Mutation analysis in 129 genes associated with other forms of retinal dystrophy in 157 families with retinitis pigmentosa based on exome sequencing

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Purpose: Mutations in 60 known genes were previously identified by exome sequencing in 79 of 157 families with retinitis pigmentosa (RP). This study analyzed variants in 129 genes associated with other forms of hereditary retinal dystrophy in the same cohort.

Methods: Apart from the 73 genes previously analyzed, a further 129 genes responsible for other forms of hereditary retinal dystrophy were selected based on RetNet. Variants in the 129 genes determined by whole exome sequencing were selected and filtered by bioinformatics analysis. Candidate variants were confirmed by Sanger sequencing and validated by analysis of available family members and controls.

Results: A total of 90 candidate variants were present in the 129 genes. Sanger sequencing confirmed 83 of the 90 variants. Analysis of family members and controls excluded 76 of these 83 variants. The remaining seven variants were considered to be potential pathogenic mutations; these were c.899A>G, c.1814C>G, and c.2107C>T in BBS2; c.1073C>T and c.1669C>T in INPP5E; and c.3582C>G and c.5704–5C>G in CACNA1F. Six of these seven mutations were novel. The mutations were detected in five unrelated patients without a family history, including three patients with homozygous or compound heterozygous mutations in BBS2 and INPP5E, and two patients with hemizygous mutations in CACNA1F. None of the patients had mutations in the genes associated with autosomal dominant retinal dystrophy.

Conclusions: Only a small portion of patients with RP, about 3% (5/157), had causative mutations in the 129 genes associated with other forms of hereditary retinal dystrophy.

Retinitis pigmentosa (RP, OMIM 268000) is the most common and highly heterogeneous genetic group of hereditary retinal degeneration diseases, affecting one in about 3,500–5,000 individuals worldwide [1-3]. So far, mutations in over 60 genes have been reported to be responsible for about half of nonsyndromic RP [4-6]. Phenotypic and molecular genetic overlap has been observed in different forms of retinal degeneration [7,8]; for example, RP might be the main sign of syndromic RP or other related diseases [9-11]. Mutations in a few genes have been shown to cause different forms of retinal dystrophy [7], while a few genes originally held responsible for other forms of retinal dystrophy have been found to cause RP as well [12]. Systematic analysis of all genes responsible for other forms of retinal dystrophy in patients with RP is limited, especially in Chinese cohorts [13,14]. Therefore, systemic evaluation of the frequency of mutations in all genes responsible for other forms of retinal dystrophy, apart from known RP genes, would be valuable.

Our previous whole exome sequencing study detected potential pathogenic mutations in 73 known genes in 86 of 157 patients with RP [15-17]. Due to the highly heterogeneous and genetically and clinically complicated features of RP, mutations in the genes related to more severe or syndromic diseases might be ignored, and mutations in previously analyzed genes might be mistakenly used in molecular diagnosis and clinical evaluation. Therefore, it might be interested to know if mutations in genes associated with other forms of retinal dystrophy may also contribute to RP. In the present study, variants in 129 genes responsible for other forms of retinal dystrophy were analyzed based on the exome data set of the same cohort of 157 patients.

METHODS

This study conformed to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the Zhongshan Ophthalmic Center. Written informed
consent was obtained from the participants or their guardians before the collection of their clinical data and preparation of genomic DNA from their peripheral venous leukocytes. The cohort of 157 probands with RP, including 34 autosomal dominant RP, 27 autosomal recessive RP, 10 X-linked RP, and 86 sporadic RP, were the same as described in our previous study [15]. All 157 probands were initially diagnosed as non-syndromic RP. The criteria for defining RP in the families was based on the oral descriptions of the features by the probands and their family members, such as poor vision and night blindness, or the clinical examination of the other RP patients; the clinical features of RP patients were similar to those of the probands and included attenuated retinal artery and pigment abnormality in the mid-peripheral retina. Whole exome sequencing was performed by the NimbleGen SeqCap EZ Exome (44M) array and Illumina Genome Analyzer II platform with a sequencing depth of 60-fold, as described previously [15,18,19].

A total of 221 genes were listed in RetNet, accessed on January 27, 2014. Of these, 60 of the 62 genes with known association with RP had been analyzed in our previous study; the remaining two were not analyzed because EMC1 was not captured and FSCN2 (MIM: 607643) was confirmed not to be an RP gene [20]. Of the remaining 159 genes, the following 30 were not included in this study: 1) nine genes associated with age-related macular degeneration, since no evidence shows that these genes were associated with monogenic retinal diseases; 2) seven genes encoded by mitochondrial DNA that were not captured by the NimbleGen SeqCap EZ Exome (44M) array; and 3) CHM (MIM: 300390), CYP4V2 (MIM: 608614), and 12 genes associated with Leber congenital amaurosis (LCA) that were independently analyzed in our previous studies [16,17,21]. Ultimately, 129 genes were included in this study. All genes screened in the earlier studies and in the current study are listed in Appendix 1.

Variants in the 129 genes were selected based on the data set from whole exome sequencing. Candidate variants were filtered out with the following multiple-step bioinformatics analysis: 1) the SNPs and short indels in the exon region were filtered against data from dbSNP132, the 1000 Genome Project, HapMap, the YH project, and the Exome Variant Server, removing minor allele frequency (MAF) values that were greater than 0.01, the frequency based on the incidence of RP (1/4000), and the fraction of heterozygotes in Hardy–Weinberg equilibrium; 2) excluded non-coding variants without altering splicing sites predicted by BDGP; 3) excluded the synonymous variants without altering splicing sites in the genes; 4) excluded missense variants predicted to be benign by both Polyphen-2 and SIFT in autosomal dominant and X-linked genes; and 5) compared with the whole exome sequencing data of 633 individuals in ethnic-matched regions without retinal degeneration.

Sanger sequencing was used to confirm the candidate variants with primer pairs targeting the fragments encompassing the candidate variants. Primers (Appendix 2) were designed using Primer3 [22]. The procedures used for amplification, sequencing, and analysis of the target fragments were as previously described [18,19]. Segregation analysis of potential pathogenic variants were performed in available family members. Candidate causative mutations were validated in 192 normal Chinese individuals.

RESULTS

Multiple-step bioinformatics analysis was used to select 90 candidate variants from the 129 genes from the whole exome sequencing of the 157 patients. Of the 90 variants, 83 (92%) were confirmed by Sanger sequencing, while the other 7 (8%) with the low sequencing depth were false-positives. Of the 83, 76 (Appendix 3) were considered as unlikely causes of RP for the following reasons: 1) mutations that are more likely to be pathogenic have already been detected in one of the 72 genes related to RP and LCA or CHM in our previous studies [15-17]; 2) the genotype did not match the patterns of inheritance; 3) the variants did not segregate with the disease; 4) the variants were found in controls or in the 1000 Genome/Exome Variant Server database with a frequency higher than the prevalence rate of RP as causative mutations; 5) the variants were unlikely or less likely to be pathogenic mutations based on previous reports; 6) the phenotype did not match the genotype; and 7) the other allele for compound heterozygous variants if one of them was confirmed to be a false-positive. The remaining seven variants involved three genes: BBS2 (MIM: 606151), INPP5E (MIM: 613037), and CACNA1F (MIM: 300110); these were considered to be potentially causative in five patients (Figure 1 and Table 1), including three patients with homozygous (1) or compound heterozygous (2) mutations in genes associated with an autosomal recessive trait, and two patients with hemizygous mutation in genes associated with an X-linked trait. Limited cosegregation analysis and family structure did not reject the association of the mutations with RP (Figure 1). The carriers with the heterozygous mutant alleles were evaluated as asymptomatic for the disease through follow-up examinations and revisits by phone calls. All 7 mutations were absent in the 192 normal individuals (Table 1).

Clinical data of the five patients with RP are summarized in Table 2. Ocular signs and symptoms in all five patients met the diagnosis of RP (Figure 2). Two patients (RP237...
and RP240) had BBS2 mutations; one had RP alone with a missense mutation and a nonsense mutation, while the other one had BBS syndrome (misdiagnosed as RP) and a homozygous nonsense mutation. The patient (RP374) with INPP5E mutations had typical RP but without noticeable extraocular or systemic anomalies (unfortunately, a brain CT...
| Gene       | Inheritance | Patient ID | Chromosome | Start position | Nucleotide change | Amino acid change | State | Computational prediction | Allele frequency in 1000 G | Patients | NC | Reported |
|------------|-------------|------------|------------|----------------|-------------------|-------------------|-------|--------------------------|----------------------------|-----------|----|----------|
| BBS2       | AR          | RP237      | chr16      | 56536626       | c.899A>G          | p.D300G           | Het   | PoD D                    | 1/2184 1/314 0.384 | Novel    |    |          |
|            |             |            | chr16      | 56530975       | c.1814C>G         | p.S605*           | Het   | - -                      | 1/2184 1/314 0.384 | Novel    |    |          |
|            |             | RP240      | chr16      | 56518732       | c.2107C>T         | p.R703*           | Homo  | - -                      | 1/2184 1/314 0.384 | Novel    |    | Deveault et al. 2011 |
| INPP5E     | AR          | RP374      | chr9       | 139327693      | c.1073C>T         | p.P358L           | Het   | PoD D                    | 1/2184 1/314 0.384 | Novel    |    |          |
|            |             |            | chr9       | 139324862      | c.1669C>T         | p.R557C           | Het   | PrD D                    | 1/2184 1/314 0.384 | Novel    |    |          |
| CACNA1F    | XL          | RP401      | chrX       | 49071594       | c.3582C>G         | p.Y1194*          | Hemi  | - -                      | 1/2184 1/314 0.384 | Novel    |    |          |
|            |             |            |            |                | c.5704-5C>G       | Splicing defect   | Hemi  | SSA -                     | 1/2184 1/314 0.384 | Novel    |    |          |

Note: #, These variants were absent from the Exome Variant Server database and were evaluated in 2184 alleles of autosomal genes from 1000 Genome database. †, In the 157 patients, 109 are male and 48 are female, and there are 205 alleles for genes on the X chromosome. ‡, In the 327 controls, 175 are male and 152 are female, and there are 479 alleles for genes on the X chromosome. The following abbreviations are used: AR, autosomal recessive; AD, autosomal dominant; XL, X-linked; P/SS, Polyphen-2/Splice Site Prediction; 1000 G, 1000 Genome; NC, normal control; Het, heterozygous; Hom, homozygous; Hemi, hemizygous; PrD, probably damaging; PoD, possibly damaging; D, damaging; SSA, splicing site abolished; not applicable.
Table 2. Clinical features of the five patients with mutations identified in this study.

| Patient ID | Gene     | Variations                  | Inheritance | Gender | Age at exam | First symptom | Visual acuity OD/OS | Fundus examination | Electroretinography responses | Other systemic symptom          |
|------------|----------|-----------------------------|-------------|--------|-------------|-----------------|---------------------|---------------------|-----------------------------|-------------------------------|
| RP237      | BBS2     | c.[899A>G]; 1814C>G        | Sporadic    | F      | 14          | EC              | 0.1/0.1            | ARA; PBSL           | Extinguished; Extinguished   | None                          |
| RP240      | BBS2     | c.[2107C>T]; [1073C>T]    | Sporadic    | M      | 13          | PV; NB          | 0.1/0.2            | ARA; SP             | Extinguished; Extinguished   | Polydactyly; Obesity; Diabetes mellitus |
| RP374      | INPP5E   | [1669C>T]                  | Sporadic    | F      | 20          | EC              | 0.2/0.2            | ARA; TLP; PBSL      | Extinguished; Extinguished   | None                          |
| RP401      | CACNA1F  | c.[3582C>G];[0]            | Sporadic    | M      | 9           | NA              | 0.2/0.08           | PD                  | Extinguished; Extinguished   | None                          |
| RP403      | CACNA1F  | c.[5704–5C>G];[0]         | Sporadic    | M      | 32          | PV              | 0.2/0.3            | ARA; PBSL           | Severe reduced; Severe reduced | None                          |

Note: M, male; F, female; EC, early childhood; OD, right eye; OS, left eye; PV, poor vision; NB, night blindness; ARA, attenuated retinal arteries; PD, pigment deposit; PBSL, pigment bone spicule-like; TLR, tapetal-like retinal degeneration.
scan was not available). The two patients (RP401 and RP403) with \textit{CACNA1F} truncation mutations showed generalized retinal degeneration involving both rods and cones.

**DISCUSSION**

Seven putative pathogenic mutations in 3 of the 129 genes known to associate with other forms of retinal dystrophy...
have been detected in 5 of 157 (3%, Figure 3) patients with RP based on whole exome sequencing. These mutations are predicted to be causative based on bioinformatics analysis and comparison with previously reported mutations in BBS2, INPP5E, and CACNA1F. Ocular changes in the five patients with mutations are consistent with RP (Figure 2 and Table 2). These findings suggest that a small portion of RP may be caused by mutations in genes responsible for other forms of retinal dystrophy.

Systematic analysis of genes responsible for other forms of retinal dystrophy in patients with RP has been limited. Previously, genotyping microarrays were used to analyze all known mutations in several genes associated with retinal dystrophy in patients with retinal dystrophy [23-25]. However, only a small subset of known mutations are covered by this technology, and the total diagnostic rate is less than 15% [26,27]. Systematic analysis of most of these genes in patients with RP or hereditary retinal dystrophies has been conducted using targeted-capture sequencing. The frequencies of detected mutations in these genes are from 4% to 11% [13,28,29]. Analysis of individual genes also revealed that mutations in a few genes known to associate with other forms of retinal dystrophy also cause a fraction of RP [12,30,31]. This suggests that the frequency of mutations in genes associated with other forms of retinal dystrophy in our cohort of RP is close to that reported previously.

Consistencies and inconsistencies were both evident in the associations between the phenotypes and the identified mutations in the five patients. The two patients with BBS2 mutations included one with nonsyndromic RP that has missense and nonsense mutations, while the other with Bardet-Biedl syndrome had a homozygous nonsense mutation. A missense and a nonsense mutation in BBS2 have previously been reported to be responsible for Bardet-Biedl syndrome [32,33], and two missense mutations in BBS2 have been described recently in patients with nonsyndromic RP [34]. Comparisons of these findings suggest that the phenotypes might be affected by the type of mutation. Further study is needed to clarify this interesting issue.

The patient (RP374) with INPP5E mutations had no other noticeable signs of systemic anomalies, although mutations in INPP5E have been identified in patients with MORM syndrome [35] and Joubert syndrome [36]. Unexpectedly, two patients (RP401 and RP403) with RP had hemizygous

Figure 3. Proportions of mutations in 129 genes associated with other forms of retinal dystrophy in 157 unrelated patients with retinitis pigmentosa.
truncation mutations in \textit{CACNA1F}; these kinds of mutations have been identified in patients with congenital stationary night blindness [37,38], cone-rod dystrophy [39], and Aland Island eye disease [40], but not in patients with RP. The association of \textit{CACNA1F} mutations with RP in these two patients is uncertain, although the phenotype in the patients is consistent with RP, and mutations in \textit{CACNA1F} are predicted to be null.

Overall, mutations in all known genes—including these 129 genes associated with other forms of retinal dystrophy, 72 genes related to RP and LCA, and \textit{CHM}—could be identified in less than 58% of 157 patients with RP. In each of the disease categories, mutations were detected in 71% (24/34) autosomal dominant RP, 48% (13/27) autosomal recessive RP, 80% (8/10) X-linked RP, and 53% (46/86) sporadic RP. Over 42% of the patients without identified mutations might carry other pathogenic variants in the noncoding regions of the corresponding genes, or may have gross deletion involving whole exons that might be missed by whole exome sequencing and Sanger sequencing, or may be caused by mutations in novel genes yet to be identified. The information about these patients without identified mutations is a valuable resource for seeking out new genes that are responsible for RP.

\section*{APPENDIX 1. GENOMIC INFORMATION OF ALL THE GENES RESPONSIBLE FOR OTHER FORMS OF RETINAL DYSTROPHY IN THE EARLIER STUDIES AND THE CURRENT STUDY.}
To access these data, click or select the words “Appendix 1”.

\section*{APPENDIX 2. PRIMERS USED IN SANGER SEQUENCING.}
To access these data, click or select the words “Appendix 2”.

\section*{APPENDIX 3. OTHER VARIANTS DETECTED IN 129 GENES RESPONSIBLE FOR OTHER FORMS OF RETINAL DYSTROPHY FROM THE 157 UNRELATED PATIENTS WITH RETINITIS PIGMENTOSA.}
To access these data, click or select the words “Appendix 3”.

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