Mutation analysis of the genes associated with anterior segment dysgenesis, microcornea and microphthalmia in 257 patients with glaucoma

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Abstract. Genetic factors have an important role in the development of glaucoma; however, the exact genetic defects remain to be identified in the majority of patients. Glaucoma is frequently observed in patients with anterior segment dysgenesis (ASD), microcornea or microphthalmia. The present study aimed to detect the potential mutations in the genes associated with ASD, microcornea and microphthalmia in 257 patients with glaucoma. Variants in 43 of the 46 genes, which are associated with ASD, microcornea or microphthalmia, were available in whole-exome sequencing. Candidate variants in the 43 genes were selected following multi-step bioinformatic analysis and were subsequently confirmed by Sanger sequencing. Confirmed variants were further validated by segregation analysis and analysis of controls. Overall, 70 candidate variants were selected from whole-exome sequencing, of which 53 (75.7%) were confirmed by Sanger sequencing. In total, 27 of the 53 were considered potentially pathogenic based on bioinformatic analysis and analysis of controls. Of the 27, 6 were identified in *BEST1*, 4 in *EYA1*, 3 in *GDF6*, 2 in *BMP4*, 2 in *CRYBA4*, 2 in *HCCS*, and 1 in each of *CRYAA*, *CRYGC*, *CRYGD*, *COL4A1*, *FOXC1*, *GJA8*, *PITX2* and *SHH*. The 27 variants were detected in 28 of 257 (10.9%) patients, including 11 of 125 patients with primary open-angle glaucoma and 17 of 132 patients with primary angle-closure glaucoma. Variants in these genes may be a potential risk factor for primary glaucoma. Careful clinical observation and analysis of additional patients in different populations are expected to further these findings.

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Introduction

Glaucoma, an irreversible neurodegenerative disease (1), affects ~60 million people worldwide (2). Primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) are the predominant types of glaucoma in various populations (2). Genetic factors have well-known important roles in the development of glaucoma (3-7); mutations in 7 genes (8-14) are responsible for a small portion of glaucoma (15-17), and recent studies have disclosed a number of new genes or loci associated with glaucoma (18-27). However, the exact genetic defects involved remain elusive for the majority of patients.

Glaucoma is frequently observed in patients with anterior segment dysgenesis (ASD), microcornea or microphthalmia. Approximately 50% of patients with ASD will eventually develop glaucoma (28). The incidence of glaucoma is 77% in elderly patients with relative anterior microphthalmus (cornea diameter <11 mm, axial length >20 mm) (29). Microphthalmia, which is always accompanied with microcornea, is considered a primary risk factor of angle-closure glaucoma (30). Mutations in a number of genes have been linked to ASD, microcornea and microphthalmia (31-36), and some of these were recently reported to be responsible for primary glaucoma (37,38). Systemic analysis of these genes in patients with primary glaucoma may provide an overview of the contribution of their mutations to primary glaucoma.

In our previous study, whole-exome sequencing was performed for 257 patients with primary glaucoma, where mutations in 7 known glaucoma genes were present in 7.8% of patients (15). In the present study, variants from exome sequencing for 43 genes known to be associated with ASD, microcornea or microphthalmia were selected for further analysis. Overall, 27 potential pathogenic variants in 14 of the 43 genes were identified in 28 of 257 patients with primary glaucoma, suggesting a possible association of these genes with primary glaucoma.

Materials and methods

Patients. The 257 unrelated patients with primary glaucoma, including 125 with POAG and 132 with PACG, have been described in our previous study (15). Written informed consent was obtained from the participants or their guardians prior to the collection of clinical data and peripheral venous blood samples. The study was consistent with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the Zhongshan Ophthalmic Center (Guangdong, China). Whole-exome sequencing on genomic DNA from the patients has been described in our previous study (15). In brief, the solution-based exome capture system (TruSeq Exome Enrichment kits; Illumina, Inc., San Diego, CA, USA) was applied and the average sequencing depth was set at 125-fold.

Selection of genes for analysis. Genes associated with ASD, microcornea or microphthalmia were selected based on the PubMed search (http://www.ncbi.nlm.nih.gov/) accessed on February 1, 2014. The classification of phenotypic spectrum of ASD was based on a previous review (28). The following search terms were used: [mutation AND (ASD OR Axenfeld-Rieger Syndrome OR Peters anomaly OR Peters Plus syndrome OR aniridia OR sclerocornea OR megalocornea OR microcornea OR microphthalmia)] AND ('2009/02/01' [Date-Publication]: '2014/02/01' [Date-Publication]). From all the reports identified with the associated results, only those describing genes with mutations in humans were selected for further analysis, which resulted in 46 candidate genes (Fig. 1). Of the 46 genes, 43 were included in the present study, while one (*CYP1B1*) had been analyzed in our previous study (15) and two, *PRSS56* and *MACOM*, were excluded as they were not captured by the TruSeq Exome Enrichment kit. Variants in the 43 genes were selected from whole-exome sequencing and subsequently filtered through the following steps: i) Inclusion criteria of variant selection: Variants predicted to affect the coding residue or mRNA splicing; variants with minor allele frequency <0.01 compared with the 1000 Genomes Project database accessed on September 1, 2014; missense variants predicted to be damaging by either PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) or SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) (39,40); intronic variants predicted to affect splicing site by BDGP (http://www.fruitfly.org/); nonsense variants, insertions and deletions; and heterozygous variants in genes associated with autosomal dominant diseases, compound heterozygous or homozygous variants in genes associated with autosomal recessive diseases, hemizygous variants in genes associated with X-linked recessive diseases, and both hemizygous and heterozygous variants in genes associated with X-linked dominant diseases. ii) Selected variants confirmed by Sanger sequencing were analyzed further. iii) For genes only with specific types of variants reported to be correlated with associated eye diseases, other types of variants were tentatively listed as less likely pathogenic variants. For example, missense variants in *NHS* were listed as less likely pathogenic variants as only truncation mutations in this gene had been reported to be causative. iv) The remaining variants were validated based on 192 ethnicity-matched normal controls and available family members.

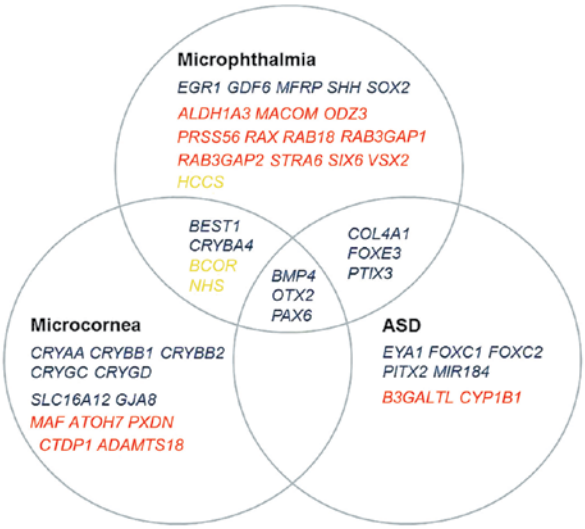

Figure 1. Genes with mutations reported in patients with anterior segment dysgenesis (ASD), microcornea or microphthalmia. Variants in 43 of the 46 genes were analyzed as *PRSS56* and *MACOM* are not captured by the TruSeq Exome Enrichment kit, and *CYP1B1* has been previously analyzed. Blue indicates genes associated with autosomal dominant diseases, red indicates genes associated with autosomal recessive diseases and yellow indicates genes associated with X-linked diseases.

Primer design. The primers used to confirm the candidate variant were designed using the Primer3 online tool (http://primer3.ut.ee/) (41). Polymerase chain reaction was used to amplify the fragments harboring the target variants. The sequence of the amplicons was determined with an ABI BigDye Terminator v3.1 Cycle Sequencing kit on an ABI3130 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA) as described previously (42).

Results

Analysis of the variants. Overall, 70 candidate variants of the 43 genes were selected from data derived from whole-exome sequencing on the 257 patients. Of the 70, 53 (75.7%) were confirmed by Sanger sequencing, while 17 were false-positives. The compound heterozygous variants in *B3GALTL* were excluded as only one was confirmed and the other was a false-positive. Fifteen variants in *NHS*, *BCOR* and *COL4A1* were tentatively categorized as less likely pathogenic variants as these types of causative mutations had not been previously reported. Six of the remaining 37 variants were excluded as they were also presented in normal individuals. Three of the remaining variants were of uncertain significance as they were detected in patients with potential pathogenic mutations in known glaucoma genes. In addition, one variant in *PAX6* was excluded as it was absent in other affected family members. Eventually, 27 potential pathogenic mutations in 14 genes were identified (Table I). Of the 27, 20 were not present in the 1000 Genomes Project or Exome Variant Server, while 7 were present in the 1000 Genomes Project and Exome Variant Server with a frequency of 2/2,184 to 1/13,006. All the 27 mutations were absent in the 192 ethnicity-matched normal controls and were predicted to be damaging to the encoded protein by bioinformatic analysis.

Table I. Potential pathogenic mutations identified in 28 unrelated Chinese patients with primary glaucoma.

Gene	Inh	Patient ID	Diagnosis	Variations		Online prediction		MAF in NC	Reported or not[a]	MAF in 1000G or EVS
				Nucleotide	Amino acid	SIFT	PolyPhen-2			
CRYAA	AD	G636	PACG	c.[307C>T];[=]	p.[R103C];[=]	D	PrD	0/384	Novel	None
CRYGC	AD	G217	PACG	c.[110G>A];[=]	p.[R37Q];[=]	D	PrD	0/384	rs140859599	1/2184, 1/13006
CRYGD	AD	G598	PACG	c.[19T>C];[=]	p.[Y7H];[=]	D	PrD	0/384	Novel	None
COL4A1	AD	G353	POAG	c.[502G>A];[=]	p.[G168R];[=]	T	PrD	0/384	rs144171664	Unknown
FOXC1	AD	G378	POAG	c.[553_555del];[=]	p.[185_185del];[=]	NA	NA	0/384	Novel	None
GJA8	AD	G462	POAG	c.[569A>G];[=]	p.[N190S];[=]	D	PrD	0/384	Novel	None
PITX2	AD	G654	PACG	c.[891C>A];[=]	p.[Q297H];[=]	T	PrD	0/384	Novel	None
SHH	AD	G408	POAG	c.[682G>A];[=]	p.[D228N];[=]	D	PrD	0/384	Novel	None
BMP4	AD	G555	PACG	c.[450C>G];[=]	p.[N150K];[=]	T	PrD	0/384	Reported[b]	None
		G370	POAG	c.[502G>C];[=]	p.[G168R];[=]	D	PrD	0/384	Novel	None
CRYBA4	AD	G644	PACG	c.[383C>T];[=]	p.[S128F];[=]	D	PrD	0/384	Novel	None
		G603	PACG	c.[413A>G];[=]	p.[E138G];[=]	D	PrD	0/384	Novel	None
GDF6	AD	G629	PACG	c.[136C>T];[=]	p.[R46C];[=]	D	B	0/384	Novel	None
		G479	POAG	c.[1271A>G];[=]	p.[K424R];[=]	T	PrD	0/384	rs121909353[c]	2/2184, none
		G539	PACG	c.[1288A>G];[=]	p.[I430V];[=]	T	PrD	0/384	Novel	None
EYA1	AD	G443	POAG	c.[35G>A];[=]	p.[R12H];[=]	T	PrD	0/384	rs74720958	1/2184, none
		G447	POAG	c.[175G>A];[=]	p.[G59R];[=]	D	PrD	0/384	rs146216506	Unknown, 1/13006
		G455	POAG	c.[585A>G];[=]	p.[I195M];[=]	D	B	0/384	Novel	None
		G543	PACG	c.[679G>C];[=]	p.[A227P];[=]	T	PrD	0/384	Novel	None
BEST1	AD	G617	PACG	c.[205T>C];[=]	p.[C69R];[=]	D	PrD	0/384	Novel	None
		G381	POAG	c.[436G>T];[=]	p.[A146S];[=]	T	PrD	0/384	Novel	None
		G664	PACG	c.[652C>A];[=]	p.[R218S];[=]	D	PrD	0/384	Reported[d]	None
		G38	PACG	c.[698C>T];[=]	p.[P233L];[=]	D	PrD	0/384	Reported[e]	None
		G402, G587	POAG, PACG	c.[763C>T];[=]	p.[R255W];[=]	D	PrD	0/384	rs372989281[f]	Unknown, 1/13002
		G663	PACG	c.[910_912del];[=]	p.[304_304del];[=]	NA	NA	0/384	Novel	None
HCCS	XL	G592	PACG	c.[175C>T];[0]	p.[R59C];[0]	D	PrD	0/286[g]	rs200354469	Unknown
		G524	PACG	c.[572A>T];[=]	p.[E191V];[=]	T	PrD	0/286[g]	Novel	None

[a]Mutations reported as pathogenic in previous studies are references: [b](46), [c](43), [d](47), [e](44) and [f](45). [g]Only the number of X chromosomes from the 192 normal individuals were calculated. Inh, inheritance; AD, autosomal dominant; XL, X-linked; PACG, primary angle-closure glaucoma; POAG, primary open-angle glaucoma; D, damaging; T, tolerated; NA, not applicable; PrD, probably damaging; B, benign; MAF, minor allele frequency; NC, normal control; 1000G, 1000 Genomes Project; EVS, Exome Variant Server.

Associations of the mutations with disease. Of the 27 mutations, 25 were heterozygous in 13 genes associated with autosomal dominant diseases, one was heterozygous and one was hemizygous in *HCCS* associated with X-linked dominant diseases, and none were present in the genes associated with autosomal recessive diseases. Five of the 27 mutations have been previously reported to be pathogenic (43-47), while the remaining 22 were novel. The 27 mutations were detected in 28 of 257 patients with glaucoma, including 11 patients with POAG and 17 patients with PACG (Table II). The distributions of the 27 mutations in POAG and PACG are illustrated in Fig. 2. Mutations in *COL4A1*, *FOXC1*, *GJA8* and *SHH* were only detected in patients with POAG, while mutations in *CRYAA*, *CRYGC*, *CRYGD*, *CRYBA4*, *PITX2* and *HCCS* were only detected in patients with PACG. Mutations in *BMP4*, *GDF6*, *EYA1* and *BEST1* were detected in the two groups of patients. Of the 27 mutations, 26 were detected in 26 patients, respectively; while the remaining mutation, a previously reported c.763C>T mutation in *BEST1* (45), was detected in 1 patient with PACG and 1 patient with POAG.

Analysis of family history. Of the 28 patients with mutations, 10 had a family history of glaucoma suggesting an autosomal dominant trait, and the other 18 were sporadic (Fig. 3). Analysis of limited family members from four families showed segregation of glaucoma with mutations in the *GDF6*, *EYA1* and *BEST1* genes (Fig. 3). In one of the five families, the patient (G443) and his daughter had the c.35G>A (p.R12H)

Table II. Clinical data of the 28 patients with potential pathogenic mutations.

Family ID	Diagnosis	Gene	Mutation[a]	Gender	Diagnosis age, years	Cornea, mm	AL, mm	BCVA	Peak IOP, mmHg	VCDR
G636	PACG	*CRYAA*	c.[307C>T];[=]	M	60	11.6/11.6	21.99/22.00	0.7/0.8	39/14	1.0/0.3
G217	PACG	*CRYGC*	c.[110G>A];[=]	F	49	11.0/11.0	NA	0.6/0.4	52/NA	0.3/0.9
G598	PACG	*CRYGD*	c.[19T>C];[=]	F	63	11.8/11.3	22.11/22.27	HM/1.0	49/20.3	0.9/0.9
G353	POAG	*COL4A1*	c.[502G>A];[=]	M	60	12.0/11.9	26.45/26.42	1.0/1.0	NA	0.7/0.8
G378	POAG	*FOXC1*	c.[553_555del];[=]	F	68	12.3/12.3	26.20/26.15	1.2/1.2	23/23	0.4/0.5
G462	POAG	*GJA8*	c.[569A>G];[=]	M	29	11.6/11.5	22.25/22.38	1.2/HM	24/22	0.6/1.0
G654	PACG	*PITX2*	c.[891C>A];[=]	F	51	NA	22.50/24.02	0.3/FC	NA[b]	0.9/0.9
G408	POAG	*SHH*	c.[682G>A];[=]	F	58	11.9/11.4	23.48/23.43	0.7/1.2	NA[b]	0.5/0.5
G555	PACG	*BMP4*	c.[450C>G];[=]	M	64	11.0/11.0	22.57/23.01	1.0/NLP	NA/32	0.7/1.0
G370	POAG	*BMP4*	c.[502G>C];[=]	F	52	11.3/11.2	23.45/23.69	0.9/1.0	33/28	0.8/0.8
G644	PACG	*CRYBA4*	c.[383C>T];[=]	F	65	11.4/11.8	21.80/21.72	0.4/1.2	NA[b]	0.3/0.6
G603	PACG	*CRYBA4*	c.[413A>G];[=]	M	70	NA/11.2	23.70/23.67	NLP/0.6	37/14	0.9/0.4
G629	PACG	*GDF6*	c.[136C>T];[=]	F	53	NA	21.29/21.29	0.5/0.2	16/54	0.3/NA
G479	POAG	*GDF6*	c.[1271A>G];[=]	F	30	10.0/10.0	26.06/25.92	0.7/0.8	22/25	0.6/0.4
G539	PACG	*GDF6*	c.[1288A>G];[=]	F	49	NA	NA	0.6/0.7	NA	0.3/0.3
G443	POAG	*EYA1*	c.[35G>A];[=]	M	30	11.0/11.0	29.05/NA	0.2/LP	30/NA	0.3/NA
G447	POAG	*EYA1*	c.[175G>A];[=]	M	56	11.8/11.3	23.73/23.55	0.6/0.7	NA[b]	0.9/0.9
G455	POAG	*EYA1*	c.[585A>G];[=]	M	32	12.4/12.4	23.90/23.95	1.5/NLP	NA[b]	0.9/1.0
G543	PACG	*EYA1*	c.[679G>C];[=]	M	56	11.5/11.5	22.25/22.30	1.2/0.9	13/35	0.3/0.4
G617	PACG	*BEST1*	c.[205T>C];[=]	F	72	11.5/11.4	24.48/23.91	0.5/0.5	NA	0.4/0.7
G381	POAG	*BEST1*	c.[436G>T];[=]	M	34	12.2/11.6	25.36/25.09	1.5/FC	48/55	0.9/1.0
G664	PACG	*BEST1*	c.[652C>A];[=]	F	47	11.6/12.1	21.24/21.33	1.0/1.2	NA[b]	0.4/0.4
G38	PACG	*BEST1*	c.[698C>T];[=]	M	20	10.5/10.0	21.38/21.38	0.5/0.2	27/32	0.5/0.7
G402	POAG	*BEST1*	c.[763C>T];[=]	M	56	12.3/12.4	25.23/25.17	0.4/0.6	40/40	0.9/0.9
G587	PACG	*BEST1*	c.[763C>T];[=]	F	68	11.2/10.6	22.17/21.91	1.0/0.2	17/39	0.5/0.9
G663	PACG	*BEST1*	c.[910_912del];[=]	F	44	NA/12.4	21.20/21.42	0.05/0.05	51/33	1.0/1.0
G592	PACG	*HCCS*	c.[175C>T];[0]	M	47	NA	22.48/22.44	0.5/0.8	36/23	0.9/0.5
G524	PACG	*HCCS*	c.[572A>T];[=]	F	80	11.0/11.0	22.62/22.75	0.1/0.3	50/9.5	0.5/0.3

[a]Nomenclature of variations is consistent with the recommendations of the Human Genome Variation Society (http://www.hgvs.org/). [b]The patients had elevated intraocular pressure prior to treatment; however, the highest intraocular pressure was unavailable in the present study. PACG, primary angle-closure glaucoma; POAG, primary open-angle glaucoma; M, male; F, female; NA, not available; AL, axial length; BCVA, best corrected visual acuity; HM, hand movement; FC, finger count; NLP, no light perception; Peak IOP, peak intraocular pressure; VCDR, vertical cup-disc ratio.

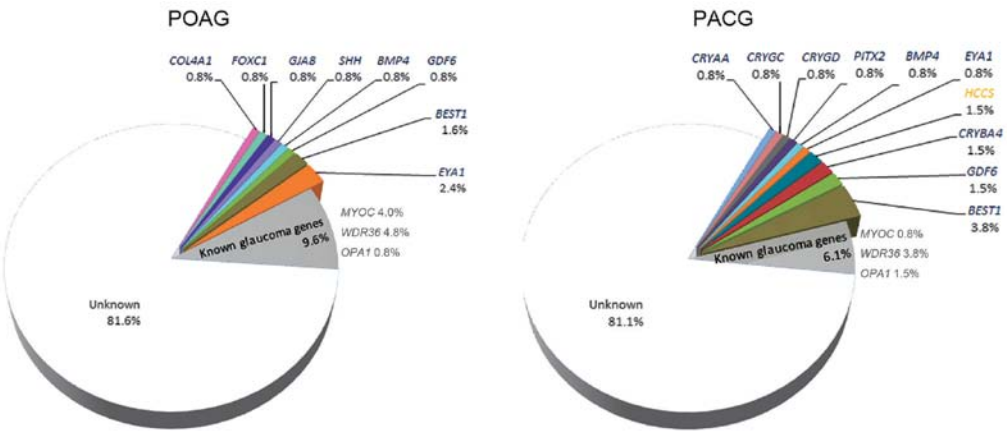

Figure 2. Distribution of the mutations in patients with primary glaucoma based on whole-exome sequencing. The gene symbols in blue indicate genes associated with autosomal dominant diseases and the gene symbol in yellow indicates the gene associated with X-linked diseases.

mutation in *EYA1*; however, the phenotype of the daughter had signs of glaucoma risk but did not meet the diagnostic criteria: Unilateral elevated intraocular pressure (18 mmHg for the right eye and 23 mmHg for the left) at the age of 11 years, but had normal visual field and retinal nerve fiber layers on optical coherence tomography. For the 29 patients

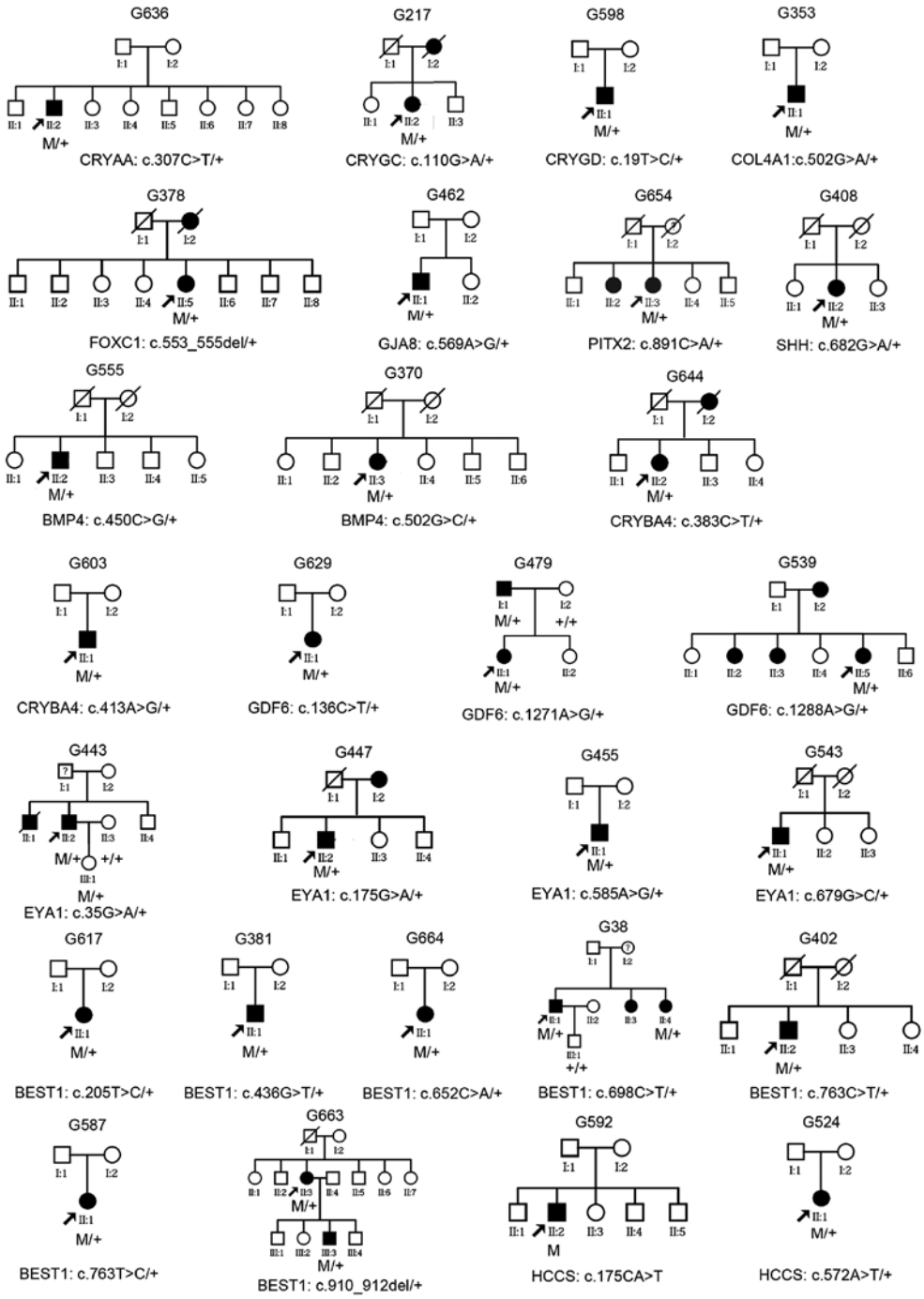

Figure 3. Family trees of the 28 unrelated patients with potential pathogenic mutations. M, mutation; +, wild-type allele.

with mutations and an initial diagnosis of primary glaucoma, other signs associated with ASD, microcornea and microphthalmia were not observed except for a slightly smaller corneal diameter (10-11 mm) in 3 patients (patients G38, G479 and G587; Table II) following careful re-examination. In addition, macular lesion with yellow-white deposits was observed in 2 patients with *BEST1* mutation and in affected family members in the two respective families.

Discussion

In the present study, 27 potential pathogenic mutations in 14 genes have been identified in 28 of 257 patients with primary glaucoma based on analysis of exome sequencing results for 43 genes associated with ASD, microcornea or microphthalmia. The 27 mutations were confirmed by Sanger sequencing and were predicted as damaging by bioinformatic analysis. Five of the 27 mutations have been previously reported to be correlated with different forms of associated ocular diseases (43-47) and the remaining 22 are novel. All the mutations were absent in normal controls and the majority of them were not present in existing human genome variant databases. Analysis of family members from five families suggests a segregation of primary glaucoma with mutations. These lines of evidence suggest that the mutations in these genes are likely to have roles in the development of primary glaucoma.

Glaucoma, secondary to ASD, microcornea or microphthalmia, has been described in patients with mutations in one of the following genes: *BEST1* (48), *BMP4* (49), *COL4A1* (50), *FOXC1* (51), *FOXE3* (52), *PAX6* (53), *PITX2* (54,55), *PXDN* (56), *PRSS56* (38), *SIX6* (37) and *VSX2* (57). The association of mutations in these genes with primary glaucoma has not been previously studied, except for a recent study in which rare and common variants in *SIX6* have been demonstrated as a risk factor for POAG (37). Such variants in other associated genes may also be risk factors for primary glaucoma. The identification of 27 rare damaging variants in 14 associated genes in 28 of the 257 patients in the present study further supports the potential involvement of these genes in primary glaucoma. By contrast, certain patients with variants in these genes may have minor or subtle changes in anterior segment, as seen in 3 (G38, G479 and G587) of the 28 patients with a relatively smaller corneal diameter. These changes may possibly be neglected or undetected, and therefore, the patients with such changes may mimic primary glaucoma. In either case, variants in these genes are possibly risk factors for primary and secondary glaucoma.

The present preliminary study provides a brief overview of variants in the 43 genes associated with ASD, microcornea and microphthalmia in patients with primary glaucoma. The identification of 27 potential pathogenic variants in genes associated with ASD, microcornea and microphthalmia in 28 of 257 patients with primary glaucoma suggests potential risk factors in the development of primary glaucoma. Further studies are expected to enrich the understanding between variants in these genes and primary glaucoma.

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