The Next Generation Sequencing Based Molecular Diagnosis of Visual Diseases

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Abstract Vision is one of the most important senses of human beings. It is estimated that 285 million people worldwide suffer from visual impairments. Although the etiology of visual impairments is both complex and multifactorial, genetic factors have been shown to play important roles. Both common and rare genetic variants have been associated with various types of inherited visual disorders. Studying the genetics of visual impairments can help to confirm or to refine clinical diagnosis, lead to better prognosis, guide the family planning, and allow targeted treatment(s). Recently developed next generation sequencing technology can generate enormous amount of sequencing data quickly at relatively low cost, and has great advantages compared to other sequencing methods. Due to the clinical and genetic heterogeneity of human visual disorders, next generation sequencing technology is a useful tool for the molecular diagnosis. In this review, we will discuss specifically the application of next generation sequencing in the molecular diagnosis of visual disorders with monogenic or oligogenic inheritance.

Keywords Next generation sequencing • Visual impairments • Molecular diagnosis • Monogenic and oligogenic inheritance

1 Introduction

Vision is one of the most important senses of human beings. More than 70% of what we learn in a lifetime comes to us through our sight [1]. It was estimated that, in 2010, 285 million people worldwide suffered from visual impairments, including 39 million with blindness and 246 million with low vision [2]. The major causes for visual impairments are uncorrected refractive errors and un-operated cataract, and the major causes for blindness are advanced cataracts and glaucoma (Table 1). Genetic factors play an important role in the etiology of visual disorders. For example, pathogenic variants in more than 230 genes are associated with retinal dystrophies

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Table 1 Prevalence and genetics of common visual impairments

Disorders	Frequency in general population	Genetics (loci/genes)
Uncorrected refractive errors	1/60 [2]	17 loci [105]
Cataract	1/70 [2]	>115 genes and loci [3, 106]
Glaucoma	1/1200 [2]	9 genes and 10 loci [91, 92]
Age-related macular degeneration	1/2400 [2]	19 loci [107]
Corneal Opacities	1/2400 [2]	>5 [108]
Diabetic Retinopathy	1/2400 [2]	34 loci [109]
Trachoma	1/2400 [2]	unknown
Retinitis Pigmentosa	1 /3000 to 1/4000 [110, 111]	64 genes and loci[a]
Retinoschisis	1/5000 to 1/25000 [112]	*RS1*[a]
Stargardt macular dystrophy	1/10,000 [113]	*ABCA4*[a]
Retinoblastoma	1/15000 to 20,000 [114]	*RB1*[a]

[a]https://sph.uth.edu/retnet/sum-dis.htm

thus far, and more than 110 genes are associated with congenital cataracts (Table 1, https://sph.uth.edu/Retnet/) [3]. Several common risk alleles/loci associated with "common complex eye disorders", such myopia, glaucoma, and the age-related macular degenerations, have been identified successfully (Table 1) [4–7]. Due to the clinical and genetic heterogeneity of visual disorders, next-generation sequencing (NGS) can be a useful tool to identify the underlying genetic causes of visual disorders. In this review, we will discuss specifically the application of NGS in the molecular diagnosis of visual disorders with either monogenic or oligogenic inheritance.

2 The Clinical Utility of Molecular Testing for Eye Diseases

Several clinical utilities are available for molecular testing of genetic eye diseases. First, molecular diagnosis helps to confirm or to refine the clinical diagnosis of the ocular disorder and expand the frequent systemic associations. Heritable eye diseases are clinically and genetically heterogeneous. Different genes can cause clinically indistinguishable appearances. Patients with the clinical diagnosis of an isolated, non-syndromic eye disorder may have other syndromic features not obviously present or easily identified during the initial ophthalmological evaluation [8, 9]. Therefore, molecular diagnosis helps to enhance an accurate prognosis and to improve patient management. For example, a patient diagnosed with non-syndromic infantile-onset retinal dystrophy was found to carry pathogenic variants in *IQCB1*, which is the causal gene for Senior-Loken syndrome. Because of the molecular finding, this patient's renal disease was anticipated before any kidney disease was known and treated/managed in a pre-planned manner [10]. For another

example, identification of pathogenic variants in the *RB1* gene for patients with retinoblastoma reduces the otherwise costly and timely surveillance examinations [11]. Third, a firm molecular diagnosis can guide the family planning and reproductive decisions. For example, parents carrying mutations causing retinoblastoma, a malignant and potentially lethal tumor of the retina in infancy and childhood, may choose either preimplantation genetic diagnosis to ensure the delivery of a baby without such disease, or prenatal diagnosis to guide further management [12, 13]. Fourth, molecular diagnosis allows gene-specific treatment. For examples, several clinical trials have shown that gene replacement strategies are able to improve the vision of children with Leber congenital amaurosis (LCA) caused by mutations in *RPE65*, although the long-term effects need monitoring and perhaps improved techniques [14–16]. Finally, even identifying the unique cause alone of a visually impairing condition will relieve the anxiety and psychological burdens of many patients and their families who may believe that other environmental factors or misbehaviors caused the condition.

3 Methodology

This chapter focuses on NGS-based molecular diagnosis of eye diseases. However, other sequencing methods are still being used for the molecular diagnosis of eye diseases. Understanding the advantages and limitations of each method should help us to choose appropriate tests to meet the specific needs of different clinical situations. PCR followed by Sanger sequencing is considered the gold standard sequencing method and typically is used when the clinical presentation of the patient is specific and/or a small number of candidate genes associate with the differential diagnosis. For examples, eye diseases such as retinoblastoma, vitelliform macular dystrophies (VMD), familial exudative vitreoretinopathy (FEVR), and anophthalmia and microphthalmia (AM) have distinct clinical presentations and each has a small number of causal genes, and thus are suitable for Sanger sequencing [17–20]. However, Sanger sequencing is too costly when a large number of genes or exons may be associated with the disease. Also Sanger sequencing cannot be used to accurately detect CNV and low level mosaicism. In addition, unexpected SNPs on the primer can undermine the PCR based amplification of target regions (allele drop off). Microarray-based methods typically cover a large number of targeted mutations that are common for certain diseases. For examples, APEX-(arrayed primer extension) based tests covering hundreds of reported mutations have been used widely for the molecular diagnosis of several types of retinal dystrophies, such as LCA, Retinitis pigmentosa (RP), Bardet-Biedl syndrome (BBS), and Alström syndrome (ALMS) [21–23]. Although it is both cost and time effective, the diagnosis rate of array-based methods is relatively low. It detects only a fixed number of known variants and cannot identify novel or recently reported variants, which are frequently found in eye diseases that have high locus and allelic heterogeneity.

Recently, high resolution melting (HRM) has been used for high throughput genetic screening of RP [24]. It has low cost, rapid turnaround time, and is able to detect novel variants. However, it is suitable only for the analysis of medium-sized genes. Homozygosity mapping is a genetic method to identify disease-associated loci, especially in consanguineous families. It has been used widely to identify novel disease-causing genes and/or pathogenic variants in known genes underlying several diseases, including genetically heterogeneous eye diseases [25–27]. Because it involves genotyping of related family members and it helps only to map the disease-associated loci, homozygosity mapping is not commonly offered as a diagnostic test but is used to identify disease-associated genomic regions. Some clinical diagnostic laboratories, such Baylor Miraca Genetics Laboratories, do offer whole exome SNP array together with WES as a quality control method and to identify potential disease-associated loci.

NGS has been used widely for the molecular diagnosis of eye diseases. NGS methods can be classified into two categories: targeted NGS or whole genome sequencing (WGS). Targeted NGS methods enrich target genomic regions first before high throughput sequencing, while WGS require no prior target enrichment. Targeted NGS methods, including panel based NGS and whole exome sequencing (WES), are often used in molecular diagnostic studies of eye diseases. The first step of targeted NGS is to enrich the target genomic regions. The target enrichment strategies include multiplex PCR, molecular inversion probes (MIP), array hybridization capture, and in solution hybridization capture [28]. Different sequencing platforms, such as HiSeq, Ion Torrent, 454 GS, and single-molecular sequencing by Pacific Biosciences, were invented for high throughput sequencing [29–31]. For detailed comparison between different capture methods and different sequencing platforms, please refer to Part One review of this book and the references [32].

Compared with other methods, NGS has high throughput, low per-base cost, and the ability to detect novel variants and copy number variations (CNVs). It is particularly useful when the clinical presentation of patients is non-specific and many candidate genes/exons associate with the differential diagnosis. Specifically, panel-based NGS methods include a set of genes relevant to the specific diagnosis, while WES aims to interrogate the coding regions within the whole genome. Comparing with panel-based NGS, WES generally has higher total cost and lower average coverage depth. However, WES has the ability to detect mutations in recently identified disease genes or to discover novel genes associated with diseases. Several current limitations of NGS exist. First, the sequence quality of NGS at highly repetitive and/or GC rich regions is not optimal. Currently the NGS reads are not long enough to correctly map repetitive/homologous sequences. For example, *RPGR* gene, especially its open reading frame 15 (ORF15), is a mutational hot sport for X-linked RP [33]. However, the ORF15 has repetitive sequences that cannot be accurately sequenced and mapped by conventional targeted capture NGS [34]. To overcome this challenge, long range PCR followed by NGS and targeted alignment can be used to only amplify and map the target region. The GC-biases

may be introduced during PCR amplification in the NGS library preparation process, and several improvements have been made to reduce such bias [35–37]. Second, NGS has relatively higher total sequencing costs. Third, NGS generates large volume of data and requires intensive computations for data analysis. Fourth, NGS has not yet been considered as the golden standard method to detect structural variations (SVs) and other types of genomic rearrangements. WGS has been reported to be more accurate than targeted NGS to call SVs and CNVs, partly because WGS has more uniform coverage then targeted NGS [38]. However, recent reports suggest that CNVs and Alu insertions can also be reliably detected by panel based NGS in clinical diagnostic laboratories [39]. Fifth, because NGS (especially WES or WGS) covers a large number of genes not apparently relevant to the patient's clinical presentations and is able to identify numerous genetic variants that may not be interpretable, potential ethical and psychological issues, including how to report incidental findings and how much emphasis to place on variants with uncertain clinical significance, may arise. This is less of a problem for targeted panel NGS which focus one genes directly relevant to the clinical presentations. Nevertheless, targeted NGS is becoming the most used method in the molecular diagnosis of eye diseases and is being rapidly improved.

4 Retinal Dystrophies

The retina is a complex tissue lining the inside of the back two-thirds of the eye that senses light and converts light into electrical signals. Inherited retinal dystrophies (RD) represent a group of heterogeneous eye disorders that are genetically determined and usually highly symmetric. The clinical presentations of RD vary from late onset mild visual impairment to congenital blindness. RD can be either non-syndromic, that is, occurring in the retina and adjacent tissues alone, or syndromic, thus are consistently associated with other systemic, extraocular features. To date, mutations in more than 200 genes are known to cause RD with either Mendelian or non-Mendelian (e.g., mitochondrial) inheritance (https://sph.uth.edu/Retnet/sum-dis.htm). The genes frequently mutated in each RD are summarized as Table 2. Due to wide clinical and genetic heterogeneities, more than 20 retinal diseases involved in the differential diagnosis of RD [40]. Collectively, it is estimated that RD affect 1 in every 2000 individuals, or more than 3 million people worldwild [41]. In the past few years, NGS has been applied widely for the molecular diagnosis of patient cohorts with various non-specific or specific RD, such as retinitis pigmentosa (RP) (Fig. 1), Stargardt macular dystrophy (STGD), Leber congenital amaurosis, familial exudative vitreoretinopathy, Usher syndrome (USH), and Bardet-Biedl Syndrome (BBS). The gene enrichment methods and results of recently published studies on NGS-based molecular diagnosis of RD are summarized as Table 3.

Table 2 Causal genes and estimated contributions to specific type of retinal dystrophies

Disorders	Fractions of total cases contributed by causal genes
AR RP including Usher and BBS (50–60% of RP)	*USH2A* (17%), *ABCA4* (5.6%), *PDE6B* (4–5%) [110]
AD RP (30–40% of RP)	*RHO* (25%), *RP1* and *PRPF31* (5%) [110]
X-linked RP (5–15% of RP)	*RPGR* (75%), *RP2* (7–10%) [110]
AR cone-rod dystrophy	*ABCA4* (16%), *EYS* (3%), *CERKL* (1%) [115]
AR cone dystrophy	*KCNV2* (13%), *ABCA4* (4%), *CNGB3* (3%) [115]
AD cone and cone-rod dystrophy	*GUCY2D* (8%), *CRX* (4%), *GUCA1A* (3%) [115]
X linked cone and cone-rod dystrophy	*RPGR* (53%), *OPN1LW/OPN1MW* (8%), *CACNA1F* (3%) [115]
Achromatopsia	*CNGB3* (59%), *CNGA3* (36%), *PDE6C* (3%) [115]
Leber congenital amaurosis	*CEP290* (15%), *GUCY2D* (12%), *CRB1* (10%) [116]
Bardet-Biedl syndrome	*BBS1* (23%), *BBS10* (20%), *BBS2* (8%) [117]
Usher syndrome type I	*MYO7A* (29–50%), *CDH23* (19–35%), *PCDH15* (11–19%) [118]
Usher syndrome type II	*USH2A* (55–90%), *GPR98* (3–6%) [118]

AR: autosomal recessive, *AD:* autosomal dominant

4.1 Diagnostic Rates

In clinical settings, the diagnostic rate for a clinically naïve diagnosis has become an important parameter for clinical geneticists to make decision on whom and where to send the test. The published molecular diagnostic rates depend on several factors (Table 3). First, the number of target genes covered by the panel NGS method affects the diagnostic rate. Gene panels covering updated lists of genes for specified types of RD tend to have higher diagnostic rates. In addition, it is well recognized that patients with certain clinical diagnoses may actually carry pathogenic variants in genes that not typically associated with that diagnosis [8, 9]. For example, patients diagnosed with LCA were frequently found to carry pathogenic variants in *ALMS1*, the causal gene for Alstrom syndrome. Therefore, a larger panel or even WES that covers more RD genes relevant to the differential diagnosis may have a better chance to detect variants in the "unexpected" genes and consequently have a higher diagnostic rate. Second, the methods and data quality, as well as analytical methods affect the diagnostic rate. Higher read depth, more accurate sequencing quality, and less human errors are the basis of high detection rate. Also, improved algorithms to detect both SNVs and CNVs using the same NGS data set can lead to higher diagnostic rate than methods that only focus on SNVs [42]. Third, the theoretical percentages of cases that can be explained by mutations in currently known disease genes vary among different diseases. For example, complete sequencing of the 9 known USH genes can explain as high as 72–90% of USH cases [43, 44]. Consistently, the published NGS diagnostic rates for USH cohorts are generally higher than other types of RD, ranging from 70–100% [45–47]. Whereas current known genes are estimated to account for 50% of FEVR cases, and the recent NGS

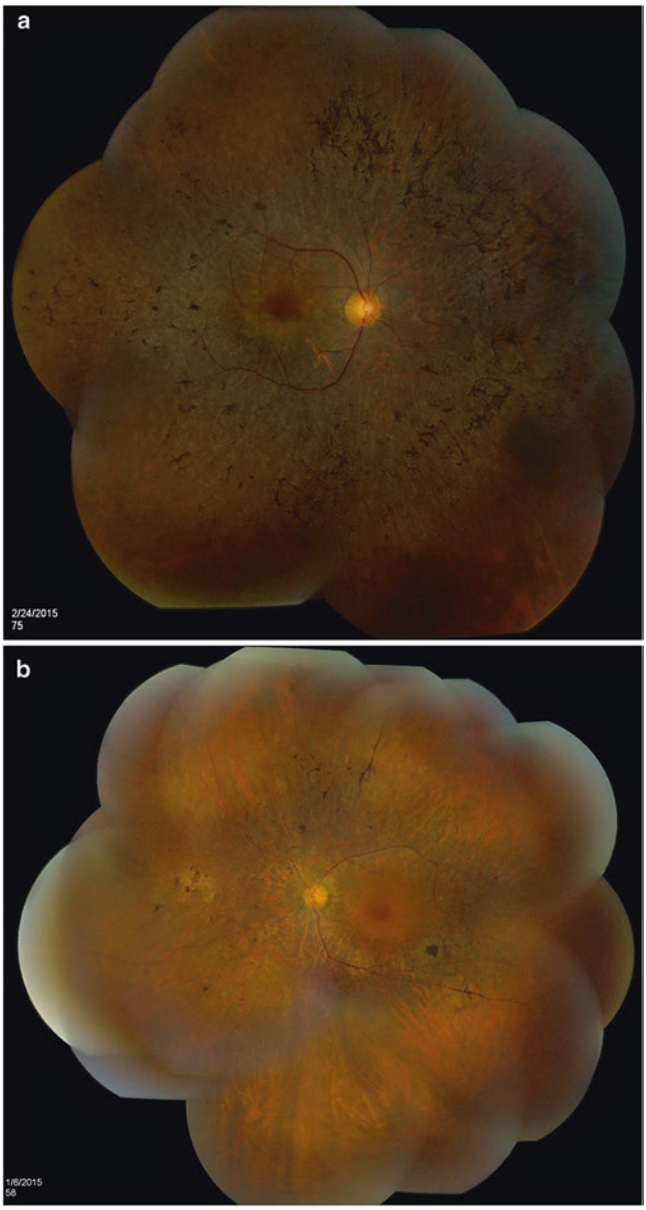

Fig. 1 (**a**) Photomontage of the left fundus of a 46 year old female with advanced retinitis pigmentosa. Note the pigment migration into the retina (creating the clumps and the linear formations around retinal vessels, so-called 'bone spicules'), the vascular attenuation, and the diffuse pallor of the optic nerve. No distinguishing characteristics separate which of the numerous genes is responsible for the progressive destruction of the retinal pigment epithelium. (**b**) Photomontage of the left fundus of a 60 year old female with advanced retinitis pigmentosa. Note the relative paucity of pigment deposits, but the atrophy of the retinal pigment epithelium, the vascular attenuation of both arteriolar and venular systems, and the diffuse pallor of the optic nerve

Table 3 Summary of published studies on NGS based molecular diagnosis of retinal dystrophies

	Clinical presentation	Cohort size	Target genes	Enrichment method	Diagnosis rate
Simpson et al. (2011) [119]	RP	5	45	Array hybridization	60% (3/5)
Neveling et al. (2012) [120]	RP	100	111	Array hybridization	36% (36/100)
Fu et al. (2013) [51]	arRP	31	163	Solution hybridization	39% (12/31)
Wang et al. (2014) [121]	RP	123	163	Solution hybridization	37% (45/123)
Wang et al. (2014) [39]	RP	65	66	Solution hybridization	82% (53/65)
Xu et al. (2014) [122, 123]	RP	157	189	Solution hybridization	53% (84/157)
Zhao et al. (2015) [50]	RP	82	186	Solution hybridization	60% (49/82)
Zernant et al. (2011) [124]	STGD	159	*ABCA4*	PCR	33% (53/159)
Strom et al. (2012) [125]	STGD	9	Whole Exome	Solution hybridization	67% (6/9)
Fujinami et al. (2013) [126]	STGD	79	*ABCA4*	PCR	47% (37/79)
Zhang et al. (2014) [127]	STGD	5	Whole Exome	Solution hybridization	100% (5/5)
Zaneveld et al. (2015) [128]	STGD	88	213	Solution hybridization	67% (59/88)
Coppieters et al. (2012) [65]	LCA	17	16	PCR	18% (3/17)
Wang et al. (2013) [8]	LCA	179	163	Solution hybridization	40% (72/179)
Licastro et al. (2012) [129]	Usher	12	Whole Exome/9[a]	Solution hybridization/ PCR[a]	42% (5/12)
Salvo et al. (2015) [49]	FEVR	92	163	Solution hybridization	49% (45/92)
Yoshimura et al. (2014) [46]	Usher type 1	17	9	PCR	94% (16/17)
Bujakowska et al. (2014) [45]	Usher type 1	47	Not mentioned	Solution hybridization	70% (33/47)
Qu et al. (2015) [47]	Usher	5	103	Array hybridization	100% (5/5)
Xing et al. (2014) [130]	BBS	5	144	Solution hybridization	100% (5/5)

(continued)

Table 3 (continued)

	Clinical presentation	Cohort size	Target genes	Enrichment method	Diagnosis rate
O'Sullivan et al. (2012) [131]	RD	50	105	[b]	42% (21/50)
Schorderet et al. (2012) [132]	RD	23	63	Solution hybridization	52% (12/23)
Shanks et al. (2012) [133]	RD	36	73	Array hybridization	25% (9/36)
Chen et al. (2013) [134]	RD	25	189	Array hybridization	56% (14/25)
Glockle et al. (2013) [41]	RD	170	105	Solution hybridization	91% (155/170)
Watson et al. (2014) [135]	RD	20	162	Solution hybridization	60% (12/20)
Huang et al. (2014) [136]	RD	179	252	Solution hybridization	55% (99/179)
Liu et al. (2015) [137]	RD	20	316	Array hybridization	55% (11/20)

[a]Two methods were used in one study
[b]Unclear

study on a FEVR cohort reported about 49% of diagnosis rate [48, 49]. Fourth, different ethnic groups have different mutation spectrums. For example, the diagnostic rate of RP patients is reported to be 40% in China but 60% in Northern Ireland [50, 51]. It has also been reported that Chinese and European ancestry LCA patients have different mutation spectrums [52]. It is possible that some populations may carry more variants in known disease loci than other populations, leading to higher diagnostic rate. Fifth, the diagnostic rate may be lower than expected if the patient cohort had been "prescreened" by other molecular diagnostic methods, such as Sanger sequencing on frequently mutated genes [8]. In the practice of medicine, clinical geneticists commonly order specific target gene tests first and then order NGS tests if the results of target gene tests are unrevealing. Therefore, several patient cohorts published in NGS studies have been "prescreened" to a certain extent. Lastly, the detail and specificity of the clinical characterization of the referred patients and the underlying genetic heterogeneity of the patient cohort can affect the success of a firm molecular diagnosis.

4.2 Inheritance Models

Most RD, whether ocular alone or part of a syndromic complex, are single gene disorders. However, several forms of oligogenic inheritance have been reported in RD and should be considered when interpreting the NGS data, especially for those

genes in the same biological pathway. For example, in digenic inheritance (DI), two pathogenic variants in each of the two unlinked loci are required to cause diseases. The first human example of DI was reported in 1995 when, individuals from three families were found to have double heterozygous pathogenic variants in two unlinked photoreceptor-specific genes, *ROM1* and *PRPH2*, and developed RP [53]. It was later suggested that wild type *ROM1* and *PRPH2* proteins form functional heterotetramers, while null *ROM1* and mutant *PRPH2* lead to the reduced levels of *ROM1*/*PRPH2* heterotetramers, and the unstable *PRPH2* homotetramers likely cause disorganization of photoreceptor cell outer segments and consequently cause RP phentypes [54]. Schäffer 2013 provided a comprehensive summary of DI associated with various human disorders [55]. In triallelic inheritance (TI), three pathogenic variants in two different unlinked loci are required to cause disease. TI was first described in humans in BBS, when three pathogenic variants in *BBS2* and *BBS6* genes cause BBS [56]. Later, *BBS4* gene has also been reported to participate in TI of BBS [57]. However, the prevalence of TI cases in BBS is debated [58, 59]. In addition, modifiers may affect the severity of RD clinical presentations. For examples, a common allele p.A229T in *RPGRIP1L* gene is associated with photoreceptor cell loss in ciliopathies [60]. Polymorphisms of both *RPGRIP1L* and *IQCB1* gene can modify X-linked RP caused by RPGR mutations [61]. Variants in the *PDZD7* gene modify the retina dystrophy in Usher syndrome [62]. Lastly, It has been reported that patients may have pathogenic variants in more than one RD genes [41]. Indeed, several published reports of oligogenic inheritance lack further repeats or functional validations. Nevertheless, recognizing variant forms of oligogenic inheritance will improve the accuracy of interpretation in the molecular analyses of RD.

4.3 Pathogenic Variants

Most published NGS-based molecular diagnostic methods aim to capture both coding regions and the flanking intronic regions. Consequently, most studies focus on the analysis of coding changing variants, including nonsense, small insertions/deletions, canonical splice site, and missense changes. Professional societies, such as the American College of Medical Genetics and Genomics and the Association for Molecular Pathology, have published guidance to help the interpretation of sequence variants [63]. However, other non-coding variants have been reported to cause RD and should not be overlooked. First, deep intronic and synonymous changes are well known causes of RD. For example, the c.2991 + 1655A > G change in intron 26 of *CEP290* gene is one of the most frequent pathogenic variants found in LCA, accounting for about 21% of LCA cases. This intronic change creates a new splice-donor site and inserts a cryptic exon, which leads to a premature stop codon downstream of exon 26 [64]. To assure that this variant is included in the targeted NGS analysis, either specific primers or probes should be designed for the target capture, or directed Sanger sequencing should complement NGS [8, 65]. Similarly, several deep intronic and synonymous changes were identified in *ABCA4* gene and

cause Stargardt disease. These changes lead to inclusion or deletion of sequence segments in the mRNA transcripts [66]. In addition, a deep intronic change c.7595-2144A > G was recently found in *USH2A* gene to cause Usher syndrome type 2 [67]. In all these examples, RNA sequencing was used to identify abnormal mRNA transcripts resulting from intronic or synonymous changes. To increase the diagnostic rate further, RNA sequencing may be used in parallel with DNA sequencing to identify novel intronic or synonymous changes that affect mRNA splicing, especially for cases of recessive disorders that have only one copy of a pathogenic variant without the second mutant allele and/or for cases that have candidate disease loci localized by genetic mapping. Second, exonic deletions/duplications and copy number variations (CNVs) have been reported to cause in RD. For example, deletions/duplications were reported to account for 26% of Usher syndrome cases with monoallelic mutations in *USH2A* gene [68]. Large rearrangements and duplications in *PCDH15* gene were identified in 13% of Usher syndrome Type 1 families who had been screened for mutations in the five known USH1 genes [69]. A common Alu insertions in *MAK* gene in Jewish RP patients have also been described before [39]. Although many clinical diagnostic laboratories offer deletion/duplication analysis by methods such as multiplex ligation-dependent probe amplification (MLPA) or array-based CGH analysis (aCGH), it will be ideal to detect deletions/duplications using the same NGS data set for single nucleotide variations (SNVs) analysis. Several bioinformatics approaches have been developed to detect CNVs from NGS data [9, 70]. However, the performance of CNVs analysis in clinical settings is still suboptimal because of high error rates caused by short reads, genome complexity, and large coverage variations of targeted NGS data. Recently, Feng, et al. reported the systematic detection of exon deletions with NGS data from clinical samples with RD and other inherited disorders [42]. CNV analysis with NGS data can be improved further by increasing both reads length of NGS and data quality, and advanced bioinformatics tools. Lastly, several genomic regions involved in RD may not be captured easily and analyzed by NGS. As one classic example, the ORF15 of the *RPGR* gene is a mutational hotspot in RP. However, the ORF15 sequence is highly repetitive and hard to be analyzed by conventional NGS [33, 34]. To overcome this limitation, long range PCR followed by NGS can be applied.

5 Retinoblastoma

Retinoblastoma is the single most common malignant ocular tumor in childhood, affecting about 1 in every 14,000 to 18,000 live births in the United States (National Cancer Institute). Retinoblastoma is caused predominantly by biallelic mutations in *RB1* gene. In the developed world, retinoblastoma presents most frequently as sudden onset of strabismus or as a “white pupil”, either in the parent’s observation or in an informal photograph. In heritable forms of retinoblastoma, affected individuals typically have tumors in each eye, usually multicentric, diagnosed at younger age, and germline *RB1* mutations. While in non-heritable form, children typically have

unilateral tumors that include somatic mutations in *RB1*. Various types of mutations in *RB1*, including SNVs, CNVs, genomic rearrangements, and non-coding regulatory changes, have been reported in retinoblastoma tumors [71]. Promoter methylation of *RB1* also plays a role in the development of retinoblastoma [72]. In addition, genetic factors other than *RB1* gene, such as miRNAs, *MYCN* gene amplification, and polymorphisms in *MDM2* and *MDM4* gene, may contribute to the development of retinoblastoma [73–76].

The current molecular methods for the diagnosis of retinoblastoma include Sanger sequencing and allele-specific PCR for SNVs, MLPA and quantitative multiplex PCR (QMPCR) for insertions/deletions and CNVs, and a methylation assay to detect hypermethylated *RB1* promoters [11]. Due to the allelic heterogeneity of retinoblastoma, a stepwise molecular diagnostic approach has been proposed [77]. An mRNA analysis will determine the consequence of genomic variants affecting transcripts. Recently, NGS has been applied to the molecular diagnosis of retinoblastoma [78–80]. Compared with other methods, NGS can detect unbiasedly the low-level mosaic variants that are found frequently in sporadic retinoblastoma [78, 79]. NGS-based methods are also able to detect both SNVs and CNVs in retinoblastoma [80]. Because of the wide mutational spectrum of *RB1* and the current limitations of NGS, NGS combined with other complementary methods in a stepwise manner may be the optimal molecular strategy for retinoblastoma diagnosis.

6 Congenital Cataracts

Congenital cataracts (CCs) refers to opacification of parts of or the entire lens present at birth; when involving the entire cortex of the lens or some parts of the axial view of the incoming image, the cataract may lead to severe visual impairment. CCs are estimated to affect 1 to 6 babies in every 10,000 live births; at least half of CCs are associated with genetic factors [81–84]. CCs can present either as an isolated non-syndromic event or as one part of syndromic conditions. To date, more than 20 and 90 genes are associated with non-syndromic and syndromic CCs, respectively [3, 85]. The locus heterogeneity makes CCs as ideal genetic disorders for NGS-based molecular diagnoses.

Surprisingly, only a few mutational surveys on CC cohorts have been reported. Sanger sequencing and single strand conformation polymorphism (SSCP) have been used to screen for mutations in CC cohorts from India and from Denmark [86–88]. Recently, an NGS panel covering more than 110 CC genes was applied on 36 individuals with syndromic or non-syndromic bilateral CCs and was reported to detect the genetic causes of CCs in 75% of individuals, suggesting the high detection rate [3]. In this study, identification of unexpected genetic causes in patients with apparent CCs investigated at an early age had altered the clinical management and consequent genetic counseling. In another recent study, WES identified the genetic causes for half of the 18 families with CCs [89]. As a summary, the high genetic heterogeneity of CCs warrants NGS as the optimal molecular diagnostic method.

7 Glaucoma

Glaucoma is a visual impairment caused by retinal ganglion cell loss, optic nerve excavation and atrophy, and subsequent visual field defects [90]. Early-onset (adolescent) forms of glaucoma are typically inherited. Early-onset forms of glaucoma affect about 1 in every 2500 to 20,000 individuals [91]. Glaucoma is clinically heterogeneous and can be classified into several types, such as primary congenital glaucoma, juvenile/primary open-angle glaucoma, and primary angle-closure glaucoma. Currently, common risk variants in 10 loci and rare variants in 9 genes have been associated with glaucoma (Table 1) [91, 92]. Gene-gene/gene-environment interactions and epigenetics may also contribute to the pathogenesis of glaucoma [91]. Recently, Huang et al. applied WES and Sanger sequencing for the molecular diagnosis of 683 unrelated patients with primary glaucoma [93]. They used WES to analyze seven glaucoma genes and identified mutations in 20 out of 257 patients. In addition, they used Sanger sequencing on the *MYOC* gene and identified mutations in 5 of the remaining 426 patients. Compared to other inherited eye disorders, the diagnostic rate of WES based testing on glaucoma (8%) in this study is low. More genetic studies on glaucoma will garner better insight on the genetic contributions to glaucoma.

8 Challenges

NGS is able to generate enormous amounts of data, which in turn bring substantial analytical challenges. One emerging concern is that false positives may arise by chance as the amount of genomic data increases. Indeed, recent evaluations of previously reported "mutations" suggest that many of them are frequent in general populations and thus are not likely to cause diseases, including eye disorders [94–98]. To overcome this challenge, more detailed and stringent standards for the classification of variant pathogenicity are needed [63]. Further, curation of general or disease-specific variant databases will clarify the pathogenicity of variants currently felt to have uncertain or unknown significance [99–101].

Ethic, legal, and social issues also arise in the genomic era [102]. The practice of genetic testing should be carefully regulated, and genetic counseling should be provided by the attending physician or genetic counselors to maximize the benefit and reduce the risks to the consultant and the family [103]. Patients' understanding and needs should be met to provide optimal health care [104].

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