

# An Ophthalmic Targeted Exome Sequencing Panel as a Powerful Tool to Identify Causative Mutations in Patients Suspected of Hereditary Eye Diseases

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**Purpose:** We evaluate the power of a next-generation sequencing-based ophthalmic targeted sequencing panel (NGS-based OTSP) as a genetics-testing tool for patients suspected of a wide range of hereditary eye diseases.

**Methods:** NGS-based OTSP encompasses 126 genes with identified mutations that account for the majority of Chinese families with hereditary eye diseases. A total of 568 probands suspected of having hereditary eye diseases underwent genetic testing by OTSP with targeted phenotype-driven analysis.

**Results:** NGS-based OTSP detected 329 potential pathogenic variants in 62 genes. These mutations might represent the genetic cause in 52% (293/568) of probands suspected of having hereditary eye diseases. Within the disease subgroups, the detection rates were 61% (124/202) for retinal degeneration disease, 53% (35/66) for eye tumors, 49% (53/108) for retinal vessel disease, 46% (13/28) for retinal detachment, 33% (19/58) for significant refractive error, 35% (16/46) for optic atrophy, 48% (11/23) for anterior segment dysgenesis, and 59% (22/37) for other hereditary eye diseases. These detection rates are comparable to those obtained in our previous study performed with whole exome sequencing. Mutations in the same gene were detected in different forms of hereditary eye diseases. The average turnaround time for OTSP is 30 days, and the average cost is 139 USD per patient.

**Conclusions:** NGS-based OTSP is a powerful tool for routine clinical genetic diagnostic testing in patients suspected of having hereditary eye diseases.

**Translational Relevance:** NGS-based OTSP can be used as a routine clinical test to improve the genetic counseling and medical care of patients suspected of having hereditary eye diseases.

**摘要**

**目的:** 评估基于新一代测序的眼科靶向捕获系统 (OTSP), 作为临床常规筛查工具在各种眼遗传病基因突变检测中的效率。

**方法:** 我们设计了一种眼科靶向测序捕获系统, 覆盖了中国眼遗传病患者中最常见的 126 个致病基因。基于这一系统, 我们对 568 名疑似患有各种眼遗传病患者的基因组 DNA 进行了致病基因突变筛查及分析。

**结果:** OTSP 在 62 个基因中检测到 329 个可能的致病变异。这些变异可解释 52% 患者的致病原因 (293/568), 在眼遗传病的不同亚类中突变检出率如下: 遗传性视网膜变性 61% (124/202)、眼肿瘤 53% (35/66)、视网膜血管疾病 49% (53/108)、视网膜脱离 46% (13/28)、严重屈光不正 33% (19/58)、视神经病变 35% (16/46)、眼前段发育不良 48% (11/23)、其它多种罕见遗传性眼病 59% (22/37)。OTSP 的突变检出率与我们既往用全外显子组测序检出率相当。同一基因的不同突变可以导致不同亚类的眼遗传病。OTSP 作为临床常规基因突变检测的平均检测周期约 30 天, 每例患者的检测成本是 138 美金, 较全外显子测序更快速和便宜。

**结论:** 基于新一代测序的 OTSP 是一种高效眼遗传病基因突变筛查工具, 可以用于多种眼遗传病患者的常规临床检测。

**相关转化:** 基于新一代测序的 OTSP 的常规临床应用, 有助于协助疑似眼遗传病患者的诊断、遗传咨询和医疗保健。

## Introduction

Hereditary eye diseases are a phenotypically heterogeneous group of ocular conditions caused by gene defects and often lead to irreversible visual impairment.<sup>1-4</sup> Hereditary eye diseases are the most common causes of blindness and account for 50% to 70% of cases of childhood blindness or severe vision loss.<sup>5,6</sup> One-third of entries in the Online Mendelian Inheritance in Man (OMIM) database include a clinical synopsis that contains terms referring to the structure or function of the eye,<sup>7</sup> and hundreds of causative genes of hereditary eye diseases currently are identified in OMIM (available in the public domain at <http://www.ncbi.nlm.nih.gov/omim/>) and Retinal Information Network (RetNet; available in the public domain at <http://www.sph.uth.tmc.edu/Retnet/>). Although genetic diagnosis is important to determine a prognosis and provide genetic counseling and gene-specific therapy, identifying a causative mutant among hundreds of genes remains a challenge in individual probands in clinical settings, especially in patients with hereditary eye diseases, which exhibit great heterogeneity in terms of genotypes and phenotypes.<sup>1-4</sup> In recent decades, different methods have been developed to directly identify genetic defects. Targeted mutation detection is the cheapest and most robust method to detect known variants among multiple samples, but identifying novel vari-

ants using this technique is difficult.<sup>8</sup> Sanger sequencing is best suited for diseases with highly distinct clinical conditions caused by just genes with few exons, and evaluating hundreds of hereditary eye disease genes by Sanger sequencing is impractical due to its high cost and long turnaround time.<sup>9</sup> Microarray-based phenotype-specific single-nucleotide polymorphism panels are a powerful tool for screening multiple known gene mutations at a low cost and with a short turnaround time. However, the increasing number of causal alleles limits the use of this approach.<sup>10</sup>

High-throughput next-generation sequencing (NGS) massively increases sequencing output by allowing simultaneous analysis of billions of DNA fragments in a single experiment. Therefore, this approach is expected to overcome the technical limitations of other genetic tests for hereditary eye diseases. NGS-based genetic tests, such as disease-specific gene panels, whole exome sequencing (WES), and whole genome sequencing (WGS), have been applied to detect gene defects responsible for different types of hereditary eye diseases, such as inherited retinal disease,<sup>11,12</sup> family exudative vitreoretinopathy,<sup>13</sup> early-onset high myopia,<sup>14</sup> congenital cataract,<sup>15</sup> and infantile nystagmus syndrome.<sup>16,17</sup> However, NGS-based genetic tests are not yet considered routine clinical examinations for patients with suspected hereditary eye diseases in China

mainly for the following two reasons. First, in diseases, such as hereditary eye diseases, that exhibit great heterogeneity in terms of their genetic basis, mode of inheritance, and clinical phenotype, it is difficult for most ophthalmologists to select an appropriate diagnostic panel. Second, although WES and WGS provide good coverage of known disease-associated human genes, their high costs and long turnaround times limit their application in a clinical setting.<sup>18,19</sup> Therefore, there is a need for an NGS-based diagnostic panel that can overcome these two major problems and serve as a routine clinical genetic test for a wide range of hereditary eye diseases.

In a series of previous studies based on Sanger sequencing and WES of genomic samples obtained from thousands of families, we identified a large number of potential pathogenic mutations in genes responsible for different forms of hereditary eye diseases.<sup>15,20–35</sup> Based on the results of these and other related reports, we designed an NGS-based ophthalmic targeted sequencing panel (NGS-based OTSP) that targets 126 genes commonly mutated in Chinese families with different forms of hereditary eye diseases. These genes accounted for 95% of the causative genes detected in our previously analyzed cases. We aimed to evaluate the power of NGS-based OTSP as a routine genetic test to identify molecular pathogenic variants in patients suspected of having a range of hereditary eye diseases in a clinical setting.

## Methods

### Participants

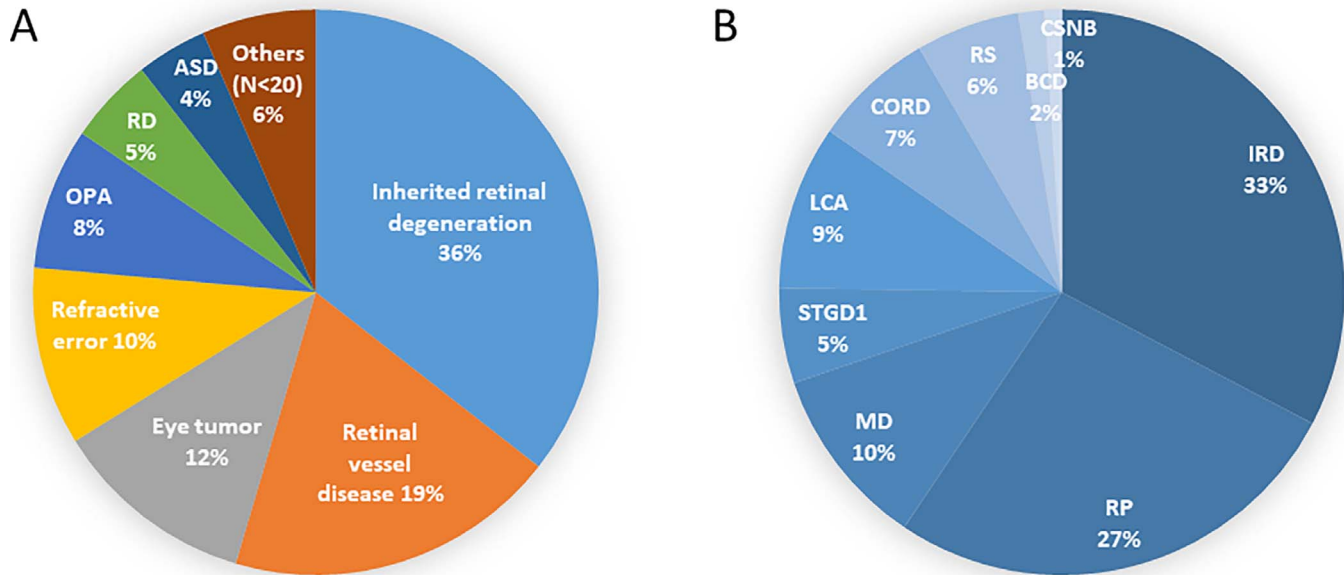
This study was approved by the institutional review board of Zhongshan Ophthalmic Center, Sun Yat-sen University. Written informed consent that complied with the tenets of the Declaration of Helsinki was obtained from all patients or their guardians before enrollment in this study. A total of 568 probands were referred for genetic testing at Zhongshan Ophthalmic Center from September 2016 to October 2017. Referrals for NGS-based OTSP were given primarily by ophthalmologists with different subspecialties, including pediatric and genetic eye diseases, medical and surgical retina and vitreous care, cataracts, glaucoma, cornea care, strabismus and amblyopia, ocular oncology, and optometry, as well as general outpatient clinics. The basic information and initial diagnoses of the probands were collected from the Hospital Information System (HIS) and outpatient records.

### Target Gene Sequencing

Genomic DNA was extracted from peripheral blood using Maxwell 16 DNA Purification Kits (Promega, Madison, WI) according to the manufacturer's standard protocol. In our previous studies, we detected genetic mutations in thousands of patients with a wide range of ophthalmic genetic diseases, which allowed us to determine the spectrum of phenotypes and genotypes of a wide range of ophthalmic genetic eye diseases by Sanger sequencing and WES.<sup>15,20–35</sup> Based on the spectrum, a custom-designed capture panel, that is, NGS-based OTSP, was designed to capture the exons of 126 genes (Supplementary Table S1) that were mutated most frequently and that accounted for over 95% of the genes in which mutations were detected in our patients suspected of having a range of hereditary eye diseases. Genomic DNA was fragmented into approximately 200 base-pair (bp) fragments with a Bioruptor Plus (Diagenode, Liege, Belgium), and then used to generate a paired-end library with a KAPA HTP Library Preparation kit (Roche, Basel, Switzerland). Library capture was completed using a NimbleGen SeqCap EZ Choice Library SR V5 kit (Roche), and the library was sequenced with an Illumina NextSeq550 Mild output v2 kit (150 PE) on an Illumina NextSeq550 Analyzer (Illumina, San Diego, CA). The average depth of the target region was  $\times 250$ . The image analysis and base calling were performed using the Illumina Pipeline to generate the raw data.

### Variant Evaluation

Strand NGS software (Karnataka, India) was used to analyze the sequencing data and detect candidate mutants according to the manufacturer's instructions. The National Center for Biotechnology Information (NCBI) database (NCBI build 19) was used as an alignment reference. All single-nucleotide variants (SNVs) and small insertions and deletions (indels) were annotated and filtered using the NCBI RefSeq Gene, NCBI dbSNP 146, dbSNP138 InDels, 1000 Genomes Project, ExAC database<sup>36</sup> and the Human Genome Mutation Database (HGMD; Professional Version, Qiagen, Germany).<sup>37</sup> Variants with minor allele frequencies (MAF)  $> 0.01$  were filtered out. Nine bioinformatics software programs, including SIFT\_pred (dbNSFP), Polyphen2\_HDIV\_pred (dbNSFP), Polyphen2\_HVAR\_pred (dbNSFP), LRT\_pred (dbNSFP), MutationTaster\_pred (dbNSFP), MutationAssessor\_pred (dbNSFP), FATHMM\_pred (dbNSFP), MetaSVM\_pred (dbNSFP), and MetaLR\_pred



**Figure 1.** Description of the patient population. (A) Eight subgroups of diseases were included in this study. Diseases affecting fewer than 20 patients were categorized as the “other group.” (B) The subgroup comprising inherited retinal degeneration diseases was further divided into nine types of disease subgroups.

(dbNSFP), were used to predict which amino acid substitutions would affect the structures and functions of proteins. Variants with positive predictions from fewer than three types of software were filtered out. Every variant that could potentially affect a splice site was evaluated by the online Human Splicing Finder tool (available in the public domain at <http://www.umd.be/HSF3/HSF.shtml>).<sup>38</sup> A probable molecular diagnosis was made based on the identified potential pathogenic variants (PPVs) when the following criteria were met: (1) a gene with variants was associated with the proband’s phenotype or diagnosis as described in the HIS or outpatient records, (2) the genotype and frequency of the variant were consistent with the expected mode of inheritance of the disease and genes, (3) or the variants of the genes were addressed as disease-causing mutations (DMs) in HGMD, (4) the variants were confirmed by Sanger sequencing, (5) the variants were further supported by segregation analysis when the proband’s family members were available, and (6) all PPV were automatically and/or semimanually classified by InterVar (available in the public domain at <http://wintervar.wglab.org/>)<sup>39</sup> according to the guidelines of the American College of Medical Genetics and Genomics (ACMG)<sup>40</sup> when benign variants were ruled out.

### Statistical Analysis

$\chi^2$  analysis was used to calculate the difference in the detected rate of difference subgroup diseases

among this study, our previous study and other reports.  $P < 0.05$  was considered statistically significant. Statistical analyses were performed with Excel 2018 (Microsoft Corp., Redmond, WA).

## Results

### Proband Demographics

Of the 568 probands who were referred to receive the NGS-based OTSP test, there were 382 males and 186 females (average age of all probands,  $14.48 \pm 14.11$  years; range, 0.39–77.40). According to the first or principal diagnoses in the HIS and outpatient records, these probands suffered from a wide range of hereditary eye diseases (Fig. 1A), including 202 (36%) with inherited retinal degeneration (IRD; Fig. 1B), 54 (27%) with retinitis pigmentosa (RP), 21 (10%) with macular degeneration (MD), 19 (9%) with Leber congenital amaurosis (LCA), 14 (7%) with cone-rod dystrophy (CORD), 12 (6%) with retinoschisis (RS), 11 (5%) with Stargardt disease (STGD1), two (1%) with congenital stationary night blindness (CSNB), and three (1%) with Bietti crystalline corneoretinal dystrophy (BCD). There were 66 (33%) probands without a good diagnosis, but who were included in the subgroup as IRD, 108 (19%) with retinal vessel disease (such as familial exudative vitreoretinopathy [FEVR], retinopathy of prematurity, Coats disease,

and persistent hyperplastic primary vitreous), 66 (12%) with eye tumors, 58 (10%) with significant refractive errors (high myopia or high hyperopia), 46 (8%) with optic atrophy (OPA), 28 (5%) with retinal detachment (RD), 23 (4%) with anterior segment dysgenesis (ASD, including aniridia and primary congenital glaucoma), and 37 (6%) with other rare hereditary eye diseases (17 with congenital motor nystagmus, 12 with congenital lens malformations, six ocular albinism, and two with strabismus). Approximately 46% (259/568) of the probands were referred by two ophthalmologists from the section for Pediatric and Genetic Eye Diseases, and the remaining 54% (309/568) were referred by 53 ophthalmologists from other sections.

### Validation of the NGS-Based OTSP Test for Hereditary Eye Diseases

To validate the NGS-based OTSP test for hereditary eye diseases, we used eight DNA samples obtained from probands with different hereditary eye diseases. Among these individuals, the following 12 mutations in eight genes were detected by WES in our previous study: c.1666-1\_1666del in *COL11A1* (NM\_080629.2), c.626G>C in *NYX* (NM\_022567.2), [c.2783G>A]:[c.139del] in *GUCY2D* (NM\_000180.3), [c.1556T>C]:[c.396-11C>G] in *CNGA3* (NM\_001298.2), [c.3106G>A]:[c.983A>T] in *ABCA4* (NM\_000350.2), c.2476\_2477del in *RPGR* (NM\_001034853.1), [c.1090C>T]:[c.458del] in *IQCB1* (NM\_001023570.2), and c.641\_644del in *SPATA7* (NM\_018418.4). All mutations were detected by OTSP.

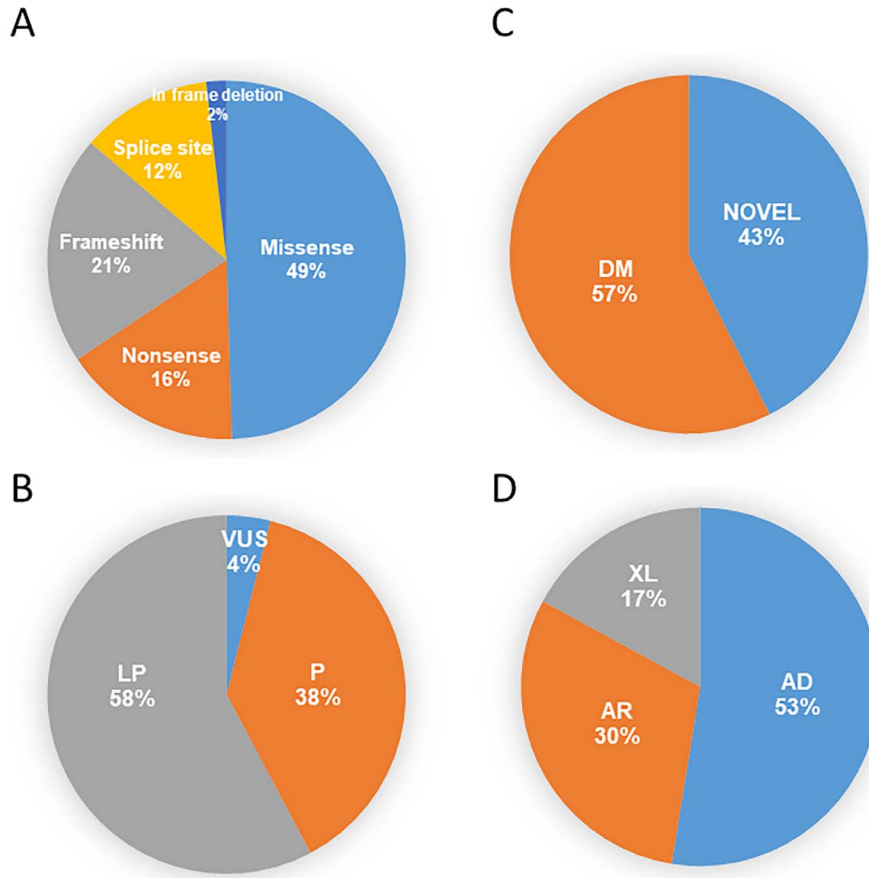
### Potential Pathogenic Variants Detected by the NGS-Based OTSP Test

In total, 329 PPVs were detected in 293 of the 568 probands, and these variants were located in 62 of the 126 targeted genes (Supplementary Table S2). Of these 329 variants, 163 (50%) were missense variants, 68 (21%) were frameshift variants, 53 (16%) were nonsense variants, 39 (12%) were splice site variants, and six (2%) were in frame deletions (Fig. 2A). Based on the ACMG/Association for Molecular Pathology (AMP) guidelines,<sup>40</sup> these 329 mutations were classified as pathogenic variants (126, 38%), likely pathogenic variants (190, 58%), and variants of unknown significance (VUS; 13, 4%; Fig. 2B). When we referred to the HGMD database, 57% (189/329) of the mutations have been designated previously as known disease-causing variants, and 43% (140/329) were novel (Fig. 2C). Of the 293

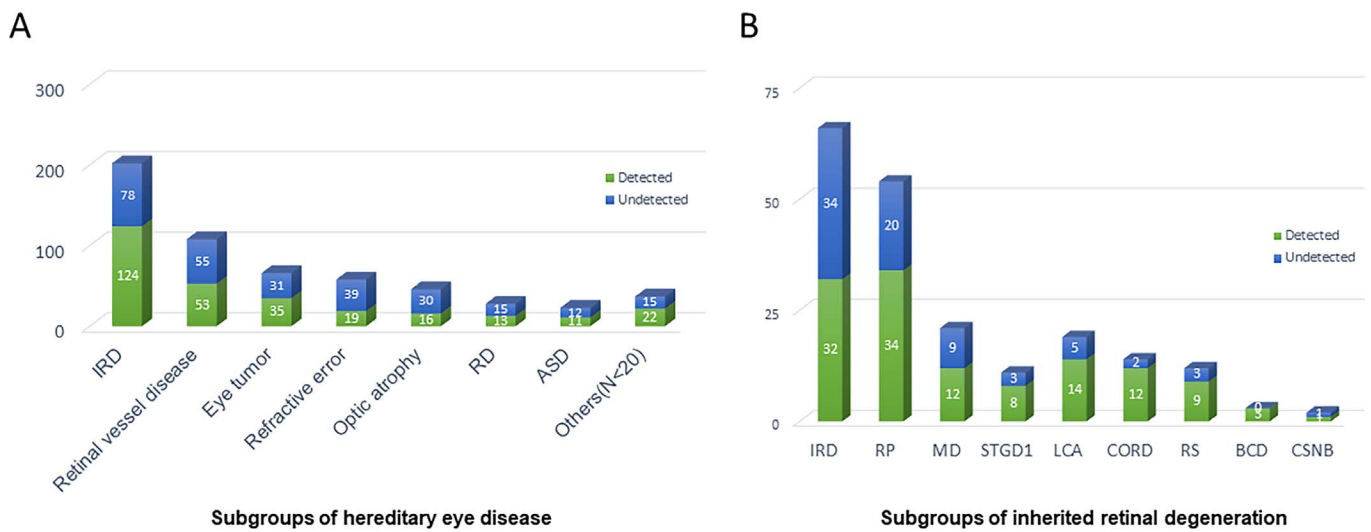
probands with these mutations, the diseases were transmitted as autosomal dominant traits in 154 families (53%, 154/293), autosomal recessive traits in 89 (30%, 89/293), and X-linked traits in 50 (17%, 50/293, Fig. 2D). There were nine probands with mutations in recessive genes carrying one variant (Supplementary Table S2).

### Molecular Diagnosis Rates of NGS-Based OTSP for Different Diseases

Although the overall molecular diagnostic rate for all probands was 52% (293/568), this diagnostic rate varied according to the types of hereditary eye diseases as follows (Fig. 3A): 61% (124/202) for IRD, 49% (53/108) for retinal vessel disease, 53% (35/66) for eye tumors, 33% (19/58) for significant refractive error, 35% (16/46) for OPA, 46% (13/28) for RD, 48% (11/23) for ASD, and 59% for other rare hereditary eye diseases (those with fewer than 20 patients, including congenital motor nystagmus [53%, 9/17], congenital lens malformation [58%, 7/12], ocular albinism [67%, 4/6], and strabismus [100%, 2/2]). The rates of different diseases in the subgroup of inherited retinal degeneration were: 48% (32/66) for IRD, 63% (34/54) for RP, 57% (12/21) for MD, 73% (8/11) for STGD1, 74% (14/19) for LCA, 86% (12/14) for CORD, 75% (9/12) for RS, 100% (3/3) for BCD, and 50% (1/2) for CSNB. For most hereditary eye diseases, the diagnostic rates determined based on NGS-based OTSP were consistent with those found by WES or Sanger sequencing in our previous studies as well as those found in other reports (Supplementary Table S3).<sup>11,13,14,16,20,21,23-28,34,41-44</sup> The only statistically significant difference ( $P < 0.05$ ) was observed for optic atrophy (Supplementary Table S3); the molecular diagnostic rate for this disease was 35% according to NGS-based OTSP, whereas the rate was 10% according to Sanger sequencing in our previous study as well as in another study. The molecular diagnostic rate of NGS-based OSTP was similar between referrals from ophthalmologists from the section of Pediatric and Genetic Eye Diseases (56%, 146/259) and referrals from other sections (50%, 155/309). The most frequently mutated genes (those with diagnostic rates  $>1.5\%$  in this population) were *RBI* (6%, 34/568), *FZD4* (3.5%, 20/568), *ABCA4* (3.5%, 20/568), *LRP5* (3.2%, 18/568), *RS1* (2.8%, 16/568), *TSPAN12* (2.5%, 14/568), *OPA1* (2.3%, 13/568), *CNGA3* (1.9%, 11/568), and *PAX6* (1.9%, 11/568; Table 1). Three patients harbored PPVs in different genes, including *GRM6* c.1537G>A+*TSPAN12*



**Figure 2.** Characteristics of the 329 PPVs in 62 genes. (A) Composition of the PPVs. (B) Classifications of 329 PPVs according to the ACMG/AMP guidelines. (C) Among the 329 PPVs, 57% are recorded in the HGMD as diseasing-causing (DM) and the remaining PPVs are novel. (D) PPVs detected in genes associated with diseases that are transmitted as AD (53%), AR (30%), and XL (17%) traits. P, pathogenic; LP, likely pathogenic; AD, autosomal dominant; AR, autosomal recessive; XL, X-linked



**Figure 3.** Mutation detection rate in probands. (A) Detection rate in different subgroups of diseases. (B) Detection rate in different subgroups of inherited retinal degeneration.

**Table 1.** Mutated Genes in Different Forms of Hereditary Eye Diseases

Gene (detection rate>0.5%)	IRD	Retinal vessel disease	Eye tumor	Refractive error	OPA	RD	ASD	Others (N<20)	Total patient number	Rate (568)
<i>RB1</i>	0	0	34	0	0	0	0	0	34	6.0%
<i>FZD4</i>	2	16	0	1	0	1	0	0	20	3.5%
<i>ABCA4</i>	20	0	0	0	0	0	0	0	20	3.5%
<i>LRP5</i>	0	14	0	3	0	1	0	0	18	3.2%
<i>RS1</i>	11	3	0	0	0	2	0	0	16	2.8%
<i>TSPAN12</i>	1	9	0	3	0	1	0	0	14	2.5%
<i>OPA1</i>	1	0	0	0	12	0	0	0	13	2.3%
<i>CNGA3</i>	11	0	0	0	0	0	0	0	11	1.9%
<i>PAX6</i>	0	1	0	0	1	0	7	2	11	1.9%
<i>FBN1</i>	0	0	0	2	0	0	1	5	8	1.4%
<i>NDP</i>	1	4	1	0	0	2	0	0	8	1.4%
<i>BEST1</i>	5	1	0	0	1	0	0	0	7	1.2%
<i>CYP4V2</i>	6	0	0	0	0	0	0	0	6	1.1%
<i>USH2A</i>	6	0	0	1	0	0	0	0	7	1.2%
<i>GPR143</i>	0	0	0	0	0	0	0	6	6	1.1%
<i>RPGR</i>	6	0	0	0	0	0	0	0	6	1.1%
<i>COL2A1</i>	0	0	0	2	0	3	0	0	5	0.9%
<i>ALMS1</i>	5	0	0	0	0	0	0	0	5	0.9%
<i>KIF11</i>	0	3	0	0	0	2	0	0	5	0.9%
<i>CHM</i>	4	0	0	0	0	0	0	0	4	0.7%
<i>EYS</i>	3	0	0	0	0	0	0	0	3	0.5%
<i>FRMD7</i>	0	0	0	0	0	0	0	4	4	0.7%
<i>IQCB1</i>	4	0	0	0	0	0	0	0	4	0.7%
<i>RP1</i>	4	0	0	0	0	0	0	0	4	0.7%
<i>BBS2</i>	2	0	0	0	0	0	0	1	3	0.5%
<i>CACNA1F</i>	2	0	0	1	0	0	0	0	3	0.5%
<i>COL11A1</i>	0	1	0	0	0	2	0	0	3	0.5%
<i>GRM6</i>	1	0	0	2	0	0	0	0	3	0.5%
<i>GUCY2D</i>	2	0	0	1	0	0	0	0	3	0.5%
<i>LCA5</i>	3	0	0	0	0	0	0	0	3	0.5%
Genes detected rate <0.05	0	0	0	0	0	0	0	41	41	7.2%

Mutant genes detected in only one kind of disease are highlighted in green. Mutant gene detected in different kinds of disease are highlighted in blue. FEVR (familial exudative vitreoretinopathy) genes are highlighted in red.

c.1537G>A in #13427, *COL11A1* c.4177C>T+ *LRP5* c.1996G>A in #11926, and *LRP5* c.4105\_4106del+ *FZD4* c.313A>G in #14731. Further clinical and genetic validation studies are needed to determine the pathogenetic reasons for the results observed in these patients. Of the 62 genes with mutations, 20 had mutations that were associated with different forms of

diseases and affected 52.90% of the probands with identified mutations (155/293).

### Cost-Effectiveness Evaluation of OTSP

The average turnaround time for the OTSP test was  $30 \pm 10$  days and ranged from 12 to 58 days. The cost of detecting mutations in samples was approx-

imately \$138 (USD), which included library construction (~\$95), NGS (~\$31), and Sanger sequencing (~\$12). The patients were charged \$282, which covered the costs of labor, management, and data analysis. This cost was lower than that of a 334 gene-panel designed by the Saudi group to target vision disease (cost, \$150)<sup>19</sup> and lower than that of a 2742-gene panel (cost, \$360) designed to detect pediatric disease.<sup>18</sup> Therefore, NGS-based OTSP is affordable and reasonably efficient.

## Discussion

In this study, we evaluated the effectiveness of the NGS-based OTSP in 568 probands with a wide range of hereditary eye diseases and obtained an overall molecular diagnosis rate of 52%. In most hereditary eye diseases observed in this study, the detection rates by NGS-based OTSP were roughly consistent with those by WES or Sanger sequencing in previous studies. The average turnaround time was 30 days, and the cost was US\$138 per sample. The turnaround time is still shortening with the upgrade of the software, and 7 days is in practice now. This NGS-based OTSP test not only is as reliable as other standard genetic tests, but also faster and more affordable than most other genetic tests. Another significant advantage of NGS-based OTSP is that the referring physician does not need to worry about which gene panel should be chosen or what disease name is most appropriate. The widespread application of this kind of genetic test may offer ophthalmologists new opportunities to re-examine the phenotypic variations of gene-specific diseases and reclassify diseases based on genetics. This test also will provide valuable information allowing genotypes and phenotypes to be better associated, especially in terms of causative mutations versus benign variants. This NGS-based OTSP test could be used as a routine clinical diagnostic test in most hereditary eye diseases, although it may need to be updated in the future.

NGS-based tests have been applied successfully to detect mutations in genes responsible for various types of hereditary eye diseases.<sup>11–17,20,21,28,29,31,32</sup> Given their high accuracy and sensitivity for detecting variants when compared to exome sequencing,<sup>45</sup> panels that specifically target genes associated with one or a group of diseases have been used in several studies including studies of IRD,<sup>11</sup> FEVR,<sup>13</sup> and infantile nystagmus syndrome.<sup>16</sup> However, hereditary eye diseases are characterized by high degrees of phenotypic and genetic heterogeneity, and atypical

presentations may prevent clinicians from choosing appropriate panels. Moreover, the lack of uniformity in panel design limits the application of panel tests in a clinical setting. Although WES and WGS have the advantage of being able to detect variants in almost all known genes, their higher costs and longer turnaround times may hinder their application as routine clinical tests in clinical settings in developing countries, such as China. Targeted exome sequencing seems to be a reasonable alternative to WES/WGS in terms of its usefulness in routine clinical examinations because it can achieve high accuracy and sensitivity at lower cost and with a shorter turnaround.<sup>45</sup> Indeed, to improve clinical genetic diagnosis, the Saudi group and Shen independently developed targeted exome sequencing panels that included genes for most known Mendelian diseases (3000 genes were targeted by the Saudi group, and 2742 genes were targeted by Shen);<sup>18,19</sup> these panels acquired reasonable diagnostic rates for different genetic diseases. Based on our previous study,<sup>15,20–32,34,35</sup> we designed NGS-based OTSP that targets the 126 genes that are mutated most frequently in Chinese probands with hereditary eye diseases. In our population, the majority of clinically relevant mutants were detected in AD (53%), followed by AR (30%) and XL (17%). The proportions are highly consistent with those reported by Shen,<sup>18</sup> which was based on a Chinese population and detected mutants in AD (53%), followed by AR (31%), and XL (15%). The vision panel of the Saudi group covered 334 genes at a cost of US\$150 and yielded a detection rate of 52% in 418 probands with different hereditary eye diseases. Our panel exhibited the same detection rate (52%), but at a lower cost (US\$138) because of its smaller size (126 genes). Hence, NGS-based OTSP is a tool with a high detection yield that is relatively inexpensive and can be used for routine clinical genetic testing for patients suspected of having a wide range of hereditary eye disease, especially in the developing countries, such as China.

Although Sanger sequencing is the gold standard for confirming a gene variant, it carries the risk of allele dropout events,<sup>46</sup> which can be caused by amplification bias resulting from rare genetic variations located within the binding region of a primer,<sup>47</sup> or elongation blocking by the secondary structure of the amplicons during polymerase chain reaction (PCR) sequencing.<sup>48</sup> In contrary, the NGS-based OTSP test is advantageous because it partially resolves these problems. For example, there was one proband (#10836) in whom no mutation in *OPAI* was



detected by Sanger sequencing in our previous study.<sup>34</sup> However, a heterozygous substitution in *OPAI*, c.2661G>A, caused a splicing shift and was detected by the NGS-based OTSP, confirmed by additional Sanger sequencing using a different pair of primers, and then further shown to cosegregate with optical atrophy in the proband's family. In this study, optical atrophy was the only disease for which the rate of molecular diagnosis was significantly higher for NGS-based OTSP (35%) than for traditional Sanger sequencing (10%). Whether allele dropout was the major reason for the difference in detecting *OPAI* requires further study.

Mutations in the FEVR-causative genes, including *FZD4*, *LRP5*, *TSPAN12*, *NDP*, and *KIF11*, are the most common causes of hereditary eye diseases, and accounted for 22% (65/293) of probands with detected mutations and 11% (65/568) of all probands analyzed in this study. These mutations were detected most frequently in retinal vessel disease (43%, 46/108 patients) and also were present in seven of the 28 probands who were initially diagnosed with retinal detachment. Patients with FEVR are at high risk of retinal detachment,<sup>49,50</sup> but how often retinal detachment may be associated with FEVR is unknown, and corroboration by genetic diagnostic evidence is especially rare. Our results confirmed that FEVR is a common primary cause of retinal detachment. Additionally, mutations in the genes responsible for FEVR were identified in seven probands with high myopia, four with retinal degeneration, and one who was previously diagnosed with retinoblastoma. These results vividly demonstrate the unexpectedly substantial level of heterogeneity of the phenotypes and genotypes that are associated with hereditary eye diseases and the advantages of our NGS-based OTSP over panels that target individual diseases, such as RP, LCA, CORD, and FEVR as well as lens and cornea disease. The OTSP presented here is a better choice for identifying clinically relevant mutants in patients with atypical phenotypes or novel clinical presentations.

Due to technical and design limitations, this NGS-based OTSP may not detect several types of rare variants, such as structural, copy number, upstream or downstream cis-element, or deep intron causative variations, and mutations in genes that are not included in the test. Mutations embedded in areas with high GC or repeat regions (e.g., *PRGR*) represent another weakness because the covering rate by this OTSP is not 100%. Additionally, the pathogenicity of some mutations may need further

validation, especially in genes in which there is a large amount of variation in the general population and genes for which a limited number of reported causative mutations have been reported to date. Variant-based clinical re-evaluations of key signs and segregation analyses of family members may provide valuable information to resolve this problem. Furthermore, although the most commonly mutated genes, which are responsible for a wide range of hereditary eye diseases, are covered by this NGS-based OTSP test, the number of probands with certain diseases, such as congenital cataract, primary congenital glaucoma, congenital motor nystagmus, ocular albinism, and strabismus, was limited in this study. Therefore, the power of the OTSP test for diagnosing some of these diseases may need to be evaluated further in additional probands.

Our NGS-based OTSP test is a powerful tool that can identify causative mutations in probands with a wide range of hereditary eye diseases in routine clinical practice.

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