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Molecular Genetic Analysis of Two Functional Candidate Genes in the Autosomal Recessive Retinitis Pigmentosa, RP25, Locus

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ABSTRACT *Purpose*: To identify the disease gene in five Spanish families with autosomal recessive retinitis pigmentosa (arRP) linked to the RP25 locus. Two candidate genes, *EEF1A1* and *IMPG1*, were selected from the region between D6S280 and D6S1644 markers where the families are linked. The genes were selected as good candidates on the basis of their function, tissue expression pattern, and/or genetic data. *Methods*: A molecular genetic study was performed on DNA extracted from one parent and one affected member of each studied family. The coding exons, splice sites, and the 5′ UTR of the genes were amplified by polymerase chain reaction (PCR). For mutation detection, direct sequence analysis was performed using the ABI 3100 automated sequencer. Segregation of an *IMPG1* single nucleotide polymorphism (SNP) in all the families studied was analyzed by restriction enzyme digest of the amplified gene fragments. *Results*: In total, 15 SNPs were identified of which 7 were novel. Of the identified SNPs, one was insertion, two were deletions, five were intronic, six were missense, and one was located in the 5′ UTR. These changes, however, were also identified in unaffected members of the families and/or 50 control Caucasians. The examined known *IMPG1* SNP was not segregating with the disease phenotype but was correlating with the genetic data in all families studied. *Conclusions*: Our results indicate that neither *EEF1A1* nor *IMPG1* could be responsible for RP25 in the studied families due to absence of any pathogenic variants. However, it is important to notice that the methodology used in this study cannot detect larger deletions that lie outside the screened regions or primer site mutations that exist in the heterozygous state. A role of both genes in other inherited forms of RP and/or retinal degenerations needs to be elucidated.

KEYWORDS candidate genes; genetic diseases; interphotoreceptor matrix; mutation screening; retinitis pigmentosa

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INTRODUCTION

Retinitis pigmentosa (RP: OMIM #268000) is one of the most debilitating hereditary retinal disorders that cause severe visual impairment and has

an incidence of approximately 1/3500.[1] RP is characterized by a high genetic heterogeneity and may be inherited as an autosomal recessive (ar), autosomal dominant (ad), digenic or X-linked trait (XL). Autosomal recessive RP (arRP) is the most common form of RP worldwide and accounts for ~39% of cases in Spain.[2] To date, 21 loci were found to be responsible for arRP (http://www.sph.uth.tmc.edu/retnet/). The genes causing arRP for four of these loci still remain to be identified.[3–6]

The RP25 locus has been mapped between markers D6S257 and D6S1570, a region that spans approximately 16.1 cM in four Spanish families with arRP.[6] Recently, three additional families have been mapped to the same region.[7]

Several loci with retinal dystrophy phenotypes have been mapped to the pericentromeric region of chromosome 6 (6q14-q21). Autosomal dominant Stargardt-like disease (STGD3),[8] autosomal dominant macular dystrophy (ADMD),[9] cone-rod dystrophy (CORD7),[10] Leber congenital amaurosis type 5 (LCA5),[11] and benign concentric annular macular dystrophy (BCAMD),[12] are located in the overlapping region with RP25 while North Carolina macular dystrophy (MCDR1),[13] and progressive bifocal chorioretinal atrophy (PBCRA)[14] are in the non-overlapping region (Fig. 1).

Recently, the gene responsible for STGD3 and ADMD has been identified.[15] Subsequently, *RIM1* has been reported as the causative gene for CORD7 (Fig. 1).[16] However, the genes for LCA5 and RP25 loci are not yet identified. Both LCA5 and RP25 could be allelic, even though it is highly likely that more than one mutation is involved in their etiology.

To date, a number of candidate genes including *ELOVL4* and *RIM1* were excluded as the disease-causing genes for the RP25 locus.[8,17–21]

In order to identify the causative gene for the RP25 locus, we selected five Spanish families that are linked to the telomeric region of the RP25 between markers D6S280 and D6S1644 where there is an overlap between RP25 and LCA5 loci (Fig. 1). This region spans ~7 cM and contains at least 60 genes of which eukaryotic elongation factor 1 alpha 1 (*EEF1A1*), and interphotoreceptor matrix proteoglycan 1 (*IMPG1*) were selected as good candidate genes. *EEF1A1* and *IMPG1* are located at 74.3 and 76.7 Mb telomeric on chromosome 6, respectively.

EEF1A1 was shown to be expressed in the ciliary structure of the *Trypanosoma brucei* along with 20 other

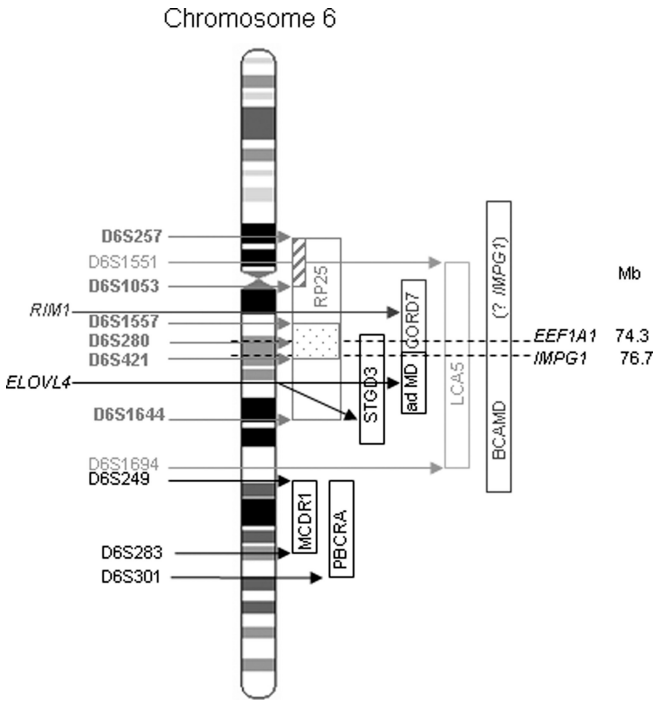

FIGURE 1 **Schematic representation of chromosome 6, showing the retinal disease loci in the region and the location of candidate genes screened (on the right side). The hatched area in the RP25 locus indicates the overlap between the Spanish and the Pakistani families, and the dotted area depicts the region where all Spanish families are linked.**

proteins (Dawe H, Mckean P, Gull K, and Gaskell S, personal communication) of which two, RP2 gene for XLRP and BBS5 gene for Bardet-Biedl syndrome 5, were previously reported as being responsible for other retinal degenerations.[22,23] According to the data obtained from the NCBI database, *EEF1A1* was shown to be expressed in the retina, retinal pigment epithelium (RPE), and choroid. On the other hand, *IMPG1* that encodes a major proteoglycan of the interphotoreceptor matrix (IPM) was shown to be preferentially expressed in the retina by both rod and cone photoreceptor cells.[24,25]

Mutation screening of the two genes in five Spanish families linked to the RP25 locus is reported here. One of the single nucleotide polymorphisms (SNPs) was also assessed by restriction digest analysis to follow the segregation pattern of *IMPG1* in these families.

MATERIALS AND METHODS

Families and DNA

Five Spanish families, one consanguineous and four non-consanguineous, were included in the study. An informed consent was obtained from all participants for clinical and molecular genetic studies. The

study conformed to the tenets of the Declaration of Helsinki.

PCR Amplification of Candidate Genes

Twenty-five pairs of primers were designed using Primer 3 Out put program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to screen the coding regions together with splice sites (GT/AG) and the 5′ UTR of *EEF1A1* and *IMPG1* (Table 1).

Each PCR was performed in 25-μl reaction mixture containing genomic DNA (100 ng), primers (0.4 μM each), $MgCl_2$ (1.5–2.5 mM; Bioline, UK), deoxynucleoside triphosphate (dNTPs; 0.2 mM; Promega, UK), 1 $\times$ PCR buffer (Bioline, UK) and Taq polymerase (0.5 U; Bioline, UK). Amplification reactions were performed under the following conditions: 3 min of denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C to 59°C (Table 1) for 30 s, extension at 72°C for 30 s, and a further extension step at 72°C for 5 min.

For GC-rich areas, buffer 3 (containing 22.5 mM $MgCl_2$; supplied by Boehringer Manheim GmbH, Germany) together with dimethyl sulfoxide (DMSO) 5% or Ready mix (AB-0795; ABgene, UK) were used.

Mutation Detection

Sequence analysis was performed after using Montage PCR cleanup kit (Millipore, UK) to purify the PCR products. Sequencing reactions were performed as follows: 2 μl of the cleaned PCR products was mixed with 0.5 μl dye terminator (Big Dye Terminator V3.1; Applied Biosystems Cheshire, UK), 0.5 μl primer (0.4 μM), 4.5 μl dH_2O, and 2.5 μl sequencing buffer version 3.1. These were performed under the following conditions: initial denaturation at 96°C for 3 min followed by 25 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 5 s and then 4 min at 60°C, and finally a further extension at 4°C for 2 min. After sequencing, products were cleaned with Montage sequencing reaction cleanup kit (Millipore) and then run on automated fluorescence DNA sequencer (ABI 3100, Perkin Elmer, Foster City, CA, USA), according to the manufacturer's instructions.

Restriction Enzyme Analysis

To follow the segregation of *IMPG1* in our families, a cosegregation study on all family members was performed using restriction enzyme digestion analysis. Exon 13 primer pairs were used to PCR-amplify a 773-bp product as described above, which was subsequently digested for an hour at 37°C with 1 unit of *MsI* I enzyme (New England BioLabs, UK) and analyzed on 2% agarose gel (Sigma Genosys, UK). A total volume of 20 μl containing 2 μl 10X buffer 2, 0.2 μl 1X bovine serum albumin (BSA) to enhance the enzyme activity, 10 μl PCR product, 0.5 μl of the *MsI* I enzyme, and 7.3 μl distilled water was used. The recognition sequence for the enzyme was 5′ . . . CAYNN NNRTG . . . 3′, which was recognized by the wild-type allele.

RESULTS

Mutation Screening

All changes were assigned a nucleotide number starting at the first translation base of *EEF1A1* and *IMPG1* according to the GenBank entries NM_001402 and NM_001563, respectively.

EEF1A1

Mutation screening of *EEF1A1* led to the identification of five genetic variants, of which four were novel (Table 2). One of the five identified changes was a T>C transition in the 5′ UTR (position −43), which was reported previously in the SNP database (Table 2). Of the four newly identified SNPs, two were intronic and another two were silent changes. The two noncoding changes included T>C transitions at IVS5+28 and +71, which were detected with an allele frequency of 41.6% and 33.3%, respectively, in 100 control chromosomes. The remaining coding changes were G>A transitions that resulted in synonymous substitutions (K444K and Q459Q) in exon 7. However, these silent changes were not segregating with the disease phenotype and were detected with an allele frequency of 49.6% in 100 control chromosomes.

IMPG1

Ten single nucleotide changes were identified in *IMPG1*, three of which were novel (Table 2). Of the three novel changes, one was insertion of single base pair (A) at position (IVS2-212). A novel deletion of one base pair (T) at position (IVS6-9) was also detected in four of the studied families but did not cosegregate with the disease phenotype. The remaining unreported SNP was an intronic A > T transversion at IVS5+75. Of the previously reported changes, two were intronic

TABLE 1 **Primers Designed for Amplification of *EEF1A1* and *IMPG1***

Gene	Exon no.	Sequence (5′.....3′)	Product size (bp)	$MgCl_2$	Temp.
EEF1A1	Exon 1A F	GGCCAAGATCTGCACACTG	620	1.5 mM	59°C
	Exon 1A R	TTTCACGACACCTGAAATGG			
	Exon 1B F	AGGGGTTTTATGCGATGGAG	425	1.5 mM	58°C
	Exon 1B R	TCACTAGTTCTGGGGAAATCAC			
	Exon 2 F	AAGTGGAAACTGCCAATTAAGG	358	1.5 mM	58°C
	Exon 2 R	TCCCTGTCAACTCTCCAAATG			
	Exon 3 F	CTTTATCCCAAAAGGCTTGC	496	1.5 mM	58°C
	Exon 3 R	TTACGGGTGACTTTCCATCC			
	Exon 4 F	AACATGCTGGAGCCAAGTG	396	1.5 mM	58°C
	Exon 4 R	CGGGTTTGAGAACACCAGTC			
	Exon 5 F	TGGTGGTAAGTTGGCTGTAAAC	454	1.5 mM	58°C
	Exon 5 R	GGTTCAGGATAATCACCTTGG			
	Exon 6 F	AAATGACCCACCAATGGAAG	483	1.5 mM	58°C
	Exon 6 R	TCATATCACGAACAGCAAAGC			
	Exon 7 F	TCCACCTTTGGGTAAGGATG	395	1.5 mM	58°C
	Exon 7 R	TGGTCCACAAAACATTCTCC			
IMPG1	Exon 1 F	CACCTGAGGGAAAGACAAGC	368	1.5 mM	57°C
	Exon 1R	TCAATTGGTAGCCTTGTTGG			
	Exon 2 F	TAGCAGTTGCACCACGGTAG	480	1.5 mM	57°C
	Exon 2 R	TGTGGCTAAATGACAGAACTGG			
	Exon 3 F	TTTCCCAAATGGCTCAAAAG	584	1.5 mM	57°C
	Exon 3 R	AACCCTTACGTTGTGGAAACC			
	Exon 4 F	TGCAAAATAATATGGTACAGTCAGG	261	1.5 mM	57°C
	Exon 4 R	GTGGCTGACATAAAATCCTACAG			
	Exon 5 F	TTTTTGCATTTTTCCTCAATG	498	2.5 mM	57°C
	Exon 5 R	TTTTTGCATTTTTCCTCAATG			
	Exon 6 F	GAAAAAGCATATTGAATTTTGACC	274	3.5 mM	57°C
	Exon 6 R	TGGTTTATCCATTCTCTTTTCCTG			
	Exon 7 F	GCCTTCATAATCCACTTCTTGAG	330	3.5 mM	57°C
	Exon 7 R	TCGCCGTAAGGGGTTTTATGTC			
	Exon 8 F	CATTTTCAGCTGTTCCCCTAAC	296	1.5 mM	57°C
	Exon 8 R	GTTTCCAGGATTTGGCAGAG			
	Exon 9 F	TGAACAAACAAAAGAGACAATGG	274	3.5 mM	57°C
	Exon 9 R	CATCAAAAAGTAATGGGCTTATCC			
	Exon 10 F	GATATTCTCTCCGAGCCCATC	487	1.5 mM	57°C
	Exon 10 R	TGATCGACTTTAGAAGACCCAAG			
	Exon 11 F	CCGCATATTTCAACCTGGAC	268	1.5 mM	57°C
	Exon 11 R	AAAGGGGATTTTGCTCTGTTC			
	Exon 12 F	TCAATGGATGTTATCCTTTTAGAAC	358	1.5 mM	57°C
	Exon 12 R	GGATGGCTTTGTCACTGGTC			
	Exon 13 F	AAATGATCTACGCAAATGCTATG	771	3.5 mM	57°C
	Exon 13 R	GCTTGGCGGTTTGTTTCTAC			
	Exon 14 F	AAAATCACAGCCATCCATCTC	563	1.5 mM	57°C
	Exon 14 R	TTTGATTTTTGTGGCCTAAAG			
	Exon 15 F	TGGTCTCTTGCCTTTGACTG	384	1.5 mM	57°C
	Exon 15 R	AAAACCATGGGTTGAAAGGAC			
	Exon 16 F	TCCAACTCAAACGGAAGACACA	364	1.5 mM	57°C
	Exon 16 R	AAATGTCACCCCCTTAAAACAG			
	Exon 17 F	CATAAATGGCAAGCACATCC	492	1.5 mM	57°C
	Exon 17 R	TTTCAGGGAAGGTGGAAGC			

T > C transitions, one was a deletion of one base pair at IVS14+52, and the other four were missense changes. Two of the missense changes were nonconservative substitutions in exons 13 and 15, respectively, and the other two were conservative changes in exons 15 and 16, respectively (Table 2).

In total, 15 SNPs were identified, of which 7 were novel. Four of the 15 SNPs changed the encoded amino

TABLE 2 **The Changes Detected in *EEF1A1* and *IMPG1***

Gene	Sequence variation	AA change	Allele frequency	Published in SNP database or not
EEF1A1	−43T > C	—		rs2073465
	IVS5 + 28T > C	—	41.6%	Not published
	IVS5 + 71T > C	—	33.3%	Not published
	c.1742G > A	K444K	49.6%	Not published
	c.1788G > A	Q459Q	49.6%	Not published
IMPG1	IVS2-212insA	—	16.5%	Not published
	IVS4-90T > C	—		rs1341568
	IVS5 + 75A > T	—	33.3%	Not published
	IVS6-9delT	—	50%	Not published
	IVS9-48C > T	—		rs17802616
	c.1682C > G	H518D		rs3734311
	IVS14 + 52delA	—		rs3215818
	c.2240C > T	R703W		rs10943299
	c.2262G > A	R710H		rs3734313
	c.2412G > A	S760N		rs3778005

acid, one was an insertion and two were deletions of one base pair. These changes, however, were also identified in unaffected members of the families and/or in 100 control chromosomes. By examining the haplotypes data from the identified SNPs, there were no shared haplotypes in the families studied and no crossover identified that may refine the region.

Restriction Enzyme Analysis

The results of restriction digest analysis of rs3734311 SNP in exon 13 of *IMPG1* showed that it was not segregating with the disease phenotype but was correlating with the genetic data in all members of the studied families. All members who carried the mutant allele (G/G) showed only one band at the expected size (773 bp) because the enzyme recognized only the wild-type allele. However, members who carried the wild-type allele in a homozygous manner (C/C) showed a completely digested product of the expected size (385 bp). Moreover, heterozygous members who had both the mutant and the wild-type allele presented with two products at the expected size (773 and 385 bp) (Fig. 2).

DISCUSSION

Herein we report the screening of two candidate genes for mutations in five Spanish families linked to the RP25 locus. One of the previously reported SNPs was examined by restriction digest analysis to analyze the segregation of *IMPG1* in our families. All novel sequence variations were tested in at least 100 control chromosomes of Spanish origin.

Because of the rarity of the RP25 form of RP, it is reasonable to hypothesize that there may be a founder effect among the five Spanish families. However, by examining the SNPs from this work and previously identified SNPs in the region,[21] there was no shared haplotype.

We previously proposed that the gene responsible for RP25 could be either in the region between D6S257 and D6S1053 where there is an overlap of the linkage data of both Spanish and Pakistani families or between

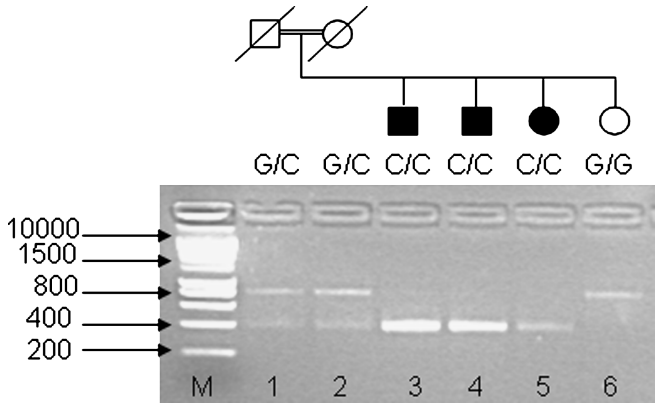

FIGURE 2 **Cosegregation study of RP5 family using *MsI* I restriction enzyme. Heterozygous parents (lanes 1 and 2) show partially digested products (773 and 385 bp) representing both the mutant and the wild-type alleles. Lanes 3–5 (affected individuals) show a digested product (385 bp) depicting the wild-type allele (C/C). Lane 6 shows a completely undigested product (773 bp) representing the mutant homozygous allele (G/G). M is 10 kb smart ladder.**

D6S1557 and D6S421 where six Spanish families were linked further down on chromosome 6 (Fig. 1).[21] However, we cannot rule out the possibility that the gene could be in the region between D6S280 and D6S1644 where five of our Spanish families are in overlap with LCA5 locus (Fig. 1).

Clinical heterogeneity is not an infrequent finding in retinal degenerations. For example, ABCR gene has been reported as the gene responsible for both recessive Stargardt macular degeneration and arRP.[26,27] Similarly, different mutations in the peripherin/RDS gene result in RP, macular dystrophy, cone-rod dystrophy, pattern dystrophy, and central areolar choroidal dystrophy.[28–33] Moreover, LCA genes have been reported to be the cause for other retinal dystrophies. For example, *GUCY2D* mutations (LCA1) have been identified in autosomal dominant cone-rod dystrophy,[34] *RPE65* and *CRB1* mutations cause both arRP and LCA,[35,36] and mutations in the CRX gene were reported to cause autosomal dominant cone-rod dystrophy, LCA, and late-onset dominant RP.[37]

It is conceivable that mutations in different sites cause different structural alterations in the predicted protein, predisposing to varying phenotypes.[38] Thus, there is a possibility that a similar situation between LCA5 and RP25 could exist.

In our study, *EEF1A1* and *IMPG1* were selected as good functional candidates where both LCA5 and RP25 loci overlap. In mammals, *EEF1A1* is expressed in two isoforms, termed EEF1A1 and EEF1A2. The two isoforms are 92% identical at the amino acid level and have been characterized in both human and rodents.[39] Although *EEF1A1* is expressed in every tissue examined, *EEF1A2* is expressed only in the terminally differentiated cells of brain muscle and heart.[40] *EEF1A1* is the human orthologoe for the protein that was shown to be expressed in the cilia of the *Trypanosoma brucei* proteome (Dawe H, Mckean P, Gull K, and Gaskell S, personal communication), which made the gene an excellent candidate. It has been previously reported that successful transmission of several proteins from the cell body to the outer segment of the photoreceptor cells depends on transport along a modified cilium and that defective passage of certain molecules results in retinal degeneration associated with RP.[41] For example, the BBS5 gene has been identified by comparative genomics based on the fact that the gene responsible for BBS should encode proteins involved in basal body/flagellar assembly.[23] Similarly, we screened *EEF1A1* based on the assumption that it has a role in maintenance of an integral cilia in the flagellated *Trypanosoma*.

Meanwhile, it has been reported that *IMPG1* represented an excellent candidate for chromosome 6q linked retinopathies based on its chromosomal localization and the potential role of IPM molecules in retinal adhesion and in maintenance of photoreceptor polarization, orientation, turnover, and viability.[42,43] Screening *IMPG1* for mutations in patients with STGD3, MCDR1, PBCRA, CORD7, and LCA5 revealed no sequence alterations.[10,11,42] However, mutation screening of *IMPG1* in BCAMD patients revealed L579P amino acid substitution, which may play a causal rule.[12] Thus, to understand the role of IPM in the normal and diseased human retina it was crucial to screen *IMPG1* in our families. We used rs3734311 SNP to follow the segregation of *IMPG1* in the studied families. The results of restriction digest analysis showed that there is no crossover and that *IMPG1* lies in the vicinity of the RP25 locus. Subsequently, we sequenced all the coding regions and the 5′ UTR of the gene to search for any pathogenic variants.

In conclusion, we screened *EEF1A1* and *IMPG1* for mutations in arRP families linked to the RP25 locus. Our results showed that the studied genes cannot be involved in the pathogenesis of RP25 due to the absence of pathogenic changes. However, a role of both genes in other inherited forms of RP and/or retinal degenerations mapping to the same chromosomal region needs to be elucidated. Future work involving screening of other candidate genes and/or recruiting additional families in order to narrow down the genetic interval is underway.

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