New COL6A6 variant detected by whole-exome sequencing is linked to break points in intron 4 and 3′-UTR, deleting exon 5 of RHO, and causing adRP

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Purpose: This study aimed to test a newly devised cost-effective multiplex PCR assay for the molecular diagnosis of autosomal dominant retinitis pigmentosa (adRP), as well as the use of whole-exome sequencing (WES) to detect disease-causing mutations in adRP.

Methods: Genomic DNA was extracted from peripheral blood lymphocytes of index patients with adRP and their affected and unaffected family members. We used a newly devised multiplex PCR assay capable of amplifying the genetic loci of RHO, PRPH2, RP1, PRPF3, PRPF8, PRPF31, IMPDH1, NRL, CRX, KLHL7, and NR2E3 to molecularly diagnose 18 index patients with adRP. We also performed WES in affected and unaffected members of four families with adRP for whom a disease-causing mutation was previously not found.

Results: We identified five previously reported mutations (p.Arg677X in the RP1 gene, p.Asp133Val and p.Arg195Leu in the PRPH2 gene, and p.Pro171Leu and p.Pro215Leu in the NHO gene) and one novel mutation (p.Val345Gly in the RHO gene) representing 33% detection of causative mutations in our adRP cohort. Comparative WES analysis showed a new variant (p.Gly103Arg in the COL6A6 gene) that segregated with the disease in one family with adRP. As this variant was linked with the RHO locus, we sequenced the complete RHO gene, which revealed a deletion in intron 4 that encompassed all of exon 5 and 28 bp of the 3′-untranslated region (UTR).

Conclusions: The novel multiplex PCR assay with next-generation sequencing (NGS) proved effective for detecting most of the adRP-causing mutations. A WES approach led to identification of a deletion in RHO through detection of a new linked variant in COL6A6. No pathogenic variants were identified in the remaining three families. Moreover, NGS and WES were inefficient for detecting the complete deletion of exon 5 in the RHO gene in one family with adRP. Carriers of this deletion showed variable clinical status, and two of these carriers had not previously been diagnosed with RP.

Retinitis pigmentosa (RP) is the most common form of inherited retinopathy [1-3], affecting more than 1.5 million people worldwide. RP displays all three modes of Mendelian inheritance—autosomal dominant (adRP), autosomal recessive (arRP), and X-linked (XLRP)—as well as digenic [4] and mitochondrial inheritance modes [5,6]. In particular, adRP has been associated with mutations in nearly 20 genes [6-8]. Over the last two decades, candidate genes associated with adRP in individual patients have been screened for mutations in different populations, using a variety of methods, including single-strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and denaturing high-performance liquid chromatography (DHPLC) followed by direct genomic sequencing. Our group previously employed these methods to achieve molecular diagnosis of 23.5% (52 of 221) of families with adRP in a Spanish population. More recently, mutation arrays have been used for surveys, accounting for identification of disease-causing mutations in 15.1% of patients with adRP [9].

There is growing interest in genetic diagnostics within current clinical practice, and next-generation DNA sequencing (NGS) technology is becoming increasingly necessary to characterize mutations that cause monogenic disease [10-12]. Several studies have reported massively parallel sequencing of RP candidate genes [13-18]. Clinical NGS molecular testing surveys of patients with RP have revealed that most adRP-causing mutations are detected in fewer than a dozen candidate genes [7,8].

We recently developed a cost-effective method of testing for mutations in 12 common adRP-associated genes that account for more than 95% of known mutations [19]. To improve our adRP molecular testing approach for routine use, we devised a new simple and robust multiplex PCR assay capable of amplifying the genetic loci of RHO (GeneID: 6010; OMIM 180380), PRPH2 (GeneID: 5961; OMIM 179605),
The study was conducted in 21© 2015 Molecular Vision

We first conducted a pilot GeneID: 1406; OMIM From GenBank, we obtained the genomic TM 21 - TM 4 1

For each six-plex obtained per sample without male-to-male disease transmission, family members with or without male-to-male transmission. In families Declaration of Helsinki and approved by the internal clinical HD-OCT; Carl Zeiss). All patients gave informed consent was examined with ocular coherence tomography (Cirrus Spain), and full-field electroretinography. Macular anatomy testing with a Humphrey system 745I (Carl Zeiss, Barcelona, Spain), and fundus examination, fundus photography, visual field pressure measurement, biomicroscopic slit-lamp examination and acuity measurement with Snellen optotypes, intraocular and fundus examination, fundus photography, visual field testing with a Humphrey system 745I (Carl Zeiss, Barcelona, Spain), and full-field electroretinography. Macular anatomy was examined with ocular coherence tomography (Cirrus HD-OCT; Carl Zeiss). All patients gave informed consent before participation. This study adhered to the ARVO state patients with retinitis pigmentosa who had been admitted to the Terrassa Hospital or sent from other Spanish hospitals. All patients underwent a full ophthalmic and electrophysi A family with adRP was defined as a family with adRP who had been previously excluded for adRP-causing mutations by multiplex PCR and NGS. In one family with adRP, WES revealed a new genetic variant in the RP-unrelated gene COL6A6 (GeneID: 131873), which segregates in a three-generation family with adRP with a variable clinical phenotype. However, this COL6A6 genetic variant was considered to be linked to the deletion in RHO rather than causative of adRP. The present article also discusses the advantages and limitations of mutation detection using NGS and a comparative WES approach.

METHODS

Patients and DNA samples: The study was conducted in patients with retinitis pigmentosa who had been admitted to the Terrassa Hospital or sent from other Spanish hospitals. All patients underwent a full ophthalmic and electrophysiological [20] examination that included best-corrected visual acuity measurement with Snellen optotypes, intraocular pressure measurement, biomicroscopic slit-lamp examination and fundus examination, fundus photography, visual field testing with a Humphrey system 745I (Carl Zeiss, Barcelona, Spain), and full-field electroretinography. Macular anatomy was examined with ocular coherence tomography (Cirrus HD-OCT; Carl Zeiss). All patients gave informed consent before participation. This study adhered to the ARVO statement on human subjects, conducted in accordance with the Declaration of Helsinki and approved by the internal clinical research ethics committee (CEIC) of Terrassa Hospital, Spain.

A family with adRP was defined as a family with at least two affected members in successive generations, with or without male-to-male transmission. In families without male-to-male disease transmission, family members were considered to have adRP if men and women showed similar clinical phenotypes or if an XLRP-linked association was previously excluded by linkage analysis of known XLRP loci. However, the possibility of XLRP linkage with complete penetrance in women in these families should not be discarded [8]. We obtained DNA from 18 adRP index cases. In 12 cases, DNA was isolated from peripheral blood lymphocytes via an automatic process using the MagNA Pure Compact Instrument (Roche, Barcelona, Spain) following the manufacturer’s protocol. The other six DNA samples were obtained from the index cases of families with adRP who had been referred from centers in the EsRetNet network.

Oligonucleotide design and multiplex PCR amplification from gDNA: From GenBank, we obtained the genomic reference sequences for 11 adRP-associated genes: CRX, IMPDH1, KLHL7, NRL, NR2E3, PRPF3, PRPF8, PRPF31, PRPH2 (RDS), RHO, and RPI. The oligonucleotide primers for multiplex PCR (Appendix 1) were designed using the Oligo 7.41 program (Molecular Biology Insights Colorado Springs, CO), with the aim of amplifying most of the coding exons and flanking splice junctions from each gene. The thermodynamic-based program MFEprimer-2.0 was also used to check PCR primer specificity and compatibility [21,22]. To obtain each library, we used 44 pairs of primers divided into six-plex. Each forward and reverse primer included an M13 tag at the 5′-end for posteriori sequencer adaptor insertion (Appendix 1). Multiplex PCR reactions were performed in a 25-µl reaction mix containing 20 ng gDNA as the template, using the Qiagen Multiplex PCR Kit (Qiagen, Barcelona, Spain) supplemented with a 0.5X final concentration of Q-Solution (Qiagen) and following the manufacturer’s recommendations. Appendix 1 presents the specific plex conditions and primers used.

Chimerical sample preparation: We first conducted a pilot experiment to evaluate the appropriateness of the multiplex PCR method. This experiment comprised a parallel sequencing run of five chimerical samples prepared using DNA samples from several patients previously molecularly diagnosed with adRP. Each chimerical sample contained three point mutations distributed within its six-plex (Table 1), which helped evaluate the efficacy of this approach for successfully detecting genetic variations.

Library construction and sample-specific DNA barcode for parallel NGS: For each six-plex obtained per sample via the multiplex PCR assay, the DNA concentration was measured using an Epoch™ Microplate Spectrophotometer combined with the Take3™ Multi-Volume Plate (Izasa, Barcelona, Spain), following the manufacturer’s instructions. The samples were then pooled at equal concentrations. To
simultaneously test several samples in a single sequencing run, each sample library was constructed using a specific adaptor. Each adaptor included a unique sequence of ten nucleotides (called the molecular identifier, MID) to distinguish each sample after NGS. The MID adaptors were inserted into the DNA fragments using a limited-cycle PCR (20 cycles at 95 ºC for 30 s, 50 ºC for 30 s 72 ºC for 1 min), with a short M13-sequence tag included at the 5’-end of the specific primers. From the 454 Standard MID set (Roche), the present study used MIDs 1 to 5 (corresponding to chimerical samples 1 to 5; Table 1), and MIDs 1 to 18 (corresponding to the 18 analyzed index patients with adRP), which were compatible with the amplicon library protocol. The DNA libraries from each sample were then pooled so they could be clonally amplified through emulsion PCR and sequenced using the GS 454 Junior platform (Roche).

Clonal amplification of DNA libraries and NGS: Next, the DNA libraries generated from the multiplex system were clonally amplified. An emulsion PCR (emPCR) technique was performed following the instructions of the emPCR Amplification Method Manual-Lib-A (Roche). Once the DNA libraries possessed the sequencing adaptors (or MID adaptors) and had gone through the clonal amplification process, they were loaded into a PicoTiter sequencing plate (PTP). The sequencing run was prepared as described in the Sequencing Method Manual (GS Junior Titanium Series, Roche). DNA library sequencing was performed for 200 nucleotide cycles (approximately 500 bases) using a GS 454 Junior instrument (Roche) following the manufacturer’s protocols.

Data analysis and variant annotation: The data from a sequencing run were processed using the GS Run Processor application to convert raw images into signal intensity values. The data processing steps were configured as part of the sequencing run using the Instrument Procedure Wizard, as described in the GS Sequencer application manual (Roche). Appendix 2 presents the data processing pipeline, specifying the options required to perform data processing with respect to what should be processed (the NGS libraries).

The processed and quality-filtered reads were then analyzed with the GS Amplicon Variant Analyzer (AVA) for the multiplex PCR assay analysis. The amplicons (excluding adaptors and MIDs) were used as the reference to align amplicon reads, and template-specific portions of the fusion primers were regarded as the forward and reverse primers. The known mutations in the selected samples were defined as substitutions relative to the reference sequence. Correspondence between the samples and MID tags was specified, and since the same MID was present in both orientations, an “either” encoding multiplexer was used to demultiplex the reads.

All sequence variants were named according to the Human Genome Variation Society’s recommended guidelines, using the A of the ATG translation initiation codon as nucleotide +1. Each HCDiff was qualified as a single-nucleotide polymorphism (SNP) or a disease-causing mutation. Analysis for pathogenicity of genetic variants was performed using the SIFT, PolyPhen2, and MutationTaster algorithms.

| Chimerical sample | Gene | Variant | Protein change |
|-------------------|------|---------|----------------|
| 1                 | RHO  | c.119C>T| p.Leu40Pro     |
|                   | PRPF31| c.735C>T| None (c.735C>T) |
|                   | IMPDH1| c.926G>C| p.Arg309Pro    |
|                   | RHO  | c.644C>T| p.Pro215Leu    |
| 2                 | IMPDH1| c.962C>T| p.Ala321Val    |
|                   | CRX   | c.425A>G| p.Try141Cys    |
|                   | RHO  | c.1040C>T| p.Pro347Leu   |
| 3                 | PRPF8 | c.6968_6988del21bp| p.Val2325fsX2329 |
|                   | RPI   | c.2115delA| p.Lys705fsX712 |
|                   | PRFP31| c.669_770insA| p.Lys257fsX277 |
| 4                 | PRPF3 | c.1466C>A| p.Ala489Asp   |
|                   | RPI   | c.2038C>T| p.Arg677X     |
|                   | RHO  | c.217_219delAAC| p.Asn73del   |
| 5                 | PRPF31| c.328_330delATC| p.Ile109del  |
|                   | PRPH2 | c.641G>A| p.Cys214Tyr    |
Genetic variants that affected the splicing process were analyzed using NetGene2, NNSPLICE v0.9, Human Splicing Finder (HSF), and RESCUE-ESE.

**WES analysis:** Of the 18 families previously studied with gene expression and genetic variant analysis, we constructed NGS libraries from the plate, and column-purified using the High Pure PCR Product Purification Kit (Roche). These samples were then submitted to StabVida (Oeiras, Portugal) for direct PCR Product Purification Kit (Roche). These samples were then subjected to cluster generation for the HiSeq2000 following the manufacturer’s instructions. Whole-exome sequencing was performed using the Illumina Genome Analyzer (KAPA Biosystems, Carlsbad, CA) and Coffalyser.Net software version 2.5p1. Appendix 4 presents the average reads per amplicon obtained for each gene. High numbers of reads were obtained from all amplicons for all genes—except the product of that probe. For family RPT65, a heterozygous RHO deletion was detected using 1.5% agarose gel electrophoresis of the PCR fragments obtained using a forward primer (5′-TCA CGG TCG TGA GGG TCC A-3′) that targets exon 4 and a reverse primer (5′-TGC CTC CTC CAC CTC TAG C-3′) targeting the 3′-untranslated region (UTR) of RHO (NCBI Ref Seq NG_009115.1).

**RESULTS**

Parallel mutation detection assay in adRP-associated genes by multiplex PCR and NGS: We constructed NGS libraries that contained more than 40 amplicons obtained by multiplex PCR of six-plex. These libraries were used to establish a mutation detection assay covering a gene panel of 11 candidate genes commonly associated with adRP. To evaluate the appropriateness of the multiplex PCR method, we first performed a pilot experiment consisting of a parallel sequencing run of chimerical samples 1 to 5.

Capillary Sanger sequencing and MLPA: Amplified products of mutation-positive or ambiguous samples were recovered from the plate, and column-purified using the High Pure PCR Product Purification Kit (Roche). These samples were then submitted to StabVida (Oeiras, Portugal) for direct sequencing on a 3730XL ABI DNA sequencer (Applied Biosystems, Foster City, CA) using the Big Dye terminator V1.1 DNA sequencing kit and the following PCR primers. The reactions for obtaining sequences of 100 nt × 2 (paired-end) were subsequently performed in the HiSeq2000 (Illumina, San Diego, CA) following the manufacturer’s instructions.

Multiplex ligation-dependent probe amplification (MLPA) reactions were performed using the SALSA® MLPA® P235 Retinitis probemix kit (MRC-Holland, Amsterdam, the Netherlands), which is designed to detect deletions/duplications of one or more sequences from RHO 3q21.3, RPP8 q11.2, IMPDH1 7q32, and PRPF31 19q13.4. Analyses were performed using Peak Scanner v1.0 (Applied Biosystems, Carlsbad, CA) and Coffalyser.Net software (MRC-Holland, Amsterdam, the Netherlands). Heterozygous deletions of recognition sequences were expected to produce a 35–50% reduced relative peak area for the amplification product of that probe. For family RPT65, a heterozygous RHO deletion was detected using 1.5% agarose gel electrophoresis of the PCR fragments obtained using a forward primer (5′-TCA CGG TCG TGA GGG TCC A-3′) that targets exon 4 and a reverse primer (5′-TGC CTC CTC CAC CTC TAG C-3′) targeting the 3′-untranslated region (UTR) of RHO (NCBI Ref Seq NG_009115.1).

Retinal COL6A6 gene expression and genetic variant screening in families with adRP and the control population: To investigate retinal COL6A6 expression, we performed PCR amplification of partial exons 2 and 3 from retinal PCR Ready First Strand cDNA (Amsbio, Barcelona Spain). Standard gel electrophoresis was used to detect the PCR fragment that was positive in cDNA and absent from gDNA. The novel genetic variant c.307G>A that causes a p.Gly103Arg change in COL6A6 was investigated in 120 index patients with molecular undiagnosed adRP and 200 controls using real-time PCR with fluorescence resonance energy transfer (FRET) probes. The following primers and probes were used: forward, 5′-CCT GGA GGC T-p (synthesized by Tib Molbiol, Berlin, Germany).
The adRP-associated point mutations found with the Sanger method were detected in the five chimerical samples (Table 2). These mutations were detected with a mean variation of 48.1% (range, 33.9% to 60.0%) with a resulting confidence level of 99.9% [24]. All point mutations were successfully detected with a read count of >30×, with the exception of the RP1 gene-related point mutations. For these mutations, only 15 reads were counted from chimerical sample 3 and 13 reads from chimerical sample 4. However, the primary mutations c.2115delA and c.2038C>T were still effectively detected, showing total variations of 40.0% and 53.9% in chimerical samples 3 and 4, respectively. These values showed a confidence level close to 99.9%, as is required for molecular diagnosis [23,24].

### Molecular diagnosis of patients with adRP by multiplex PCR and NGS

To investigate the clinical use of our previously described multiplex PCR approach, we used this approach to analyze 18 patients with an uncharacterized adRP-causing mutation. This analysis was performed through three distinct NGS runs, each containing a library constructed using DNA from six patients. Appendix 5 shows the average data from NGS runs. NGS data analysis revealed six different mutations (Table 3), which were confirmed by capillary Sanger sequencing. We detected the novel genetic variant c.1034T>G (Val345Gly) in RHO, which segregated in family RPT100 (Figure 1) and was also tested by capillary Sanger sequencing. In our survey using the multiplex PCR and NGS approach, successful molecular diagnosis was achieved in six (33.3%) of the 18 index patients with adRP.

### Assay for molecular diagnosis of adRP families by WES

Of the families with adRP for which a disease-causing mutation was not detected by the multiplex PCR approach, four families (SJD3, RPT65, RPN83, and RPN89) were chosen to undergo comparative WES analysis. The aim was to investigate the usefulness of this technique in molecular diagnosis of adRP and/or to find new adRP-associated genes. We used a trio strategy to find variants that were shared by two related patients with RP but absent in an unaffected family member. The two affected relatives analyzed with WES were selected to be as distantly related as possible to avoid the maximum common genetic variants. WES analysis of the four families with adRP revealed an average of 80 new genetic variants (not annotated in a database) and 110 annotated genetic variants, with a frequency of <0.01. One candidate genetic variant—c.307G>A (p.Gly103Arg) in the COL6A6 gene, which was found only in family RPT65—was identified as a strong candidate based on cosegregation in a four-generation family with adRP, absence in controls, and in silico analysis (see below). No obvious pathogenic genetic

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**Table 2. Mutations detected for each of the chimeric samples using multiplex PCR and next-generation sequencing.**

| Chimerical/MID | Gene | Variant | % Variant | Reads per amplicon |
|---------------|------|---------|-----------|--------------------|
| 1 RHO         | PRPF31 | c.669_770insA | 35.0 | 314 |
| 1 RHO         | PRPF31 | c.6968_6988del21bp | 48.7 | 394 |
| 1 IMPDH1      | RHO   | c.644C>T | 33.9 | 149 |
| 1 CRX         | RHO   | c.1040C>T | 51.2 | 84 |
| 2 PRPF8       | RP1   | c.2115delA | 40.0 | 15 |
| 2 PRPF8       | PRPF31 | c.669_770insA | 35.0 | 314 |
| 2 PRPF8       | RHO   | c.217_219delAAC | 58.1 | 74 |
| 3 PRPF3       | RHO   | c.2115delA | 40.0 | 15 |
| 3 PRPF3       | PRPF31 | c.328_330delATC | 45.4 | 326 |
| 3 PRPF3       | PRPH2 | c.641G>A | 50.5 | 309 |
| 4 PRPF3       | PRPF31 | c.328_330delATC | 45.4 | 326 |
| 4 PRPF3       | PRPH2 | c.641G>A | 50.5 | 309 |
| 5 PRPF3       | PRPF31 | c.328_330delATC | 45.4 | 326 |
### Table 3. Variants causing aDRP that were found in 18 patients studied using the multiplex-PCR approach.

| Sample   | Gene | Variant                  | % Variant | Reads per amplicon | Protein change     | HGMD*   |
|----------|------|--------------------------|-----------|--------------------|--------------------|---------|
| RPT65**  | RHO  | (g.9281_10108del)        | –         | –                  | Truncated          | New     |
| RPT100   | RHO  | c.1034T>G                | 44.6      | 65                 | p.Val345Gly        | New     |
| RPN235   | RP1  | c.2029C>T                | 62.5      | 19                 | p.Arg677X          | CM991103|
| RP208    | PRPH2| c.518A>T                 | 64.3      | 68                 | p.Asp173Val        | CM941209|
| RP227    | RHO  | c.512C>T                 | 63.0      | 54                 | p.Pro171Leu        | CM910335|
| Tek14    | PRPH2| c.584G>T                 | 42.9      | 163                | p.Arg195Leu        | CM032999|
| RPN240   | RHO  | c.644C>T                 | 53.9      | 127                | p.Pro215Leu        | CM003954|

*Human Gene Mutation Database  **Genetic variant not detected by multiplex PCR and NGS
variants were identified in the remaining three families, and further analysis of these families is ongoing.

New genetic variant in COL6A6 detected by WES cosegregates in one family with adRP: Appendix 6 shows the candidate genetic variants found by WES in family RPT65, which were validated using direct capillary Sanger sequencing and segregated in the family. The new genetic variant c.1034T>G (p.Gly103Arg) in COL6A6 was the only variant in the family that cosegregated with the phenotype (Figure 2). The presence of the genetic variant c.1034T>G in the RPT65 family was validated by FRET assay (Appendix 7). We also used FRET to screen for this genetic variant in 120 index patients with adRP (in which the adRP-causing mutation is still unknown) and in 200 controls, none of whom exhibited the p.Gly103Arg genetic variant in COL6A6. PCR amplification of a fragment containing the juxtaposing sequences of exons 2 and 3 from a retinal cDNA sample revealed retinal expression of COL6A6.

Exon 5 deletion in RHO linked to the new genetic variant of COL6A6: The COL6A6 gene was located in chromosome 3 only 1 Mbp away from RHO loci, a gene where most adRP-causing mutations are identified. Thus, we performed MPLA analysis to determine whether there was an abnormal copy number of genomic DNA sequences at the RHO locus. The results showed a nearly 30% reduction (not shown) in one probe that targets exon 5 of RHO, suggesting a deletion in this locus. Direct genomic sequencing of the complete RHO gene revealed a deletion beginning in intron 4 (g.9281_10108del) encompassing all of exon 5 and 28 bp of the 3'-UTR. PCR amplification was also performed to detect this RHO deletion and to segregate it within family RPT65 (Figure 2B). This deletion (g.9281_10108del) was present in all patients and carriers of the new COL6A6 genetic variant, showing the linkage between both variants. Moreover, unaffected members of the family did not carry either of these genetic variants, showing no recombinants for the variants within the RPT65 family.

Ophthalmic examination: Table 4 and Table 5 show the clinical features of the families RPT100 (Figure 1) and RT65 (Figure 2), who carried novel rhodopsin mutations found in our survey. Initially, we knew only the II:1 branch of the

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Figure 1. Pedigree and Sanger sequencing of family RPT100. A: Pedigree of the adRP family RPT100, which carries the mutation p.Val345Gly in RHO. Capillary Sanger sequencing showed either the presence of the mutation (+) or of the wild-type allele (−). Squares and circles represent men and women, respectively. The open symbols represent unaffected family members, while the completely filled symbols represent patients with retinitis pigmentosa (RP). B: Fluorogram representation of the Sanger sequencing of mutation c.1034T>G in the RHO gene.
RPT65 family, who had been ophthalmologically examined and followed for years in our hospital (Table 5). They showed early onset of symptoms, including the appearance of night blindness during their early 20s. Fundus examination showed bone spicules, attenuated blood vessels, and optic disc pallor that worsened with age. Visual field testing revealed important visual field constriction in the patients’ 20s, with normal function of the foveal photoreceptors allowing good central vision up until the end-stage of the disease (Figure 3 and Figure 4). Affected family members who were previously diagnosed with RP (descendants of II:1) experienced a slow rate of vision loss and had a more pericentral pattern of degeneration and pigment deposition.

Soon after discovering the p.Gly103Arg variant in COL6A6 among the descendants of II:1, we found in the central region of Spain a family branch of II:2 who had recently noticed the disease in their family. Member IV:4 was clinically diagnosed with RP after an ophthalmic

| Family member | Age (years) | Onset of NB | Current VFC | Current VA/BE | Current ERG | Funduscopy |
|---------------|-------------|-------------|-------------|---------------|-------------|------------|
| II-2          | 57          | 25          | <10° central | 10/200         | Not detectable | Typical RP |
| II-3          | 54          | 5           | <10° central | 10/200* 20/50** | Not detectable | Typical RP |
| II-4          | 50          | 14          | <10° central | 20/30* LP**    | Not detectable | Typical RP |
| III-3         | 28          | 10          | <10° central | 20/30         | Not detectable | Typical RP |
| III-4         | 23          | 12          | <10° central | 20/30         | Not detectable | Typical RP |

NB, night blindness; VFC, visual field constriction; VA, visual acuity; BE, both eyes; LP, light perception *right eye **left eye
examination prompted by experiencing certain vision difficulties in semidarkness. Segregation analysis with FRET and capillary sequencing of the \textit{COL6A6} genetic variant in the family branch descendent of II:2 (Figure 2) showed that the affected member IV:4 and the undiagnosed III:8 and III:10 were carriers. These undiagnosed carriers III:8 and III:10 were ophthalmologically examined after detection of the mutation. Their fundus examinations showed typical RP signs, although the patients stated that they were unaware of the disease. Some family members reported that they had not noticed any visual impairment in the obligate carrier II:2, who died at the age of 83 years. Finally, we found that all carriers of the novel \textit{COL6A6} genetic variant p.Gly103Arg in this family were also carriers of the 827-bp deletion (g.9281_10108del) in \textit{RHO} (Figure 2).

**DISCUSSION**

In the present study, we analyzed 18 families with adRP to detect mutations in the genetic loci most commonly associated with adRP. We previously devised a robust and rapid

| Family member | Age (years) | Onset of NB | Current VFC | Current VA/BE | Current ERG | Funduscopy |
|---------------|-------------|-------------|-------------|---------------|-------------|------------|
| III-3         | 61          | 14          | <10° central| 20/30         | Not detectable | Typical RP |
| III-10        | 43          | N/A         | <10° central| 20/25 25/25   | N/A                | Typical RP |
| IV-2          | 37          | 10          | <10° central| 20/20         | Not detectable | Typical RP |
| IV-3          | 36          | 13          | <10° central| 20/20         | Not detectable | Typical RP |
| IV-4          | 9           | 9           | <10° central| 20/20         | Severe loss of amplitude in scotopic, maxima, and photopic responses | Typical RP |

NB, night blindness; VFC, visual field constriction; VA, visual acuity; BE, both eyes, N/A not applicable

Table 5. Clinical features of adRP family RPT65 carrying the g.9281_10108del mutation in \textit{RHO}.

Figure 3. Lower macular sensitivity is shown with age. Microperimetric map image (MAIA, Topcon) of II:1 and son IV:2 (from family RPT65) who show normal sensitivity in the central macula (green area) and decreased sensitivity at the peripheral macula (red area). Thirty-six stimulus locations covering the central 10° field were tested.
method that uses multiplex PCR and an NGS benchtop platform, and that can be performed in any routine molecular genetics laboratory. This method is simple and cost-effective for the analysis of individual or few samples, which matches the current demand of laboratories in clinical hospitals. Our estimated cost for the analysis of six samples in a parallel run is approximately €200 using a GS Junior platform. Using this method revealed the disease-causing mutation in 33.3% of Spanish families with adRP, even when the mutation was new (p.Val345Gly in \textit{RHO}). There are several possible explanations for the lack of detection of a disease-causing mutation in >60% of Spanish families who were analyzed for the most common adRP-associated genes, here and in previous studies [9,25]. Some of these families may actually have XLRP with female carriers who express the clinical phenotype of RP [8].

It is also possible that the mutation causing adRP is in an unknown gene or otherwise escapes the detection methods used. We reevaluated the families with adRP for mutation detection and selected those with male-to-male disease transmission as well as families that had been previously excluded for XLRP by linkage analysis.

In an attempt to find new adRP-associated mutations, three years earlier we had analyzed five index patients by DNA target capture and NGS using a panel of 448 genes, including those known to be associated with RP [26]. However, the present cost of WES is nearly ten times lower than it was when the previous candidate gene panel was performed. The advancements and cost decreases in WES analysis led us to evaluate this methodology for possible routine molecular diagnostics of adRP. The large number of individual genetic variants necessitates analysis using at least trios of family individuals. Thus, the standard cost of €850 per sample should be at least tripled for WES analysis. Moreover, to minimize the common genetic variants, the analysis should include patients with the most minor possible relationship (e.g., different family generations). This limits the potential of WES as molecular diagnosis for use only in families with a large number of individuals available (both patients and unaffected family members). Additionally, characterization of a disease-causing mutation usually requires costly and time-consuming work for segregation of the identified candidate genetic variants (an average of 80). This limits the use of WES in routine molecular diagnosis and circumscribes its use in the detection of new genetic disease-causing variants. We used WES to analyze genetic variants in the II:1 branch of family RPT65. Comparative analysis of the new

Figure 4. In the upper two panels, an OCT image of a 5-mm horizontal scan shows a distinct and continuous IS/OS line, correlating with the 20/20 visual acuity in the right eye of patient IV:2 from family RPT65. In the lower two panels, intraretinal cysts in the intermediate retina and abnormally structured IS/OS line correlate with the mother’s (II:1) poorer visual acuity in the right eye (20/30).
genetic variants that were present in patients and absent in the unaffected family member revealed a list of 21 candidate variants—of which only c.307G>A (p.Gly103Arg) in the COL6A6 gene cosegregated with the clinically diagnosed patients with RP. This variant was also present in the obligate III:10 carrier and in family member II:8 who was not previously diagnosed with RP. We positively characterized this new genetic variant as a possible cause of adRP, showing that it was absent in the 200 analyzed controls. We also demonstrated that the COL6A6 gene was expressed in the retina. However, no functional analysis was performed because, presumably, the COL6A6 protein [27] plays a structural role in the retina rather than a function that is measureable in vitro.

Based on these data and the standard criteria used for finding rare disease-causing variants using WES [28-30], the p.Gly103Arg variation in the COL6A6 gene could be considered a strong candidate for the cause of adRP in the RPT65 family. Moreover, in silico analysis of this genetic variant by different approaches (including SIFT, PolyPhen, and MutationTaster) all predicted pathogenicity for the change p.Gly103Arg in the COL6A6 protein. This result could be interpreted as the final point in a WES analysis that results in the identification of a novel disease-causing mutation in a new adRP-associated gene. When possible, the complete characterization of a novel gene associated with a genetic disease should involve a functional test. However, the positive data obtained by WES are sufficient that COL6A6 could be considered a marker for disease-causing in this family.

The locus of COL6A6 in chromosome 3 is nearly 1 Mbp from the rhodopsin gene, where most adRP-causing mutations have been found. In this family, RHO had been previously screened for mutation, but no RHO gene mutation was detected either with DGGE or with NGS analysis. As has been previously pointed out [29,31-33], the usefulness of these techniques may be limited in the case of a mutation that encompasses intronic regions or deletions that affect the amplification or target capture. Sanger sequencing of the complete RHO gene in the family revealed an 827-bp deletion (g.9281_10108del) in intron 4 that encompassed all of exon 5 and 28 bp of the 3′-UTR of RHO. In detection methods that rely on PCR amplification, such as DGGE and multiplex PCR, the reverse primer used was placed over only the deleted 3′ region of RHO, and thus, only the wild-type allele was amplified. However, revision of the read coverage in our NGS assays of the RHO-deleted region showed that the results were insufficient to detect the deletion mutation.

If translated to the protein, the mutant allele g.9281_10108del may cause a pathogenic mechanism through a dominant-negative effect. If this mutant RHO is not translated or is processed by a nonsense-mediated mRNA decay (NMD) mechanism, it could lead to a haploinsufficiency effect. Similar to this deletion of exon 5 of RHO, two Scottish families with adRP reportedly showed a G-to-A transition in the highly conserved AG dinucleotide in the 3′-acceptor splice site of exon 4 of RHO [34]. This mutation, revealed by illegitimate transcription analysis of circulating blood lymphocytes, shows an aberrant RNA product in which exon 4 is spliced with an adenine of the 3′-UTR of RHO, thus excluding exon 5 plus 143 bp of the 3′-UTR [35]. However, there is currently no evidence that this mutant is translated to a protein.

The RP-affected carriers of the mutation g.9281_10108del in RHO showed a phenotype that included early-onset night blindness and bone spicules, with slow disease progression. These patients with RP showed relatively well-conserved visual function compared with most other patients carrying an RHO mutation—for example, the p.Val345Gly carriers, in whom RP causes early visual impairment. However, mild or even recessive RP phenotypes caused by RHO mutations have been previously reported in carriers of some mutations that predict splicing alteration or a truncated protein [36-41]. Revision of the RP phenotype associated with mutations in PRPF8 (a splicing pre-mRNA factor) leads to an early-onset severe alteration in the peripheral retina, but the patients generally retain good visual acuity until the fifth or sixth decade of life [42,43]. These RP phenotypes are comparable with the ones presently observed in patients with the RHO gene deletion of exon 5. It can be speculated that there is a convergent pathogenic mechanism caused by RHO haploinsufficiency, with a lack of rhodopsin due to an untranslated mutated protein and an insufficient RHO splicing rate caused by a mutation in PRPF8.

**APPENDIX 1. MULTIPLEX-PCR CONDITIONS FOR NGS TARGET ENRICHMENT (PLEX A TO F).**

To access the data, click or select the words “Appendix 1.”

**APPENDIX 2. WORKFLOW OF THE DATA PROCESSING SCHEME IN THE GS JUNIOR SYSTEM FOR AMPLICON ANALYSIS. THE BLOCKS IDENTIFY THE VARIOUS DATA ACQUISITION, DATA PROCESSING, OR DATA ANALYSIS APPLICATIONS AND THEIR OUTPUTS.**

To access the data, click or select the words “Appendix 2.”
APPENDIX 3. SEQUENCE READS OBTAINED BY NGS IN THE PARALLEL RUN OF THE CHIMERICAL SAMPLE.

To access the data, click or select the words “Appendix 3.”

APPENDIX 4. AVERAGE NUMBER OF READS PER AMPLECTRON FOR EACH GENE ANALYZED.

To access the data, click or select the words “Appendix 4.”

APPENDIX 5. AVERAGE NUMBER OF READS PER AMPLECTRON FOR EACH ANALYZED GENE AMONG THE 18 PATIENTS.

To access the data, click or select the words “Appendix 5.”

APPENDIX 6. VARIANTS SELECTED FOR SEGREGATION STUDIES.

To access the data, click or select the words “Appendix 6.”

Bold type line shows the only identified genetic variant that co-segregated with RP in the RPT65 family.

APPENDIX 7. FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) METHOD USED FOR SCREENING THE GENETIC VARIANT C.307G>A IN COL6A6 IN ADRP INDEX PATIENTS AND A CONTROL SPANISH POPULATION.

To access the data, click or select the words “Appendix 7.”

FRET fluorogram shows a sample that was wild-type (red peak) and one that was heterozygous (blue double peak) for the genetic variant.

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