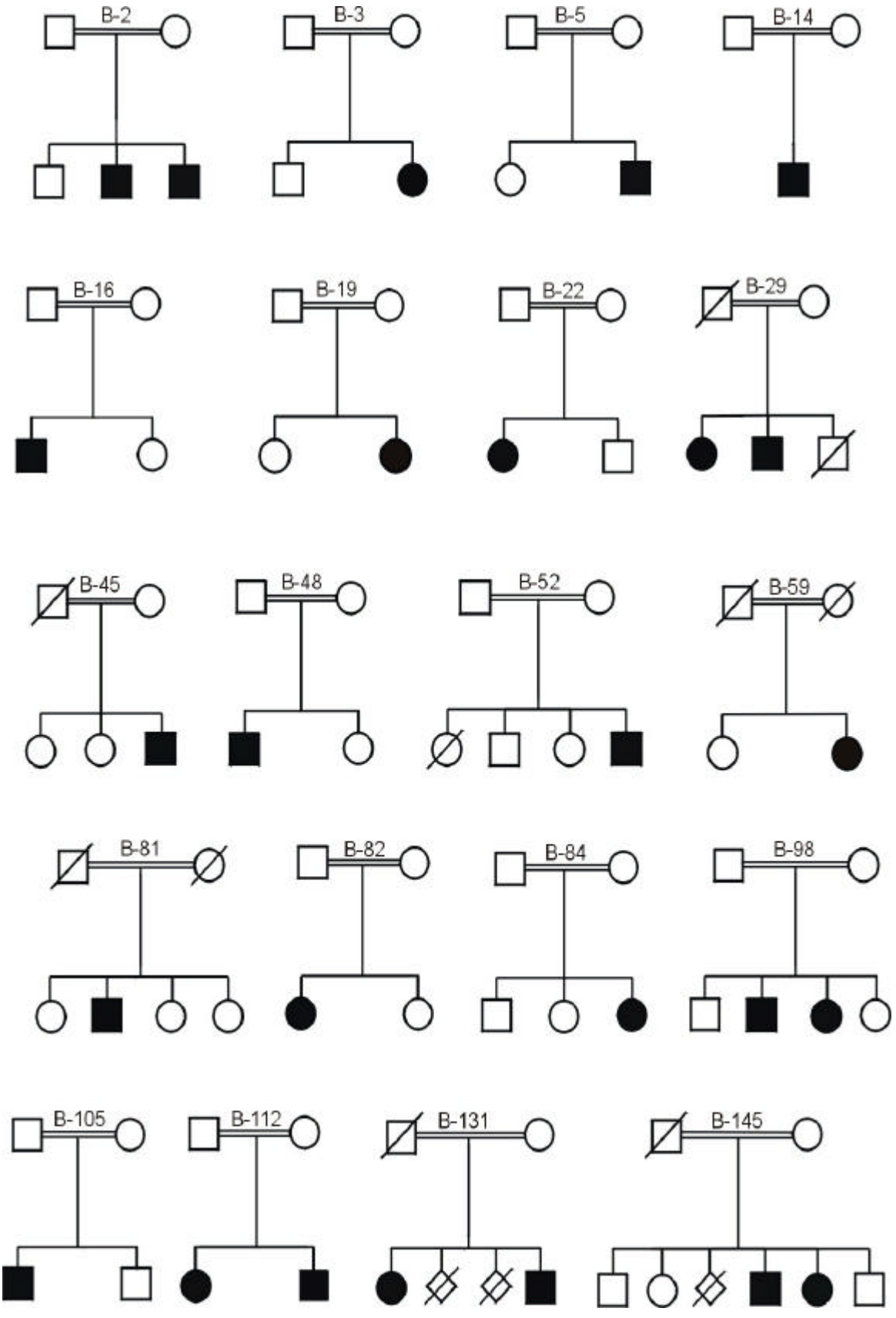
Les dades obtingudes a partir dels estudis realitzats en els últims anys han permès definir diverses entitats clíniques dins la RP. Es tracta d'una malaltia degenerativa i progressiva, existint un cert solapament de símptomes entre la RP i altres distròfies de la retina que compliquen el seu diagnòstic. Cal considerar que moltes de les famílies incloses en aquest treball han estat diagnosticades en un estadi avançat de la malaltia degut a que els pacients amb aquest tipus d'afectació no són conscients de presentar una malaltia fins que aquesta no es fa molt evident, complicant de forma extraordinaria el diagnòstic clínic de la RP. Quan l'estudi dels pacients coincideix en un estadi inicial de la malaltia també existeixen una sèrie de dificultats alhora d'establir el diagnòstic clínic, ja que moltes de les alteracions que permeten diferenciar aquesta patologia d'altres degeneracions de la retina

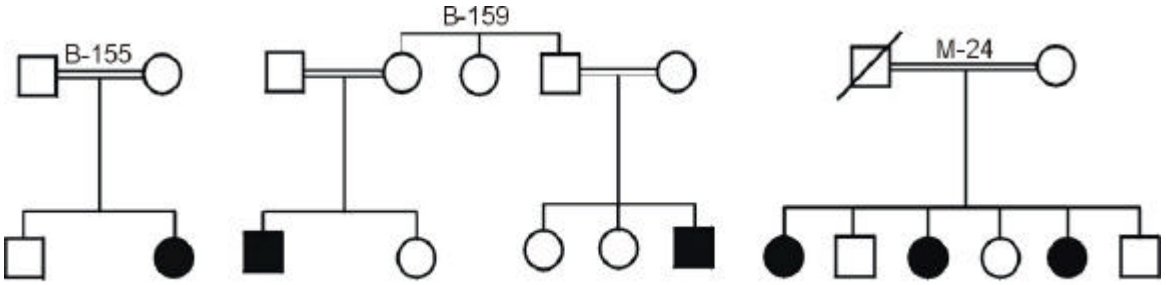
perifèriques no es manifesten fins que la malaltia progressa i assoleix estadis més avançats. Totes aquestes complicacions presents en el diagnòstic clínic d'aquesta malaltia queden reflectides en algunes de les famílies que han estat seleccionades a l'inici d'aquesta tesi, on a posteriori han estat caracteritzades per presentar retinosi punctata albescens, amaurosi congènita de Leber, coroïderèmia i RP amb PPRPE. També hi ha famílies on les successives examinacions oftalmològiques al llarg del temps han permès determinar l'existència de diversos fenotips en diferents membres de la mateixa família, manifestant l'elevada heterogeneïtat clínica característica de la RP.

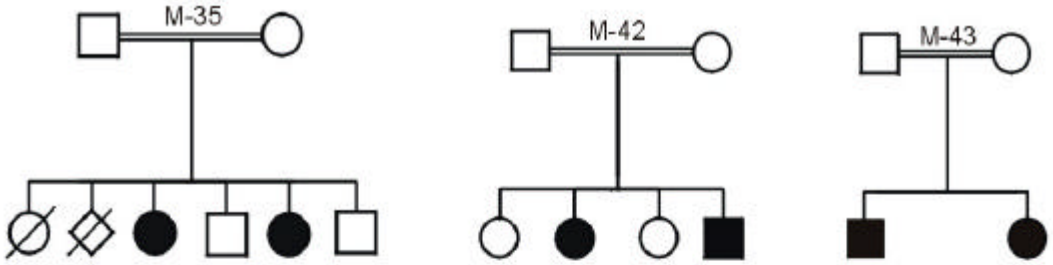
En l'Estat Espanyol, la RP és una patologia amb un percentatge molt elevat de casos en els que només existeix un sol afectat en la família i amb absència de consanguinitat (40%) (Ayuso i col., 1997; 2001), en aquests pacients és difícil determinar el tipus d'herència ja que podrien estar inclosos en qualsevol dels tres patrons d'herència mendelians (AD, AR, i LX). Tots aquests casos en la RP caracteritzats per presentar una herència incerta se'ls defineix com RP simplex, i es postula que la majoria d'aquests casos podrien estar emmascarant el percentatge real de RP amb un patró d'herència autosòmic recessiu.

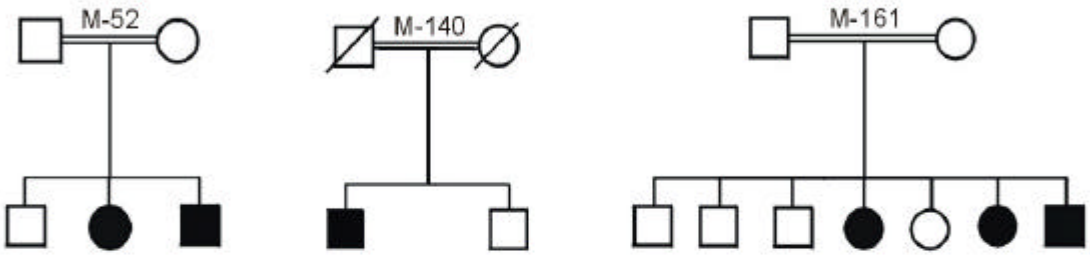
En aquesta tesi s'han inclòs un total de 93 famílies diagnosticades clínicament per presentar RP, que es troben distribuïdes en 51 famílies consanguínies (Figura 18) i 43 famílies no consanguínies (Figura 19). Les famílies amb consanguinitat són definides per presentar, com a mínim, un sol membre afectat de RP en la mateixa generació, mentre que les famílies no consanguínies es caracteritzen per la presència de més d'un membre afectat de la malaltia en la mateixa generació, tot i que en aquest darrer grup també s'ha inclòs alguna família que mostrava antecedents de la patologia a nivell familiar malgrat presentar un sol afectat en la mateixa generació, i que alhora no seguia els criteris definits per una herència autosòmica dominant o una herència lligada al cromosoma X. En aquest estudi també s'han inclòs 4 casos inicialment diagnosticats de RP, malgrat que amb posteriors examinacions clíniques es va rectificar el diagnòstic inicial confirmant la presència d'una clínica corresponent a retinosi punctata albescens (Figura 20). Finalment, s'han seleccionat 27 famílies afectades amb la síndrome d'Usher tipus II, diagnosticades per exploracions oftalmològiques, examinacions audiomètriques i proves vestibulars. Considerant que la síndrome d'Usher sempre segueix un patró d'herència autosòmic recessiu, en aquest cas no s'ha establert cap criteri de classificació pel què fa al tipus d'herència (Figura 21).

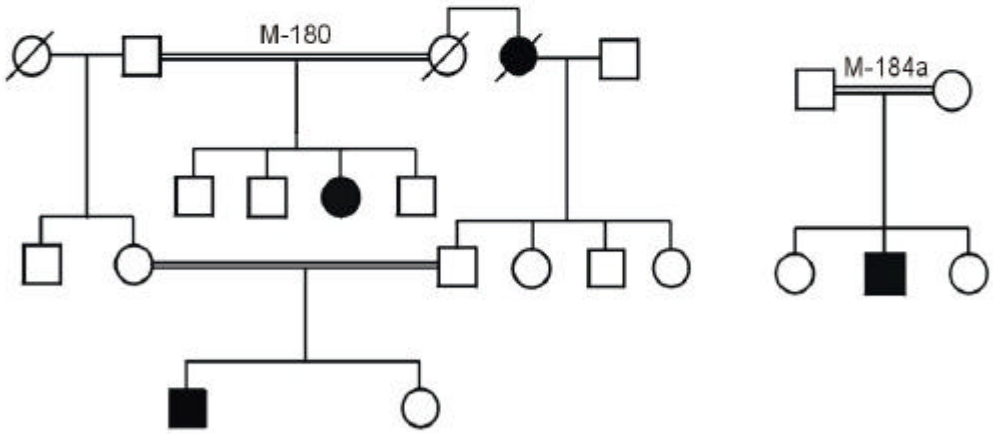
Figura 18. Famílies consanguínies amb RP seguint un patró d'herència autosòmic recessiu.

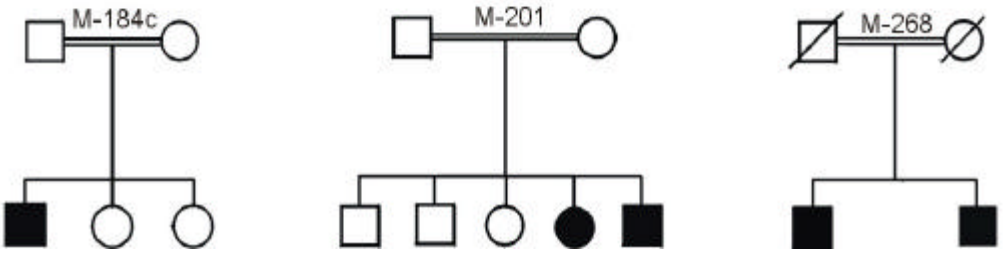



B-155
B-159
M-24


M-35
M-42
M-43


M-52
M-140
M-161


M-180
M-184a


M-184c
M-201
M-268

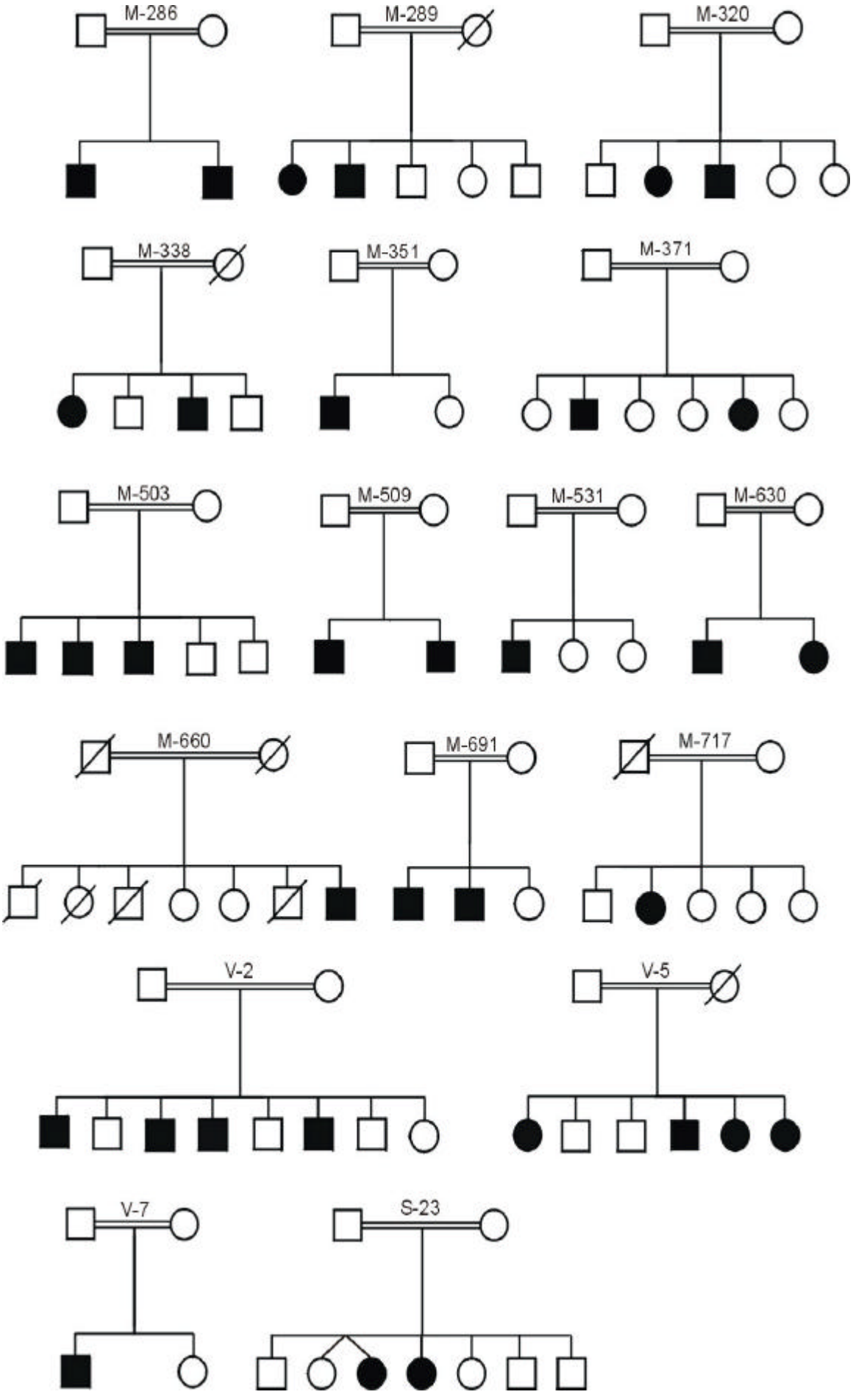
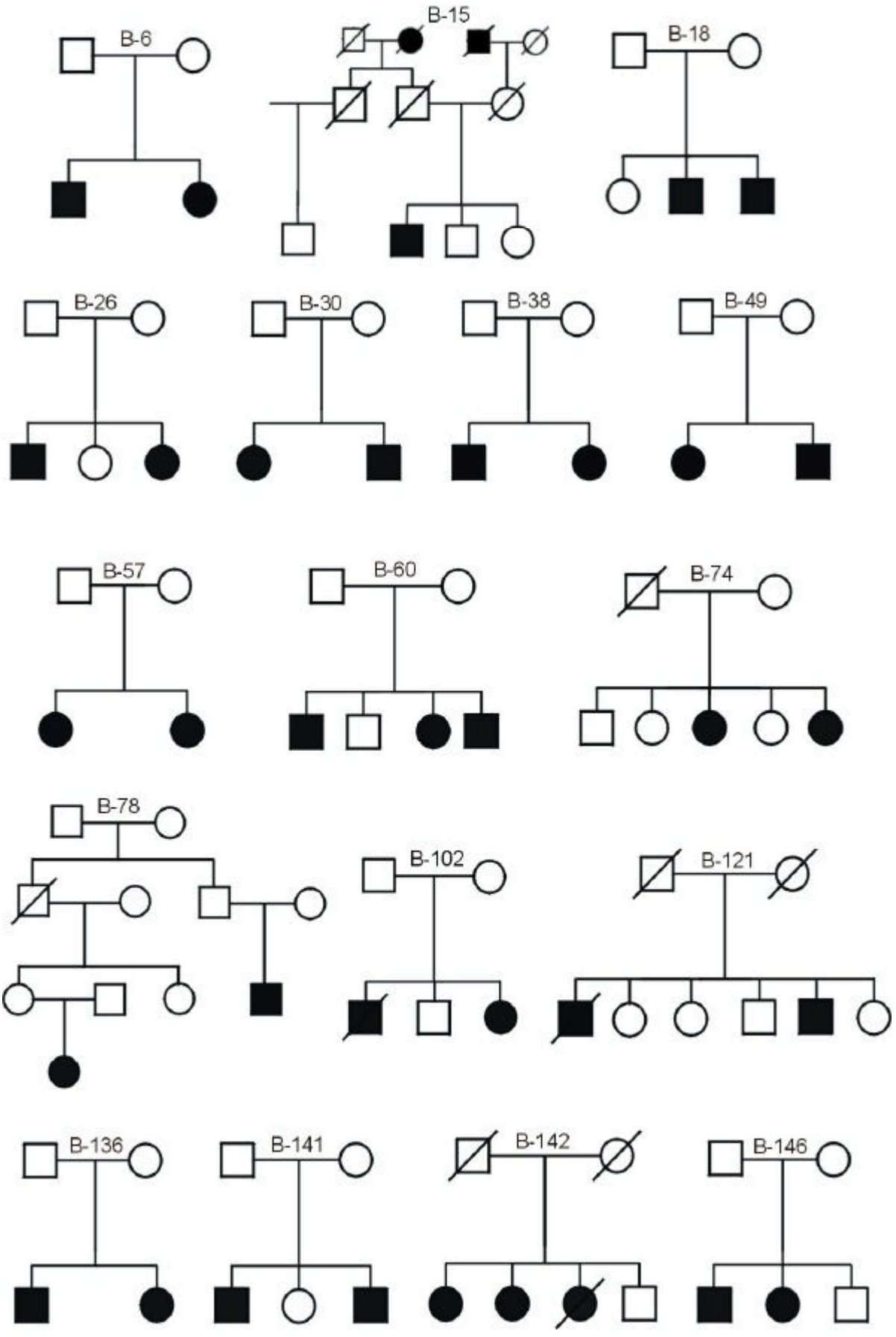
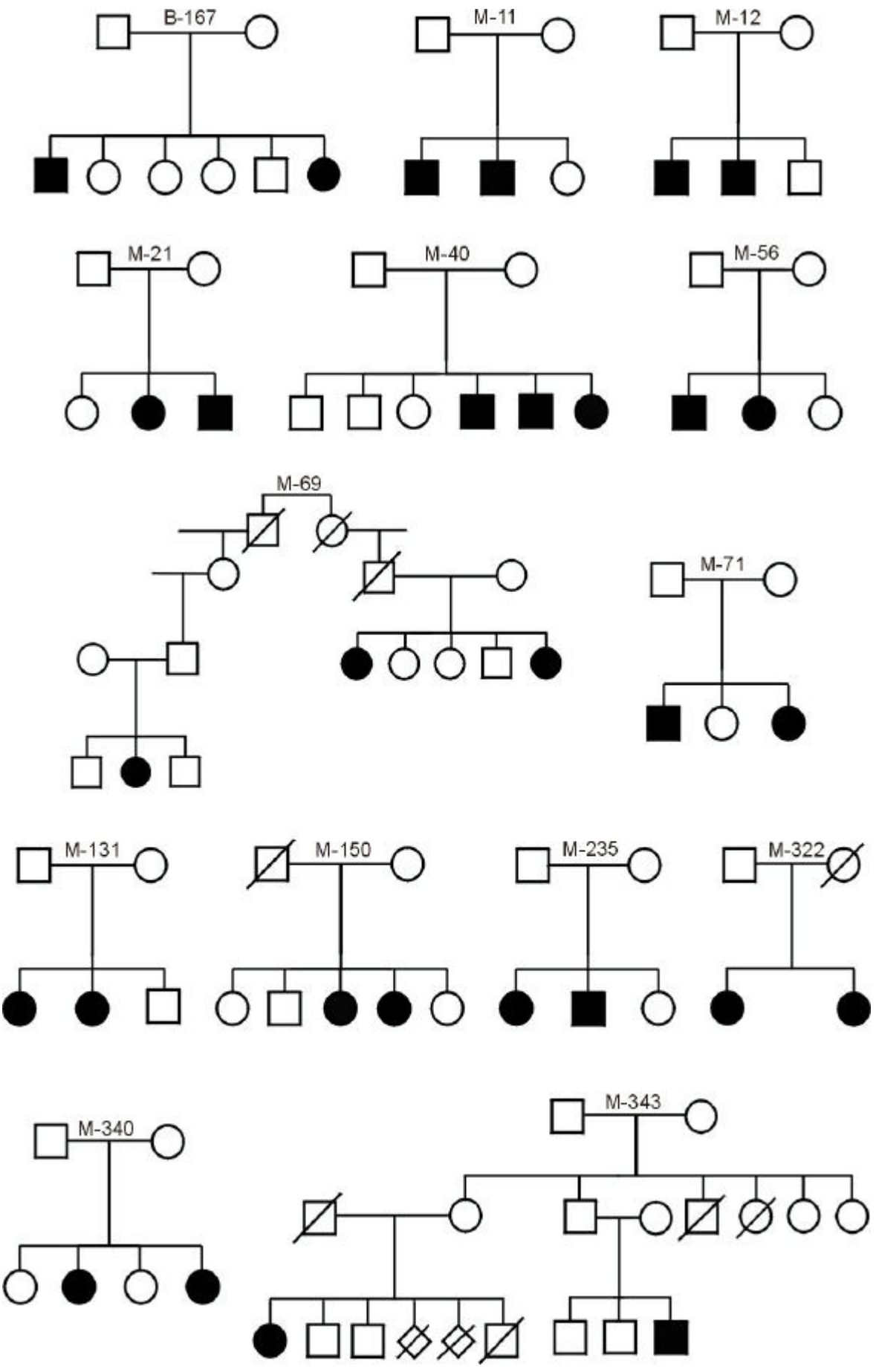

M-286
M-289
M-320
M-338
M-351
M-371
M-503
M-509
M-531
M-630
M-660
M-691
M-717
V-2
V-5
V-7
S-23

Figura 19. Famílies no consanguínies amb RP incloses en aquest treball.




B-167
M-11
M-12
M-21
M-40
M-56
M-69
M-71
M-131
M-150
M-235
M-322
M-340
M-343

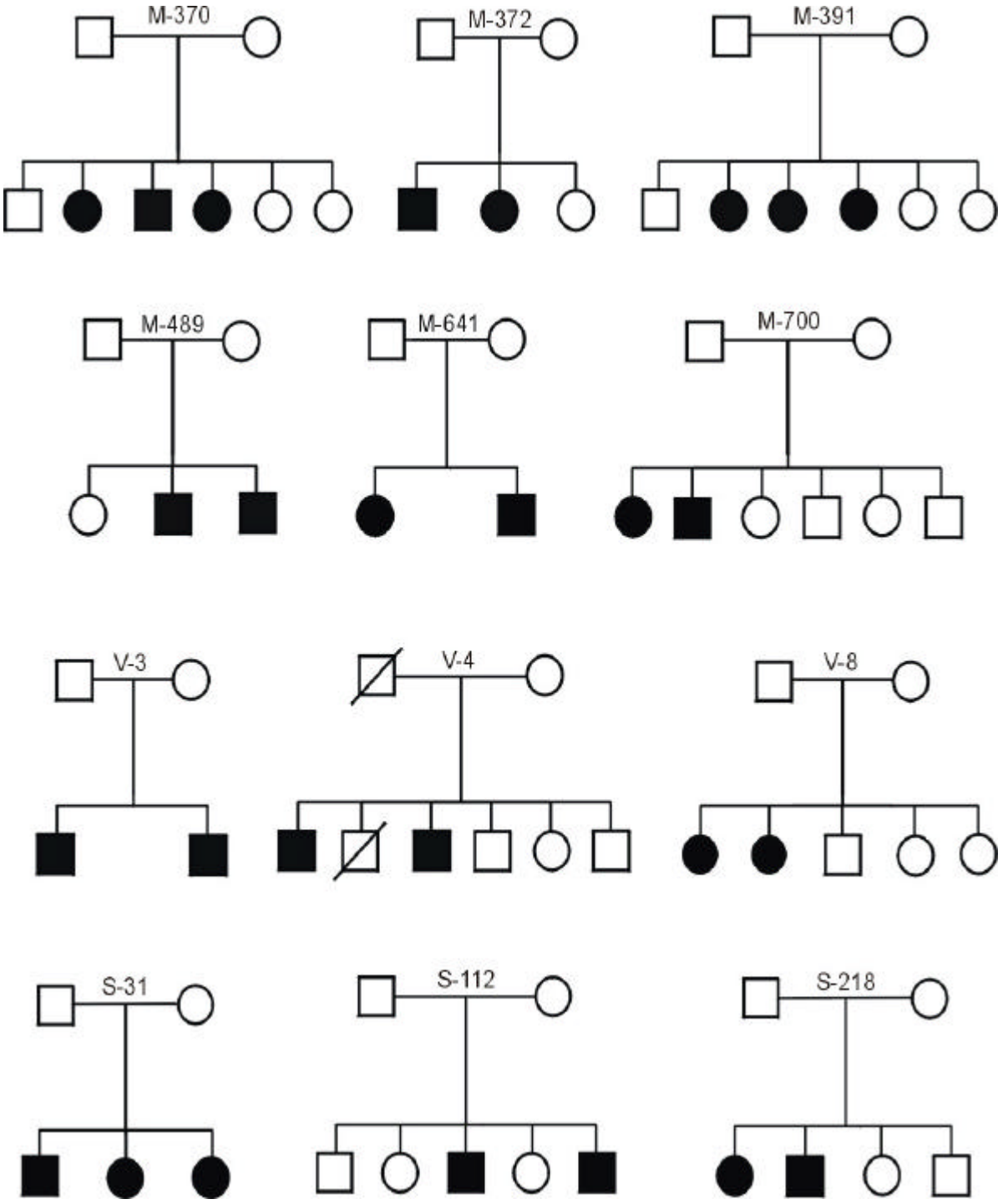

M-370
M-372
M-391
M-489
M-641
M-700
V-3
V-4
V-8
S-31
S-112
S-218

Figura 20. Famílies amb retinosi punctata albescens.

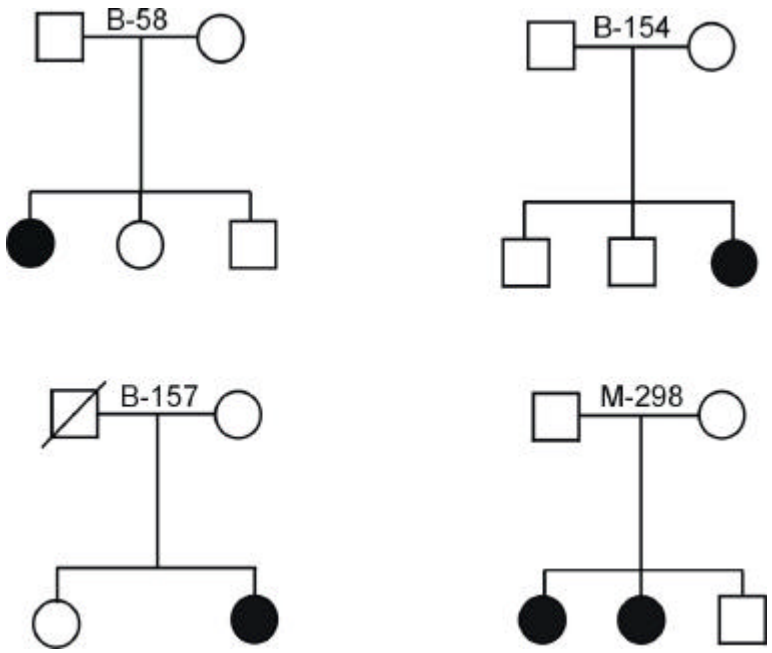
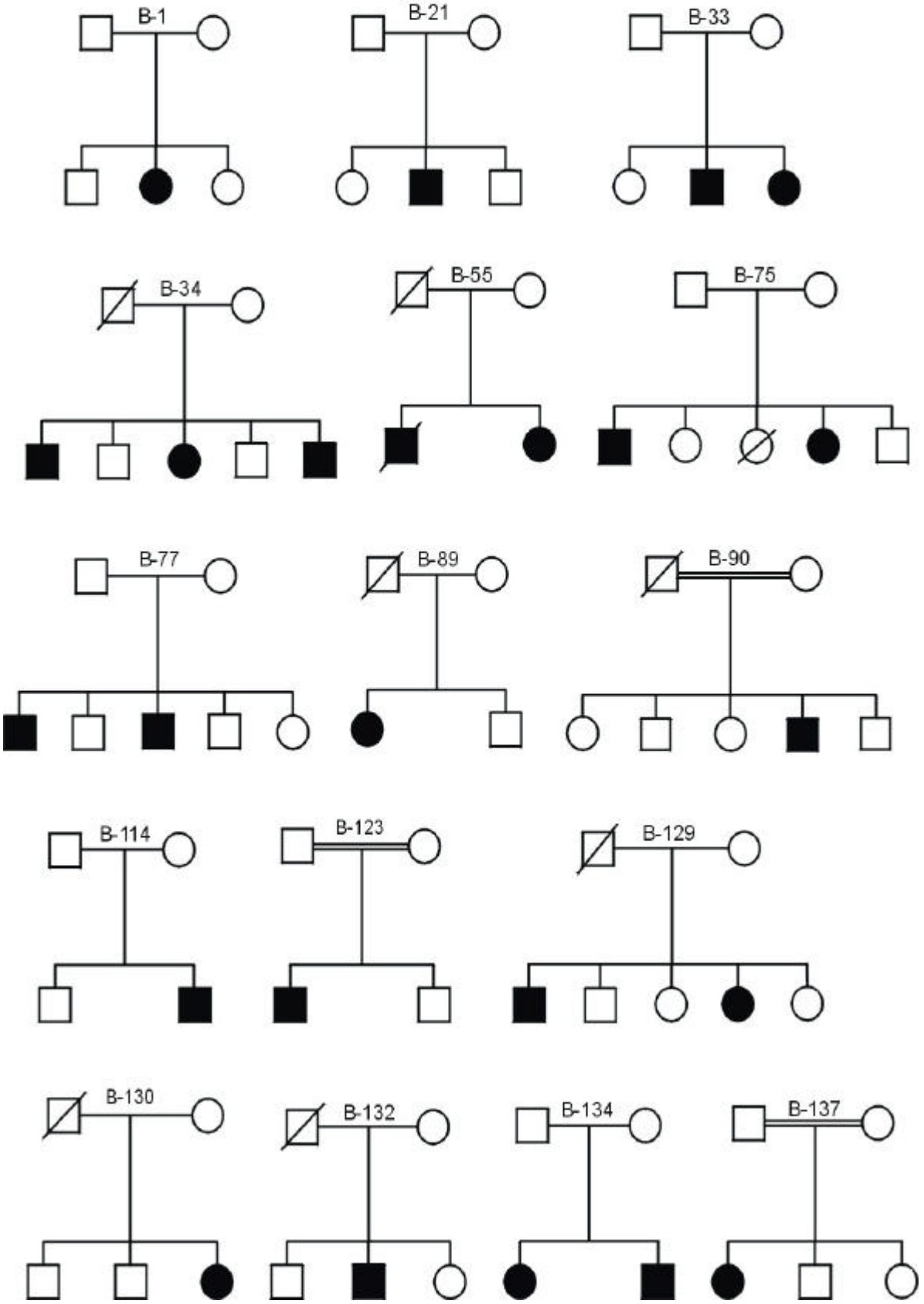
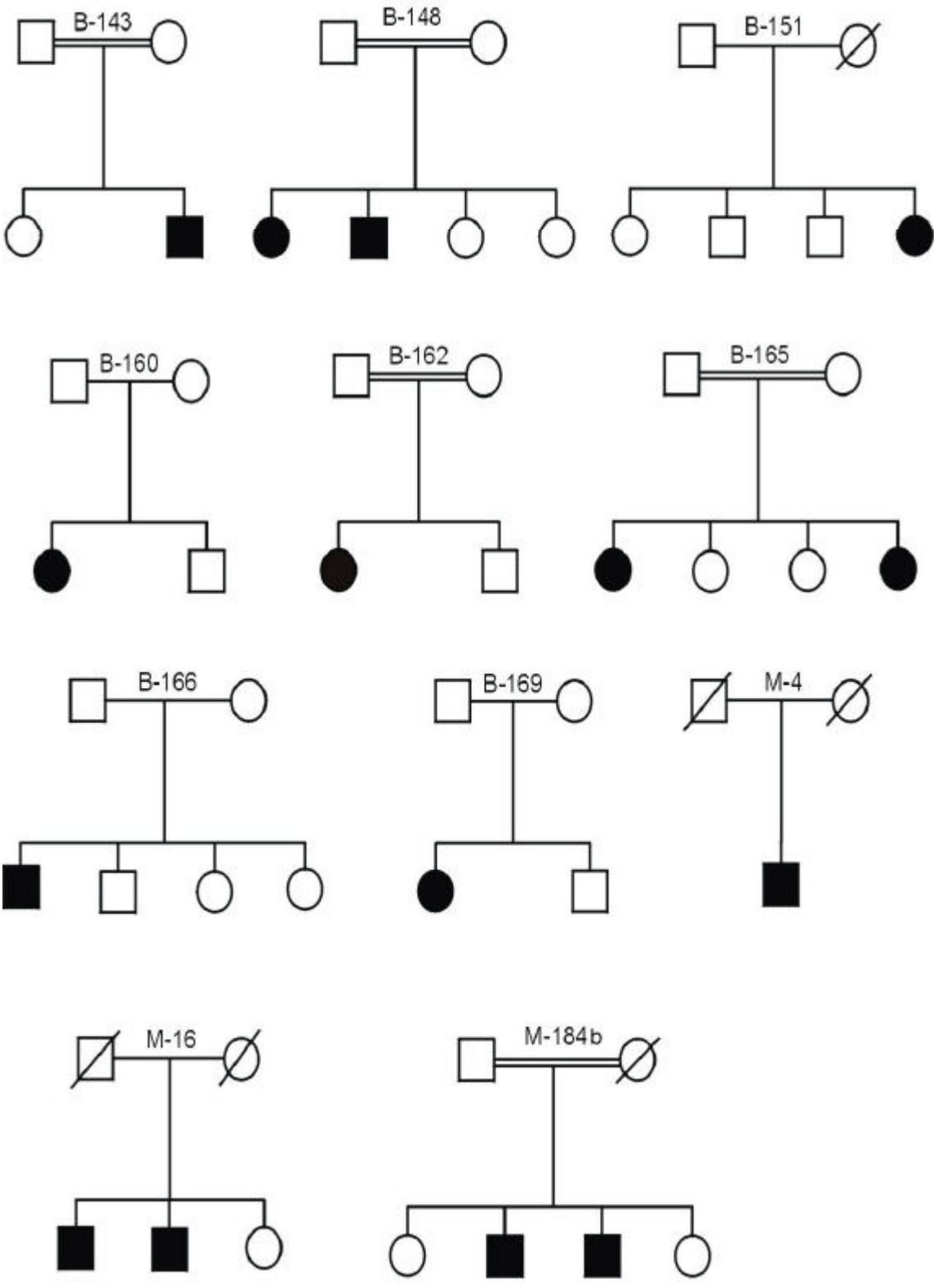


Figura 21. Famílies amb la síndrome d'Usher tipus II seleccionades per l'estudi del gen USH2A.




B-143
B-148
B-151
B-160
B-162
B-165
B-166
B-169
M-4
M-16
M-184b

4. RESULTATS

4.1. ESTUDI MOLECULAR DEL GEN RLBP1

4.1.1. " Evaluation of RLBP1 in 50 autosomal recessive retinitis pigmentosa and 4 retinitis punctata albescens Spanish families".

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Ophthalmic Genetics 2001; 22: 19-25

Inicialment, tots els gens identificats en la RP codificaven proteïnes implicades en el mecanisme de la fototransducció que es dóna en els fotoreceptors, la degeneració dels quals és considerat el defecte primari en aquesta patologia. A posteriori, es va suggerir que la degeneració dels fotoreceptors observada en la RP podria ser una conseqüència de l'alteració dels mecanismes implicats en el cicle visual que tenen lloc en l'EPR, ja que existeix un íntim contacte entre aquest i els fotoreceptors. Per aquest motiu, es va determinar que els gens que codifiquen per les proteïnes del cicle visual podrien ser considerats candidats a estudiar en la patologia de la RP. El gen RLBP1 és un gen que codifica per una de les proteïnes implicades en el cicle visual. En aquest treball s'ha realitzat la búsqueda de mutacions en el gen RLBP1 per avaluar la seva possible implicació en les famílies afectades de RPAR i de retinosi punctata albescens seleccionades com a candidates per aquest estudi mitjançant l'anàlisi de cosegregació i/o homozigositat.

Ophthalmic Genetics 1381-6810/01/
US$ 16.00

Ophthalmic Genetics – 2001, Vol. 22
No. 1, pp. 19–25

Accepted 13 September 2000

Evaluation of RLBP1 in 50 autosomal recessive retinitis pigmentosa and 4 retinitis punctata albescens Spanish families

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Abstract Defects in retinal vitamin A metabolism or in genes expressed in the retinal pigment epithelium (RPE) are related to non-syndromic retinitis pigmentosa (RP). The RLBP1 gene encodes the cellular retinaldehyde-binding protein which, in the RPE and Müller cells of the retina, is thought to play a role in retinoid metabolism and visual pigment regeneration. We describe a study of the involvement of the RLBP1 gene in 50 autosomal recessive retinitis pigmentosa (ARRP) and four retinitis punctata albescens Spanish families. Cosegregation and homozygosity studies using an intragenic polymorphism and three close markers (D15S116, D15S127, and D15S130) ruled out RLBP1 as the cause of ARRP in 26 pedigrees. In the remaining families, SSCP analysis of the coding region and sequencing of the abnormal migrating bands did not detect any disease-causing mutation. These results indicate that mutations in the RLBP1 gene are not responsible for the ARRP or retinitis punctata albescens in this set of Spanish families. We did, however, identify two frequent polymorphisms (3'UTR + 167 G > T, T: 0.23 and G: 0.77; IVS6 + 20 T > C, T: 0.36 and C: 0.64), a silent substitution (S218S), and a rare variant (5'UTR-101 G > A).

Key words RLBP1 gene variants; ARRP

Introduction Retinitis pigmentosa (RP) is the term applied to a clinically and genetically heterogeneous group of retinal degenerations.

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Acknowledgements:
We wish to thank S. Goldflam and J.W. Crabb for providing the CRALBP c-DNA probe. This work was supported by FIS (99/0010-03) and Fundació ONCE.

Its prevalence has been estimated to be 1:3000–1:5000.[1] RP is clinically characterized by night blindness and constriction of visual fields, leading to legal or complete blindness. Fundus examination late in the disease shows attenuated retinal vessels, pale optic nerve head, and bone-spicule pigmentary deposits. Photoreceptor degeneration causes an abnormal or absent electroretinogram. Retinitis punctata albescens is a progressive retinal degeneration that can be distinguished from RP primarily by the fundus appearance, characterized by numerous punctate whitish-yellow spots. There is also a diffuse degeneration of the retinal pigment epithelium (RPE), which begins peripherally and progresses toward the macula.[2]

RP can be inherited in either an autosomal dominant (ADRP), autosomal recessive (ARRP), X-linked (XLRP), or digenic mode. Mutations in several genes (5 for ADRP, 7 for ARRP, 2 for XLRP, and 1 digenic form) have been found to cause nonsyndromic RP and additional human RP loci have been mapped by linkage analysis (http:/www.sph.uth.tmc.edu/Retnet/).

In a previous study, we analyzed different RP genes in a panel of ARRP Spanish families.[3] In the present study, we focused on the RLBP1 gene which is localized on chromosome 15q26 and has eight exons. The first exon is entirely untranslated and both exon 2 and exon 8 contain untranslated regions.[4] Recessive mutations in this gene have been recently identified in a few patients with recessively inherited retinitis punctata albescens.[5–7]

The RLBP1 gene encodes the CRALBP protein (cellular retinaldehyde-binding protein). This protein is an abundant cytosolic component in the RPE and Müller cells of the retina, where it is thought to play a role in retinoid metabolism and visual pigment regeneration. CRALBP can also serve as a substrate carrier, modulating the interaction of 11-cis-retinol with visual cycle enzymes for visual pigment regeneration in photoreceptor cells. In-vitro studies have shown that CRALBP stimulates the oxidation of 11-cis-retinol and retards its esterification, suggesting that CRALBP may influence the partitioning of 11-cis-retinol in the RPE between storage as a retinyl ester or oxidation to 11-cis-retinaldehyde for the regeneration of visual pigment in photoreceptor cells.[8,9]

The purpose of this study was to evaluate the involvement of RLBP1 in 50 ARRP and four retinitis punctata albescens Spanish families. Several polymorphic markers identified within the gene or in its close vicinity were used in cosegregation studies and in homozygosity analyses. Patients from families in whom the involvement of RLBP1 could not be excluded or were noninformative for the markers were further analyzed for mutations in the RLBP1 coding region using SSCP and sequencing analysis.

Patients and methods

FAMILIES This study comprised 50 ARRP families (25 consanguineous and 25 nonconsanguineous pedigrees) and four families with retinitis punctata albescens. The patients included in this study had unaffected parents and at least one affected sibling or were the offspring of a con-

sanguineous mating. Most of the families were examined ophthalmologically at either the Hospital de la Santa Creu i Sant Pau in Barcelona or the Fundación Jiménez Díaz in Madrid. All of the patients were assessed with best-corrected visual acuity, slit-lamp biomicroscopy, color vision test, ocular tension, perimetry, and electroretinography (ERG). In addition, both direct and indirect ophthalmoscopy were performed.

ANALYSIS OF DNA MARKERS Genomic DNA was obtained as described by Miller et al.,[10] with minor modifications. The intragenic PvuII polymorphism was analyzed by Southern blot using a cDNA probe.[11] D15S116, D15S127, and D15S130 were amplified by PCR and resolved on a 40% polyacrylamide gel according to the ECL technique. These markers were used to evaluate cosegregation between RLBP1 and ARRP in the families. Homozygosity at either of these markers in the affected members of the consanguineous families was also considered a criterion to assess RLBP1 involvement.

PCR-SSCP ANALYSES OF THE RLBP1 GENE RLBP1 exons 2–8 were PCR-amplified from human genomic DNA. Exon 8 was analyzed in two overlapping fragments because of its large size (8a and 8b). The primer pairs that were used (Table 1) were described previously by Saari et al.[9] PCR amplification was performed in a final volume of 25 µl, comprising 1.5 mM $MgCl_2$, 10 mM of each dNTP, and 10 pmol of the primers. PCR conditions were as follows: 6 min at 94°C; 30 cycles (30 s at 94°C followed by 30 s at an annealing temperature that ranged from 55°C to 57°C depending on the fragment and 2 min at 72°C); 10 min at 72°C.

For SSCP analyses, 3 µl of the PCR product were mixed with 6 µl of 95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 0.5 M EDTA solution. The samples were then denatured by incubation at 95°C

TABLE 1. Primers used in the analysis of the RLBP1 gene.

Primers 5′→3′	*Position**	*Length*
2F-TGAGATCCACAGTTCTGAGAC 2R-AGGAGAGCCCTGGAGGACA	3,901–4,093	193 bp
3F-GAACTGAAGGTCTGAGCAGG 3R-CAGGAGAGAGAATGCAGTCA	4,251–4,540	290 bp
4F-CTCATCACCTGTGTGTCCTGCC 4R-GAGAGCGGATAGCATCCTCATG	5,631–5,972	342 bp
5F-CTTCTGAGTCCCACTAGGAGG 5R-CCAGTAGAGGCCAGGGTTGA	7,710–8,035	326 bp
6F-CCTCAGGACCTCAAGCCTTA 6R-CTGCAAGCACCATGAAAGGA	11,050–11,359	310 bp
7F-AATGAGTGGGAGCCTCTGAG 7R-CCCTCTTGTCTCATTGTCTGG	12,146–12,358	213 bp
8aF-CTCCTGCTCAGTTCTGTCTC 8aR-ACTTGAGAACAGGGTGACACC	12,540–12,889	350 bp
8bF-AAGGGCTGCTTGAGATGACTG 8bR-CACCAGGACTGCTGCCTTTG	12,810–13,190	381 bp

*Genbank database. Accession L34219.

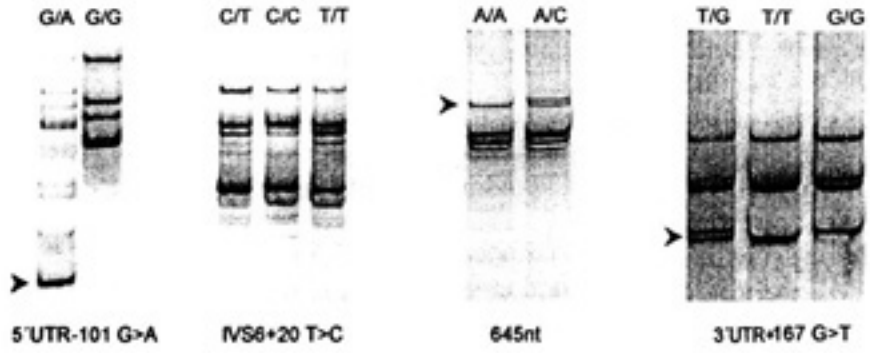

Fig. 1. SSCP pattern of the different substitutions found in the RLBP1 gene.

for 5 min and placed on ice. Electrophoresis was carried out using a non-denaturing 12% polyacrylamide gel. Every sample was analyzed under two different running temperatures (room temperature and 4°C). After electrophoresis, the gels were silver-stained. Fragments that showed abnormal resolution patterns were automatically sequenced (ABI 310® Fluorescent DNA Sequencer, Perkin Elmer Cetus, Norwalk, CT, USA).

Results Cosegregation and homozygosity analyses did not rule out the RLBP1 gene as the cause of RP in 30 families (9 consanguineous RP, 17 nonconsanguineous RP, and 4 punctata albescens). Therefore, one affected member from each of these families was screened for mutations in the RLBP1 coding regions. We identified: i) a G-to-A substitution at the acceptor splice site of intron 1 (5′ UTR-101 G > A)(Given that this change did not segregate with the disease in this family (M-24) and was not present in 70 RP chromosomes or in 100 control chromosomes, this change can be classified as a rare variant.); ii) an A-to-C change at position 654 that resulted in a silent mutation at residue 218 (S218S) in a consanguineous family (B-22); and iii) two new frequent polymorphisms 3′UTR + 167 G > T and IVS6 + 20 T > C (In 100 control chromosomes, the frequencies of the different alleles were T: 0.23/G: 0.77 for the polymorphism in the 3′UTR region and T: 0.36/C: 0.64 for the polymorphism in intron 6.). Figure 1 shows the SSCP pattern of these four different changes.

Discussion Although a number of loci and genes are known to be involved in ARRP, individually they account for a very small number of cases. The identified genes causing ARRP are usually expressed in the photoreceptors of the neuroretina. However, a set of proteins involved in the retinol cycle and associated with retinal dystrophies has been described (RPE65, RDH5, RBP4, and CRALBP). RPE65 encodes an RPE-specific protein that may function in vitamin A metabolism. Mutations in this gene cause several different phenotypes ranging from severe rod-cone dystrophy to a retinal dystrophy and Leber's congenital amaurosis.[12–15] Mutations in RDH5, the gene encoding 11-cis-retinol dehydrogenase, have been found in patients with fundus albipunctatus.[16] A retinol deficiency in a family with compound heterozygous missense mutations in the gene for serum retinol-binding protein (RBP4) has also been described.[17] Although retinol transport was severely impaired in the two affected siblings and there was severe

RPE degeneration in the posterior segment, most tissues including the anterior segment appeared mildly or not affected.

The RLBP1 gene encodes the CRALBP protein, which shows positive immunoreactivity in RPE and Müller cells. CRALBP is a water-soluble intracellular protein that interacts closely with 11-cis-retinol dehydrogenase. Moreover, CRALBP acts as a substrate transporter for several other proteins in the retinoid metabolism.

In this work, we studied the involvement of the RLBP1 gene in a set of 50 ARRP and four retinitis punctata albescens Spanish families. The lack of cosegregation and/or homozygosity between the disease and different polymorphic markers allowed us to exclude the RLBP1 gene as the cause of ARRP in 44% of the families (24 out of 50). In the remaining 26 ARRP and the four retinitis punctata albescens families, we analyzed the entire coding region of the RLBP1 gene. SSCP analysis of the coding region and sequencing of the abnormal migrating bands did not detect any disease-causing mutation. These results indicate that mutations in the RLBP1 gene are not responsible for the ARRP or the retinitis punctata albescens seen in this set of Spanish families. We did, however, identify two frequent polymorphisms (3′UTR + 167 G > T, IVS6 + 20 T > C), a silent mutation (S218S), and a rare variant (5′UTR-101 G > A).

Maw et al.,[5] in a set of 14 Indian pedigrees of nonsyndromic ARRP, identified a substitution in the untranslated exon 2, a silent mutation in exon 4, and a missense mutation in exon 5. The mutation that led to a nonfunctional CRALBP protein was present in a homozygous state in the four affected siblings of one of the consanguineous families. Burstedt et al.[6] reported a founder mutation in the RLBP1 gene (Arg234Tpr) of the patients of seven families affected with Bothnia dystrophy, a type of retinitis punctata albescens found in a restricted area of northern Sweden. Morimura et al.[7] screened 324 unrelated patients with ARRP, isolated RP, retinitis punctata albescens, Leber's congenital amaurosis, or a related disease. In that wide range of samples, only four different mutations occurring in the RLBP1 gene were detected in three of the 28 patients with retinitis punctata albescens. One of those patients was homozygous for the Swedish founder mutation and had also been included in the Swedish study. The two remaining patients were of European ancestry.

The RLBP1 gene has now been analyzed in many patients from different populations with ARRP or an allied retinal degeneration. It seems to be a rare cause of recessive retinal disease. The five different pathogenic mutations reported to date have been found in homozygous and in compound heterozygous forms in patients with clinical manifestations similar to those found in patients with RP accompanied by small yellow or yellow-white deposits scattered over the fundus. Thus, the phenotype produced by RLBP1 mutations seems to be covered by the term 'retinitis punctata albescens'.

The four Spanish patients with retinitis punctata albescens included in the present study had a photoreceptor degeneration with numerous punctate whitish-yellow dots in the fundus, while the complete sequencing of the coding region of RLBP1 gene did not demonstrate any pathogenic mutation. In addition, Morimura et al.[7] found muta-

tions in only three of 28 unrelated patients with this phenotype and other authors[18,19] have documented cases caused by defects in either the RDS gene or the rhodopsin gene. All these data reinforce the fact that retinitis punctata albescens is a term used by ophthalmologists that encompasses a genetically heterogeneous group of diseases.

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4.2. ESTUDI DELS GENS CANDIDATS RGR, CRBP1 I CRB1

4.2.1. " Study of the involvement of the RGR, CRBP1 and CRB1 genes in the pathogenesis of autosomal recessive retinitis pigmentosa".

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Journal of Medical Genetics 2003; 40: e89 (online mutation report)

L'anàlisi de gens candidats és una de les estratègies utilitzades per l'estudi de la RP. Dos dels criteris establerts per la selecció de gens com a candidats per ser estudiats és que les proteïnes codificades presentin una funció important en el procés de la visió o que codifiquin proteïnes expressades en els teixits afectats per la RP, encara que la funció d'aquestes proteïnes sigui desconeguda en el procés de la visió. Seguint aquesta estratègia, aquest treball s'ha centrat en l'estudi molecular de tres gens candidats en la patologia de la RP en famílies que segueixen un patró d'herència autosòmic recessiu i que provenen totes elles de població Espanyola. S'han considerat com a candidats: i) els gens CRBP1 i RGR, seleccionats per codificar proteïnes expressades en l'EPR i per participar en el cicle visual que va dirigit a la regeneració de la rodopsina, i ii) el gen CRB1 seleccionat per codificar una proteïna expressada en la part neural de la retina, de funció encara desconeguda en el context del procés visual.

ONLINE MUTATION REPORT

Study of the involvement of the *RGR*, *CRPB1*, and *CRB1* genes in the pathogenesis of autosomal recessive retinitis pigmentosa

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J Med Genet 2003;**40**:e89(http://www.jmedgenet.com/cgi/content/full/40/7/e89)

Retinitis pigmentosa (RP), which occurs in about 1 in 3000-7000 people in Spain, is inherited in an autosomal dominant manner in 12% of cases, in an autosomal recessive way in 39%, and in an X linked manner in 4% of cases.[1] This leaves 41% of RP cases with a simplex form and 4% in which the transmission pattern is unclear.

The different genes that have been implicated in retinal degeneration are known or assumed to be expressed in the photoreceptor cells of the retina or in the retinal pigment epithelium (RPE). The large number of RP genes identified can be grouped into a number of functional classes: (1) proteins of the visual cascade, (2) proteins of the visual cycle, (3) photoreceptor cell transcription factors, (4) proteins related to catabolic processes, and (5) genes of unknown function.

Previous studies performed in autosomal recessive retinitis pigmentosa (ARRP) Spanish families have shown that genes coding for recoverin,[2] rhodopsin, rod outer segment membrane protein and peripherin/RDS,[3] S antigen and the gamma subunit of rod cGMP-phosphodiesterase,[4] interstitial retinol binding protein,[5] the alpha subunit of rod cGMP-phosphodiesterase and NRL,[6] and the retinaldehyde binding protein[7] do not play a role in this disorder. However, mutations in the beta subunit of the rod cGMP-phosphodiesterase gene,[8–11] in the ATP binding cassette receptor gene,[12] in the *TULP1* gene,[13] in the alpha subunit of the rod cGMP gated channel,[14] and in the *USH2A* gene[15] have been detected in a small percentage of Spanish ARRP families. These data indicate that other genes play a part in the degeneration process of the retina in the remaining families.

We analysed the involvement of three additional genes, the RPE retinal G protein coupled receptor (*RGR*), the cellular retinol binding protein (*CRBP1*), and the crumbs homologue 1 (*CRB1*) (table 1) in 92 ARRP Spanish families.

RGR is an integral membrane protein that is expressed in the cytoplasm of RPE and Müller cells.[16] It is a member of a large family of G protein coupled receptors and shows considerable overall homology to the visual pigments and retinochromes. Under light conditions, RGR converts all-*trans*-retinal to 11-*cis*-retinal, whereas the reverse isomerisation occurs within rhodopsin.

CRBP1 belongs to a family of cytosolic proteins whose members bind various hydrophobic ligands. This protein is a component in the retinal pigment epithelium where it is believed to function in intracellular storage and transport of retinol.

The *CRB1* gene is expressed in human retina and brain. It exhibits alternative splicing at its 3′ end and the four classes of mRNA are predicted to encode four different proteins, two of which are found in human retina.[17] Given its homology to the *Drosophila* Crumbs protein that is required for polarity and adhesion in embryonic epithelia, it has been postulated that the role of CRB1 in vertebrate photoreceptors may be in cell adhesion and photoreceptor morphogenesis. A role in localising the phototransduction complex to the apical membrane of the photoreceptors has been proposed owing to the specific expression of CRB1.

MATERIALS AND METHODS

Families

This study comprises 92 ARRP families including four families with retinitis punctata albescens (50 consanguineous and 42 non-consanguineous pedigrees). Most of the families were examined ophthalmologically at the Hospital de la Santa Creu i Sant Pau in Barcelona or at the Fundación Jiménez Díaz in Madrid. The clinical diagnosis of RP was based on ophthalmological examination including measurements of visual acuity, ophthalmoscopy, dark adaptation, ocular tension by air tonometer, perimetry, and electroretinogram amplitudes according to ISCEV protocols.[18]

METHODS

Blood samples were obtained after informed consent was given and genomic DNA was extracted from leucocytes of peripheral blood from each patient.[19] We used single strand conformation analysis (SSCP) to screen all exonic sequences (including intron-exon boundaries) containing the open reading frame of the *RGR*, *CRBP1*, and *CRB1* genes. Primers

Key points

- Autosomal recessive retinitis pigmentosa (ARRP) is a genetically heterogeneous form of retinal degeneration. The genes for the RPE retinal G protein coupled receptor (*RGR*) and the crumbs homologue 1 (*CRB1*) have been reported to be the cause of ARRP. Although no mutations in the cellular retinal binding protein gene (*CRBP1*) have been reported, we have considered this gene as a candidate for ARRP.
- SSCP analysis and DNA sequencing of the entire coding regions of these genes were performed in 92 ARRP Spanish patients. Several exonic and intronic single nucleotide polymorphisms were detected in the *RGR* and *CRBP1* genes. However, no disease causing mutations were found, suggesting that these genes are most probably not involved in the disease in this set of ARRP Spanish pedigrees. In contrast, the mutational analysis of the *CRB1* gene allowed the identification of a number of rare sequence variants and intronic polymorphisms and of seven pathogenic mutations.
- The ocular phenotype of RP patients harbouring these mutations confirms that considerable clinical heterogeneity is associated with mutations in the *CRB1* gene.

Table 1 Characteristics of the three genes included in the study

Gene name	Symbol	OMIM ID	No exons	Chr location	Size (kb)	Mutations report (references)
RPE retinal G protein coupled receptor	*RGR*	600342	7	10q23	14.8	22
Cellular retinol binding protein 1	*CRBP1*	180260	4	3q21-q22	21	–
Crumbs homologue 1	*CRB1*	604210	12	1q31-q32.1	>40	20, 21, 25, 26

Table 2 Results of mutation analysis at the genomic DNA level of the *RGR* and *CRBP1* genes

Gene	Sequence variation	AA change	Minor allele Frequency (%)
RGR	nt 19 C>T*	Leu7Leu	8
	nt 27 C>T*	Thr9Thr	49
	nt 459 C>T*	Tyr153Tyr	38
	nt 722 C>T*	Ser241Phe	2.6
	IVS6+5 A>G*	-	0
	nt 615 G>A	Lys205Lys	-
CRBP1	5′UTR-134 C>T	-	10
	5′UTR-37 C>T	-	6

*Reported in reference 22

corresponding to the complete coding sequence of the *RGR* and the *CRBP1* genes were designed according to GenBank entries NT_033890 and NT_005832 to yield PCR products in the range 200-350 bp. The 28 primer sets described by Hollander *et al*[20 21] were used to amplify the complete coding region including two promoter regions of the *CRB1* gene. PCR amplification was performed in a final volume of 25 µl containing $MgCl_2$ (1-2 mmol/l depending on the fragment amplified), 200 µmol/l of each dNTPs, 0.2 µmol/l of each primer, 0.5 units of *Taq* DNA polymerase (Ecogen) in the recommended buffer, and 100 ng of genomic DNA. The thermocycling conditions were 94°C for six minutes, followed by 30 cycles at 94°C for 30 seconds, from 53-63°C depending on the fragment for 30 seconds, and 72°C for two minutes, followed by a 10 minute final extension step at 72°C.

After amplification, mutation analysis was carried out using SSCP under two different conditions combining acrylamide concentration, running temperatures, and voltage. Fragments that showed abnormal patterns of migration by SSCP were analysed on an automated sequencer (ABI Prism 310, Applied Biosystems).

On detection of a sole mutant allele, the patient sample was subjected to direct sequence analysis of the remaining exons and of the promoter regions of the corresponding gene.

RESULTS

All deleterious mutations and genetic variants were assigned a nucleotide number starting at the first translated base of the *RGR* and *CRBP1* genes according to the GenBank entries U14910 and NM_002899, respectively. The corresponding accession numbers for the *CRB1* gene were AY043324 or AY043325 for isoforms I or II, respectively. Deletions were names in accordance with the HUGO recommendations.

RPE retinal G protein coupled receptor (*RGR*)

A PCR-SSCP strategy was used to screen each of the seven exons of the *RGR* gene in the 92 unrelated patients under study. SSCP band shifts were detected in DNA encompassing exons 1, 4, and 6. Sequencing of these fragments allowed the identification of six single base pair substitutions (table 2). Four of these single nucleotide changes were C>T transitions, three of which corresponded to previously described frequent polymorphisms.[22] The remaining C>T substitution causing the missense change Ser241Phe was present in nine unrelated ARRP patients (eight carriers and one homozygote). Family studies in these nine ARRP families indicated that all the affected RP patients were carriers of the Ser241Phe in the four

Table 3 Results of mutation analysis at the genomic DNA level of the *CRB1* gene

Mutations	Sequence variation	Cd	Exon	Effect	Cosegregation
M-717*	nt 478^81 ins G	160-161	2	168 Stop	+
M-489*	nt 614 T>C	205	2	Ile205Thr (nc)	+
M-641*	nt 2244^47 del 3bp	749	7	delSer	+
B-102*	nt 2671 T>G	891	7	Cys891Gly	+
M-69/M-641	nt 2843 G>A	948	9	Cys948Tyr	+
B-15*	nt 2882^88 del 3bp	962	9	delLeu	+
B-102/M-69*	nt 3299 T>C	1100	9	Ile1100Thr (nc)	+
Rare sequence variants					
M-43	nt 867 C>T	289	4	Thr289Met (nc)	-
V-4/M-489	nt 1410 G>A	470	4	Leu470Leu (scc)	-
B-141	nt 1647 T>C	549	6	Asn549Asn (scc)	-
M-180*	nt 2035 C>G	679	6	Gln679Glu (nc)	-
B-50	nt 2306 G>A	769	7	Arg769His (c)	-
M-68	nt 3171 C>T	1057	9	Asn1057Asn (scc)	-
M-40	nt 3992 G>A	1331	11	Arg1331His (c)	-
M-69	5′UTR-268 G>A				
Polymorphic intronic variants					
	T/A		IVS1-12		-
	T/A*		IVS2+42		-
	T/G*		IVS4-53		-
	T/G*		IVS4-64		-

*Not previously reported change; nc: non-conservative change; scc: synonymous codon change; c: conservative change

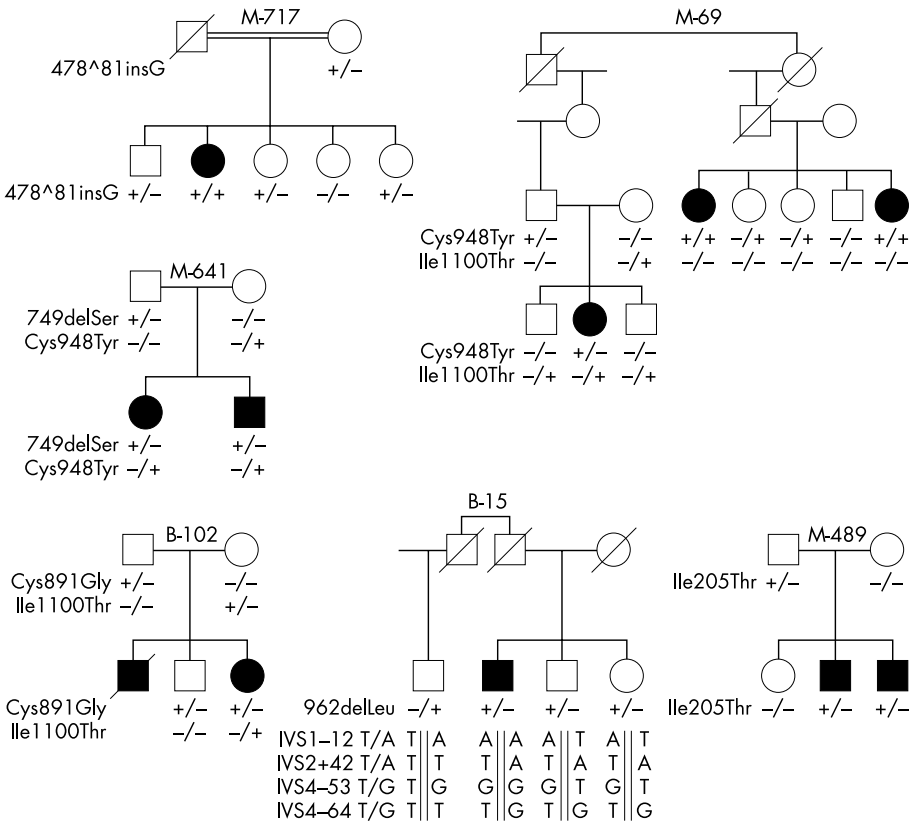

Figure 1 Pedigrees of the Spanish ARRP families in which *CRB1* mutations have been identified.

non-consanguineous pedigrees, whereas cosegregation with the disease phenotype could be excluded in the five consanguineous families. In the control group (190 chromosomes), we found five alleles with Ser241Phe. A new silent variation (Lys205Lys) was identified in a carrier state in the two affected brothers of an ARRP family. A patient showing a punctata albescens phenotype was a carrier of an A>G transition in intron 6 (IVS6+5 A>G). Segregation analysis in the patient's family showed no cosegregation with the disease phenotype. This change was not observed in 190 control chromosomes.

Cellular retinol binding protein 1 (*CRBP1*)

The mutational screening of the four exons and their flanking regions of the *CRBP1* gene in 92 unrelated patients allowed the identification of two single base pair substitutions in the amplimer containing exon 1 (table 2). These single nucleotide changes were C>T transitions in the 5′ UTR region (position –37 and position –134) and correspond to two new polymorphisms with a minor allele frequency of 6% and 10%, respectively.

Crumbs homologue 1 (*CRB1*)

A total of 19 germline sequence variants were observed in this study, including seven pathogenic mutations, eight rare sequence variants, and four intronic polymorphisms (table 3). Seven mutations meet the criteria of pathogenicity, namely, absence in controls and segregation with the disease within the family. Six out of seven are novel: the non-conservative change (Ile>Thr) located in two different *CRB1* codons (205 and 1100, respectively); two in frame deletions causing the loss of serine (cd 749) and leucine (cd 962) residues; an amino acid changing variant Cys891Gly and one out of frame deletion caused by the insertion of a guanine between codons 160-161 creating a stop codon in position 168. We also observed the previously reported Cys948Tyr mutation. Fig 1 shows the pedigrees of the ARRP families in which these mutations were identified.

In addition to deleterious mutations, we detected eight rare sequence variants that include three synonymous codon changes (Leu470Leu, Asn549Asn, and Asn1057Asn), two conservative amino acid changes (Arg769His and Arg 1331His), two non-conservative amino acid changes (Thr289Met and Gln679Glu), and a G to A substitution at position –268 of the 5′ UTR region (table 3). Family studies in all these eight substitutions excluded cosegregation with the disease phenotype. We identified four intronic variants (IVS1-12 T/A, IVS2+42 T/A, IVS4-53 T/G, and IVS4-64 T/G), all of them with a >1% frequency in the general population, which were regarded as polymorphisms.

DISCUSSION

RGR is a seven transmembrane domain receptor, a close relative of rhodopsin, found in the support cells for the photoreceptors, the RPE, and the Müller glia. Unlike rhodopsin, the RGR protein is coupled to all-*trans*-retinal that is isomerised to 11-*cis*-retinal upon light exposure.[23] The essential role of RGR in the process of vision was reinforced when (1) RP associated mutations in the *RGR* gene were described by Morimura *et al*[22] and (2) the phenotype of mice with targeted disruption of *Rgr* was described.[24] Two mutations, a number of other changes less likely to be pathogenic, and four frequent polymorphisms were found in the mutational screening of the *RGR* gene performed by Morimura *et al*,[22] which included a large group of patients with photoreceptor degeneration. In our group of Spanish ARRP patients, we found three of the previously described polymorphisms with similar allelic frequencies, two variations (IVS6+5 A>G and Lys205Lys) and the Ser241Phe substitution (table 2). Morimura *et al*[22] found the latter change heterozygously in two cases of recessive RP, two simplex cases, and one of 95 unrelated normal controls; one simplex case was

Table 4 Mutations described in the *CRB1* gene

Mutation type	Location	Nucleotide change	Effect	Reference
Missense				
1	cd 144	TTC > GTC	Phe144Val	26
2	cd 161	GCC > GTC	Ala161Val	20
3	cd 205	ATA > ACA	Ile205Thr	This report
4	cd 250	TGT > TGG	Cys250Trp	20
5	cd 383	TGT > TAT	Cys383Tyr	26
6	cd 433	TAT >TGT	Tyr433Cys	21
7	cd 480	TGT > CGT	Cys480Arg	26
8	cd 480	TGT > GGT	Cys480Gly	26
9	cd 681	TGT > TAT	Cys681Tyr	26
10	cd 745	ACG >ATG	Thr745Met	20
11	cd 764	CGT > TGT	Arg764Cys	20, 21, 26
12	cd 837	GAC > CAC	Asn837His	21
13	cd 891	TGC > GGC	Cys891Gly	This report
14	cd 894	AAC > AGC	Asn894Ser	21
15	cd 948	TGT > TAT	Cys948Tyr	20, 21, 26, this report
16	cd 1041	ATG > ACG	Met1041Thr	20
17	cd 1071	CTC > CCC	Leu1071Pro	20
18	cd 1100	ATA > AGA	Ile1100Arg	21
19	cd 1100	ATA > ACA	Ile1100Thr	This report
20	cd 1181	TGC > CGC	Cys1181Arg	21
21	cd 1205	GGA > AGA	Gly1205Arg	26
22	cd 1317	AAC > CAC	Asn1317His	26
23	cd 1321	-	Cys1321Ser	25
24	cd 1354	GCC > ACC	Ala1354Thr	21
Nonsense				
1	cd 403	TCA > TGA	Ser403Stop	20, 21
2	cd 801	AAG > TAG	Lys801Stop	21
3	cd 995	GAG > TAG	Glu995Stop	20
4	cd 1111	GAA > TAA	Glu1111Stop	21
5	cd 1332	TGC > TGA	Cys1332Stop	26
6	cd 1333	GAG > TAG	Glu1333Stop	21
Frameshift				
1	cd 37	del T	-	26
2	cd 86-87	ins GT	-	26
3	cd 143-144	del GATTC	-	26
4	cd 160-161	ins G	168 Stop	This report
5	cd 204-207	del AAATAGG	-	21, 26
6	cd 749	del 3bp	del Ser	This report
7	cd 729	ins Alu	-	20
8	cd 812-813	ins polyA	-	26
9	cd 850-851	del GGCT	-	26
10	cd 871	ins T	-	26
11	cd 962	del 3bp	del Leu	This report
12	cd 1115	del GGTTTCATTA	-	25
Splice site				
1	nt 2978+5	G>A	-	20, 21
2	nt 4013+1	G>T	-	21

homozygous Ser241Phe. We found the same substitution in nine unrelated ARRP patients (eight carriers and one homozygote). The pathogenic significance of this variant is difficult to assess because although there is a higher frequency of this variant in ARRP patients (10/182 alleles) compared with the frequency in control population (5/190 alleles) ($p=0.0005$), no cosegregation exists between the variant Ser241Phe and the disease in the Spanish consanguineous families.

CRBP1 is the carrier protein involved in the intracellular transport of retinol. Analysis of the visual cycle in *CRBP1* knockout mice suggests that the binding protein participates in a process that drives diffusion of all-transretinol from photoreceptor cells to RPE, perhaps delivering vitamin A to lecithin-retinol acyltransferase for esterification. This alleged involvement of the *CRBP1* gene in the visual process and the lack of mutational studies of this gene in patients affected by retinal degenerations prompted us to screen ARRP patients for pathological mutations in the four exons and flanking sequences of the *CRBP1* gene. We detected no disease causing mutation in our set of families but two new single nucleotide polymorphisms were found both in the 5′ UTR region of the gene (table 2). These polymorphisms were observed in controls as well as in unaffected family members.

The recent work of two groups of investigators[20 21 25 26] has identified a number of mutations in the *CRB1* gene in patients affected by (1) a form of autosomal recessive RP (RP12), characterised by a preserved para-arteriolar retinal pigment epithelium (PPRPE) and by a severe loss of vision at age <20 years, (2) Leber congenital amaurosis, (3) RP with Coats-like exudative vasculopathy, and (4) a severe form of RP with common features. Table 4 summarises the reported mutations in the *CRB1* gene.

Bearing in mind these findings, we undertook the study of the *CRB1* gene in our set of ARRP families. Overall, seven pathogenic mutations were detected, six of which are reported for the first time. In addition, a number of rare sequence variants and intronic polymorphisms were identified.

The insertion of a G residue between nucleotides 478-481 was found in a homozygous state in the affected patient of a consanguineous family (M-717 in fig 1). This sequence alteration generates a stop signal in codon 168. All the remaining asymptomatic members of this family were heterozygous carriers of this mutation or homozygous for the wild type allele.

Table 5 Clinical findings in patients with mutations in the *CRB1* gene

Family	Mutations in *CRB1* gene	Age	Age of onset	Visual field	Visual acuity	Refraction	Fundus	ERG	Other symptoms
M-717	478^81insG	52 y	15 y	<5°	Light perception		Pale papilla Constricted arterioles Salt and pepper pigmentation in mid periphery and in posterior pole, more abundant around the macula	Extinguished	Hyperopia Nystagmus
M-69	Cys948Tyr Cys948Tyr	55 y	1 y	Absolute scotoma	Amaurosis		Difficult to evaluate owing to opacities		Nystagmus Dense cataracts Microphthalmus Corneal leukoma secondary to keratoconus
M-69	Cys948Tyr Cys948Tyr	48 y	1 y	Absolute scotoma	Amaurosis		Difficult to evaluate owing to opacities		Nystagmus Dense cataracts Microphthalmus Corneal leukoma secondary to keratoconus
M-69	Cys948Tyr Ile1100Thr	21 y	3 y	<5°	0.1 RE 0.2 LE	+2+1.5 100° RE +1.5+1 80° LE	Bone spicule pigmentation Pale papilla Constricted arterioles	Extinguished	Nystagmus at 7 mth
M-641	Cys948Tyr 749delSer	11 y	4 mth	Central scotomae	0.3 RE 0.1 LE	+7.00-2.5 30° RE +7.00-1.00 150°LE	Bone spicule pigmentation in periphery Pale papilla Constricted arterioles PPRPE in temporal periphery	Extinguished	Strabismus
M-641	Cys948Tyr 749delSer	8 y	6 mth	Annular scotoma Peripheral sensitivity	0.1 RE 0.2 LE	+2.5+2 93° RE +2+2 98° LE	Bone spicule pigmentation in periphery Pale papilla Constricted arterioles PPRPE in temporal periphery	Extinguished	Congenital nystagmus
B-102	Cys891Gly Ile1100Thr	15 y	4 y		0.3 RE 0.2 LE		Bone spicule pigmentation Normal macula Pale papilla Vascular constriction	Extinguished	Hyperopia
B-15	962delLeu	54 y	13 y	RE: absolute scotoma LE: <5°	0.3 RE 0.2 LE	-0.25-0.25 170° RE -0.25-1.25 101° LE	Focal pigmentation in posterior pole Normal papilla Diffuse chorioretinal atrophy	Extinguished	Cataracts
M-489	Ile205Thr	37 y	At birth	Absolute scotoma	Light perception		Bone spicule pigmentation Pale papilla Narrow vessels	Extinguished	Nystagmus Keratoconus Franceschetti sign
M-489	Ile205Thr	35 y	At birth	Absolute scotoma	Light perception		Bone spicule pigmentation Pale papilla Narrow vessels	Extinguished	Nystagmus Franceschetti sign

Clinical findings of the affected patient (table 5) show an RP pattern with an early onset and a severe loss of vision under the age of 20. Given that the PPRPE phenotype can only be identified in the early/middle stages of the disease, the present fundus examination of this patient with advanced RP does not allow us to exclude a previous typical RP12 pattern.

A homozygous point mutation (G>A nucleotide 2843) causing the substitution Cys948Tyr was found in two RP sisters of family M-69 (fig 1). The clinical findings in these patients were consistent with a diagnosis of LCA (table 5). This substitution was also present in another branch of this family where the affected RP subject carried a non-conservative change Ile1100Thr in addition to Cys948Tyr. Clinical data of this patient (table 5) indicate an early onset of typical RP with macular symptoms. An extensive search for PPRPE signs yielded a negative result. The Cys948Tyr mutation was also identified in the two patients from family M-641 (fig 1), who also had a deletion of a serine residue in position 749. Ophthalmological examination of both affected sibs showed an RP12 phenotype with PPRPE, night blindness, and loss of visual field before the age of 10 years. Nystagmus and hyperopia were also observed as described in cases with an RP12 phenotype. Since the cysteine residue in position 948 is involved in the formation of disulphide bridges in the 14th EGF-like domain of *CRB1*, the correct folding of this domain may be impaired by changes affecting this position. The mutation Cys948Tyr, which is the *CRB1* mutation that is most frequently found, has been described in patients with Leber congenital amaurosis (LCA), with RP characterised by a preserved para-arteriolar retinal pigment epithelium, and in patients who had RP with Coats-like exudative vasculopathy. The three homozygous Cys948Tyr patients described to date were diagnosed with LCA. This clinical phenotype has also been associated with the presence of Cys948Tyr in combination with a frameshift mutation, with a missense mutation, and in patients in whom only the mutated allele Cys948Tyr has been found.[20 21 26] The identification, in the present work, of two LCA patients (M-69 family) who are homozygous Cys948Tyr reinforces the view that Cys948Tyr is a mutation that leads to a severe phenotype when present homozygously, resulting in the complete loss of function of CRB1.[21]

The inheritance of Cys948Tyr in combination with Ile1100Thr (family M-69) is associated with an early onset RP phenotype without PPRPE whereas the coinheritance of Cys948Tyr and 749delSer (family M-641) manifests as an RP12 with PPRPE. These cases may have residual CRB1 function as postulated by den Hollander *et al*,[21] who identified four PPRPE patients who carried Cys948Tyr in combination with other missense mutations.

A new point mutation causing the substitution of the cysteine residue in position 891 by a glycine was found in compound heterozygosity with a non-conservative change (Ile1100Thr) in the proband of family B-102. Ophthalmological examination of this patient (table 5) showed a typical RP with an early onset and a rapid progression of the disease. Cys891 is a conserved residue located in the 13th EGF-like domain of *CRB1* and its substitution, impairing the formation of disulphide bridges, probably causes domain misfolding with secondary deleterious effects on the global conformation of the protein. The mutation Ile1100Thr that cosegregates in this family affects the same isoleucine residue found by Hollander *et al*[21] to be mutated (Ile1100Arg) in a LCA proband.

A deletion of three nucleotides leading to the 962 del Leu of the laminin 3 motif was found, in a heterozygous state, in the proband of family B-15. Sequence analysis of the entire *CRB1* ORF did not show any additional mutation. The disease in this patient may be the result of an additional missing mutant *CRB1* allele (a point mutation in the non-coding regions of the *CRB1* gene or a large genomic rearrangement that would not be detected with the methodology used). Clinical findings in this patient (table 5) meet all the standard definitions of retinitis pigmentosa except for the fundus appearance. He had night blindness from an early age, with a progressive visual field loss and a non-recordable ERG. The most striking finding in the fundus of this patient is the extensive atrophy of the retinal pigment epithelium and choroid. Choroid atrophy is widespread leaving areas of bare sclera, although other areas are still spared. There is little dispersion in pigment scattered all across the fundus. The pigment does not tend to assume the spicular configuration of deposits in typical RP. The disc is not pale but the retinal arteries are thin. This choroideremia (CHM)-like fundus prompted us to analyse the segregation of three intragenic markers of the *REP-1* gene (two SNPs located in exons 5 and 9 and a VNTR in exon 14) in the members of family B-15. Linkage between these informative markers and the disease phenotype can be excluded (data not shown). Thus, the diagnosis of a CHM associated with *REP-1* abnormalities can be rejected.

A novel non-conservative missense mutation, Ile205Thr, was identified in the affected brothers of family M-489 (fig 1). The Ile205Thr mutation changes an apolar residue conserved through evolution by a polar amino acid, in the 5th epidermal growth factor (EGF)-like domain of the CRB1 protein. The mutational analysis of the entire coding region and promoter sequences of the *CRB1* gene failed to detect any additional mutation. The ophthalmological investigations of these affected members in family M-489 showed an LCA pattern defined by congenital blindness, nystagmus, extinguished ERG, and the presence of Franceschetti sign in both patients (table 5).

Six out of the seven rare sequence variants identified in this study have been previously reported in Leber congenital amaurosis patients.[26] Arg769His and Arg1331His were identified by Lotery *et al*[26] in control chromosomes and we were able to exclude cosegregation with the disease phenotype in our families. The lack of cosegregation has also been shown in the three synonymous codon changes (Leu470Leu, Asn549Asn, and Asn1057Asn) previously reported in LCA probands, indicating that these substitutions are not pathogenic. Two non-conservative amino acid changes (Thr289Met and Gln679Glu) were identified in Spanish ARRP patients. Thr289Met was initially considered to be related to LCA by Lotery *et al*,[26] who found this change in an affected proband. Nevertheless, we regard this change as non-pathogenic in line with the family studies that indicate a lack of cosegregation in the Spanish ARRP family (data not shown). A similar conclusion can be drawn for Gln679Glu, a previously unreported sequence variant identified in the present study.

Four intronic single nucleotide polymorphisms were found among the study participants. The most common change, located 12 bp upstream from the start of exon 2, has been previously reported to be identically distributed among the alleles of LCA probands and controls.[26] Similar results were obtained in our Spanish population. Analysis of the three new variants indicates that they represent polymorphisms in the human population, suggesting no particular relationship to retinal degeneration.

In conclusion, the data reported here provide (1) strong evidence against a direct involvement of the *RGR* and *CRBP1* genes in our set of Spanish autosomal recessive retinitis pigmentosa families and (2) data to establish the implication of the *CRB1* gene in the development of different types of retinal degeneration. The wide range of phenotypes associated with *CRB1* mutations underlines how the relationship between pathogenic mutations and disease phenotype is becoming increasingly complex. Further molecular and biochemical studies to elucidate the function of this protein will help us to define the events that result in blindness and will provide insights into the physiology of vision.

ACKNOWLEDGEMENTS

This work was supported by Fondo de Investigación Sanitaria (PI020052), Fundaluce (Fundación de lucha contra la ceguera), and ONCE (Organización Nacional de Ciegos de España). The authors belong to the "Grupo Multicéntrico Español para el Estudio de RP".

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4.3. ESTUDI DEL GEN USH2A

4.3.1. " Prevalence of 2314delG mutation in Spanish patients with Usher syndrome type II (USH2)".

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Ophtalmic Genetics 2000; 21:132-128

La síndrome d'Usher és un desordre genètic que s'hereta seguint un patró autosòmic recessiu. En els últims anys, en aquesta patologia s'ha determinat l'existència d'heterogeneïtat genètica i clínica, augmentant la seva complexitat a nivell molecular. La identificació d'una mutació causant de la patologia que expliqui un percentatge elevat de casos és molt important en aquest tipus de malalties heterogènies. En el cas de la síndrome d'Usher tipus II, entre el 74-90% dels casos presenten lligament al locus USH2A, la detecció de la mutació c.2299delG en el gen USH2A (prèviament definida com 2413delG) explica un percentatge important de casos, que varia segons l'origen de la població estudiada (Eudy i col.,1998; Dreyer i col., 2000; Weston i col., 2000). En aquest treball en col·laboració amb altres centres de l'Estat Espanyol, s'ha determinat la presència de les delecions descrites (c.2299delG, c.2898delG i c.4338-9delCT) i la seva freqüència en la síndrome d'Usher tipus II en famílies amb origen espanyol.

Ophthalmic Genetics 1381-6810/00/
US$ 15.00

Ophthalmic Genetics – 2000, Vol. 21, No. 2, pp. 123-128

Accepted 21 April 2000

Prevalence of 2314delG mutation in Spanish patients with Usher syndrome type II (USH2)

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Abstract The Usher syndrome (USH) is a group of autosomal recessive diseases characterized by congenital sensorineural hearing loss and retinitis pigmentosa. Three clinically distinct forms of Usher syndrome have so far been recognized and can be distinguished from one another by assessing auditory and vestibular function. Usher syndrome type II (USH2) patients have congenital moderate-to-severe nonprogressive hearing loss, retinitis pigmentosa, and normal vestibular function. Genetic linkage studies have revealed genetic heterogeneity among the three types of USH, with the majority of USH2 families showing linkage to the USH2A locus in 1q41. The USH2A gene (MIM 276901) has been identified: three mutations, 2314delG, 2913delG, and 4353-

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Acknowledgements: The authors would like to thank the Fondo de Investigaciones Sanitarias (FISS nº 98/0338), the ONCE, and the Fundación ONCE for financial support. We are also grateful to the participating patients and their family members, and to the FAARPEE for their help and cooperation.

54delC, were initially reported in USH2A patients, the most frequent of which is the 2314delG mutation. It has been reported that this mutation can give rise to typical and atypical USH2 phenotypes. USH2 cases represent 62% of all USH cases in the Spanish population, and 95% of these cases have provided evidence of linkage to the USH2A locus. In the present study, the three reported mutations were analyzed in 59 Spanish families with a diagnosis of USH type II. The 2314delG was the only mutation identified in our population: it was detected in 25% of families and 16% of USH2 chromosomes analyzed. This study attempts to estimate the prevalence of this common mutation in a homogeneous Spanish population.

Key words Usher syndrome type II; USH2A gene mutations; 2314delG mutation

Introduction The Usher syndrome (USH) is a hereditary disorder that combines congenital sensorineural hearing loss, retinitis pigmentosa (RP), and variable vestibular symptoms. This syndrome is the most frequent cause of combined deafness and blindness in adults. The prevalence of USH among children born with hearing impairment is estimated at 3-6%. This condition is both phenotypically and genotypically heterogeneous. Three clinical forms of USH distinguishable by the severity of the hearing loss and the extent of the vestibular involvement have been reported.[1] Type I (USH1) presents profound congenital deafness and vestibular dysfunction. In type II (USH2), the hearing loss is moderate-to-severe and nonprogressive, with normal vestibular function. Finally, type III (USH3) is characterized by a progressive sensorineural hearing loss and variable vestibular symptoms.[2,3]

The genetic heterogeneity of the USH syndrome is demonstrated by the presence of at least eight distinct genetic loci. About 90-95% of the families with Usher type II show linkage to chromosome 1q32-q41 (USH2A).[4-7] Recently, a novel locus for USH2 (USH2B) was mapped to chromosome 3p23-p24.2 in a Tunisian consanguineous family.[8]

The gene responsible for USH2A has been identified. This gene expresses a putative extracellular matrix protein. Moreover, three different mutations (2314delG, 2913delG, and 4353-54delC) which cause a frameshift with premature stop codon have been identified exclusively in patients with USH2A.[9] The most frequent is the 2314delG mutation, which was detected by Eudy et al.[9] in individuals mainly from northern Europe; one of the remaining patients, homozygous for the 2314delG mutation, was from Spain. Recently, Liu et al.[10] reported a higher prevalence and phenotypic variability of the 2314delG mutation.

USH type II is believed to be the most common of the three types of Usher syndrome. It accounts for 62% of all USH cases in the Spanish population. This study reports the results of linkage analysis and screening of the three mutations listed above in individuals with USH type II from several regions of Spain.

Materials and methods

SUBJECTS This study includes 59 families with at least one USH type II-affected individual, referred to us from different Spanish regions. The diagnosis of USH2 was established on the basis of the clinical data obtained from ophthalmic and audiovestibular examination. The family history and any potential nongenetic causes of hearing impairment were carefully evaluated. Patients who seemed to have atypical USH2 were not included in this study.

DNA ANALYSIS DNA from each affected patient and from several nonaffected family members was extracted from peripheral blood leukocytes. Of the 59 families with USH2 analyzed in this study, 18 were subjected to haplotype and linkage analysis. The remaining 41 USH2 families were not subjected to segregation analysis because of the small size of the family or because a blood sample was collected only from the affected individual.

Haplotype and linkage analysis Linkage analysis was performed before refinement of the critical region and identification of the USH2A gene by Eudy et al.[9] Polymorphic markers surrounding the USH2A locus (D1S217, D1S419, D1S237, D1S474, and D1S229) on chromosome 1q[7,11,12] were used. Haplotype identity between individuals who share the 2314delG mutation were analyzed using markers D1S237 and AFM 114XF2.[13] The PCR products were electrophoresed through 8-12% polyacrylamide gel and stained with silver. Pairwise and multipoint linkage analysis were performed using the MLINK and LINKMAP subroutines of version 5.1 of the LINKAGE program.[14] Penetrance was assumed to be 0.99 and the mode of inheritance was assumed to be autosomal recessive.

Mutation detection A search for the three known mutations (2314delG, 2913delG, and 4353-54delCT) was carried out in all the available affected subjects from the 59 families. DNA was amplified by PCR using the primers reported by Eudy et al.,[9] except for the 2314delG mutation since the primer pairs indicated by these authors yield too large a PCR product to be correctly studied by SSCP. The following primers were used to amplify products for 2314delG mutation detection: direct 5'GAACAAATTCTGCAATCCTC3' and reverse 5'ACTGCCCTGTCTTAGC ATTA3', which yield a fragment of 179bp. These PCR primer pairs were designed using the program Primer3 Old Version (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The single-strand conformational polymorphism (SSCP) assay was carried out on 12% nondenaturing polyacrylamide gels. Samples showing an anomalous pattern of mobility in SSCP were sequenced according to the fluorescent dideoxy terminator method and analyzed using an ABIPRISM DNA sequencer.

Results and discussion

In 17 (94.4%) of 18 USH type II families, linkage analysis with the five above-listed polymorphic markers provided evidence of cosegregation of the disease with the USH2A

locus. For the 1q-linked cases, the markers D1S237 and D1S474 showed significant maximum lod scores (5.945 and 4.890, respectively) with the USH2 disease locus at zero recombination distance. Subsequent refinement of the USH2A region[7,12,13] allowed us to use closer markers and to improve reliability of indirect genetic testing in our set of USH2 families. Thus, segregation studies with the marker AFM144XF2 showed complete cosegregation between the analyzed marker and the disease phenotype in two informative families showing obligate recombinant events with the most distal marker on 1q (D1S229). We have not observed any linkage disequilibrium between USH2 phenotype and polymorphic markers used in linkage analysis. The low genetic heterogeneity associated with this form of the syndrome indicates that >90% of all Spanish patients with Usher syndrome type II are presumed to carry USH2A mutations, and emphasizes the importance of mutational analysis in USH type II cases.

Of the three mutations investigated in this study, only the 2314delG mutation was identified in our population. It was detected in 15 of 59 families (25%). Four unrelated probands, three of them belonging to consanguineous families, presented this deletion in a homozygous state; the remaining 11 patients were identified as heterozygous for the 2314delG mutation. Our data reveal that 19 (16%) of the 118 USH2 chromosomes analyzed presented the 2314delG mutation. Neither the 2913delG nor the 4353-54delCT mutation was identified in any of the patients in our series. There was a similar distribution of the 2314delG mutation between the different regions of Spain. Non-common alleles of the markers D1S237 and AFM114XF2 on the 2314delG chromosome were obtained. Most haplotypes were also found in the control population. Previous studies with closely linked genetic markers have failed to reveal haplotype identity over a much shorter genomic distance,[9,10] suggesting that this mutation has arised independently in different populations. Nevertheless, to obtain suitable data from haplotype-sharing analysis within and between populations, further observations with intragenic markers in no-2314delG and mutated chromosomes from larger populations are necessary.

The percentage of families and alleles with the 2314delG mutation in our series as revealed by SSCP analysis is similar to that reported by Eudy et al.[9] using heteroduplex analysis. Those authors detected the 2314delG in 21 (22%) of the 96 Usher type IIa patients, eight of whom were identified as homozygous for the mutation. With regard to the remaining two mutations investigated, Eudy et al. identified each of them in only one heterozygous USH2A patient. Recently, Liu et al.[10] reported a higher prevalence of the 2314delG mutation in 23 families with typical and atypical USH, from the United Kingdom and China, who were screened by combined SSCP/heteroduplex analysis. Those authors identified the 2314delG mutation in 12 (52%) of the families studied, eight (62%) of 13 cases with typical UHS2, and four (40%) of 10 families with atypical USH. Atypical USH was described by Liu et al.[10] as a condition consisting of bilateral sensorineural progressive hearing loss, variable vestibular problems, and retinitis pigmentosa. Patients belonging to this class could be classified as USH3 on the basis of the clinical findings reported by Pakarinen et al.[3] USH type III

seems to be especially frequent in Finland, but is considered a rare clinical entity in other populations. Thus, to investigate the three reported mutations in the USH2A gene, we only analyzed Spanish patients with typical USH2 findings. The frequency of the 2314delG mutation in our ethnically homogeneous Spanish population is similar to that obtained by Eudy et al.[9] in a heterogeneous population. However, a higher level of detection for this mutation was reported by Liu et al.[10] in patients with a typical USH phenotype living in the United Kingdom. The DNA tested for these mutations in the USH2A gene accounted for 18% of the ORF;[10] therefore, while the prevalence of other mutations in the whole gene sequence could be reported, it appears that the 2314delG mutation is the most frequent USH2A gene mutation in the different populations.

Although lower frequencies were initially reported, Usher type II accounts for 62% of the USH cases in Spain. In our series of families with Usher syndrome, Usher type I represented more than half of the remaining non-USH2 patients, while unclassified patients accounted for ~13% of all USH cases.[15] While USH1B subtype made up only 67% of USH1 cases in our population[16] and a wide spectrum of MYO7A mutations have been detected in Spanish Usher Type I families,[17-19] data reported here support the low genetic heterogeneity and high frequency of 2314delG in USH2 patients from different origin, and emphasize the significance of this mutation for further management of USH2 patients, especially in those areas related with genetic counselling. Furthermore, on the basis of phenotypic differences between patients with the 2314delG mutation, as Liu et al.[10] have reported, the screening of this mutation could provide information for an accurate diagnosis and genetic counselling in our unclassified USH families, and may be helpful in elucidating the physiological and pathological role of the protein expressed by the USH2A gene.

Note: The previously reported mutations by Eudy et al. (1998) have been subjected to a change of nomenclature, as Weston et al. have recently published (Am J Hum Genet 2000;66:1199-1210). Thus the 2314delG mutation has become 2299delG.

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4.3.2. " Mutations in USH2A in Spanish patients with autosomal recessive retinitis pigmentosa: high prevalence and phenotypic variation".

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Journal of Medical Genetics 2003; 40: e8 (online mutation report)

En els estudis inicials, es va descriure el gen USH2A com el responsable d'un elevat número de casos afectats amb la síndrome d'Usher tipus II. En un estudi posterior amb famílies amb RPAR, es va identificar en el gen USH2A la mutació de canvi de sentit p.C759F en un 4.5% dels casos analitzats. Per primera vegada, s'associava el gen USH2A a dos fenotips diferents, mostrant una vegada més l'heterogeneïtat genètica característica de la RP. Per aquest motiu, en aquest treball es va plantejar determinar la prevalença d'aquesta mutació p.C759F en població espanyola afectada de RPAR i avaluar el seu compromís amb la patogènia de la RP no sindròmica.

ONLINE MUTATION REPORT

Mutations in *USH2A* in Spanish patients with autosomal recessive retinitis pigmentosa: high prevalence and phenotypic variation

S Bernal, C Ayuso, G Antiñolo, A Gimenez, S Borrego, M J Trujillo, I Marcos, M Calaf, E Del Rio, M Baiget

J Med Genet 2003;**40**:e8(http://www.jmedgenet.com/cgi/content/full/40/1/e8)

Retinitis pigmentosa (RP), which occurs in about one in 3000-7000 people in Spain, is inherited in an autosomal dominant manner in 12% of cases, in an autosomal recessive way in 39%, and in an X linked manner in 4% of cases. This leaves 41% of RP cases with a simplex form and 4% in which the transmission pattern is unclear.[1]

Direct analyses of rhodopsin, the alpha and gamma subunits of rod cGMP-phosphodiesterase, periferin/RDS, rod outer segment membrane protein, recoverin, guanilate cyclase activating protein, S antigen, interstitial retinol binding protein, and NRL have failed to detect any disease causing mutation in non-syndromic ARRP Spanish families. Mutations in the beta subunit of the rod cGMP-phosphodiesterase gene,[2–5] in the ATP binding cassette receptor gene,[6] and in the *TULP1* gene[7] account for a small percentage of Spanish ARRP families. These data indicate that genes other than these may be involved in the remaining families, emphasising the genetic heterogeneity of the disease and reinforcing the hypothesis that in ARRP a number of genes rather than one major gene will account individually for a small number of cases.

The recent report that a missense mutation in the *USH2A* gene (C759F) is present in 4.5% of patients with non-syndromic ARRP[8] prompted us to analyse the involvement of this mutation in a large set of Spanish ARRP families. A complete mutational analysis of the coding region of the *USH2A* gene was performed in all cases in which the C759F allele was found. Additional mutations were identified in the *USH2A* gene in non-syndromic ARRP patients. Interestingly, two C759F homozygotes belonging to a consanguineous ARRP family had no RP symptoms and no hearing impairment.

Key points

- The *USH2A* gene has been shown to be associated with Usher syndrome type II and recent data indicate that mutations in this gene could also cause non-syndromic autosomal recessive retinitis pigmentosa (ARRP). We have screened our panel of 196 unrelated ARRP patients for the presence of the C759F missense mutation in the *USH2A* gene.
- We have identified compound heterozygotes with C759F and nonsense, splicing, or missense mutations in the *USH2A* gene that manifest as recessive retinitis pigmentosa without hearing loss. We found four C759F homozygotes belonging to two different consanguineous families; two of them were non-syndromic RP affected patients while the remaining two had no RP symptoms and no hearing impairment.
- When considering the information presented here together with that in previous reports, a picture emerges of considerable phenotypic heterogeneity arising from mutations in *USH2A* which ranges from distinct types of Usher syndrome, through non-syndromic retinitis pigmentosa, to unaffected subjects. The wide range of phenotypes associated with usherin mutations underlines how the relationship between pathogenetic mutations and disease phenotype is becoming increasingly complex.

PATIENTS AND METHODS

A group of 196 unrelated ARRP patients plus four cases of retinitis punctata albescens were studied. The patients were diagnosed at the Hospital de la Santa Creu I Sant Pau (Barcelona) at the Fundación Jiménez Diaz (Madrid) or at the Hospital Virgen del Rocio (Sevilla). The clinical diagnosis of RP was based on ophthalmological examination including measurements of visual acuity, ophthalmoscopy, dark adaptation, perimetry, and electroretinogram amplitudes.

One hundred blood donors (200 chromosomes) were used as controls to evaluate the frequency of the mutations and polymorphisms found in the patient sample. Blood samples were obtained after informed consent was given and genomic DNA extracted from these blood samples was used to assess the involvement of the C759F mutation in the *USH2A* gene. Intronic PCR primers flanking exon 13[9] were used for PCR amplification and direct sequencing. All PCR reactions were carried out on a PTC-200 Peltier Thermal Cycler. The PCR products were sequenced using the Thermosequenase Primer Cycle sequencing Kit 7-deaza dGTP (Amersham Pharmacia Biotech UK limited, Buckinghamshire, UK). The sequences were analysed on an automated sequencer Li-Cor DNA Analyser Gene Reader 4200 (Li-Cor Inc, Lincoln, NE, USA).

In all cases in which a C759F allele was found, a mutational analysis of the *USH2A* gene coding region was performed. Primers were designed from the consensus intronic sequences (GenBank accession numbers AF091973-AF091889) of the *USH2A* gene as described previously.[9] PCR products were sequenced as described above.

To establish the haplotype for the C759F alleles, six single nucleotide polymorphisms within the *USH2A* gene, exons 2-21, were selected as previously described.[10] Haplotype analysis was performed on DNA samples from all members of the six families depicted in fig 1. The establishment of haplotypes enabled us to exclude the existence of large genomic deletions in the *USH2A* gene in the affected RP patients in these families.

RESULTS

In the group of 196 ARRP patients analysed, we identified one homozygote and eight carriers of the C759F (TGC-TTC) missense mutation.

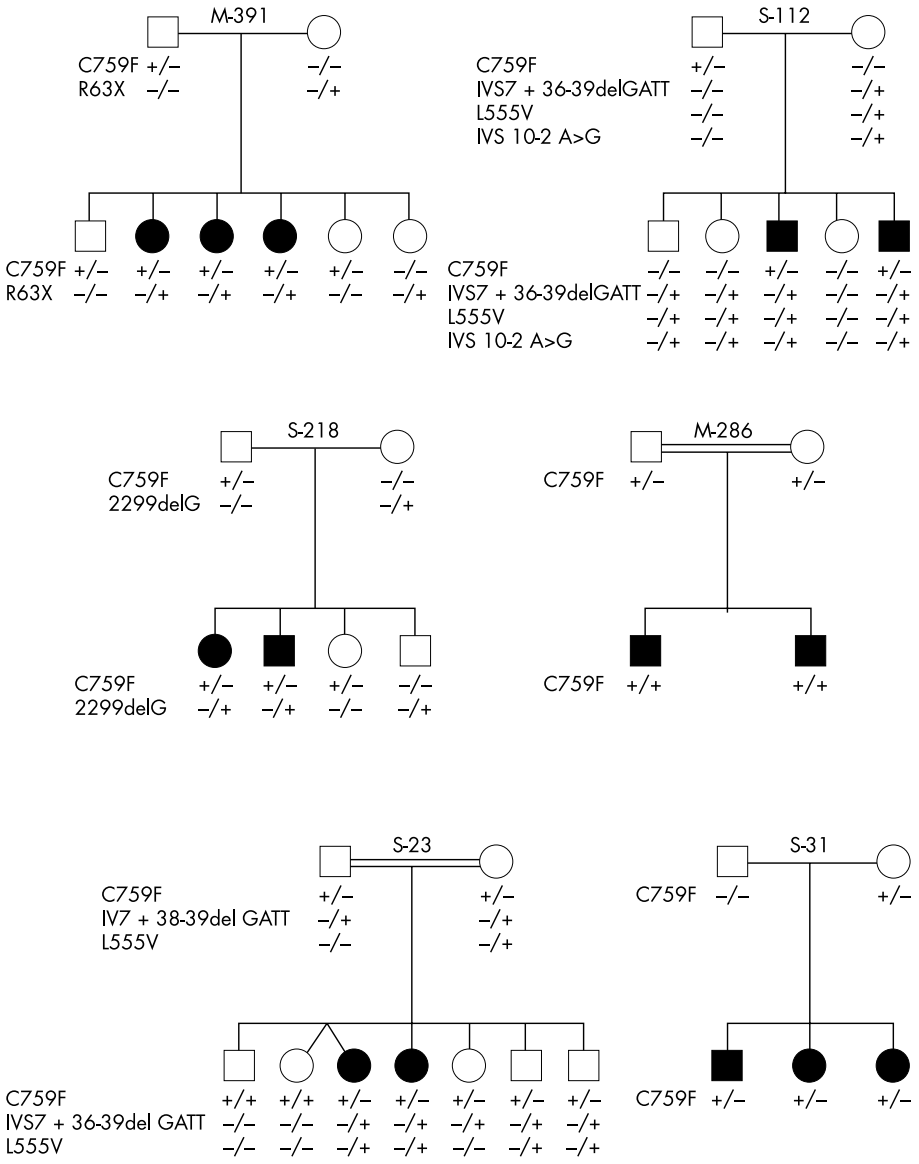

Figure 1 Pedigrees of ARRP families with mutations in the *USH2A* gene.

In three out of eight heterozygotes, no family members were available for study. In the remaining cases (five carriers and one homozygous patient), the screening for this mutation was extended to other family members and we identified 21 additional carriers (seven were RP affected patients and 14 were unaffected members of the families) and three additional homozygous cases: one case was the ARRP affected brother of the previous homozygote (M-286) and in another family (S-23) two were unaffected subjects (fig 1).

A complete mutation study of the coding regions of the *USH2A* gene was performed in (1) one ARRP patient from each of the six families depicted in fig 1, (2) two unaffected ARRP subjects homozygous for C759F from family S-23, and (3) the three ARRP patients who were heterozygous for C759F with no family members available for study.

In family M-391, in which six subjects were carriers of the C759F mutation, we identified the second mutated allele in the three ARRP affected daughters. The C to T transition at nucleotide 187 leads to a premature stop codon at position 63 (R63X). One healthy sister was heterozygous for this mutation and the two remaining unaffected sibs were heterozygous for C759F. In order to assess hearing status, the ARRP patients underwent pure tone audiometry which showed a hearing acuity within the normal range.

In family S-112, the father and the two affected RP brothers were heterozygous for C759F. We identified three variations in one maternal allele: a previously described polymorphism (IVS7 + 36-39del gatt), a replacement of a leucine residue by a valine in codon 555 owing to a C to G transversion at position 1663 in exon 10 (L555V), and an IVS10 –2 A>G change affecting the acceptor splice site of intron 10. This maternal chromosome was inherited along with the C759F mutation in the two affected RP patients. Two unaffected sibs carried the maternal mutant allele only, whereas the remaining healthy sister had no mutations in the *USH2A* gene.

In family S-218, the affected RP sibs were compound heterozygotes. They had inherited a paternal C759F allele and a maternal 2299delG mutated allele. Two unaffected brothers were heterozygous for these mutations, respectively.

C759F was found in a homozygous state in two affected RP members (aged 50 and 54) of a consanguineous family (M-286). Upon clinical examination and pure tone audiometry, they were found to have hearing acuity within the normal range for their age.

S-23 is also a consanguineous family with two RP affected females aged 44 and 41 years. These patients and two asymptomatic brothers, aged 35 and 27 years, showed an identical pattern: the C759F paternal allele and the maternal allele

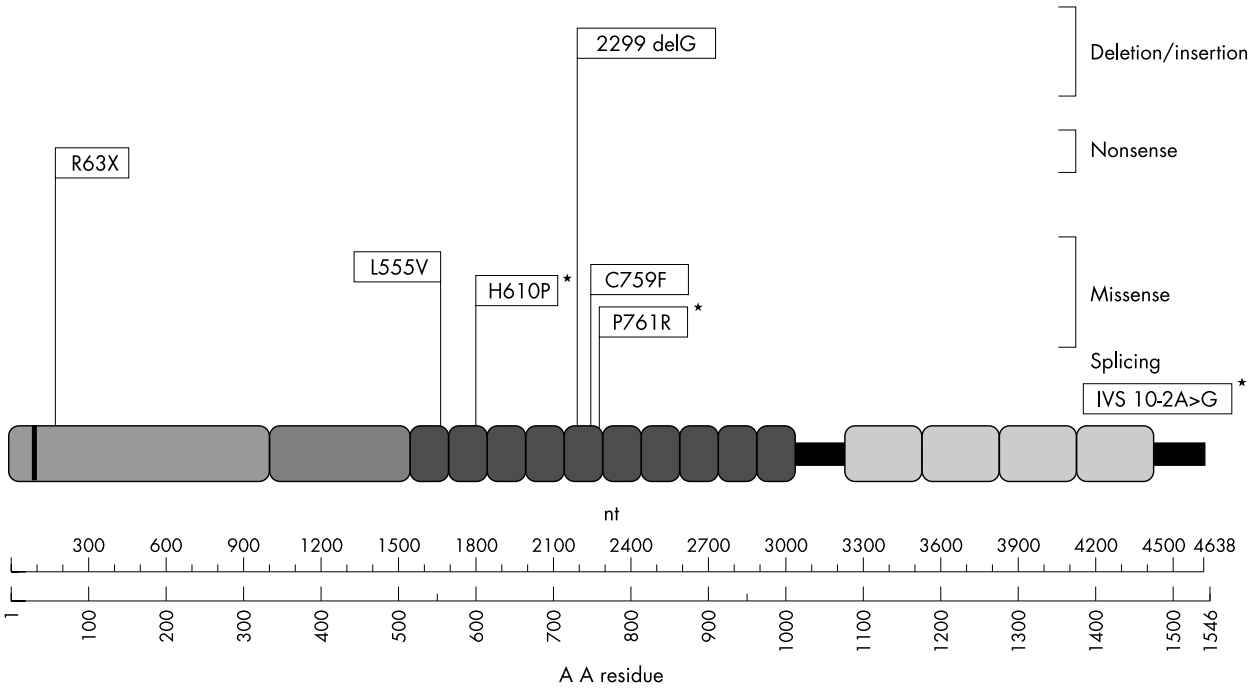

Figure 2 Schematic representation of the location within the usherin protein of the mutations identified in Spanish ARRP patients. *Indicates novel mutations.

containing the missense mutation (L555V) and the polymorphism (IVS7 + 36-39del gatt) identified in family S-112. A healthy C759F heterozygous female aged 39 also carried the intron 7 polymorphism, whereas two other unaffected sibs, aged 45 and 44 years, in whom neither L555V nor the intron 7 polymorphism was detected, were C759F homozygotes. A thorough otological and ophthalmological investigation of these two subjects ruled out RP symptoms and hearing impairment. We resampled and reanalysed all the members of this family to exclude the possibility of sample mix up.

In family S-31 in whom the three RP affected sibs were heterozygous for the C759F mutation, the analysis of the coding regions of the *USH2A* gene failed to detect any additional mutation.

The mutational analysis of the *USH2A* gene in the three ARRP patients, who were carriers of the C759F mutation and had no family members available for study, allowed the identification of the second mutated allele in one patient, a replacement of a histidine residue by a proline (codon 610) owing to a A to C transversion at position 1829 in exon 10.

During the course of the study, a novel missense mutation in exon 13, P761R, was identified in an ARRP patient. Mutational analysis of the remaining exons failed to detect any other changes.

The locations within the usherin protein of the identified mutations in our ARRP patients are indicated in fig 2.

We identified two alleles with the C759F mutation in a panel of 200 control chromosomes. The missense mutations L555V and P761R were not found whereas a frequent polymorphism (IVS7+36-39 del gatt) was present in 4% of the alleles.

Haplotype analysis within the *USH2A* gene was performed on all family members in fig 1. Only one haplotype was found to be associated with the C759F mutation. This was the only haplotype found in the four C759F homozygotes, as expected. The remaining affected ARRP patients carried two different haplotypes and were heterozygous for at least two out of six SNPs. Thus, the presence of a large deletion can be excluded.

DISCUSSION

Since the identification of the *USH2A* gene,[11] a number of authors have screened the complete gene in Usher II patients from different ethnic backgrounds.[9 12–15] The results obtained illustrate the high frequency of mutations in the *USH2A* gene in patients meeting the clinical criteria for Usher type II as well as in cases with an atypical USH phenotype.[12 13]

Recently, Rivolta *et al*[8] indicated that mutations in the *USH2A* gene could cause a significant proportion of non-syndromic recessive RP given that the C759F allele by itself accounts for approximately 4.5% of the 224 ARRP cases included in the study. We screened our panel of 196 unrelated ARRP patients for the presence of the C759F allele and we found the mutation in nine cases. The frequency of C759F in Spanish patients (4.6%) is thus similar to that reported in patients from North America.[8]

When investigating the second mutated allele in carriers of C759F, six additional mutations were identified, three of which have not been reported previously (fig 2). The mutations that led directly or indirectly to premature termination of translation, resulting in the disruption of the function of the USH2A protein, were (1) the nonsense mutation R63X, which occurs at a CpG dinucleotide, (2) the deletion 2299delG, which most commonly causes Usher syndrome type IIa, and (3) a novel splicing mutation (IVS10 –2 A>G), which alters the acceptor splice site of intron 10.

The missense mutations identified, two previously described (L555V and C759F) and two novel (H610P and P761R), affect the laminin type epidermal growth factor-like (LE) domains of the protein. LE domains have been shown to be folded protein modules containing looped structures stabilised by disulphide bridges. Substitutions of cysteine residues involved in these structures such as C759F will result in abnormal folding of the LE domain, affecting the properties of the protein. As for the H610 P mutation in exon 10, the replacement of a basic residue by a neutral and hydrophobic amino acid probably has an effect on the function of usherin. A similar conclusion can be drawn for the P761R mutation affecting the same LE domain as C759F, where a conserved

and neutral residue is replaced by a basic amino acid. The L555V missense mutation replaces a leucine by a valine residue; both residues are neutral amino acids located in the first LE domain and the effect, if any, of this substitution on the structure and/or function of the protein remains to be explored.

Rivolta *et al*[8] identified two non-syndromic ARRP patients who were compound heterozygotes with C759F and previously reported frameshift mutations, which indicates that the frameshifts do not cause Usher type II, but only non-syndromic RP if they are inherited together with the missense change C759F. We can confirm this assumption because in Spanish patients additional compound heterozygotes with C759F and nonsense, splicing, or missense mutations are associated with identical phenotypic features, reinforcing the hypothesis that mutations in the *UHS2A* gene can result in ARRP without hearing loss.

The four C759F homozygotes found in the present study belonged to two families, M-286 and S-23 (fig 1). M-286 was a consanguineous family in which the two RP sibs were homozygotes for the C759F mutation. Upon clinical examination and pure tone audiometry, they were found to have hearing acuity within the normal range for age. More interestingly, S-23 was also a consanguineous family with two RP affected females. These patients as well as three asymptomatic sibs were carriers for the C759F mutation whereas two additional unaffected sibs were C759F homozygotes. A thorough otological and ophthalmological investigation of these two subjects ruled out RP symptoms and hearing impairment, which may indicate that homozygosity for C759F does not cause disease.

USH2A has been shown to harbour mutations causing not only Usher syndrome type II,[9 11–15] but also atypical Usher syndrome[12 13] and non-syndromic ARRP.[8] When the data reported here are considered, the phenotypic variation arising from different mutations in *USH2A* becomes extraordinarily pronounced, ranging from distinct types of Usher syndrome, through non-syndromic RP, to unaffected subjects.

There are many instances where different mutations in the same gene can result in diverse phenotypes. The *USH2A* gene is another example of such phenotypic variation of a Mendelian condition. In this work, we have described the presence of the missense C759F mutation associated with extreme phenotypic variation among unrelated subjects, that is, the symptomatic homozygous cases of family M-286 *v* the asymptomatic homozygous subjects from family S-23. A particularly striking example of phenotypic heterogeneity related to the *USH2A* gene is illustrated by the case of monozygotic twins who were homozygous for 2299delG.[12]

The possible mechanisms involved in this phenotypic variation are usually presented as assumptions or speculations and only exceptionally rely on proven data. One such exception has been the finding of triallelic inheritance in the Bardet-Biedl syndrome (BBS).[16] BBS is a genetically heterogeneous disorder characterised by multiple clinical features, including pigmentary retinal dystrophy, which is considered to be an autosomal recessive condition. The authors reported the presence of three mutant alleles of the *BB2* and *BB6* genes in affected subjects. They also detected unaffected subjects who carried two *BBS2* mutations but no *BBS6* mutation, suggesting that BBS may not be a single gene recessive disease but a complex trait requiring three mutant alleles to manifest the phenotype. Based on this model, we can speculate that a similar phenomenon may underlie the extremely different phenotypes of the C759F homozygotes reported in the present work. These symptomatic homozygous patients would carry a third mutated allele of an unidentified gene whereas the asymptomatic homozygotes would lack the third abnormal allele required to manifest the disease. In line with the BBS model, C759F heterozygous symptomatic patients would carry two additional mutated alleles of the unidentified gene.

The wide range of phenotypes associated with usherin mutations underlines how the relationship between pathogenetic mutations and disease phenotype is becoming increasingly complex, rendering the Mendelian concept of monofactorial disease causation increasingly untenable for a growing number of diseases. Further molecular and biochemical studies to elucidate the function of this protein in the development and maintenance of the retina and cochlea will help to define the events that result in deafness and/or blindness and will provide insights into the physiology of vision and hearing.

ACKNOWLEDGEMENTS

This work was supported by grants from FIS (99-0010-03), ONCE/Fundación ONCE, and Fundaluce. The authors belong to Grupo Multicéntrico Español para el Estudio de RP. We are grateful to Orland Diez for his helpful comments.

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4.4. ESTUDI DEL GEN USH2A EN LA RPAR NO SINDRÒMICA I EN MALALTS AMB LA SÍNDROME D'USHER TIPUS II

En aquest apartat es presenten els resultats de l'anàlisi molecular del gen USH2A en grups de pacients amb fenotips diferents: RP no sindròmica amb un patró d'herència autosòmic recessiu (RPAR), retinosi punctata albescens (RPA) i síndrome d'Usher tipus II.

Pacients

Un grup de 85 famílies afectades de RPAR, 4 amb retinosi punctata albescens i 27 famílies amb almenys un membre afectat amb la síndrome d'Usher tipus II han estat seleccionades per l'estudi molecular del gen USH2A.

El diagnòstic clínic de la RP s'ha basat en un estudi oftalmològic exhaustiu, seguint els criteris establerts pels protocols ISCEV (Marmor i Zrenner, 1998-99). Aquests estudis clínics s'han efectuat en els Servei d'Oftalmologia de l'Hospital de la Santa Creu i Sant Pau (Barcelona) i de la Fundación Jiménez Díaz (Madrid). El diagnòstic de la síndrome d'Usher tipus II s'ha efectuat en els Serveis d'Oftalmologia i d'Otorrinolaringologia dels mateixos hospitals.

Mètodes

L'obtenció del DNA a partir de leucòcits de sang perifèrica dels diferents pacients s'ha realitzat seguint el mètode d'extracció salina (Miller i col., 1988). La regió codificant del gen USH2A està dividida en 21 exons. L'amplificació dels diferents exons junt amb les regions intròniques que els delimiten es realitza per la tècnica de la PCR, un total de 24 fragments són analitzats. Per les diferents amplificacions es van utilitzar les parelles de encebadors descrites per Weston i col. (2000). Únicament, els exons 2 i 13 van ser dividits en diversos fragments solapats degut al seu tamany. Els encebadors dissenyats pels diferents fragments que inclouen la regió codificant dels exons 2 i 13 són combinats amb l'ús dels encebadors intrònics ja descrits per Weston i col. (2000) (Taula 5). Els programes dissenyats per l'amplificació dels diferents fragments que inclouen tot l'exó 13 i l'exó 2 són exposats en la taula 6 i en la taula 7, respectivament.

Després de l'amplificació dels diferents fragments es procedeix a la búsqueda de mutacions per la tècnica d'anàlisi de polimorfismes en la conformació de cadenes senzilles (SSCP, *single-strand conformation polymorphism*) en la que l'electroforesi vertical és sotmesa a dues condicions diferents, combinant segons el fragment analitzat la concentració d'acrilamida, la temperatura i el voltatge.

Els fragments que mostraven un patró de mobilitat anormal per la tècnica del SSCP eren directament seqüenciats en un seqüenciador automàtic model ABI Prism 3100 Avant.

Tots els casos en els que únicament era detectat un dels dos al·lels causants de la patologia eren sotmesos directament a seqüenciació automàtica de la restant regió codificant del gen USH2A per tal d'identificar el segon al·lel causant de la malaltia.

Resultats

L'anàlisi molecular del gen USH2A ha revelat la presència de 27 canvis diferents, incloent 13 mutacions patològiques (Taula 8) i 14 variants no associades a malaltia (Taula 9).

Les mutacions observades es classifiquen en 7 mutacions que impliquen canvis en la pauta de lectura, 3 sense sentit i 3 de canvi de sentit. La cosegregació amb la malaltia dins de cada família i l'absència en un grup control de 95 individus indiquen que les mutacions identificades en el gen USH2A segueixen els criteris de patogeneïtat.

Set de les 13 mutacions són descrites per primera vegada: dos mutacions sense sentit (p.Y506X i p.E1492X), i 5 mutacions que causen alteració en la pauta de lectura amb aparició d'un codó de parada prematur (c.545_6delAA; c.947_54dup8; c.1211_4delA; c.2135delC; Gran deleció). Sis d'aquestes mutacions eren identificades en el grup de pacients afectats amb la síndrome d'Usher tipus II i només una es corresponia amb un cas de RPAR no sindròmica.

En el grup de pacients amb la síndrome d'Usher tipus II s'han identificat 11 mutacions diferents. Les mutacions s'han detectat en 12 de les 27 famílies afectades amb la síndrome d'Usher tipus II, establint que les mutacions detectades expliquen el 44% dels pacients amb Usher II. En set d'aquestes 12 famílies van ser identificats els

dos al·lels mutats, mentre que en les 5 famílies restants només va ser identificada una mutació en estat heterozigot. En un total de 54 al·lels estudiats amb síndrome d'Usher tipus II es van detectar 19 al·lels mutats associats a malaltia (35%). En 4 dels 85 pacients amb RPAR no síndròmica van ser detectades 3 mutacions diferents. Tots aquests pacients presentaven la mutació en estat heterozigot. És important destacar que les 85 famílies amb RPAR seleccionades per aquest estudi havien estat prèviament determinades per ser normals per la mutació p.C759F. L'estudi va ser ampliat a nivell familiar en tots aquests pacients afectats amb RPAR no sindròmica (Figura 22) i amb síndrome d'Usher tipus II (Figura 23) per avaluar la cosegregació de la mutació detectada amb la malaltia.

Taula 5. Encebadors dissenyats per l'amplificació dels diferents fragments que inclouen els exons 2 i 13 del gen USH2A.

Fragments	Seqüència encebadors 5'->3'	Tamany (pb)
Exó 2		
2AF*	GCCTGGGATGAGCTTCAG	342
2AR	GGCAAAGATCAACATTTCAATGACC	
2BF	TGCTTTATCAGGAGGAGAATGC	375
2BR	TTCTTGTCTGGTGTGATGCAGC	
2CF	TGAAAGTATTCAGTTCTGTACC	349
2CR*	GGTTTGGAATTCAGGCTGA	
Exó 13		
13AF*	GCAGTAGCATTGTTTGTGTCTC	468
13AR	CTTATCACAGTTGCAAGGCACAC	
13BF	GAAGGGAGACAGTGCAATAAATG	439
13BR*	GTAGAAGCCACAAACCAGAAAC	

***** Descrits per Weston i col., 2000

Taula 6. Programes dissenyats per l'amplificació dels diferents fragments de l'exó 13 del gen USH2A.

Programes PCR		**Temperatura (ºC)**	**Temps**
Fragments 13A i 13B			
Desnaturalització		94	6 min.
30 cicles	Desnaturalització	94	30 seg.
	Hibridació	55	30 seg.
	extensió	72	2 min.
Extensió final		72	10 min.

Taula 7. Programes per l'amplificació dels tres fragments que formen l'exó 2 del gen USH2A. Cadascun dels tres fragments solapats presenten una temperatura d'hibridació diferent.

Programa PCR		**Temperatura (ºC)**	**Temps**
Fragments 2A, 2B, 2C			
Desnaturalització		94	6 min.
30 cicles	Desnaturalització	94	30 seg.
	Hibridació	57, 59, 50	30 seg.
	extensió	72	2 min.
Extensió final		72	10 min.

Taula 8. Caracterització dels 13 canvis patològics a nivell genòmic, de la proteïna i del fenotip associat identificat en aquest treball.

	nucleòtid alterat (cDNA)	exó	efecte proteïc	domini de la proteïna	nº al·leles	fenotip associat
Mutacions que alteren la pauta de lectura						
1	c.545_6delAA	3	p.K182fsX44	Tromoposdin-1(Tn)	1	RPAR
2	c.947_54dup8	6	p.Q316fsX22	LamN tipus VI	1	Usher II
3	c.1211_4delA	7	p.N405fsX2	LamN tipus VI	1	Usher II
4	c.2135delC	12	p.S712X	4^{art} EGF	1	Usher II
5	c.2299delG[a]	13	p.E767fsX20	5^{è} EGF	8	Usher II / RPAR
6	c.2431_2delAA[d]	13	p.K811fsX10	5^{è} EGF	1	Usher II
7	Gran deleció	9-14	-	-	2	Usher II
Mutacions sense sentit						
1	c.100 C>T[b]	2	p.R34X	Tromoposdin-1 (Tn)	1	Usher II
2	c.1518 T>A	8	p.Y506X	LamN tipus VI	1	Usher II
3	c.4474 G>T	21	p.E1492X	?	1	Usher II
Mutacions de canvi de sentit						
1	c.1434 G>C[c]	8	p.E478D	LamN tipus VI	1	Usher II
2	c.2137 G>C[b]	12	p.G713R	4art EGF	2	RPAR
3	c.2276 G>T[b]	13	p.C759F	5è EGF	3	Usher II

Mutacions descrites per: **a**. Eudy i col.,1998; **b**. Dreyer i col.,2000; **c**. Adato i col.,2000; **d**. Najera i col., 2002 .

(?) regió en l'extrem C-terminal de la proteïna usherina d'homologia desconeguda

Taula 9. Descripció de canvis no patològics en el gen USH2A, identificats tant en la RPAR com en la síndrome d'Usher tipus II.

nº canvis no patològics	nucleòtid alterat	codó afectat	exó	cosegregació familiar	autor
1	c.373 A/G	p.T125A	2	-	Weston i col., 2000
2	c.504 G/A	p.T168T	3	-	Dreyer i col., 2000
3	c.573 G/A	p.V191V	3	-	No descrit
4	IVS3-80 T/C	-	IVS3	-	Weston i col., 2000
5	c.688 G/A*	p.V230M	4	-	Dreyer i col., 2000
6	IVS7+36_9delgatt	-	IVS7	-	Weston i col., 2000
7	c.1419 C/T	p.T473T	8	-	Weston i col., 2000
8	IVS9+34 C/A	-	IVS9	-	Weston i col., 2000
9	c.1931 A/T	p.D644V	11	-	Weston i col., 2000
10	c.2109 T/C	p.D703D	12	-	Weston i col., 2000
11	c.2523 C/A	p.S841Y	13	-	No descrit
12	IVS15+35 A/G	-	IVS15	-	Weston i col., 2000
13	c.4371 G/A	p.S1457S	20	-	Weston i col., 2000
14	c.4457 A/G	p.K1486R	21	-	Weston i col., 2000

(*) Canvi descrit en la bibliografia com una mutació amb significat patològic.

Figura 22. Estudi a nivell familiar de la cosegregació de les mutacions identificades en el gen USH2A en pacients afectats de RPAR no sindròmica.

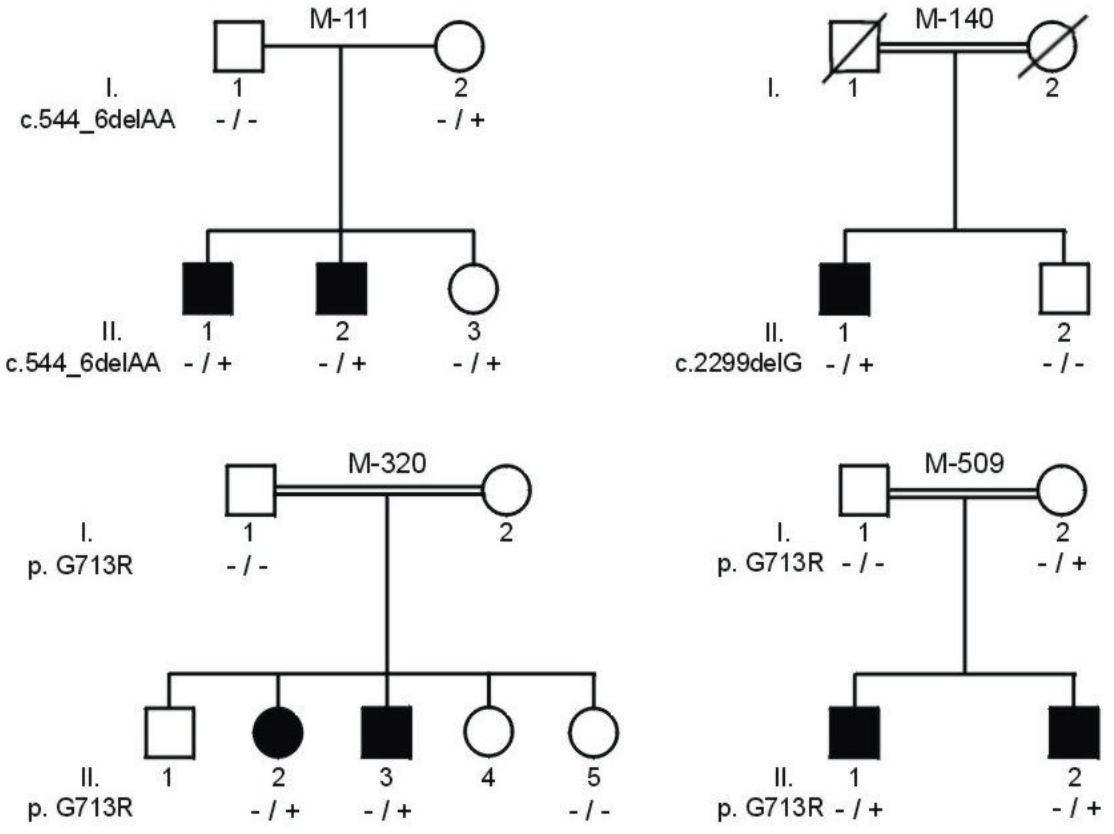
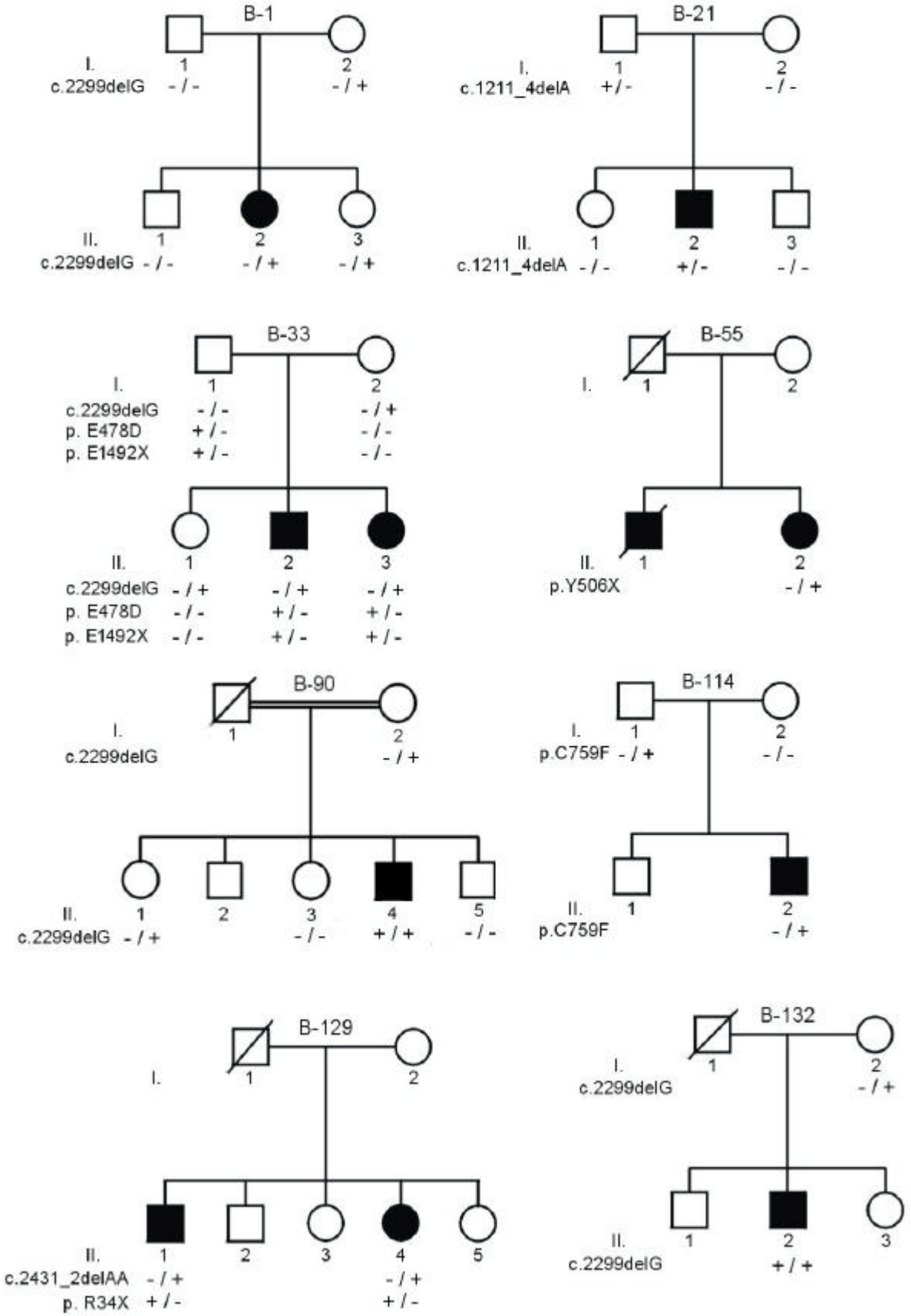
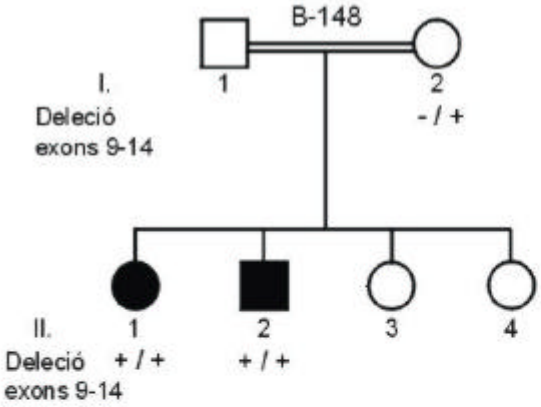
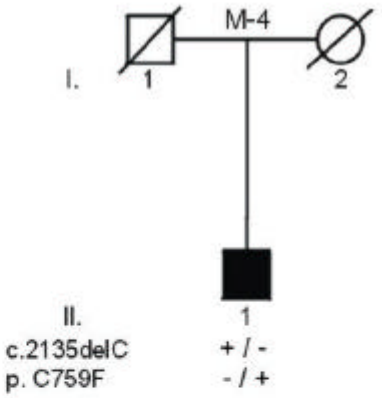


Figura 23. Cosegregació de les mutacions en el gen USH2A identificades en famílies afectades amb la Síndrome d'Usher tipus II.




B-148
I.
1
2
Deleció
exons 9-14
- / +
II.
1
2
3
4
Deleció + / +
exons 9-14
+ / +
+ / +


M-4
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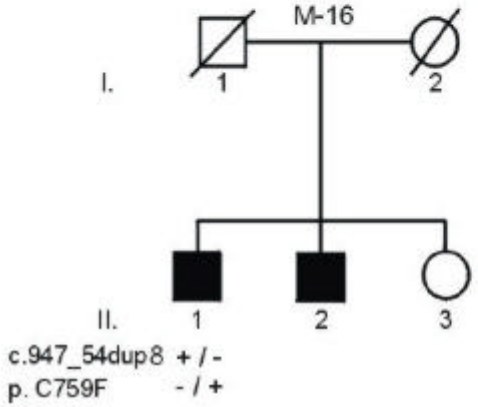
1
2
II.
1
c.2135delC
p. C759F
+ / -
- / +


M-16
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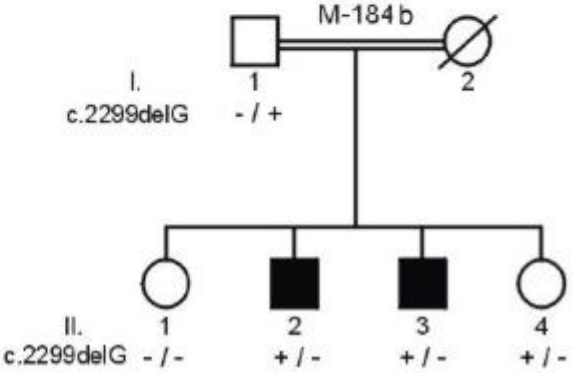
1
2
II.
1
2
3
c.947_54dup8 + / -
p. C759F
- / +


M-184 b
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1
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c.2299delG
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II.
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c.2299delG - / -
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