
María González-del Pozo,1,2 Nereida Bravo-Gil,1,2 Cristina Méndez-Vidal,1,2 Ignacio Montero-de-Espinosa,3 José M Millán,2,4,5 Joaquín Dopazo,2,6,7 Salud Borrego,1,2 and Guillermo Antinólo1,2,6,*

1Department of Genetics, Reproduction and Fetal Medicine, Institute of Biomedicine of Seville, University Hospital Virgen del Rocío/CSIC/University of Seville, Seville, Spain
2Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Spain
3Department of Ophthalmology, University Hospital Virgen Macarena, Seville, Spain
4Grupo de Investigación en Enfermedades Neurosensoriales, IIS-La Fe, Valencia, Spain
5Unidad de Genética y Diagnóstico Prenatal, Hospital Universitario La Fe, Valencia, Spain
6Genomics and Bioinformatics Platform of Andalusia (GBPA), Seville, Spain
7Computational Genomics Department, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain

Mutations in USH2A are a common cause of Retinitis Pigmentosa (RP). Among the most frequently reported USH2A variants, c.2276G>T (p.C759F) has been found in both affected and healthy individuals. The pathogenicity of this variant remains controversial since it was detected in homozygosity in two healthy siblings of a Spanish family (S23), eleven years ago. The fact that these individuals remain asymptomatic today, prompted us to study the presence of other pathogenic variants in this family using targeted resequencing of 26 retinal genes in one of the affected individuals. This approach allowed us to identify one novel pathogenic homozygous mutation in exon 13 of PDE6B (c.1678C>T; p.R560C). This variant cosegregated with the disease and was absent in 200 control individuals. Remarkably, the identified variant in PDE6B corresponds to the mutation responsible of the retinal degeneration in the naturally occurring rd10 mutant mice. To our knowledge, this is the first report of the identification of the rd10 mouse mutation in a RP family. These findings, together with a review of the literature, support the hypothesis that homozygous p.C759F mutations are not pathogenic and led us to exclude the implication of p.C759F in the RP of family S23. Our results indicate the need of re-evaluating all families genetically diagnosed with this mutation.

How to Cite this Article:

INTRODUCTION

Inherited retinal dystrophies (IRDs) are a heterogeneous group of disorders characterized by primary dysfunction or loss of photoreceptors leading to progressive visual impairment and blindness. Retinitis Pigmentosa (RP; OMIM #268000) represents the most prevalent clinical subtype of IRDs, affecting 1 in ~4,000 individuals. To date, more than 60 genes have been associated with RP. The USH2A gene (OMIM #608400), along with EYS (OMIM #612424), is the most commonly mutated gene in autosomal recessive RP (ARRP) [Bernal et al., 2003; Glockle et al., 2014]. Although most causative mutations associated with IRDs are rare (MAF<<0.01),...
some exceptions have been described. A frequent change is p.C759F (USH2A; rs80338902) which is responsible of ~4.5% of patients with ARRP [Rivolta et al., 2000; Seyedahmadi et al., 2004], and has been found in a heterozygotic state in 20 control individuals out of 6,503 exomes in the Exome Variant Server (EVS). This USH2A mutation is present in a large number of families with IRD in both a homozygous and heterozygous state, but the pathogenicity of the p. C759F variant has been questioned because of non-segregation in a Spanish family (S23) with two asymptomatic homozygous siblings [Bernal et al., 2003].

According to the relatively high prevalence of the p.C759F variant and the discrepancies between clinical data and genetic findings in family S23, we set out to evaluate the pathogenicity of this variant using a NGS panel of 26 retinal genes [Mendez-Vidal et al., 2014].

MATERIALS AND METHODS

Patients and Clinical Evaluation

This study involved a consanguineous Spanish family (S23) comprising two affected siblings diagnosed with ARRP and seven asymptomatic members (Supplemental online Fig. S1). All affected individuals were referred from the Ophthalmology Department to the Genetic, Reproduction, and Fetal Medicine Department. Additionally, a group of 200 ethnically matched control individuals was studied. This research project was reviewed and approved by our research ethics committee and followed the tenets of the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all participants. Clinical diagnosis of ARRP was ascertained as described [Mendez-Vidal et al., 2014]. Individual II:1 underwent ophthalmologic examination including funduscopy and electroretinography. Peripheral blood of all subjects was collected for genomic DNA isolation from leukocytes using standard procedures. A DNA sample of the affected individual II:4 was processed for gene panel sequencing.

Analysis of p.C759F Prevalence

The p.C759F mutation was screened in 200 control individuals of our cohort by Sanger sequencing and in 252 Spanish healthy exomes [Garcia-Alonso et al., 2014]. Also, we have performed an extensive literature review of families with RP harboring p.C759F, using the following search terms in PubMed: (Retinitis Pigmentosa OR USH2A) AND (C759F). We also explored other informative sources of data such as LOVD-USHbases, HGMD and OMIM.

Library Preparation and Gene Panel Sequencing

Library preparation, targeted sequencing on a 454 Roche GS Junior sequencer and data analysis were performed as described [Mendez-Vidal et al., 2014].

Assessment of the Pathogenicity of Candidate Variants

Sanger sequencing was used to verify each predicted disease-causing variant and to perform cosegregation analyses. Novel cosegregating variants were subsequently screened in 200 healthy matched control individuals by Sanger sequencing. The pathogenicity of missense substitutions was estimated by Polyphen-2 and SIFT scores, whereas the impact on disulfide bonds was predicted using the DIANNA web server.

Additional Genetic Analyses of USH2A

Additional Genetic Analyses of USH2A

USH2A regions with low coverage and the most common pathogenic intronic mutation of this gene (c.7595–2144A>G) were further analyzed by Sanger sequencing. To test if a recombination event or a large deletion had occurred in USH2A, linkage analysis in all family members was conducted using the microsatellite markers D1S2629, D1S2827, D1S229, and D1S490.

RESULTS

Clinical Data

The index patient (II:3) (Supplemental online Fig. S1) had a clinical diagnosis of ARRP characterized by night blindness as the initial symptom at age four, followed by progressive visual field constriction and decreased visual acuity by the age of 28 (OD:1/3 and OS:1/2). Fundus examination showed typical signs of RP with pale optic nerve disc, narrowed blood vessels, and bone spicule pigmentation in the periphery. Her ERG responses were not detectable, consistent with severe, generalized rod cone dysfunction. Her sister (II:4) showed an age of onset and clinical features similar to the proband. Individuals II:1 and II:2, harboring the homozygous USH2A mutation p.C759F, were clinically re-evaluated but no signs of RP were observed at the ages of 57 and 55, respectively. The ERG and fundus photography of individual II:1 (Supplemental online Fig. S2) confirmed normal visual function in this family member.

Prevalence of the p.C759F Mutation

Direct sequencing of exon 13 of USH2A in 200 matched controls showed the presence of the heterozygous p.C759F mutation in three individuals. Also, four out of 252 Spanish healthy exomes [Garcia-Alonso et al., 2014] were heterozygous for this mutation. A review of the literature showed that p.C759F is present in a total of 41 families, including 15 families with homozygous individuals (Supplemental online Table S1) and 26 with heterozygous carriers. Regarding the families with homozygous individuals, segregation analyses were performed in only five families (S23, M286, RP0332, RP0930, and RP0849) [Avila-Fernandez et al., 2010; Bernal et al., 2003]. Moreover, not all patients were screened for mutations in the entire gene and, in the majority of families, only exon 13 of USH2A was analyzed by Sanger sequencing.

NGS Data and Identification of Variants

To identify the causative variant underlying RP in Family S23, we performed NGS for individual II:4 using our panel of 26 IRD genes. This analysis generated a data output of 68 Mb and 155,475 reads with a mean length of 438 bp. This sample showed a percentage of reads on target of 83% and a mean coverage of 129×. The percentage of covered bases was 99.8% and, although this is a fairly
high value, exon 59 of USH2A remained uncovered. Application of variant calling, filtering, and annotation of the sequencing data from individual II:4 was performed as described above. Output data for each step of the analysis pipeline are shown in Supplemental online Table SII. A total of 669 SNVs and 2,479 indels were found but only four SNVs remained once the procedure was completed. Three of these four changes were the known USH2A variants p. C759F, p.H752H, and p.L555V, all previously detected and assessed in this family [Bernal et al., 2003; Aller et al., 2004], but we concluded that none were pathogenic. The remaining variant was a rare homozygous mutation not previously associated with human RP located in exon 13 of PDE6B at genomic position 4: 655986 (c.1678C>T; p.R560C) (Supplemental online Fig. S3A,B). The specific coverage for this position was 29×.

**Pathogenicity Evaluation of the PDE6B Mutation.**

Sanger sequencing was performed using primers that amplified exon 13 of PDE6B. The c.1678C>T (p.R560C) mutation cosegregated with the disease in Family S23 (Supplemental online Fig. S1) and was absent in 200 control individuals. This variant was not present in EVS, but one heterozygous allele out of 428 chromosomes was found as a result of another large scale genome sequencing project [Bieseker et al., 2009]. Although this variant has not been previously associated with RP in humans, it has been reported as the causative mutation of the retinal degeneration in the naturally occurring rd10 mutant mouse [Chang et al., 2007]. The mutated residue lies in an evolutionarily conserved domain that corresponds to the catalytic domain of the protein (Supplemental online Fig. S3C). In silico pathogenicity prediction tools showed that p.R560C is probably damaging (Polyphen-2 score = 0.997, SIFT score = 0). Additionally, the DiANNA web server predicted that the newly introduced cysteine alters the disulfide bonding by establishing a new disulfide bridge with the cysteine at position 717 (Supplemental online Fig. S3D). We conclude that this PDE6B mutation is the likely disease-causing variant in Family S23.

Given that exon 59 of USH2A was poorly covered during targeted sequencing, we performed Sanger sequencing to check this region, but no additional variants were detected. Likewise, direct full sequencing of the USH2A intronic mutation c.7595–2144A>G excluded the presence of this variant in the family. Finally, to exclude recombination events or large deletions in this gene, we carried out a linkage analysis in the family. The haplotypes showed a recombination event in individual II:5, but no other complex events were detected (Supplemental online Fig. S4). This, together with the fact that both affected and two unaffected individual (II:6 and II:7) shared the same haplotype, led us to exclude USH2A as the RP-causing gene.

**DISCUSSION**

In this report, we have applied NGS panel sequencing to identify causal gene mutations in a family with RP, in which the homozygous p.C759F variant was previously ruled out as the cause of the disease, questioning the pathogenic role of this mutation.

According to the literature, 15 families have been described harboring the homozygous p.C759F variant. In all reviewed cases, the segregation data were not shown or were inconclusive. Of those, Family S23 was one of the most interesting since p.C759F was homozygous in two healthy, currently asymptomatic, siblings [Bernal et al., 2003]. The other family reported by the same authors (M286) showed segregation of p.C759F with the disease. However, the offspring of that consanguineous family was comprised of only two affected individuals, and the probability of having obtained this result by chance alone is not negligible. Furthermore, Rivolta et al. [2000] described another two families with homozygous p.C759F but the segregation analysis was carried out only in two affected individuals of one of these families (#E685). The authors also identified the homozygous p.C759F mutation inherited by uniparental paternal disomy in one patient, whereas her affected second cousin was heterozygous for the same variant [Rivolta et al., 2002]. Although the authors concluded that the p.C759F variants in both affected individuals were likely of independent origin, the identification of a second mutant allele would help to clarify the molecular diagnosis of this complex family. Finally, another patient homozygous for p.C759F has been reported [Glockle et al., 2014], but the symptoms of his homozygous sibling were unclear. Of note, in most families, only the affected members were analyzed, which may be the reason for the absence of other occurrences of unaffected individuals homozygous for this mutation.

Furthermore, in the majority of families, only exon 13 of USH2A was analyzed by Sanger sequencing. Therefore, screening of additional USH2A regions including exons encoding for the large isoform, duplications, deletions, and the common pathogenic deep intronic variant (c.7595–2144A>G), should have been carried out to detect variants acting in cis with p.C759F. Likewise, the involvement of other genes could not be excluded. In this regard, clinical data such as the onset age of symptoms and the average rates of visual acuity and visual field loss, can be helpful to identify candidate genes. Family S23 was diagnosed of early onset RP and Patient II:4 reached legal blindness by the age of 30, which is not consistent with the typical disease progression associated with USH2A mutations [Sandberg et al., 2008]. Another factor against the p.C759F pathogenicity is its relative high allele frequency compared to other IRD-associated mutations.

Collectively, these results indicated that p.C759F is not implicated in the RP of Family S23. The application of targeted sequencing led to the identification of a predicted pathogenic homozygous PDE6B mutation (c.1678C>T; p.R560C). Notably, this variant is equivalent to the mutation that causes the IRD in the rd10 mutant mice with a homozygous p.R560C mutation in exon 13 of Pdeo6b (Pdeo6b_R560C) [Chang et al., 2007]. This is the first report of a family affected with ARRP caused by the same homozygous mutation present in the rd10 mice. The location of the variant in a domain responsible for the catalytic function of the protein and the conservation between mouse and human PDE6B may explain that the same mutation cause similar phenotypes in both species. Clinical features in affected individuals of the S23 family were consistent with those of RP caused by PDE6B mutations. Indeed, RP associated with PDE6B mutations is considered one of the earliest onset and most aggressive forms of IRD [McLaughlin et al., 1993].
Our work shows for the first time that p.C759F might not be pathogenic. Therefore, genetic re-evaluation of other reported families with homozygous individuals should be considered, paying special attention to the screening of regions that were not initially analyzed, such as additional exons encoding the long isoform, deep intronic regions and copy number variations. Moreover, the involvement of additional RP genes should not be dismissed, especially in those families whose clinical manifestations may be indicative of mutations in other loci.

ACKNOWLEDGMENTS

The authors are grateful to the families described in this study. This work was supported by Instituto de Salud Carlos III (ISCIII), Spanish Ministry of Economy and Competitiveness, Spain (PI1102923, CIBERER ACCI and CDTI FEDER EXP00052887/ITC-20111037), and the Foundation Ramon Areces (CIVP16A1856). The CIBERER is an initiative of the ISCIII, Spanish Ministry of Economy and Competitiveness. NB-G is supported by fellowship FI12/00545 from ISCIII.

INTERNET RESOURCES

RetNet: https://sph.uth.edu/retnet/home.htm
Exome Variant Server: http://evs.gs.washington.edu/EVS/
HGMD: http://www.hgmd.cf.ac.uk/ac/index.php
Polyphen-2: http://genetics.bwh.harvard.edu/pph2/
SIFT: http://sift.bii.a-star.edu.sg
DiANNA web server: http://clavius.bc.edu/~clotelab/DiANNA/

REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site.