De Novo Mutations Contributes Approximately 7% of Pathogenicity in Inherited Eye Diseases

Wei Li,[1,2] Xiang-Dong He,[3] Zheng-Tao Yang,[1,2] Dong-Ming Han,[1,2] Yan Sun,[3] Yan-Xian Chen,[4] Xiao-Tong Han,[5] Si-Cheng Guo,[2,6] Yu-Ting Ma,[1,2] Xin Jin,[2] Huan-Ming Yang,[1,2] Ya Gao,[2] Zhuo-Shi Wang,[3] Jian-Kang Li,[2] and Wei He[3]

[1]College of Life Sciences, University of Chinese Academy of Sciences, Beijing, China
[2]BGI-Shenzhen, Shenzhen, China
[3]He University, Shenyang, China
[4]Department of Ophthalmology, Peking University Shenzhen Hospital, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center, Shenzhen, China
[5]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangdong Provincial Key Laboratory of Ophthalmology and Visual Science, Guangdong Provincial Clinical Research Center for Ocular Diseases, Guangzhou, China
[6]College of Plant Protection, Hunan Agricultural University, Changsha, Hunan, China

Correspondence: Wei Li, College of Life Sciences, University of Chinese Academy of Sciences, 15 Datun Road, Beijing 100049, China; michael_weili@163.com.
Ya Gao, BGI-Shenzhen, Building 11, Beishan Industrial Zone, Yantian District, Shenzhen 518083, China; gaoya@genomics.cn.
Zhuo-Shi Wang, He University, No. 66 Sishui Street, Dongling District, Shenyang, Shi 110163, China; dr.wangzs@foxmail.com.
Jian-Kang Li, BGI-Shenzhen, Building 11, Beishan Industrial Zone, Yantian District, Shenzhen 518083, China; lijiankang@genomics.cn.
Wei He, He University, No. 66 Sishui Street, Dongling District, Shenyang, Shi 110163, China; hewei@hsyk.com.cn.

WL and XDH share first authorship.

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Purpose. The purpose of this study was to describe genotype-phenotype associations and novel insights into genetic characteristics in a trio-based cohort of inherited eye diseases (IEDs).

Methods. To determine the etiological role of de novo mutations (DNMs) and genetic profile in IEDs, we retrospectively reviewed a large cohort of proband-parent trios of Chinese origin. The patients underwent a detailed examination and was clinically diagnosed by an ophthalmologist. Panel-based targeted exome sequencing was performed on DNA extracted from blood samples, containing coding regions of 792 IED-causative genes and their flanking exons. All participants underwent genetic testing.

Results. All proband-parent trios were divided into 22 subgroups, the overall diagnostic yield was 48.67% (605/1243), ranging from 4% to 94.44% for each of the subgroups. A total of 108 IED-causative genes were identified, with the top 24 genes explaining 67% of the 605 genetically solved trios. The genetic etiology of 6.76% (84/1243) of the trio was attributed to disease-causative DNMs, and the top 3 subgroups with the highest incidence of DNM were aniridia (n = 40%), Marfan syndrome/ectopia lentis (n = 38.78%), and retinoblastoma (n = 37.04%). The top 10 genes have a diagnostic yield of DNM greater than 3.5% in their subgroups, including *PAX6* (40.00%), *FBN1* (38.78%), *RB1* (37.04%), *CRX* (10.34%), *CHM* (9.09%), *WFS1* (8.00%), *RP1L1* (5.88%), *RS1* (5.26%), *PCDH15* (4.00%), and *ABCA4* (3.51%). Additionally, the incidence of DNM in offspring showed a trend of correlation with paternal age at reproduction, but not statistically significant with paternal (P = 0.154) and maternal (P = 0.959) age at reproduction.

Conclusions. Trios-based genetic analysis has high accuracy and validity. Our study helps to quantify the burden of the full spectrum IED caused by each gene, offers novel potential for elucidating etiology, and plays a crucial role in genetic counseling and patient management.

Keywords: inherited eye diseases (IED), targeted exome sequencing, proband-parent trio, de novo mutation (DNM), genetic landscape

Inherited eye disorders (IEDs) refer to a series of disorders that affect the visual function of the anterior and posterior segments of the eye. Genetic etiology is an important cause of visual impairment in children and young adults, accounting for about 50% of childhood blindness.[1–4] IEDs involve a range of clinical subgroups that develop at different ages, including inherited retinal disease (IRD), congenital cataracts, anophthalmia/microphthalmia, ocular cancer retinoblastoma, and optic atrophy.[5–8] IRDs are mostly monogenic, with close to 300 genes (RetNet: available at https://sph.uth.edu/retnet) associated with isolated or syndromic forms, and have a broad range of dysfunction or progressive loss of photoreceptors with high clinical and genetic heterogeneity.[9] These subgroups may present as

isolated eye disorders (nonsyndromic forms) or as part of a multisystem syndrome that includes extraocular symptoms (syndromic forms).[5,10] In syndromic forms, ocular manifestations are often one of the primary features of the syndrome. For example, individuals with Marfan syndrome often present with ectopia lentis.[11,12]

Over the past 30 years, more than 500 IED-causative genes have been identified. Disease-causing variants are associated with a variety of eye diseases and have diverse inheritance patterns, including autosomal recessive/dominant, X-linked, and mitochondrial inheritance.[5] Despite the successful adoption of next-generation sequencing (NGS) in many clinical subgroups of IEDs, knowledge of systematic studies of the diagnostic utility of genetic testing across the full spectrum of eye disorders is still lacking. The development of powerful genetic tests has revolutionized the diagnosis of many genetic disorders. Panel-based NGS for genetic diagnosis has been shown to be a highly accurate, reproducible, and more sensitive technique currently used in IEDs.[13] Specifically, for genetically heterogeneous diseases such as IRDs, several studies have reported the use of targeted exome sequencing for genetic diagnostic testing in patients with IRD, which genetically solves approximately 55% to 75% of IRD cases.[14–17] However, in the studies to date, gene distribution and diagnostic yield have mainly focused on sporadic and pedigree samples of IRD, and proband-parent studies on the full spectrum of IED are lacking.

De novo mutations (DNMs) are associated with many disorders, including rare genetic disorders and common complex disorders. Considering the importance of DNMs in studies of neurodevelopmental disorders, such as schizophrenia and autism, contributing to the discovery of many causative genes and deepening the understanding of disease etiology. Therefore, a large-scale study of DNMs in IEDs is worthwhile.[18–22] The etiological role of DNMs has been investigated in several subgroups of IEDs, such as congenital cataracts, Stargardt disease, retinoschisis, etc. However, as case reports of DNM are extremely rare, most pedigree-based studies reported only a few cases caused by DNM, and the sample sizes were limited to fewer than a dozen of families.[23–26] In an earlier study, a large cohort of patients with suspected retinitis pigmentosa (RP) from Chinese ancestry identified 11 DNMs in 346 trios families, with an incidence of 3.18%, including 3 dominant DNMs and 7 recessive DNMs.[16] Currently, knowledge of DNMs is limited to a few reported types of IED, lacking a full spectrum of IED, and its incidence and etiological role may be severely underestimated.

The purpose of this study was to evaluate the clinical diagnostic utility of trio-based genetic diagnostic testing in patients with different IEDs subgroups, emphasizing the potential to inform management and develop specific health care pathways for patients with multiple IEDs. To expand our knowledge of the gene distribution, diagnostic yield, and the etiological role of DNM in diverse IED subgroups, we performed targeted exome sequencing on 1243 proband-parent trios of Chinese ancestry in 22 IED subgroups.

Materials and Methods

Enrolment, Ethical, and Consent Statement

Targeted exome sequencing was performed on 1243 proband-parent trios with diverse IED phenotypes that were recruited for genetic diagnostic studies. Recruitment and diagnostic criteria for each subgroup were confirmed by an IED specialist. Targeted exome sequencing, Sanger validation, and co-segregation analysis performed on individuals were approved by the Institutional Review Board (IRB) of the He Eye Specialists Hospital of He University in accordance with the Declaration of Helsinki. All recruited participants collected blood samples for sequencing, obtained written informed consent, and collected genomic DNA extracts. Supplementary Table S1 summarizes clinical diagnostic and pedigree information for all trios.

Targeted Exome Sequence Generation

Genetic testing workflow, including library preparation, genome alignment, variant calling, and annotation, were done as previously described.[27] A customized targeted enrichment capture panel designed by Beijing Genomics Institute (BGI-Shenzhen, China) was used to process DNA samples. The panel covers the coding regions of 792 IED-causative genes and 200-bp of flanking exon sequences (Genes list: Supplementary Table S2).

Paired-end sequencing was performed on the MGISEQ-2000 (DNBSEQ-G400) platform (MGI, Inc., BGI-Shenzhen, China). The clean sequence reads were aligned to the human reference genome (GRCH37/hg38) using the Burrows-Wheeler Aligner version 0.7.10 (BWA-MEM). Overall, the average mapped data for each sample has reached 3.05 Gb, with 99.95% of the fraction mapped. The average genome depth of the target region was more than 400 times, and the coverage of the target region greater than 100 times was close to 96%. Previously reported variants were determined using ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), or literature reported. Predictions of deleterious variants of function were performed in silico analyses, including Sorting Intolerant From Tolerant (SIFT),[28] Likelihood Ratio Test (LRT),[29] MutationTaster,[30] and FATHMM.[31] Variants were classified as pathogenic (P), likely pathogenic (LP), and variants of uncertain significance (VUS) according to the American College of Medical Genetics (ACMG) guidelines.[32]

De Novo Mutation Detection

Probands or either parents with low sequencing coverage (n = 24), or excess heterozygosity (heterozygous/homozygous ratio >1.9) and cross-contaminating trio (n = 31) were excluded.[33] Trios with unproven biological relationships between the probands and parents were excluded based on vcftools (n = 12).[34] After data quality control for each trio, only the complete 1243 IED trio were retained for single nucleotide polymorphisms (SNPs), small insertions and deletions (indels), and DNMs analysis, yielding genomewide variants in 792 IED-causative genes. We considered high-confidence DNMs to be (1) those with no mutant allele reads in either parent, and (2) those with mutant allele sequence depth (DP) ≥30 times and genotype quality (GQ) scores ≥30, and (3) those variant allele counts (ACs) ≤3, and rare (MAF = ≤0.1% in gnomAD version 2.0.1) exonic variants and splice variants in flanking exons. All exons and flanking exon SNPs and indels were classified as synonymous, missense, in-frame, or loss-of-function variants (LOF; including frameshift, nonsense, and essential splice site and flanking intron variants). Sanger sequencing was used to verify

the reliability of LOF DNMs, DNA samples were available and primers were designed. If a variant was not reported in the literature or the ClinVar database, it was classified as a novel variant.

Results

Characteristics of the Participants

A total of 1243 proband-parent trios across 22 IED subgroups are presented in Supplementary Table S1, including 720 male and 523 female probands, with a mean age of 21.49 ± 14.32 years. The majority of the phenotypes (96.54%, 1200/1243) were clinical diagnoses with isolated features, and the remaining 3.46% (43/1243) had features of syndromic (e.g. Usher syndrome, Wolfram syndrome, Axenfeld-Rieger Syndrome, etc.). All proband-parent trios could be classified into three categories based on clinical diagnosis: anterior segment disorders (ASDs; n = 195), posterior segment disorders (PSDs; n = 998), and other phenotypes (n = 50). The ASD categories include lens abnormalities (n = 173), corneal abnormalities (n = 12), and iris abnormalities (n = 10). The PSD categories include inherited retinal disease (n = 781), choroid dystrophy (n = 11), vitreoretinopathy (n = 46), retinoblastoma (n = 27), optic neuropathy (n = 116), and glaucoma (n = 17). The other phenotype categories include high myopia (n = 25) and all syndromes (n = 25).

Overall Diagnostic Yield and Molecular Genetic Findings

Potential disease-causing genotypes were identified in 605 of these probands, of which 521 were caused by hereditary mutations and 84 were caused by DNMs, and the identified disease-causative variants are shown in Supplementary Table S3. The overall diagnostic yield reached 48.67% (605/1243), and the top 12 genes (*ABCA4*, *USH2A*, *FBN1*, *BEST1*, *CYP4V2*, *EYS*, *OPA1*, *RPGR*, *RDH12*, *RS1*, *CRB1*, and *RPE65*) accounted for 50.25% (304/605) of the genetically solved probands. In the ASD category, the diagnostic yield was 51.80% (101/195), and the top 3 genes (*FBN1*, *PAX6*, and *CRYGD*) accounted for 54.46% (55/101) of the genetically solved probands. In the PSD category, the diagnostic yield was 49.80% (497/998), and the top 10 genes (*ABCA4*, *USH2A*, *BEST1*, *CYP4V2*, *OPA1*, *EYS*, *RPGR*, *RS1*, *RDH12*, and *CRB1*) accounted for 51.51% (256/497) of the genetically solved probands (Table 1).

In this cohort, 684 distinct disease-causing genotypes were identified and distributed across 108 genes, including 58 genes that are exclusively autosomal recessive, 36 genes that are exclusively autosomal dominant, 9 genes that are X-linked, and 5 genes (*BEST1*, *NR2E3*, *PROM1*, *RHO*, and *WFS1*) that contain autosomal dominant or recessive variants. Of these genotypes, 99 genotypes were detected in more than 2 probands, and the remaining 585 genotypes were detected in one proband. Among the probands with

Table 1. Number of Trio, Diagnostic Yield, and Distribution of Hotspot Genes in Each IED Category

IED Categories	Total Trios (n)	Solved Trios (n)	Diagnostic Yield	Identified Genes (n)	Total >50% (Top Genes)
1 ASDs	195	101	51.80%	25	54.46% (*FBN1*, *PAX6*, and *CRYGD*)
1.1 Lens abs	173	90	52.02%	24	54.44% (*FBN1*, *CRYGD*, and *NHS*)
1.1.1 CC	124	50	40.32%	21	52.00% (*CRYGD*, *NHS*, *PAX6*, *GJA8*, *CRYGC*, and *CRYBB2*)
1.1.2 MFS/EL	49	40	81.63%	3	92.5% (*FBN1*)
1.2 Corneal abs	12	5	41.67%	2	80.00% (*TGFBI*)
1.3 Iris abs	10	6	60.00%	1	100.00% (*PAX6*)
2 PSDs	998	497	49.80%	83	51.51% (*ABCA4*, *USH2A*, *BEST1*, CYP4V2, *OPA1*, *EYS*, *RPGR*, *RS1*, *RDH12*, and *CRB1*)
2.1 IRD	781	421	53.91%	70	53.44% (*ABCA4*, *USH2A*, *BEST1*, *CYP4V2*, *EYS*, *RPGR*, *RS1*, and *RDH12*)
2.2 CD	11	9	81.82%	1	100% (*CHM*)
2.3 VRP	46	27	58.7%	6	62.96% (*TSPAN12*, *LRP5*, and *NDP*)
2.4 RB	27	10	37.04%	1	100% (*RB1*)
2.5 ONPs	116	27	23.28%	8	62.96% (*OPA1*)
2.6 Glaucoma	17	3	17.65%	3	66.67% (*CYP1B1* and *LTBP2*)
3 Other Ps	50	7	14.00%	6	57.14% (*WFS1*, *COL2A1*, and *KIF11*)
3.1 HM	25	1	4.00%	1	100% (*KIF11*)
3.2 All syndrome	25	6	24.00%	5	50% (*WFS1* and *COL2A1*)
Total Ps	1243	605	48.67%	108	50.25% (*ABCA4*, *USH2A*, *FBN1*, *BEST1*, *CYP4V2*, *EYS*, *OPA1*, *RPGR*, *RDH12*, *RS1*, *CRB1*, and *RPE65*)

ASDs, anterior segment disorders; Lens abs, lens abnormalities, including abnormalities in the transparency (CC: Congenital cataract [n = 124]) or morphology and location (MFS/EL, Marfan syndrome [n = 16] and ectopia lentis [n = 33]) of the lens; Corneal abs, corneal abnormalities (corneal dystrophy, n = 12); Iris abs, iris abnormalities (aniridia, n = 10); PSDs, posterior segment disorders; IRD, inherited retinal disease, including albinism (n = 7), Bestrophinopathy (n = 33), Bietti crystalline corneoretinal dystrophy (n = 18), cone-rod dystrophy (n = 29), Leber congenital amaurosis (n = 49), macular dystrophy (n = 68), retinal dystrophy (n = 117), retinitis pigmentosa (n = 359), retinoschisis (n = 19), Stargardt disease (n = 57), and Usher syndrome (n = 25); CD, choroid dystrophy, including choroideremia (n = 10) and congenital iris choroidal defect (n = 1); VRP, vitreoretinopathy, including familial exudative vitreoretinopathy (n = 43) and Norrie disease (n = 3); RB, retinoblastoma (n = 27); OPNs, optic neuropathy, including optic atrophy (n = 109) and Leber hereditary optic neuropathy (n = 7); Other Ps, other phenotypes; HM, high myopia (n = 25); All Syndrome, including Axenfeld-Rieger syndrome (n = 3), Coats disease (n = 1), Microphthalmia (n = 11), morning glory syndrome (n = 1), VHL syndrome (n = 1), Bardet-Biedl syndrome (n = 2), Oguchi disease (n = 1), Stickler syndrome (n = 1), Waardenburg syndrome (n = 1), and wolfram syndrome (n = 3); Total Ps, total phenotypes (n = 1243).

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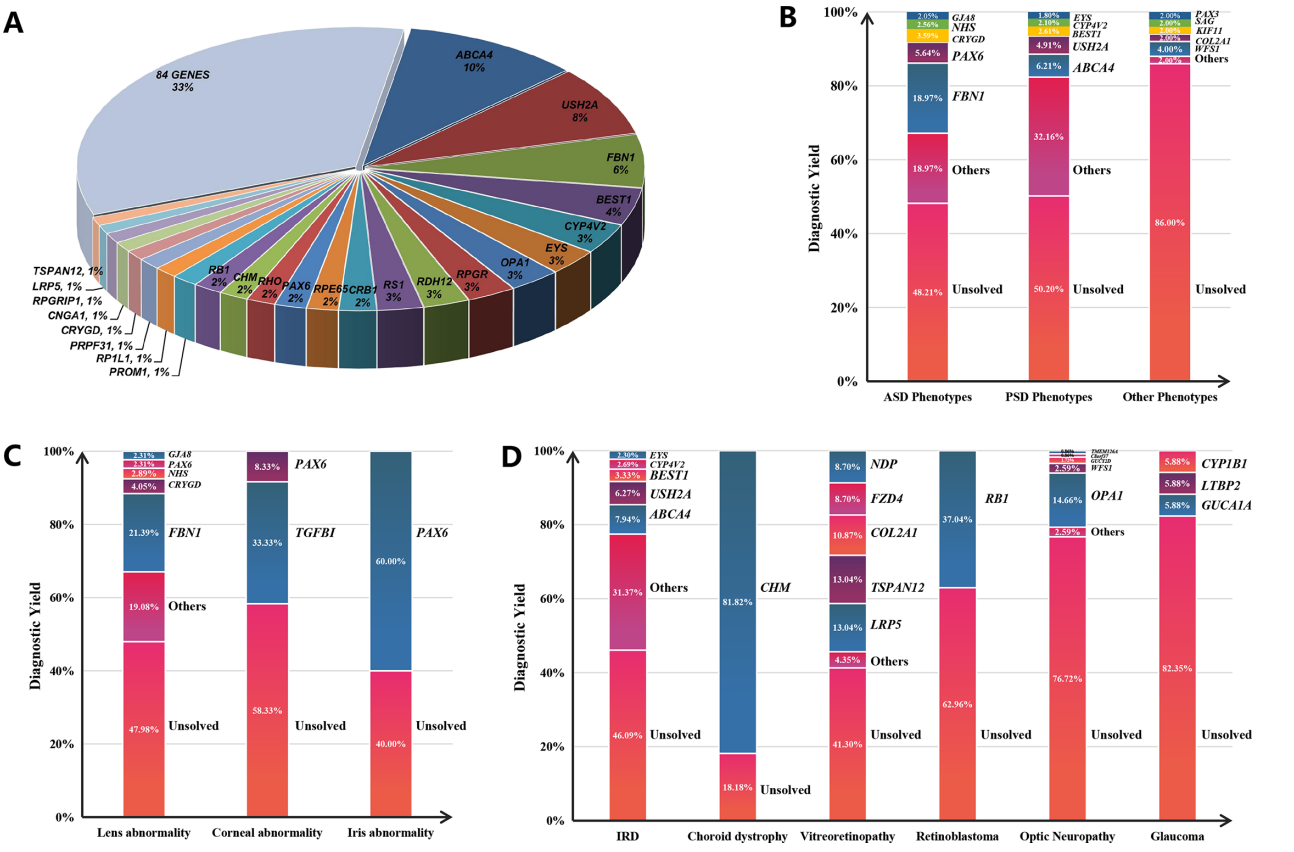

Figure 1. Distribution and diagnostic yield of IED-causative genes. (**A**) A total of 107 IED-causative genes were identified, with the top 24 genes explained 67% of the 605 genetically solved trios. (**B**) Diagnosis yield and genes distribution in ASDs, PSDs, and other phenotypic categories. (**C**) Diagnosis yield and genes distribution in three categories of ASDs. (**D**) Diagnosis yield and genes distribution in six categories of PSDs. ASDs, anterior segment disorders; PSDs, posterior segment disorders; IRD, inherited retinal disease.

a molecular diagnosis, 55.04% (333/605) were autosomal recessive (most frequent genes *ABCA4* [n = 62], *USH2A* [n = 49], *CYP4V2* [n = 22]), 35.21% (213/605) were autosomal dominant (most frequent genes *FBN1* [n = 37], *BEST1* [n = 18], and *OPA1* [n = 18]), and 9.75% (59/605) were X-linked (most frequent genes *RPGR* [n = 17], *RS1* [n = 16], and *CHM* [n = 9]). In addition, 24.92% (83/333) of autosomal recessive probands were homozygous mutations, with the rest being compound heterozygous mutations.

Of these 108 genes, the top 24 genes explain 67% (403/605) of 605 genetically solved probands (Fig. 1A). For patients in the ASD category, the most frequent genes were *FBN1* (37/195 = 18.97%), *PAX6* (11/195 = 5.64%), and *CRYGD* (7/195 = 3.59%). Of these, *PAX6* was detected in 11 probands, distributed in lens abnormalities (congenital cataract, 4/173 = 2.31%), corneal abnormalities (corneal dystrophy, 1/12 = 8.33%), and iris abnormalities (aniridia, 6/10 = 60%). *FBN1* and *CRYGD* are the most frequent causative genes for Marfan syndrome/ectopia lentis and congenital cataracts, respectively. As for PSDs, the IRD category accounted for 78.26% (781/998) trio numbers, and the most frequent causative genes were *ABCA4* (62/998 = 6.21%), *USH2A* (49/998 = 4.91%), and *BEST1* (26/998 = 2.61%). *ABCA4*-causative mutations were detected in six subgroups, including cone-rod dystrophy (n = 5), Leber congenital amaurosis (n = 1), macular dystrophy (n = 14), retinal dystrophy (n = 2), retinitis pigmentosa (n = 5), and Stargardt disease (n = 35). *USH2A* is the most frequent gene of RP (n = 359) and Usher syndrome (n = 25), with an overall diagnostic yield of 12.76% (49/384; Figs. 1B, 1C, 1D).

Incidence of DNM

Overall, 84 trios were solved by IED-causative DNMs, including 70 autosomal dominant DNMs, 6 X-linked DNMs, and 8 autosomal recessive DNMs that constituted compound heterozygous forms with another mutation from the paternal or maternal origin (Supplementary Table S4). Surprisingly, the overall diagnostic yield was 6.76% (84/1243), distributed across 17 distinct IED subgroups. The top three subgroups with the highest incidence of DNM were aniridia (4/10 = 40%), Marfan syndrome/ectopia lentis (10/49 = 38.78%), and retinoblastoma (10/27 = 37.04%). A total of 34 IED-causative DNM genes were identified, and the top 6 genes (*FBN1*, *RB1*, *PAX6*, *RP1L1*, *RHO*, and *CRX*) explained 52.38% (44/84) of the cases caused by DNM (Table 2, Fig. 2). Of these 84 disease-causing DNMs, 38 were novel, and 46 mutations were reported in the literature. Distribution of variant types in disease-causing DNMs, including 50% missense mutations (n = 42), 14.29% splices and intronic mutations (n = 12), 17.86% nonsense mutations (n = 15), 9.52% frameshift mutations (n = 8), 7.14% in-frame mutations (n = 6), and 1.19% synonymous mutations (n = 1; see Supplementary Table S4).

A set of high-confidence DNMs based on targeted exome sequencing was generated, with 1293 DNMs identified

Table 2. The Diagnostic Yield of DNM and The Distribution of Identified Hotspot Genes in per IED Subgroup

IED Subgroups	Total Trios		Solved Trios by DNM		Diagnostic Yield of DNM	Identified Genes (*n*)	Total >50% (Top Genes)
	N	Mean Age ± SD	*N*	Mean Age ± SD			
Aniridia	10	7.75 ± 6.15	4	9.38 ± 8.60	40.00%	1	100% (*PAX6*)
MFS/EL	49	9.21 ± 8.56	19	7.42 ± 5.73	38.78%	1	100% (*FBN1*)
RB	27	8.49 ± 8.03	10	6.43 ± 6.37	37.04%	1	100% (*RB1*)
All Syndrome	25	16.57 ± 10.30	3	27 ± 25.46	12.00%	2	66.67% (*WFS1*)
MD	68	28.15 ± 13.77	8	34.38 ± 16.25	11.76%	4	50% (*RP1L1*)
CRD	29	23.18 ± 13.83	3	24 ± 17	10.34%	1	100% (*CRX*)
CD	11	21.55 ± 11.94	1	–	9.09%	1	100% (*CHM*)
CC	124	6.98 ± 8.02	11	5.47 ± 5.66	8.87%	7	63.63% (*CRYBB2*, *CRYGC*, and *NHS*)
RS	19	13.42 ± 10.51	1	–	5.26%	1	100% (*RS1*)
USH	25	29.95 ± 11.05	1	–	4.00%	1	100% (*PCDH15*)
RP	359	30.48 ± 12.39	14	32.21 ± 12.87	3.90%	10	50% (*RHO*, *SNRNP200*, and *CEP290*)
ST	57	21.18 ± 11.06	2	17.5 ± 10.61	3.51%	1	100% (*ABCA4*)
Best	33	25.97 ± 13.55	1	–	3.03%	1	100% (*BEST1*)
VRP	46	14.93 ± 11.67	1	–	2.17%	1	100% (*NDP*)
LCA	49	14.22 ± 12.04	1	–	2.04%	1	100% (*IMPDH1*)
ONPs	116	17.30 ± 11.27	2	10 ± 2.83	1.72%	1	100% (*OPA1*)
RD	117	21.65 ± 14.08	2	11 ± 4.24	1.71%	1	100% (*KIF11*)
HM	25	21.63 ± 12.46	–	–	–	–	–
Glaucoma	17	23.71 ± 11.10	–	–	–	–	–
Corneal abs	12	21.75 ± 11.14	–	–	–	–	–
Albinism	7	13.93 ± 12.68	–	–	–	–	–
BCD	18	34.22 ± 9.13	–	–	–	–	–
Total Ps	1243	21.49 ± 14.32	84	16.19 ± 15.21	6.76%	34	52.38% (*FBN1*, *RB1*, *PAX6*, *RP1L1*, *RHO*, and *CRX*)

DNM, De novo mutation; *n*, number; MFS/EL, Marfan syndrome and ectopia lentis; RB, retinoblastoma; MD, macular dystrophy; CRD, cone-rod dystrophy; CD, choroid dystrophy; CC, congenital cataract; RS, retinoschisis; USH, Usher syndrome; RP, retinitis pigmentosa; ST, Stargardt disease; Best, Bestrophinopathy; VRP, vitreoretinopathy; LCA, Leber congenital amaurosis; OPNs, optic neuropathy; RD, retinal dystrophy; HM, high myopia; Corneal abs, corneal abnormalities; BCD, Bietti crystalline corneoretinal dystrophy; Total Ps, total phenotypes.

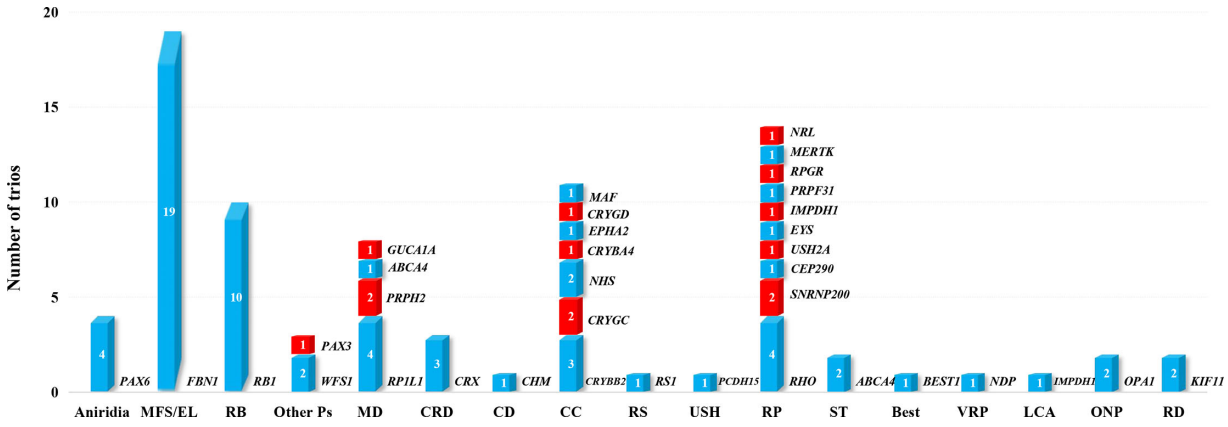

Figure 2. Number of trios and gene distribution of IED-causative DNMs in different subgroups.

across 385 genes, averaging 1.04 DNMs per trio (Fig. 3A). The majority of DNMs were missense variants (50.35%, 651/1293), 18.48% were LOF (239/1293; including 9.67% frameshift, 6.65% splice and intron, and 2.17% nonsense), 24.44% were synonymous (316/1293), and the remaining 6.73% were in-frame (87/1293). We characterized the distribution of high-confidence DNM variant types across all trios, stratified by IED subgroups and proband sex. Our study used mutational models to determine whether trios affected by the IED had more high-confidence DNM than expected.[35] All high-confidence DNMs were divided into four classes based on predicted function: synonymous, missense, LOF, and a combination of missense and LOF were defined as protein-altering DNMs. The trio of all IEDs phenotypes had a significant excess of LOF DNM (enrichment = 2.13; $P = 3.86 \times 10^{-25}$), but not excess missense DNM (enrichment = 0.813; $P = 1$), and synonymous DNM (enrichment = 0.877; $P = 0.991$). In female patients with all IEDs phenotypes ($n = 523$), the excess protein-altering DNMs (enrichment = 1.11; $P = 0.0219$) was caused by a significant excess of LOF DNMs (enrichment = 2.75; $P = 7.25 \times 10^{-23}$). In male patients with all IEDs phenotypes ($n = 720$), a significant excess of LOF DNM (enrichment = 1.68; $P = 4.53 \times 10^{-7}$) was observed (Fig. 3B).

In total, 84 probands with disease-causing DNM were distributed in 17 distinct IED subgroups, including 40 male

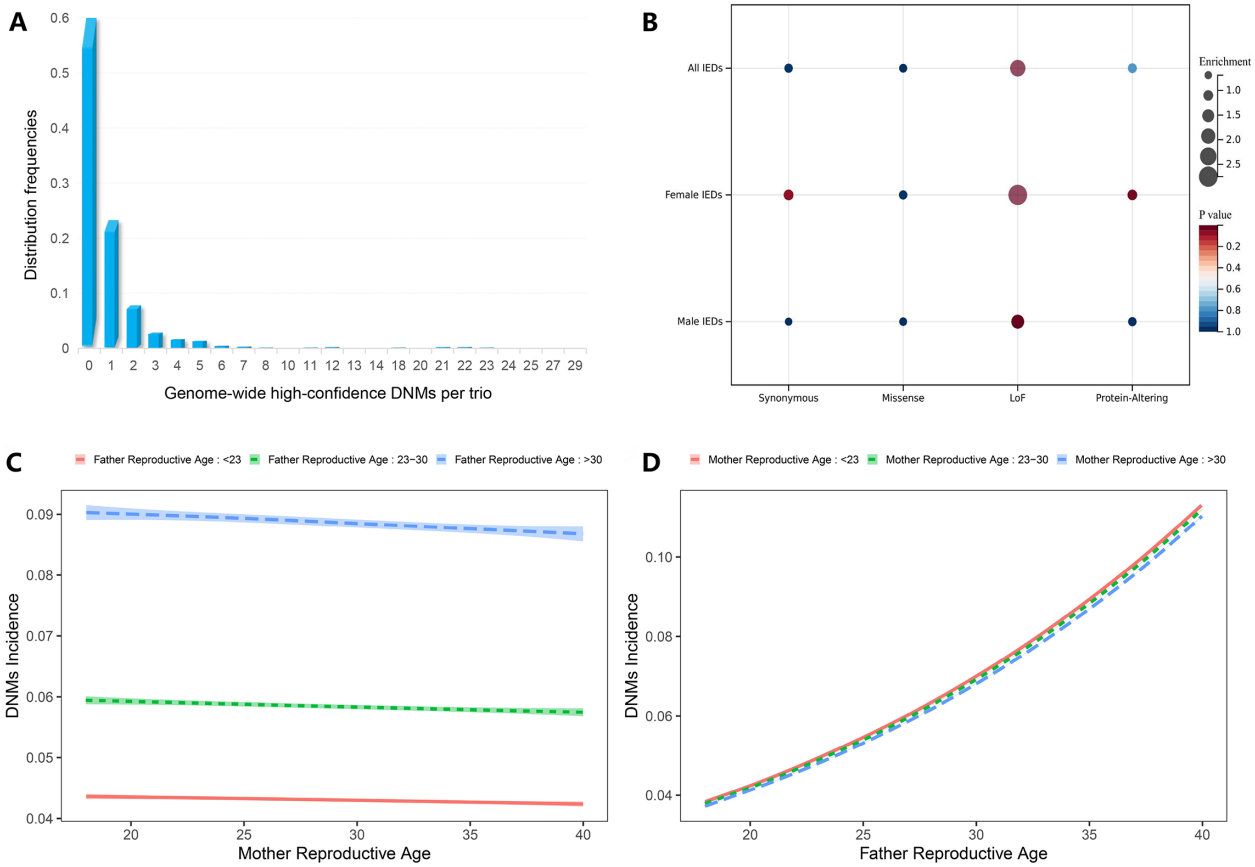

Figure 3. (**A**) The distribution frequency of the genomewide high-confidence DNM per trio. (**B**) Enrichment of DNMs by variant class for all IEDs phenotypes, females and males probands. (**C**) Correlation between mother reproductive age and the incidence of DNM in offspring. (**D**) Correlation between father;s reproductive age and the incidence of DNM in offspring.

and 44 female patients, with a mean age of 16.19 ± 15.21 years. The mean age of probands (n = 84) with a definitive molecular diagnosis of DNM was significantly lower than the mean age of all probands (1159) without DNM (21.91 ± 14.17). Among the 84 trios caused by DNM, the mean age at reproductive age of the parents was 29.45 ± 5.02 and 27.24 ± 4.45 years, respectively. Among the 1159 trios without DNM, the mean age at reproductive age of the parents was 27.99 ± 4.58 and 26.19 ± 4.43 years, respectively. These 2 categories are divided into 3 groups based on age, including ages <23 years, 23 to 30 years, and >30 years old. Using logistic regression, we found a trend, but not statistically significant (P = 0.154), for a correlation between paternal age at reproductive and the incidence of DNM in offspring. In addition, the incidence of DNM in the offspring has no correlation trend and statistical significance with the maternal age at reproduction (P = 0.959; Figs. 3C, 3D).

IED Subgroups and DNMs Contribution

A total of 22 IED subgroups were recruited, covering essentially the full spectrum of genetic eye diseases (see Tables 1 and 2). The overall diagnostic yield was 48.67%, whereas the DNM diagnostic yield reached 6.76%, which is a considerable proportion of the overall diagnostic yield. Among these 22 subgroups, the subgroup with the highest diagnostic yield was Bietti crystalline corneoretinal dystrophy (BCD; 17/18 = 94.44%) and the subgroup with the lowest diagnostic yield was high myopia (HM; 1/25 = 4%; Fig. 4A). A definite disease-causing DNM was detected in 17 of the 22 subgroups, and no convincing DNM was detected in the remaining 5 subgroups, with a DNM diagnostic yield of 0% (Fig. 4B).

In addition, we further explored the proportion of DNM in the overall diagnostic yield in distinct subgroups (Fig. 4C). The overall diagnostic yield of retinoblastoma was 37.04% (10/27), and 100% of the cases with a definite molecular diagnosis were caused by DNM, and all of them were bilateral retinoblastoma. *RB1* was the only causative gene for retinoblastoma,[36] 70% (n = 7) of variants were LOF mutations, 20% (n = 2) were missense mutations, and 10% (n = 1) were in-frame mutations. Aniridia was known to be predominantly caused by variants in *PAX6*,[37] all variant types being LOF mutations (n = 6). The overall diagnostic yield was 60% (6/10), of which the diagnostic yield of DNM accounted for 66.67% (4/6). The overall diagnostic yield of Marfan syndrome/ectopia lentis was 81.63% (40/49), of which the number of cases caused by DNM accounted for 47.51% (19/40). *FBN1* was the predominantly gene for Marfan syndrome/ectopia lentis,[11] 84.21% (n = 16) of the variants were missense, 10.53% (n = 2) were splice and intron, and 5.26% (1/19) were synonymous. Five IED subgroups, albinism (n = 7), BCD (n = 18), corneal abnormalities (corneal dystrophy, n = 12), glaucoma (n = 17), and HM (n = 25), caused entirely by paternally and maternally inherited mutations, whereas DNM had no apparent role in the etiology of the disease.

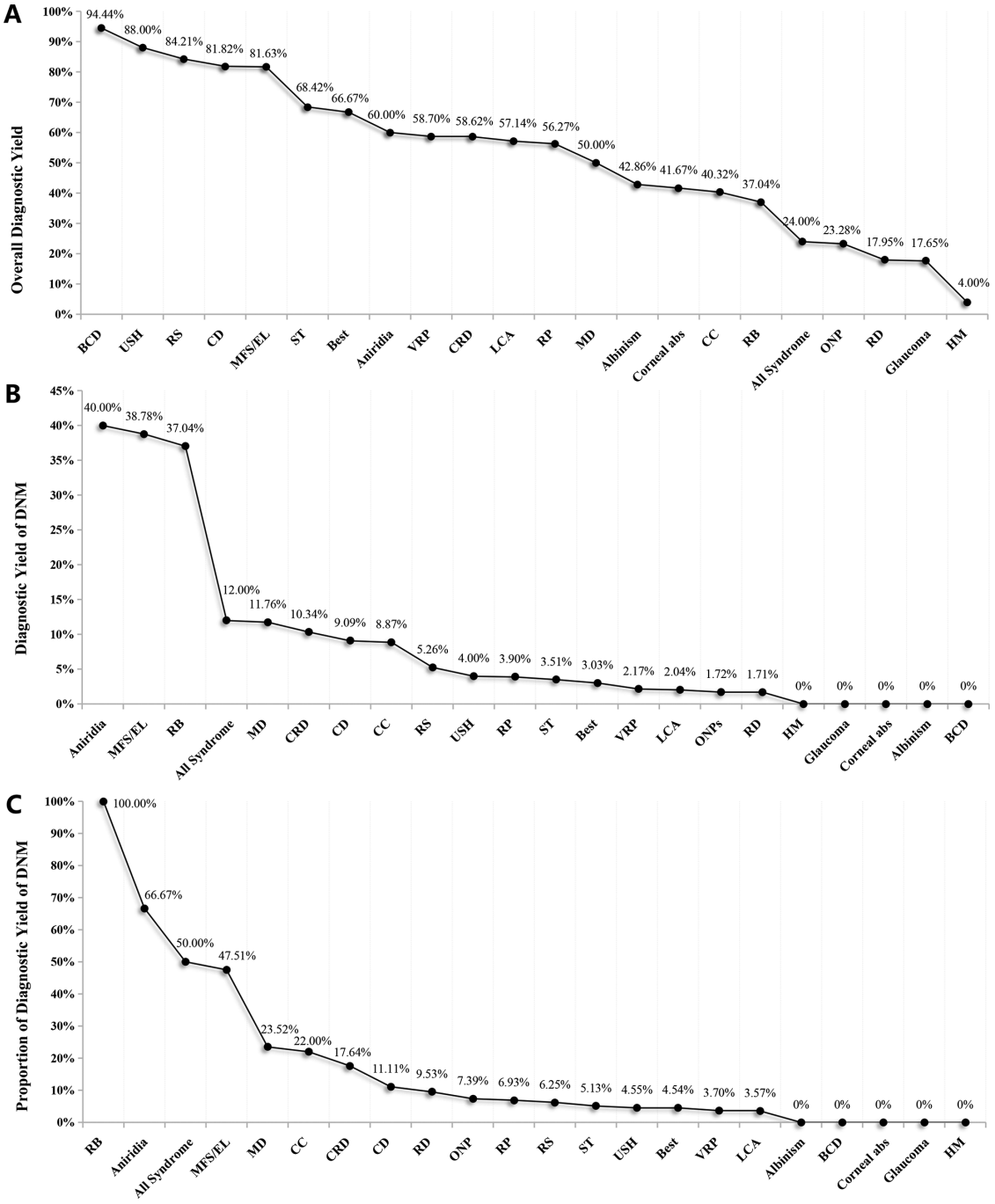

Figure 4. Statistics on the diagnostic yield of 22 IED subgroups. (**A**) Overall diagnostic yield solved by genetic testing in distinct subgroups. (**B**) Diagnostic yield solved by DNMs in distinct subgroups. (**C**) The proportion of DNM in overall diagnostic yield in distinct subgroups.

Discussion

The proband-parent trio can detect variations in mutation rates between individuals and determine the parental origin of the mutations. The data contains complete information within and between trios, avoids confounding factors caused by large families, sample stratification effects, and tests for linkage disequilibrium. As an effective diagnostic tool, trio-based genetic diagnostic testing can help confirm or rule out suspicious diagnosis, provide vital information on inheritance patterns, and assess the risk of recurrence for family members, as well as guide clinical management, appropriate referral, and surveillance decision making.[14]

In this study, 1243 proband-parent clinical records were reviewed to classify them into 3 categories: ASDs ($n = 195$), PSDs ($n = 998$), and other phenotypes ($n = 50$). In the ASD category, 88.72% (173/195) of the probands had lens abnormalities, including abnormalities of transparency (congenital cataract, $n = 124$) or morphology and location (Marfan syndrome, $n = 16$; and ectopia lentis, $n = 33$). In the PSD category, 78.26% (781/998) of the probands had inherited retinal disease, and the most prevalent subgroup was RP (n

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= 359; see Table 1). To our knowledge, this study represents the largest trio-based molecularly solved IED cohort to date. Molecular diagnosis was determined in 48.67% of probands, with 684 distinct disease-causing genotypes distributed across 108 genes. We found that the 24 most frequent genes explained 67% of the genetically solved trios. Of these 24 genes, nearly half (*ABCA4*, *USH2A*, *CYP4V2*, *RPGR*, *RS1*, *CRB1*, *RPE65*, *RHO*, *CHM*, *PRPF31*, and *CRYGD*) are in clinical trials of gene therapy, of which LUXTURNA is a commercialized gene therapy drug for the treatment of patients with IRD caused by mutations in both copies of the *RPE65* gene.[38–43]

In addition to rare inherited mutations, we propose that disease-causing DNMs may contribute to the genetic architecture of IEDs, which has not been well studied. Previous studies identified several scattered families associated with DNM in multiple IED subgroups, including congenital cataract,[2] cone-rod dystrophy,[44] high myopia,[45] macular dystrophy,[46] keratoconus,[47] retinitis pigmentosa,[48] congenital microphthalmia,[49] and Stickler syndrome.[50] Approximately 7% (6.76%, 84/1243) of the proband-parent trios, significantly higher than we expected, had a definitive molecular diagnosis due to DNM. In these subgroups, the incidence of disease-causing DNM ranged from 1.71% (optic atrophy, 2/117) to 40% (Aniridia, 4/10). Among the 84 probands detected with DNMs, 41.67% (35/84) of the probands were LOF DNMs, whereas 50% (42/84) were missense DNMs. In particular, aniridia (100%), cone-rod dystrophy (100%), retinoblastoma (70%), and congenital cataract (54.55%) were significantly enriched for LOF DNMs, whereas macular dystrophy (50%), Marfan syndrome/ectopia lentis (84.21%), and RP (57.14%) were significantly enriched for missense DNMs.

The genetic etiology of DNM in IEDs has been reported in multiple pieces of literature, but most case reports from non-East Asian populations, and systematic studies of the full spectrum of IEDs are insufficient. Our study systematically reviewed 1243 recruited IED trios of Chinese ethnicity, of whom 84 had a definitive molecular diagnosis and etiology attributable to DNM. Similar to genomic structural birth defects, missense, and LOF DNM play important roles in individual risk for IEDs, but the specificity of the genetic structure of each subgroup needs to be fully understood across different IED subgroups.[50–52] For IED probands detected with DNM, female patients have significantly increased DNM burden or more LOF DNM compared to male patients. In short, 43.18% (19/44) of female probands had a missense DNM, and 47.73% (21/44) had an LOF DNM. However, 57.5% (23/40) of male patients were missense, and 35% (14/40) were LOF DNM. Our findings suggest that differences in the genetic liability of IEDs can be explained by genomewide DNM variant associated with genetic diversity.

The incidence of DNM observed in this study (38.78%) was higher than the recognized incidence of DNM in Marfan syndrome/ectopia lentis (25%), but lower than the incidence of DNM observed in the international cohort (approximately 50%). A synonymous DNM in the *FBN1* gene (c.6354C>T, p.Ile2118Ile) was identified in proband Fam_20201073_1, and the mutation has been included in the ClinVar database as pathogenic. However, according to the ACMG guidelines, it may be defined as likely pathogenic in the absence of evidence of function. This sequence change affects codon 2118 of *FBN1* mRNA and is a "silent" variant that does not alter the amino acid sequence encoding the *FBN1* protein. The synonymous variant c.6354C>T causes in-frame skipping of exon 52 by affecting splicing patterns in Marfan syndrome, and has been reported in multiple unrelated individuals.[53–56] A similar reported synonymous variant c.4773A>G (p.Gly1591Gly) in exon 39 of the *FBN1* gene and predicted to be translational silent, alters the splice site and causes complete skipping of exon 39, resulting in Marfan syndrome.[57]

It is well known to human geneticists that individuals with phenotypes that were historically lethal in childhood (such as retinoblastoma) or were devastating to health had poor reproductive fitness (i.e. that affected patients very infrequently had children). Such diseases were mostly due to DNMs because they almost always came from unaffected parents. At the extreme, if affected individuals with a particular dominant disease do not reproduce, then all mutations must be de novo.[58,59] This is an old theory in human genetics and has been confirmed in the last 40 years as more and more disease genes were identified and mutation surveys were conducted. This is not a feature specific to ophthalmic diseases. Non-ophthalmic diseases which have long been known to have a high rate of de novo mutations are Duchenne Muscular Dystrophy (DMD; OMIM 300377)[60,61] and Neurofibromatosis type I (NF1; OMIM 162200).[62,63]

Thus, it would be predicted that *FBN1* mutations were frequent de novo because patients with Marfan syndrome/ectopia lentis (caused by *FBN1* mutations) likely have a low rate of reproduction. This explains the high rate of DNMs in the retinoblastoma gene and in the aniridia gene. In fact, almost all cases of bilateral retinoblastoma have been known since about 1960 to be due to germline DNMs, and all cases of nonhereditary unilateral retinoblastoma were known to be due to somatic DNMs.[64–66] A total of 14 bilateral and 13 unilateral retinoblastoma trios were analyzed in our studies, and DNMs in *RB1* were found in 10 bilateral cases, whereas no de novo *RB1* mutations were detected in unilateral cases. All patients with de novo *RB1* mutations developed bilateral retinoblastoma (71.4%, 10/14) as expected, and no de novo *RB1* mutation was detected in the remaining four bilateral retinoblastomas, possibly due to copy number variation or unknown complex pathogenesis. Even with dominant diseases where affected individuals do have children, it should be straightforward to figure out which cases have a DNM by just checking to see if one of the affected child's parents is affected. Of course, non-penetrance exists in some cases, and genomic variants may be inherited from phenotypically normal parents. For the *PAX6* gene that causes aniridia, disease-causing mutations were found in 6 of the 10 trios, 4 of which were caused by DNM, and 2 trios were inherited from their parents. In these 2 trios, Fam_20200086_1 was diagnosed with aniridia and congenital cataract, and it was inherited from the father who was diagnosed with congenital cataract; Fam_20200385_1 was diagnosed with aniridia and inherited from the father who was diagnosed with nystagmus. No mutation was detected in the remaining four trios, possibly due to copy number variation or other unknown genes.

To date, this first study represents the largest exploration of genetic profiles in the 1243 proband-parent trios with IEDs. This preliminary study clearly demonstrates that DNM in biologically and clinically relevant genomewide sets of genes may contribute to IED. In addition to providing unique insights into known IED genes and clinical subgroups, our results highlight DNM enrichment gene sets, as well as interactions between clinical phenotypes and variant types, which may be important for understanding the genetic

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diversity of these genes. Despite the significant number of DNMs we found in multiple IEDs subgroups, this higher proportion does not reveal a higher than reported incidence of DNM, but reflects the importance of the practice and screening results. Collectively, these novel observations and findings provide a better understanding of the genetic architecture of IEDs and serve as an efficient reference for genetic counseling and future gene therapies that may be used to treat the disease.

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