Whole-exome sequencing reveals ZNF408 as a new gene associated with autosomal recessive retinitis pigmentosa with vitreal alterations

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Abstract

Retinitis pigmentosa (RP) is a group of progressive inherited retinal dystrophies that cause visual impairment as a result of photoreceptor cell death. RP is heterogeneous, both clinically and genetically making difficult to establish precise genotype–phenotype correlations. In a Spanish family with autosomal recessive RP (arRP), homozygosity mapping and whole-exome sequencing led to the identification of a homozygous mutation (c.358_359delGT; p.Ala122Leufs*2) in the ZNF408 gene. A screening performed in 217 additional unrelated families revealed another homozygous mutation (c.1621C>T; p.Arg541Cys) in an isolated RP case. ZNF408 encodes a transcription factor that harbors 10 predicted C2H2-type fingers thought to be implicated in DNA binding. To elucidate the ZNF408 role in the retina and the pathogenesis of these mutations we have performed different functional studies. By immunohistochemical analysis in healthy human retina, we identified that ZNF408 is expressed in both cone and rod photoreceptors, in a specific type of amacrine and ganglion cells, and in retinal blood vessels. ZNF408 revealed a

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cytoplasmic localization and a nuclear distribution in areas corresponding with the euchromatin fraction. Immunolocalization studies showed a partial mislocalization of the p.Arg541Cys mutant protein retaining part of the WT protein in the cytoplasm. Our study demonstrates that ZNF408, previously associated with Familial Exudative Vitreoretinopathy (FEVR), is a new gene causing arRP with vitreous condensations supporting the evidence that this protein plays additional functions into the human retina.

Introduction

Retinitis pigmentosa [RP (MIM number 268000)] is the most common manifestation of inherited retinal dystrophy (RD), with a worldwide prevalence of 1:4000 approximately (1). It is characterized by a progressive degeneration of rod photoreceptors, leading to night blindness and constriction of the visual field, followed by the degeneration of cone photoreceptors, resulting in a total loss of vision.

There is a large variability in the age of onset, progression, retinal appearance and final visual outcome (2). RP is inherited in all Mendelian forms: autosomal dominant (20% of the cases), autosomal recessive (30%) or X-linked trait (10%). Approximately 40% of patients with RP represent isolated cases; the percentage varies among different populations (3,4). Non-Mendelian inheritance patterns such as digenic, mitochondrial or de novo mutations have been reported, accounting for a small proportion of cases (2).

To date, >70 genes have been associated with RP, being heterogeneous, both clinically and genetically. However, mutations in these genes account for the disease in little over half of all patients thus, remaining genes yet to be identified (4,5). Although the function of some of these genes has been extensively studied, it is difficult to establish a precise genotype–phenotype correlation because mutations in different RP genes can cause overlapping clinical phenotypes (6).

The ZNF408 gene encodes a zinc finger protein of 720 amino acids that is predicted to harbor 10 C2H2-type finger binding domains thought to be implicated in DNA binding.

Recently, in a large Dutch family, a heterozygous missense p.His455Tyr variant in ZNF408 has been associated with Familial Exudative Vitreoretinopathy (FEVR [MIM number 133780]), a disorder affecting the growth and development of blood vessels in the retina (7).

Interestingly, in this study novel homozygous mutations in the ZNF408 gene have been identified in two unrelated Spanish families as cause of RP. These findings support the hypothesis that different mutations, either in heterozygous or homozygous state, produce completely different phenotypes and suggest that ZNF408 may play additional roles apart from its implication in vasculature development. In this context, this is the first time the expression and cellular distribution of ZNF408 have been studied in the human retina.

Results

Previous studies

Two affected siblings (II:1 and II:2) of a Spanish family (RP-0322), whose parents came from the same and small geographic region (Fig. 1A), were diagnosed with autosomal recessive RP (arRP). In order to identify the genetic cause underlying the arRP within the family, first we performed whole-genome homozygosity mapping using high-resolution single nucleotide polymorphism (SNP)-array and further whole-exome sequencing (WES) analysis. A previous genotyping study for the index case (II:2) allowed discarding known mutations associated with arRP. Also, direct Sanger sequencing of the coding exons and flanking intronic sequences of EYS did not detect any mutated allele (8).

Genetic analysis

Homozygosity mapping and WES analysis

Whole-genome homozygosity mapping in II:1 and II:2 of RP-0322 family was performed. The two affected siblings shared four homozygous regions ranging from 2.6 to 1.8 Mb (Table 1). These regions contain a total of 193 genes, none of which were previously associated with retinal dystrophies (RD).

Subsequently, the index case (II:2) of RP-0322 family was analyzed by WES. A total of 88,299,239 reads were uniquely mapped to exonic regions with a median of coverage of 59× (mean coverage 69.5×).

After excluding those variants present in non-coding regions and common variants with a minor allele frequency (MAF) <0.005 at dbSNP, 1000 genomes (1000g) or the exome variant server (EVS) databases, we reduced the number of candidate variants underlying RP from 8133 to 276 (Table 2). Assuming an autosomal recessive inheritance pattern and taking into account a very likely common ancestry in the affected family, homozygous variants lying in the previously identified homozygous regions were prioritized (Table 2). Also, the expression in human retinal tissues was considered. With this criteria, only one likely pathogenic candidate variant remained, i.e. a frameshift variant in exon 3 of ZNF408 (NM_024741.2: c.358_359delGT; p.Ala122Leufs*2) (Fig. 1B). Due to its implication in vasculature development previously described and its expression in human retina, the ZNF408 gene was selected as a new candidate gene causing arRP.

Sanger sequencing of the specific region confirmed the presence of this variant identified by WES (Fig. 1A). This variant was not found in 374 ethnically control alleles nor in 1000g or EVS databases.

Screening of the ZNF408 gene in an arRP Spanish cohort

Following this finding, and to determine the prevalence of the ZNF408 mutations as a potential cause of arRP, mutation analysis of the ZNF408 gene was performed by Sanger sequencing in 217 additional Spanish probands affected with sporadic and/or arRP. An additional ZNF408 variant (c.1621C>T; p.Arg541Cys) was found homozygously in a sporadic case from a consanguineous Spanish RP family (II:2, RP-0976, Fig. 1A). This variant was not found in a total of 374 healthy control Spanish chromosomes and in SNP databases (1000g and EVS databases). The Arginine residue at position 541 is localized in the seventh of the 10 zinc finger domain and is highly conserved across evolution (Fig. 1B and C).

Two other novel missense variants, p.Gly492Arg and p.Gln583Lys, were identified in heterozygous state in two other arRP patients. Both missense changes were not present in 75 in-house exome datasets. These residues are highly conserved (Fig. 1C) and in silico predictions of the pathogenicity of these two missense variants assessed both of them as deleterious (Table 3).

In addition, a comparative genomic hybridization array (aCGH) with an exonic coverage (Supplementary Material, Fig. S1) was performed to discard large deletions and copy number variations (CNVs) in the ZNF408 locus in both patients. No second allele was found in these two patients.
The variants p.Gly492Arg and p.Gln583Lys are highly conserved across the evolution (Fig. 1C), rendering it possible that these variants affect ZNF408 protein function but are not responsible for the phenotype in these particular patients.

Ophthalmic examination

The age of onset in all affected individuals (II:1 and II:2, RP-0322 and II:2, RP-0976) was 30, 40 and 17 years, respectively. Night blindness was the first symptom followed by visual field loss.
and reduction of visual acuity for all patients. At the time of the last ophthalmologic examination, visual fields were symmetrically reduced from 30 to 10°. The best corrected visual acuity (BCVA) ranged from 0.8 to 0.5. Posterior subcapsular cataract was found in all cases. Fundi showed the typical changes of RP with pale optic disc, narrowed vessels and bone spicule pigmentation (Fig. 2A). Full-field electroretinogram was nonrecordable or substantially reduced. The ophthalmologic data of the patients are summarized in Table 4.

In order to discard any distinctive sign of FEVR an intravenous fluorescein angiography was performed in homozygous (Fig. 2B) and heterozygous carriers (Supplementary Material, Fig. S2). The intravenous fluorescein angiography (IVFA) for the II:1 (RP-0322) showed classic RP findings and also vitreous alterations in some areas, blurring the visibility of the fundus. Her affected sibling showed poor pigmentation in a mottled ‘salt-and-pepper-like’ pattern, especially in the midperiphery. Fluorescein enhancement along the inferior temporal arcades corresponds to retinal pigment epithelium (RPE) atrophy. Also, blurring of the fundus was found in some areas due to vitreous condensation. Similarly to the first family, IVFA of patient II:2 (RP-0976), carrying the missense p.Arg541Cys also showed typical RP fundus findings combined with vitreous condensations that clouds the visibility of the fundus (Fig. 2).

Table 3. Pathogenicity assessment of ZNF408 missense changes

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Frequency in control alleles</th>
<th>Predictions of pathogenicity</th>
<th>Align GVGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1474G&gt;A</td>
<td>p.Gly492Arg</td>
<td>0/150</td>
<td>Deleterious</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.1621C&gt;T</td>
<td>p.Arg541Cys</td>
<td>0/374</td>
<td>Deleterious</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.1747C&gt;A</td>
<td>p.Gln583Lys</td>
<td>0/150</td>
<td>Deleterious</td>
<td>Probably damaging</td>
</tr>
</tbody>
</table>

Assessment of pathogenicity for the ZNF408 missense changes. For Align GVGD, class C65 and C45 represent the highest and moderate, respectively, likelihood of a change to be pathogenic.
<table>
<thead>
<tr>
<th>Family ID</th>
<th>Patient ID</th>
<th>Genotype</th>
<th>Mutations Allele1/Allele2</th>
<th>First symptoms and course</th>
<th>Age at diagnosis (years)</th>
<th>Age of ophthalmic evaluation (years)</th>
<th>BCVA RE/LE</th>
<th>Visual Field RE/LE</th>
<th>ERG Fundus aspect</th>
<th>IVFA OCT</th>
<th>Additional findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP-0322</td>
<td>II:1</td>
<td>ZNF408</td>
<td>p.Ala122Leufs<em>2/p.Ala122Leufs</em>2</td>
<td>NB (30 year), field constriction and progressive loss of VA</td>
<td>52</td>
<td>69</td>
<td>0.5/0.8</td>
<td>Restricted to 10° central field</td>
<td>Pale optic disc, narrow vessels, moderate bone spicule pigmentation and RPE atrophy in mid periphery and macular preservation</td>
<td>No register</td>
<td>Pallor of optic disc, narrow vessels, reduced macular pigmentation, blurring fundus in some areas</td>
</tr>
<tr>
<td>II:2</td>
<td></td>
<td></td>
<td>p.Ala122Leufs<em>2/p.Ala122Leufs</em>2</td>
<td>NB (40 years), field constriction and progressive loss of VA</td>
<td>50</td>
<td>67</td>
<td>0.6/0.5</td>
<td>Restricted in superior and temporal hemifield/Restricted to 30° central field inferonasally</td>
<td>Rods abolished, mixed, cones and flicker diminished</td>
<td>No pigmentation in mottled pattern specially in midperiphery. Fluorescein enhancement along the inferior temporal arcades. Blurring of the fundus due to vitreous condensation</td>
<td>Normal optic disc, mildly attenuated vessels and poor pigmentation in lower hemiretina, macular preservation</td>
</tr>
<tr>
<td>RP-0976</td>
<td>II:2</td>
<td>ZNF408</td>
<td>p.Arg541Cys/p.Arg541Cys</td>
<td>NB, field constriction (17 years) and progressive loss of VA (38 years)</td>
<td>20</td>
<td>50</td>
<td>0.8/0.9</td>
<td>Restricted to 10° central field</td>
<td>Pale optic disc, peripapillary atrophy, vessel attenuation, both spotty and spicule pigmentation midperiphery, atrophy of the RPE and macular preservation</td>
<td>Typical RP fundus findings combined with vitreous condensations make difficult the visibility of the fundus</td>
<td>Normal morphology in the fovea, no cysts, atrophy in the periphery</td>
</tr>
</tbody>
</table>

ID, identification; BCVA, best corrected visual acuity; RE, right eye; LE, left eye; ERG, electroretinogram; IVFA, intravenous fluorescein angiography; OCT, optical coherence tomography; NB, night blindness; VA, visual acuity; RPE, retinal pigment epithelium; NCD, no clinical data.
The asymptomatic heterozygous p.Arg541Cys carriers (III:1 and III:2) from family RP-0976 were examined. No retinal vasculature abnormalities such as an avascular retina, neovascular membranes or tractional retinal detachments were observed in the retinal periphery on fundus, IVFA and optical coherence tomography (OCT) (Supplementary Material, Fig. S2).

Unfortunately, patients carrying heterozygous p.Gly492Arg and p.Gln583Lys variants were not available for performing additional ophthalmic examination.

**ZNF408 is expressed in human retina especially in both cone and rod photoreceptor cells**

ZNF408 is abundantly expressed in the human fetal eye as well as in the adult retina, suggesting that ZNF408 has an important role in eye development and visual function (7). However, its expression at the protein level and its localization in the human retina have never been investigated. To shed light on these aspects, ZNF408 protein expression and its cellular distribution were assessed using western blot and immunocytochemistry assays, respectively, on adult healthy human retina as well as in the 661W photoreceptor cell line.

In the human retina, the outer nuclear layer (ONL), ganglion cell layer (GCL) and both outer and inner plexiform layer (OPL and IPL) showed immunoreactivity against ZNF408 antibodies (Fig. 3A), being strongest at the ONL level.

In this context, ZNF408 immunolabeling at the ONL level present intense spots of immunoreactivity located around the nuclei of the photoreceptors (Fig. 3A,B,F and G) and axon terminals (Fig. 3A,B and G; arrows). Additionally, the ZNF408 immunostaining pattern exhibited a marked expression in a specific subtype of amacrine cell (Fig. 3H) and a subtype of ganglion cell of big size, with label in their entire length, including cell body, dendrites and axons (Fig. 3A,B and G). In addition, ZNF408 immunoreactivity was found in dendrites located in the IPL and in some ganglion cell axons running under the ganglion cell bodies in the nerve fiber layer (Fig. 3I).

To verify that the ZNF408 staining was located at the ONL in rod and cones and not in the Müller cells processes, we performed a double immunolabeling with anti-CRALBP antibodies, a specific marker of Müller cells that marks these cells from the outer limiting membrane to the inner limiting membrane, labeling also the RPE (Fig. 3B). Müller cell bodies were located in the innermost layer in the INL and their external processes pass around the cell bodies of all retinal cells, from the outer to the inner limiting membrane (Fig. 3B red). Double labeling with anti-CRALBP and anti-ZNF408 antibodies showed no colocalization (Fig. 3B). Higher magnifications in Figure 4A–C (arrowheads) showed absence of this colocalization verifying thus that ZNF408 is located in cell bodies, cytoplasm and myoids of photoreceptor cells but not in Müller cells.

A closer view of the ONL showed that both rods (Fig. 3F arrowheads) and cones (Fig. 3G; arrowheads) photoreceptor cells presented ZNF408 immunoreactivity. At the OPL level rod spherules (Fig. 3A,B and G; arrows) and cone pedicles (double arrows in Figs 3A,B,G and 4L) were easy to identify. Additionally, a weaker staining was observed in the inner and outer segments (Fig. 3A and G). To confirm these results, we performed double immunostaining using antibodies raised against recoverin that label cone or rod photoreceptors and some type of bipolar cells, and anti-ZNF408. In this context, it was shown (Fig. 4D–F) colocalization of anti-ZNF408 and anti-recoverin in both, cone and rod photoreceptors. Immunoreactivity in rod myoids is sharply reduced at the OLM level (arrowheads in Figs 3A,B,F and 4A–G) and only a weak immunostaining was observed in rod and cone outer segments. Double immunolabeling with anti-rhodopsin antibodies, a specific marker for rod outer segments, showed colocalization with the weak anti-ZNF408 immunoreactivity at this level (Fig. 4G–J). Cone photoreceptor cells exhibited a bright ZNF408 immunoreactivity in its cell bodies, myoids, axons and pedicles while ellipsoids and outer segments showed a lower immunostaining signal, as revealed the double immunolabeling against anti-cone arrestin (Fig. 4J–L).

As addressed above, ZNF408 is related with FEVR (7). Double immunolabeling using anti-ZNF408 and anti-collagen IV, a specific marker of blood vessels wall, was performed. Fig. 4M–O showed ZNF408 positive cells into blood vessels, which are presumably endothelial cells (Fig. 4M–O; insets). Additionally, an immunofluorescence dotted along the cross sections of blood vessels was observed (Fig. 4M–O, arrows). These results corroborate the relationship found between ZNF408 protein and FEVR patients by Collin and colleagues, supporting thus the role of ZNF408 protein in the development and maintaining of retinal blood vessels.

It is important to remark that the antibody ZNF408 specificity was confirmed by the lack of immunoreactivity in the retina when the antibody was previously preincubated with its blocking peptide (Fig. 3C).

On the other hand, we performed immunocytochemistry analysis in the mouse retinal photoreceptor-derived 661W cell line. In this context, the ZNF408 immunolabeling exhibited a nuclear and cytoplasmic punctate staining pattern (Fig. 3D and E). It is important to highlight the ZNF408 staining in nuclei, as expected given that the ZNF408 gene encodes a transcription factor that harbors 10 predicted C2H2-type finger binding domains thought to be implicated in DNA binding (7). In all cases, the staining pattern appeared in the areas of the nucleus corresponding with the euchromatin fraction (Fig. 3E). Interestingly, this nuclear distribution was also observed in the human retina as demonstrated in cones and rods (Fig. 3F and G), neurons in the INL and ganglion cells (Fig. 3A and I).

The immunoblotting analysis revealed the presence in the human retina and in the 661W cell-line samples of a prominent and specific immunoreactive band with an apparent molecular weight of approximately 67 kDa corresponding to the ZNF408 protein (Fig. 3I). We confirmed the specificity of the antibody by verifying that pre-adsorption of the antibody with the immunogenic peptide interferes with the ZNF408 detection. The observed size which was lower than the predicted for the isoform encoded by the canonical ZNF408 transcript (78.439 kDa) could be the result of a non-uniform binding of the negatively charged SDS to the protein due to its amino acid composition or the presence of a post-translational cleavage of canonical protein, among others.

**Partial mislocalization of WT ZNF408 in presence of mutant p.Arg541Cys protein**

Constructs of the wild-type (WT) and the p.His455Tyr mutant together result in mislocalization of the WT ZNF408 in the cytoplasm suggesting oligomerization of ZNF408 WT and mutant proteins (7). In order to probe the behavior of our arRP-associated p.Arg541Cys variant, similar ZNF408 WT and mutant cotransfection studies were performed. Constructs expressing N-terminal HA-tagged fusion proteins of WT_ZNF408 or the ZNF408_p.Arg541Cys mutant were generated. The analysis of >100 cells by immunocytochemistry of transiently transfected COS-1 cells revealed that localization of WT is exclusively restricted to the nucleus. The p.Arg541Cys mutant ZNF408 also mainly localizes...
Figure 3. Immunocytochemistry assays in cryofixed human retina sections and in the 661W photoreceptor cell-line. (A–C,F–H) Human retina immunolabeled with antibody against ZNF408 (green) and CRALBP (red). Nuclei are labeled with TO-PRO (blue). (A,B) ZNF408 distribution in the ONL, OPL, IPL and GCL. (B) Double immunolabeling against CRALBP and ZNF408 antibodies showed no colocalization with Müller cells. (C) No immunofluorescence was detected in section using preadsorption with the blocking peptide. (F–G) High magnification of photoreceptors showing immunoreactivity in both rods (F) and cones (arrowheads) (G). ZNF408 is expressed in cell bodies of some type of amacrine (D) and ganglion (I) cells and its dendrites in the IPL (A,B,H). Arrows in (A,B,G) indicate spherules of rods while double arrows in (A,B,G) indicate pedicles of cones. Arrowheads in A,B,G show ZNF408 immunolabeling at the border of the OLM. Arrows in (F) indicated the rod myoid and cell body. (D,E) Nuclear and cytoplasmic ZNF408 immunoreactivity in the 661W photoreceptor cell-line. Note the ZNF408 localization in the less condensed chromatin areas. (J) Immunoblotting analysis of ZNF408 protein. Human retina (from two different individual, HR1 and HR2) and 661W photoreceptor cell-line samples expressed strongly immunoreactive bands detected with ZNF408 or GAPDH antibodies (arrowheads). Protein molecular weight markers are given to the left. ZNF408 staining (peptide –) was specifically abolished when the ZNF408 antibody was preincubated with its immunogen peptide (peptide +). Scale bar A,B,C,D,E,G,H = 20, F = 10 µm.
to the nucleus, but occasionally is also present in the cytoplasm of the transfected cells (Fig. 5A and B). In both cases, the accumulation of both mutants in the cytoplasm were statistically significant. However, the p.Arg541Cys mutant did not show a statistically significant decrease in the amount of cells showing nuclear localization of the ZNF408 protein when compared with the p.His455Tyr mutant. Upon co-transfection analysis of GFP-tagged WT ZNF408 and HA-tagged p.Arg541Cys mutant ZNF408, in those
cells where p.Arg541Cys ZNF408 is located in the cytoplasm, it appears to also result in mislocalization of the WT ZNF408 (Fig. 5D). Although similar observations were found with the p.His455Tyr mutant, the amount of mislocalized WT ZNF408 protein appears to be less compared to the FEVR-causing p.His455Tyr variant (Fig. 5C and D).

Discussion
In this study, we combined homozygosity mapping and whole-exome sequencing analysis to identify two novel homozygous mutations in ZNF408 in unrelated Spanish families affected with RP. Furthermore, our functional studies provide evidence for the first association of this gene with inherited retinal dystrophy to date.

ZNF408 belongs to a large family of transcription factors characterized by an N-terminal a set domain and tandem C-terminal C2H2-type zinc fingers. This gene is highly expressed in human adult and fetal retinal tissues, as was shown by RT-PCR analysis (7), suggesting that ZNF408 could have an important role in retina development and homeostasis.

A heterozygous mutation in ZNF408 has been previously described as cause of FEVR. FEVR is a rare, autosomal-dominant inherited disorder characterized by an incomplete vascularization of the peripheral retina and/or retinal blood vessel differentiation.

However, the two RP families described herein did not show any evident sign of FEVR. Interestingly all affected members of both families presented some vitreous alterations but, the ZNF408-linked RP phenotype of our individuals is completely different from that of FEVR and thus represents a different clinical form caused by mutations in ZNF408.

Previous studies have been described posterior vitreous detachment and other vitreous alterations, including fibrillary degenerations, floaters, cottonball-like condensations, non-pigmentary and pigmentary particulation in RP patients (9–11). In our patients, the density of the vitreal alterations is higher to the previously described, blurring the fundus resembling the vitreoretinal dystrophies although the morphology is not exactly veils. Therefore, the homozygous mutations described seem to cause a consistent typical RP phenotype with minor vitreous abnormalities other than those found in FEVR patients.

Strikingly, in the RP-0322 family, we have found a homozygous frameshift mutation that it is expected to truncate the ZNF408 protein at its N-terminal, and thus predicted to result in a complete loss-of-function of ZNF408. The identification of a homozygous likely pathogenic missense variant in a second family strongly supports the implication of ZNF408 in retinal degeneration. Our data suggest that ZNF408 may have essential functions in maintenance of retinal homeostasis, in addition to its role in vasculature development.

Since FEVR is inherited as an autosomal-dominant manner and it is known that asymptomatic FEVR patients frequently have very early manifestation of the vitreoretinal findings on angiography (12), an exhaustive ophthalmic examination was performed to exclude any symptom of FEVR in heterozygous carriers of the missense variants found in this study. However, no vascular alterations were observed in the examined asymptomatic individuals.

It is intriguing how different mutations in ZNF408 could cause distinctive phenotypes of retinal disease with clearly different pathogenic mechanisms and also unequivocal inheritance patterns, such as adFEVR and arRP.

As previously described, the FEVR-associated p.His455Tyr variant could act as dominant-negative mutation. However, although the partial retention of the WT_ZNF408 by the ZNF408_p.Arg541Cys mutant was shown, this is not enough to produce a dominant-negative
activities (13). The greater the number of proteins which participate in a wide variety of cellular activities (13). The greater the number of finger domains, the higher versatility and the more fingers with specific affinity for different ligands in specific classes of proteins (14,15). ZNF408 comprises 10 of such domains that could have different and still unknown cellular functions in several parts of the organism. The presence of mutations in these different domains suggests these changes are altering the interaction of ZNF408 with specific targets, thereby resulting in the dysregulation of different target genes and subsequently are underlying either FEVR or RP.

Although further studies are mandatory to elucidate the role of ZNF408 in the human retina, it is also important to highlight the ZNF408 staining pattern localizes in the dark areas of the nucleus corresponding with the euchromatin fraction supporting the hypothesis that ZNF408 encodes a transcription factor widely expressed in the human retina. Mutations in other genes encoding transcription factors, such as the photoreceptor-specific nuclear receptor subfamily 2 group E member 3 (NR2E3) gene, which plays a critical role in the photoreceptor differentiation, have been associated with a variety of Rbds, such as RP, Goldman-Favre disease or Enhanced S-cone syndrome, and can display different clinical features and patterns of inheritance, including autosomal-dominant and autosomal recessive (16–19).

Further studies on the identification of the transcriptional targets of ZNF408 could help us to highlight new genes implicated for retinal development that could be good candidates for RD.

Here, in order to identify the disease-causing gene in a Spanish family diagnosed with autosomal recessive RP (arRP), we have performed a combined approach including whole homozygosity mapping following the manufacturer’s instructions.

Materials and Methods

Patient recruitment

Patients diagnosed with RP were recruited from the Biobank of the Fundación Jiménez Díaz Hospital (Madrid, Spain). Diagnostic criteria of RP included night blindness and/or peripheral visual loss, with visual field loss and poor visual acuity in advanced stages of the disease. A commercial arRP genotyping microarray v2.0 (AsperBiotech, Tartu, Estonia) was used in the index cases to discard known mutations (20). A total of 218 unrelated Spanish families with autosomal recessive or sporadic RP were selected. Informed consent was obtained from all patients and family members or their legal guardians involved in the study. All procedures were reviewed and approved by the Ethics Committee of the Hospital and adhered to the tenets of the Declaration of Helsinki (Fortaleza 2013).

One hundred and eighty-seven unrelated individuals of Spanish origin without RP family history were screened as controls to evaluate the frequency of the missense mutation found in this study.

Peripheral blood samples from all participating individuals were collected in EDTA tubes. Genomic DNA was extracted with an automated DNA extractor (model BioRobotEZ1; QIAGEN, Hilden, Germany) following the manufacturer’s instructions.

Genetic analysis

Homozygosity mapping

A whole-genome homozygosity mapping was performed using Affymetrix 6.0 genotyping arrays (Affymetrix, Inc., Santa Clara, CA). Arrays were processed according to manufacturer’s protocols by the Spanish Center for Genotyping (CEGEN, Santiago de Compostela, Spain). Homozygosity regions were calculated using the Linkage Disequilibrium-Hidden Markov Model algorithm (21) through the dCHIP software (22). Regions with a size >1 Mb were considered candidate regions to harbor the genetic defect.

Next-generation sequencing

WES analysis was performed by the Spanish Centre for genome Analysis (CNAG, Barcelona, Spain). The SureSelect Human All Exon Version 4 kit (Agilent Technologies, Santa Clara, CA, USA) was used for in-solution enrichment of coding exons and flanking intronic sequences following the manufacturer’s standard protocol. Sequencing was done with an Illumina HiSeq2000 sequencing platform (Illumina) generating paired-end reads up to 100 cycles.

Base calling and quality control were performed using the Illumina RTA sequence analysis pipeline. Bioinformatic analysis of the sequencing data was done by the Bioinformatic Platform for Rare Diseases (CIBERER-BIER, http://www.ciberer.es/bier/), using standard tools for quality control, sequence alignment, and variant calling in combination with our own in-house scripts including that of single nucleotide variants (SNVs) and small indels (23).

Sanger sequencing analysis

Bidirectional automatic sequencing was performed in order to confirm and segregate the obtained results by whole-exome sequencing, also to screen the ZNF408 gene in 217 additional arRP families, and to determine the frequency of one of the missense variations in the control population. Exons and exon-intron boundaries of the ZNF408 gene (NM_024741.2) were analyzed using eight oligonucleotide primers pairs designed using Primer3 software. Sequences and annealing temperatures are available on request. The PCR products were enzymatically purified with ExoSAP-it (USB, Affymetrix) and sequenced using Big Dye Terminator Cycle Sequencing Kit version 1.1 (Applied Biosystems, Carlsbad, CA). The PCR products were resolved on an automated sequencer (ABI 3130xl Genetic Analyzer, Applied Biosystems).

Sequencing results were analyzed by using Staden Package 1.7.0 software by assembling and visualizing the aligned sequences of the exons.

Prediction of the pathogenic effect of the variants

Pathogenicity of this variant was firstly assessed by co-segregation with the disease in the family, by its absence in a total of 374 healthy control Spanish chromosomes and in SNP databases (1000g and EVS databases) and by bioinformatic prediction tools (SIFT, PolyPhen2 and align-GVGD) which classified this variant as most likely damaging and by the amino acid conservation across five orthologs of the ZNF408 protein belonging to different evolutionary branches. The Clustal Omega tool and the Jalview Alignment Editor program were used to analyze the multiple sequence alignments.
Comparative genomic hybridization array
The custom aCGH 8 × 60k using Agilent SurePrint G3 CGH was designed using the Agilent eArray website (https://earray.chem.agilent.com/earray/) with an average distribution of one probe per 150 pb in the ZNF508 gene.

Briefly, genomic DNA (200 ng) from the patient and from a sex-matched control were digested by AluI and Rsal restriction enzymes for 2 h at 37°C, and the digested products were labeled with Cy3-dUTP and Cy5-dUTP fluorochromes using the Sure Tag DNA Labeling Kit (Agilent Technologies). The labeled products were purified, hybridized and washed according to Agilent protocols. The slide was scanned on a SureScan G4900DA scanner (Agilent Technologies), and the resulting TIFF images were converted by the image conversion Feature Extraction software (Agilent Technologies). Results were analyzed by Agilent CytoGenomics software v.2.7 using default analysis method—CGH v2 with the ADM-2 aberration algorithm.

Ophthalmic examination
Ophthalmic examinations were performed for all patients with mutations in ZNF408 and the available heterozygous carriers. The evaluation included visual acuity, intraocular pressure, ocular motility, pupillary reaction, biomicroscopic slit lamp examination and dilated fundus examination. Visual function was evaluated by static perimetry and Ganzfeld electrotetrogaphy according to the International Society for Clinical Electrophysiology of Vision (ISCEV) (24). An IVFA was performed.

Immunohistochemistry
The ZNF408 localization pattern was determined in cryofixed healthy adult human retina sections. They were immunostained at room temperature overnight with rabbit polyclonal antibodies to human ZNF408 from Santa Cruz Biotechnology (Santa Cruz, CA, USA, Catalog No. sc-134190) diluted at 1:50 in 0.1 m sodium phosphate buffer (pH 7.4), 0.5% Triton X-100 in the presence or absence of blocking peptide (20:1 peptide: antibody ratio; LifeTein South Plainfield NJ, USA). Double immunocytochemistry with ZNF408 were performed using it in combination with: monoclonal mouse anti-rhodopsin (Millipore Temecula, CA, USA), goat anti-recoverin (Santa Cruz Biotechnology Inc. TX, USA), goat anti-collagen IV (Millipore Temecula, CA, USA) and monoclonal mouse anti–cone arrestin (Dr MacLeish, Morehouse School of Medicine; Atlanta, GA, USA) (25,26). In addition, the 661W photoreceptor cell-line was also probed with the ZNF408 antibodies.

Furthermore, ZNF408 protein expression was assessed using western blotting on adult healthy human retinas as well as in the 661W photoreceptor cell-line. Briefly, proteins were extracted and subjected to immunoblotting analysis. Proteins (40 µg/lane) were resolved by SDS-PAGE on 4–20% polyacrylamide-gradient gels and electrotransferred to Hybond-P PVDF membranes (GE Healthcare, Buckinghamshire, UK). These were probed at 4°C overnight with the same ZNF408 antibodies used in immunocytochemistry assays at a 1:100 dilution in 25 mM Tris (pH 8.0), 150 mM NaCl, 2.7 mM KCl (TBS) in the presence or absence of blocking peptide (10:1 peptide: antibody ratio; LifeTein South Plainfield NJ, USA), or with mouse monoclonal antibodies to rabbit muscle GAPDH at a 1:1000 dilution (Millipore; Catalog No. MAB374). Thereafter, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse (Pierce, Rockford, IL, USA) IgG at a 1:20,000 dilution. Detection was performed by enhanced chemiluminescence using the SuperSignal West Dura system (Pierce).

Transfection of COS-1 cells
Constructs expressing HA-tagged or eCFP-tagged WT and mutant ZNF408 were designed using the mammalian expression vector pcDNA3-HA/DEST or pcDNA3-eCFP/DEST, both driven by a cytomegalovirus promoter.

COS-1 cells where transiently transfected using Effectene (Qiagen) according to the manufacturer’s instructions. In both cases, unpaired Student’s t-test as well as Mann–Whitney analyses were used to determine if there were statistically significant differences. Images were obtained with a Zeiss Axiolmage Z1 upright fluorescent microscope and processed with a Zeiss ApoTome slider module. All experiments were performed in duplicate (7).

Supplementary Material
Supplementary Material is available at HMG online.

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