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Heterozygous *GJA1* variants with ocular phenotype: Missense in domain but truncation out of domain

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Purpose: Oculodentodigital dysplasia (ODDD) is a group disorder caused by *GJA1* variants, of which glaucoma leading to blindness is a frequent complication of the ocular phenotype. In this study, the correlation of the *GJA1* genotype with the ocular phenotype was analyzed systematically.
Methods: *GJA1* variants were collected from in-house whole-exome sequencing data of 5,307 individuals. Potentially pathogenic variants (PPVs) were defined based on prediction of multiple in silico tools, related phenotypes, and previously established evidence. The characteristics of *GJA1* PPVs were evaluated based on our data, gnomAD, and HGMD.
Results: In total, 21 rare variants in *GJA1* were detected in 32 subjects from the study cohort. Four of the 21 variants were classified as PPVs, including two frameshift, one missense, and one in-frame deletion. The four PPVs were detected in four probands with microcornea or high hyperopia; two developed glaucoma. A systematic review of *GJA1* variants in literature suggested that most heterozygous missense PPVs are located inside the connexin domain. All truncations downstream of the connexin domain are associated with autosomal dominant disease, while most truncations within the domain are associated with autosomal recessive ODDD. Ocular signs were present in 80.0% (116/145) of patients with *GJA1* PPVs. Of the 116 patients, glaucoma was observed in 26.7% (31/116), among whom 77.4% (24/31) of cases occurred in patients ≥10 years old.
Conclusions: Eye abnormalities are the most common signs associated with *GJA1* PPVs, and they carry a high risk of developing glaucoma. The identification of *GJA1* PPVs needs further attention and clarification.

Oculodentodigital dysplasia (ODDD) is a disorder affecting multiple tissues, including ocular anomalies, nasal dysplasia, digital malformation in the form of complete syndactyly of the fourth and fifth fingers, and enamel dysplasia [1]. Ocular abnormalities are characterized by microphthalmia and microcornea that are frequently associated with secondary glaucoma [2]. Such ocular abnormalities are congenital and can be easily characterized and quantified, while secondary glaucoma can lead to severe consequences, including blindness.

Variants in *GJA1* (OMIM: 121014) are frequently associated with ODDD, rarely with syndactyly [3], heart malformations [4,5], craniometaphyseal dysplasia [6], erythrokeratodermia variabilis et progressiva [7], and palmoplantar keratoderma with congenital alopecia [8]. *GJA1*, the gap junction alpha 1 gene, is located at chromosome 6q22.31 and consists of two exons, where the second exon is the only coding exon. This gene encodes connexin 43 (CX43), a 43 kDa protein with two domains: a connexin domain and a connexin 43 domain (NCBI) [9]. Connexins comprise a family of vertebrate gap junction channel proteins that are composed of four transmembrane helices and two highly conserved extracellular loops [10,11]. Of all connexin proteins, CX43 is the most widely expressed in various human tissues [10]. Gap junction channels formed by CX43 are considered to function in direct exchanges from cell to cell and mediate crosstalk among multiple signaling pathways in cells and during development [5]. To clarify the characteristics of PPVs in *GJA1* and the correlation between glaucoma and *GJA1*, we analyzed *GJA1* variants systematically based on in-house data and published literature. Recognizing the characteristics of eye-associated potentially pathogenic variants (PPVs) in *GJA1* may be helpful for clinical genetic testing and subsequent counseling and medical intervention.

In the present study, *GJA1* variants were collected from in-house whole-exome sequencing data from 5,307 individuals with various eye conditions. Multiple in silico bioinformatics and genotype–phenotype analyses were used to define PPVs. Subsequent bioinformatics and genotype–phenotype analysis of information from online databases together with the in-house data provides a critical outline for heterozygous *GJA1* PPVs associated with ocular abnormalities. This study

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not only expends the variant spectrum of *GJA1* but also characterizes *GJA1* PPVs associated with ocular phenotypes.

METHODS

Subjects: The ocular phenotypes and venous blood samples for individuals in this study were collected by our group through a long-term, ongoing program for the genetic study of hereditary eye diseases at the Zhongshan Ophthalmic Center. Written informed consent was obtained from individuals or their guardians in accordance with the tenets of the Declaration of Helsinki and adhered to the ARVO statement on human subjects before clinical data and peripheral venous blood samples were collected. This study was approved by the institutional review board of the Zhongshan Ophthalmic Center. Genomic DNA was extracted from leukocytes from peripheral venous blood through a previously described procedure [12].

Exome sequencing analysis: Whole-exome sequencing was performed with an Agilent SureSelect Human All Exon Enrichment System (50M; Agilent, Santa Clara, CA). Enriched DNA fragments were subsequently sequenced using the Illumina HiSeq 2000 system (Illumina, San Diego, CA) with an average sequencing depth of 125X. Reads were mapped against the UCSC hg19 reference genome (Genome) using the Burrows-Wheeler Aligner (BWA). Variants calling were inferred from the Bayesian statistical algorithm based on SAMtools [13,14]. The remaining variants were annotated based on the human genome with ANNOVAR [15].

As for the remaining variants of *GJA1*, low-quality and effect-unknown variants were initially excluded, as were variants with a low read-depth (≤5 reads), variants in untranslated regions (UTRs) or in an intronic region without a significant impact on splicing, and synonymous variants. Based on the American College of Medical Genetics and Genomics (ACMG) guideline for defining PPVs [16], the remaining variants were analyzed as follows: (1) Because the prevalence of ODDD was 1 in 10 million [17], *GJA1* variants detected in the gnomAD database were excluded. (2) Missense variants were evaluated with five in silico online tools, namely, REVEL, CADD, SIFT, PolyPhen2, and PROVEAN. For REVEL and CADD [18,19], which acted as ensemble annotations for predicting the pathogenicity of missense variants, variants with a score over top 5% or top 25% percentiles of missense variants from the gnomAD database were weighted as probably or possibly damaging, respectively. Missense variants were further graded by the significance of cumulative scores based on all five in silico tools and their sequential order of association with specific ocular phenotypes. (3) Truncation variants were directly forwarded to genotype–phenotype analysis. (4) In-frame variants if the inserted or deleted residues were conserved in multiple species remained. After these analyses, the remaining variants (including frameshift variants, missense variants predicted to be damaging by at least four in silico tools, and in-frame variants) were included as PPVs for further genotype–phenotype analysis. Sanger sequencing was used to confirm the PPVs [20]. The primers used were designed by the Primer 3.0 website.

Characteristics of PPVs in GJA1:

Truncation variants—Not all truncation variants in a gene are necessarily pathogenic, although such variants have been reported to be disease-causing [21]. First, clinical data from subjects with truncation variants were evaluated to determine whether they had related phenotypes. Then, the distribution of potentially pathogenic truncation variants was plotted on the coding frame and compared with all truncation variants from gnomAD to determine whether there was a biased distribution. The significance of the distribution bias was calculated with the chi-square test.

Missense variants—For most genes, only a subset of rare variants is disease-causing and is usually evaluated as damaging or possible damaging by multiple online tools, especially for dominant disease [21]. All missense variants were initially evaluated based on their frequency in the general population from the gnomAD database, predictions using five in silico tools, and the related phenotype. Similarly, the distribution of potentially pathogenic missense variants in the present study and HGMD was plotted on the coding frame and compared with all missense variants from gnomAD to determine whether specific regional enrichment was present. The significance of the biased distribution was also calculated with the chi-square test.

Review of ocular phenotypes of GJA1 variants: Ocular phenotypes were systematically reviewed based on a search for "GJA1" in HGMD (before April 2020) and related published literature. Available full-text articles in English were retrieved from PubMed, and the ocular phenotypes were calculated.

Statistical analysis: Statistical analysis was performed with IBM SPSS Statistics Version 25.0 (IBM Corp., Armonk, NY). The allele counts of the *GJA1* variants in the present study, HGMD, and gnomAD databases were compared using the chi-square test and Fisher's exact test. A p value of less than or equal to 0.05 was considered statistically significant.

RESULTS

Heterozygous GJA1 variants identified in the present study: In total, 21 variants in *GJA1* were detected in 32 of the 5,307 unrelated individuals with various eye conditions (Appendix 1). Among the 21, four heterozygous PPVs were detected in four probands, including two truncation variants (c.738_739delTT/p.Y247Pfs*60 and c.791_792delAA/p.K264Ifs*43), one missense variant (c.124G>C/p.E42Q), and one in-frame deletion variant (c.890_892delCTT/p.S297del; Table 1). Each variant was confirmed with Sanger sequencing and was present exclusively in one proband but not in the remaining 5,306 (Figure 1A,B). All four variants are rare and absent from existing databases (gnomAD and 1000 Genomes). Two variants (c.738_739delTT/p.Y247Pfs*60 and c.890_892delCTT/p.S297del) are novel, while the other two (c.124G>C/p.E42Q and c.791_792delAA/p.K264Ifs*43) were previously reported to cause ODDD or microcornea and glaucoma [22,23], including in a different family reported by our group. The residues p.E42 and p.S297 were highly conserved in *GJA1* among multiple vertebrates (Figure 1C), and p.E42Q was predicted to be damaging by all five in silico online tools. In addition, the missense variant was located in the first transmembrane helix involved in the connexin domain, while the two truncation variants were located outside the connexin domain (Figure 2A).

The available clinical data of the four probands are summarized in Table 2. Of the four probands with heterozygous PPVs in *GJA1*, one (10651-II:2) exhibited a phenotype of ODDD, including microcornea, a narrow nose with hypoplastic alae nasi, prominent columella and thin anteverted nares together with a narrow nasal bridge, prominent epicanthic folds, and syndactyly of the fourth and fifth fingers (Figure 3). The patient's intraocular pressure was bilateral 18.0 mmHg. Of the remaining three probands, two had microcornea and glaucoma, while the third patient (5395-II:2) had high hyperopia (PHH), a disorder also called posterior microphthalmia. The best-corrected visual acuity varied between bilateral 0.2 for patient 10651-II:2 with microcornea and bilateral 1.0 for patient 5395-II:2 with high hyperopia. Glaucoma seemed a cause of deteriorating vision, as vision acuity decreased from 1.2 to finger count in the right eye of patient 10779-II:4 with glaucoma and microcornea. Available fundus photos in two patients showed mild fundus changes, including tilted optic discs, abnormal branches of the retinal vessels, and tessellated retina (Figure 3). Corneal staphyloma resulting from advanced glaucoma was observed in the right eye of patient 10779-II:4 (Table 2, Figure 3).

Characteristics of potentially pathogenic variants in GJA1: Previously, 89 heterozygous *GJA1* variants in 179 individuals from 121 families were identified as disease causing mutation (DM) based on HGMD, including three truncations, 82 missense, and four in-frame variants (Appendix 2) [1-4,7,8,22-70]. In total, 73 PPVs associated with ODDD or microcornea, including 66 missense, four in-frame, and three truncation variants, were detected in 102 probands. Of the 82 missense variants, 49 are reported to be associated with ocular phenotype, 15 are reported to have non-ocular phenotypes, and one missense variant (c.977C>T/p.T326I) is likely benign, as it was present in two subjects without related ocular phenotypes in the study cohort.

All four heterozygous truncation PPVs in *GJA1* identified to date, including three published PPVs and one novel PPV in the present study, are located downstream of the connexin domain. In contrast, four truncation variants associated with autosomal recessive ODDD in homozygous or compound heterozygous status are located inside the connexin domain, including c.6delT/p.D3Tfs*5, c.97C>T/p.R33*, c.301C>T/p.R101*, and c.442C>T/p.R148* (Appendix 3) [71-74]. Of the four probands with recessive ODDD, seven of the eight carrier parents were normal, while the other one had microphthalmia. Intriguingly, all but one truncation variant in *GJA1* from the gnomAD database was statistically significantly enriched in the connexin domain (Figure 1, p=0.000113). The only truncation variant outside the connexin domain in the gnomAD database was at the last codon leading to a loss of the stop codon with unknown effect. Therefore, these lines of evidence suggest that heterozygous truncation variants at the N-terminal region (before or inside the connexin domain) are unlikely to be pathogenic, while those at the C-terminal portion (after the connexin domain) are likely pathogenic.

In total, 82 missense variants were detected in 164 subjects from 115 families. All the 82 were absent in gnomAD, of which 49 (59.8%) were predicted to be damaging variants by at least four in silico tools and were present in 68 of the 115 (59.1%) families. The missense variants within the connexin domain and predicted to be damaging by at least four in silico tools were statistically significantly more common in the patient group than in the gnomAD database (17/713, p=2.90E-77; Appendix 4). In addition, 89.0% (73/82) of PPVs were located in the connexin domain, and 64.4% (47/73) were predicted to be damaging variants by at least four in silico tools (Appendix 4). Of the 82 missense variants, ocular phenotypes were reported to be associated with 49 variants. All but two of the 49 missense PPVs were located in the connexin domain, showing a statistically significant distribution bias compared with the variants in the gnomAD database (p=7.54E-55; Appendix 2; Appendix 4). These results indicate that heterozygous missense variants absent

Table 1. Heterozygous *GJA1* PPVs detected in our cohort.

Exon	Position	NM_000165	NM_000165	Allele	In silico prediction					gnomAD	HGMD	Ref
	at Chr6	change	Effect	Count	REVEL	CADD	SIFT	Poly-Phen-2	Provean	(v2.1) AC		
2	121,768,117	c.124G>C	p.E42Q	1	0.719	24.60	D	D	D	/	DM	[23]
2	121,768,731	c.738_739delTT	p.Y247Pfs*60	1	/	/	/	/	/	/	Novel	None
2	121,768,784	c.791_792delAA	p.K264Ifs*43	2†	/	/	/	/	/	/	DM	[22]
2	121,768,883	c.890_892delCTT	p.S297del	1	/	/	/	/	/	/	Novel	None

Note: D=damaging. "/"=not available, AC=allele count, DM=disease causing mutation, "†"=one was reported in our previous study [18]. Percentiles score of REVEL: 75%=0.612, 95%=0.882, percentiles score of CADD: 75%=23.80, 95%=27.31.

in the gnomAD database, located in the connexin domain, and predicted to be damaging by at least four in silico tools are most likely pathogenic, at least in the eye, while the heterozygous missense variants outside the connexin domain or with poor computational prediction need further analysis to evaluate their pathogenicity.

Ocular phenotypes associated with heterozygous GJA1 variants: In total, 145 patients in 98 families with heterozygous *GJA1* variants had ocular involvement, including the four families in the present study. Several specific ocular signs could be observed in 116 patients from 72 of the 98 families, including microcornea (62.9%, 73/116), microphthalmia (54.3%, 63/116), high hyperopia (6.0%, 7/116), cataracts (4.3%, 5/116), and a few rare and atypical clinical signs, including myopia, anterior eye-chamber defects, iris atrophy, and uveitis (Table 3, Appendix 2). Glaucoma, a common and severe complication secondary to microcornea and microphthalmia, developed in 31 of the 116 (26.7%) patients, including 19 with microcornea, seven with microphthalmia, and five with ODDD of unspecified ocular signs. Of these 31, glaucoma was present in seven patients under the age

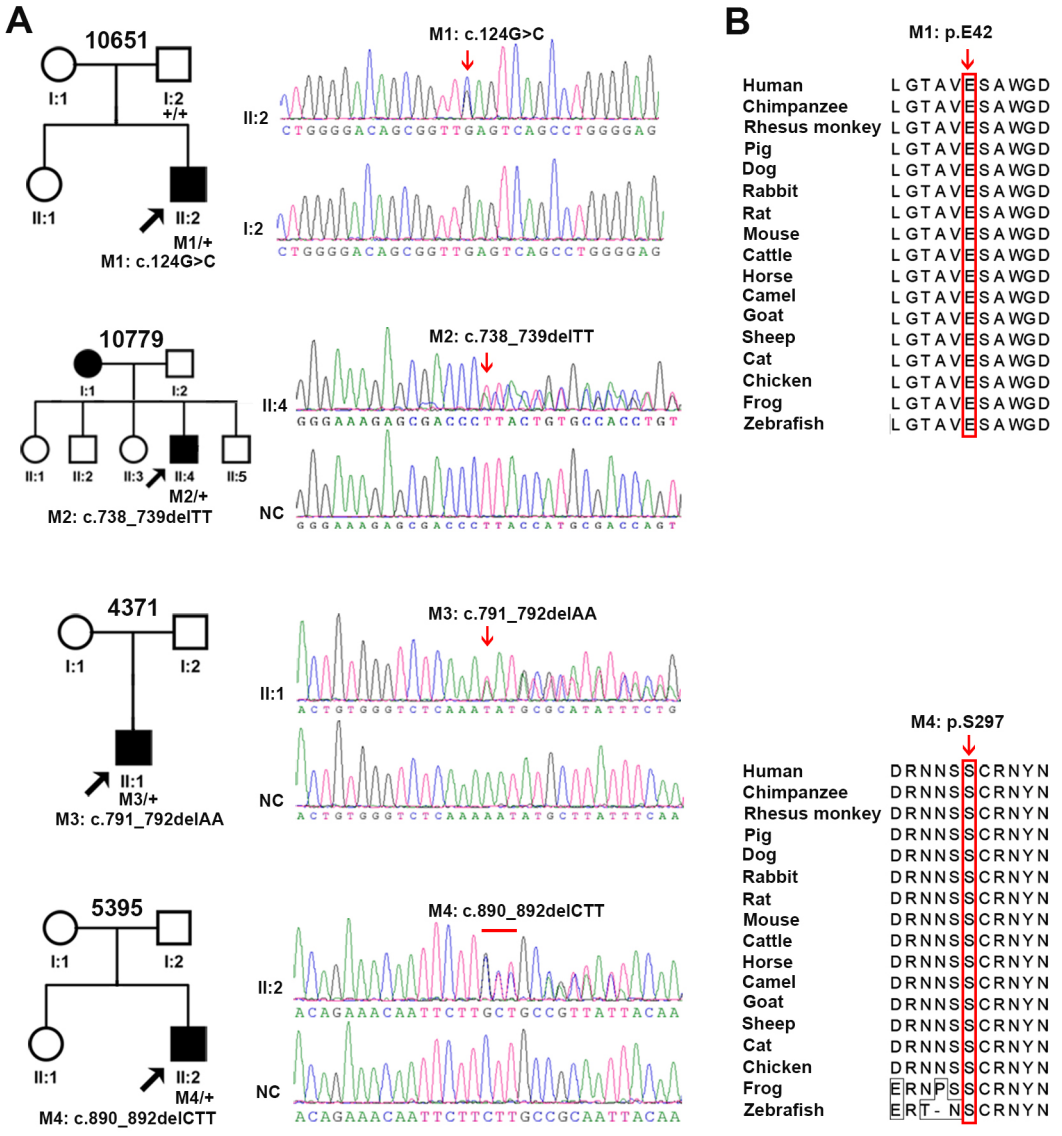

Figure 1. The pedigree and sequence chromatograms of the four probands with PPVs in *GJA1*. **A**, **B**: Mx, mutant allele. Filled squares (male) or circles (female) represent these affected individuals, and the arrow indicates the proband in each family. The accession number of the corresponding human *GJA1* transcript is NM_000165. **C**: Conservation analysis of the positions related to two missense and one in-frame deletion variants from the in-house data in multiple vertebrates.

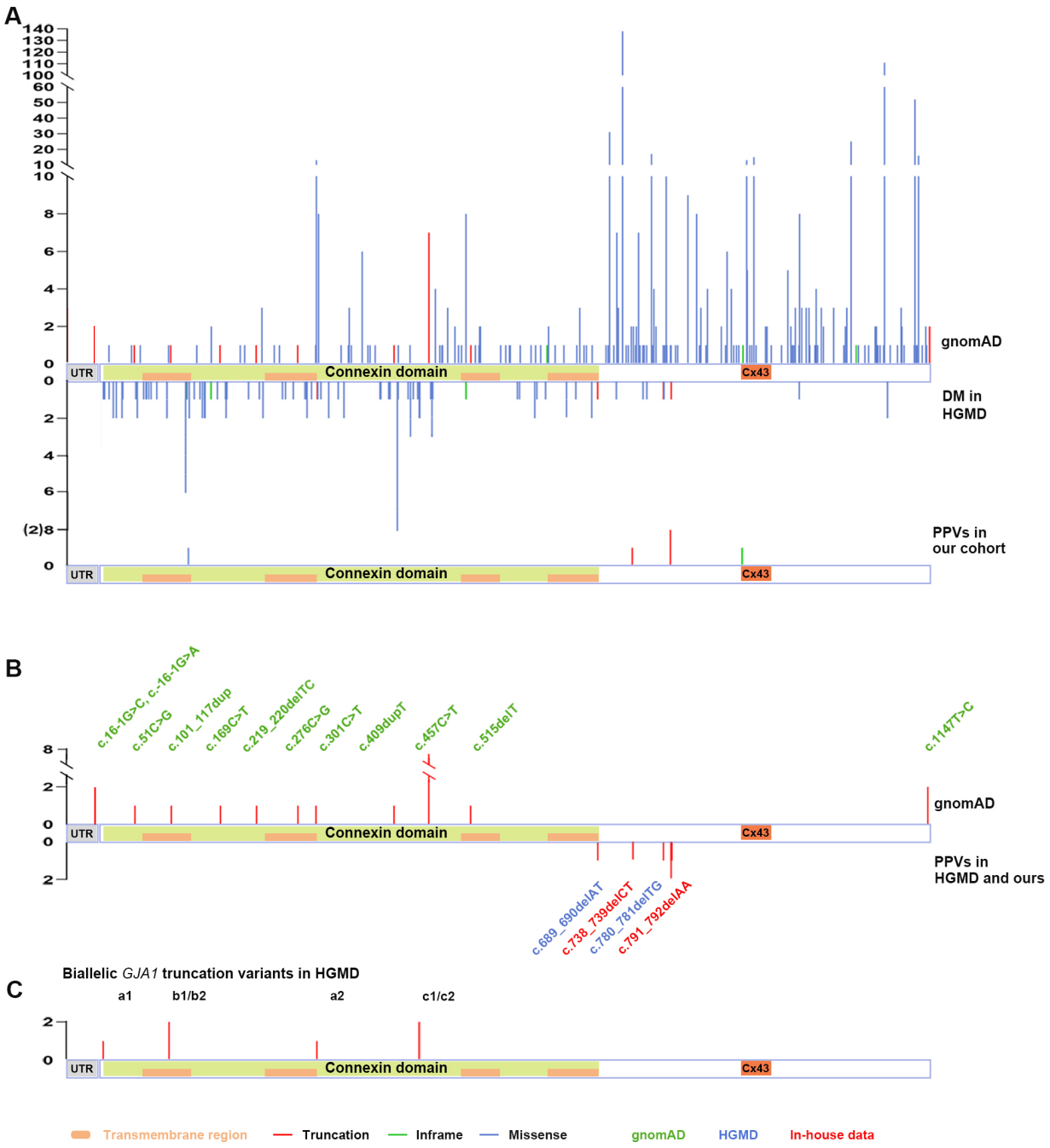

Figure 2. The distribution and allele count of heterozygous *GJA1* variants identified in the general population in the gnomAD database, DMs in HGMD, and PPVs from in-house exome sequencing data. **A**: The spectrum of variants with minor allele frequency <0.01 in the gnomAD database, disease causing mutation (DM) in human gene mutation database (HGMD), and four potentially pathogenic variants (PPVs) from in-house exome sequencing data. **B**: The distribution and allele count of truncation variants from the gnomAD database, HGMD, and the study cohort. Connexin domain: p.3–233; Cx43, connexin 43 domain: p.293–312. Transmembrane region 1: p.19–46, transmembrane region 2: p.72–100, transmembrane region 3: p.147–184, transmembrane region 4: p.206–233. **C**: The distribution of reported biallelic *GJA1* truncation variants. Variants marked with the same letters were identified in the same individual.

Table 2. Clinical data of four novel probands with heterozygous *GJA1* PPVs.

Family ID	Clinic diagnosis	NM_000165 Change	NM_000165 Effect	Gender	Age at exam	BCVA		Cornea diameter (mm)		ACD (mm)		IOP (mmHg)		AL (mm)	
						OD	OS	OD	OS	OD	OS	OD	OS	OD	OS
10,651-II:2	ODDD, MC	c.124G>C	p.E42Q	M	7 years	0.2	0.2	7.5	7.5	NA	NA	18.0	18.0	NA	NA
10,779-II:4	MC, G	c.738_739delTT	p.Y247Pfs*60	M	39 years	1.2	0.1	8.0	8.0	2.00	2.00	43.0	70.7	NA	NA
10,779-II:4§	MC, G	c.738_739delTT	p.Y247Pfs*60	M	49 years	CF	0.4	8.0	8.0	2.00	2.00	13.0	18.0	NA	21.2
4371-II:1§	MC, G	c.791_792delAA	p.K264Ifs*43	M	37 years	0.5	0.8	9.0	9.0	1.85	2.26	39.7	15.7	NA	NA
5395-II:2	PHH	c.890_892delCTT	p.S297del	F	7 years	1.0	1.0	11.9	11.9	2.88	NA	NA	NA	21.1	21.4

Note: ACD=anterior chamber depth, IOP=intraocular pressure, BCVA=best corrected visual acuity, AL=axial length, M=Male, F=Female, NA=Not available. CF=count finger, VFD=visual field defect, ODDD=oculodentodigital dysplasia, MC=microcornea, G=glaucoma, PHH=high hyperopia, yrs=years old. §, results of examinations were recorded after treatment; patient 10,779 undertook surgery for glaucoma treatment twice at the age of 39 and 43 years old. §, patient 4371 undertook surgery for glaucoma treatment at the age of 24 years old.

Table 3. Characteristic of *GJA1*-associated ocular phenotype.

GJA1-associated ocular phenotype (n=116)		
Microcornea		62.9% (73/116)
	Glaucoma	26.0% (19/73)
Microphthalmia		54.3% (63/116)
	Glaucoma	12.7% (7/63)
High hyperopia		6.0% (7/116)
Cataract		4.3% (5/116)
Glaucoma		26.7% (31/116)
	Age at diagnosis (years)	25.5±19.89
	Maximal IOP (mmHg)	36.9±11.93

of 10 years old and in 24 patients older than 10 years old (77.4%, 24/31), with an average age of onset at 25.5 years old; intraocular pressure ranged from 26 mmHg to 70 mmHg (36.90±11.93 mmHg). As glaucoma is an age-dependent blindness disease, it is expected that more of these patients will develop glaucoma later in life.

DISCUSSION

In the present study, four heterozygous *GJA1* PPVs were identified in four probands with ODDD, microcornea, or high hyperopia, two of whom developed glaucoma. Of the four, one missense PPV is located in the connexin domain, while both truncation PPVs are located downstream of the connexin domain. Combined with systematic analysis of variants in HGMD and gnomAD, these results suggest that the pathogenicity of variants in *GJA1* is position- and type-dependent. For the heterozygous truncation variants, variants inside the connexin domain are likely benign, while those downstream of the domain are likely pathogenic. However, missense PPVs are predominantly located within the connexin domain. This position- and type-dependent pattern of *GJA1* pathogenic variants resembles that of the pathogenic variants in *CRX* (Gene ID: 12951, OMIM: 602225) [21]. The exact mechanism of this pattern has yet to be explored with functional studies. A possible explanation might be that the pathogenicity of *GJA1* variants acts through a dominant-negative effect

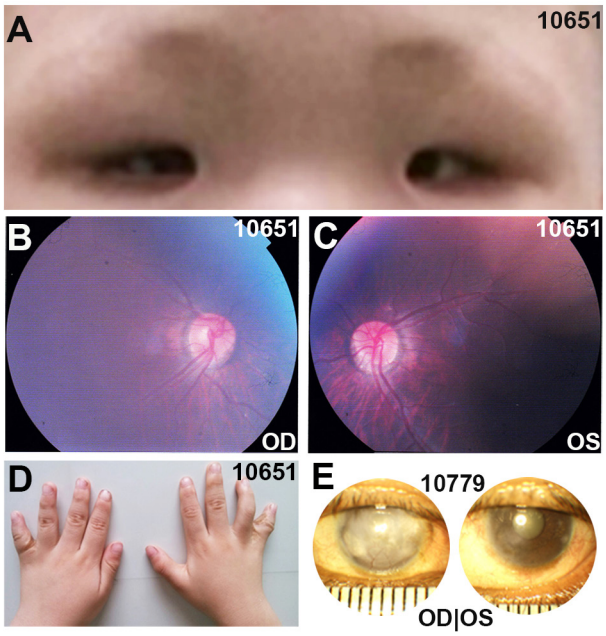

Figure 3. The facial features of one proband with ODDD and the eye appearance of the proband with microcornea and bilateral cornea opacity. **A**: Proband 10,651-II:2 showed typical facial features, including microcornea, sparse eyelashes and eyebrows, bilateral epicanthus, and ocular hypertelorism. **B, C**: Fundus imaging of the proband in 10,651 revealed increased numbers of vascular branches crossing the tilted optic disc and tessellated retina. **D**: The fourth and fifth fingers of both hands had previously been surgically released. **E**: Corneal staphyloma appeared in the right eye of proband 10,779-II:4, and bilateral microcornea and cornea opacities were also seen.

rather than haploinsufficiency of the functional protein. A null allele is likely created by truncation variants inside the connexin domain that are tolerable, while partially functional abnormal proteins generated through truncation variants outside the domain would interfere with the activity of the protein encoded by the normal allele. Proteins encoded by missense variants in the connexin domain might interact with normal proteins and then form abnormal or invalid channels, resulting in a dominant-negative effect. Proteins bearing most of the missense variants outside the domain might be tolerable and normal-like proteins (Figure 4). This type of position- and type-dependent pathogenicity may be present in many other genes in addition to *CRX* and *GJA1*. Systematic genotype–phenotype analysis based on a large data set and online databases at the individual gene level, as performed in the present study, is necessary for genes associated with

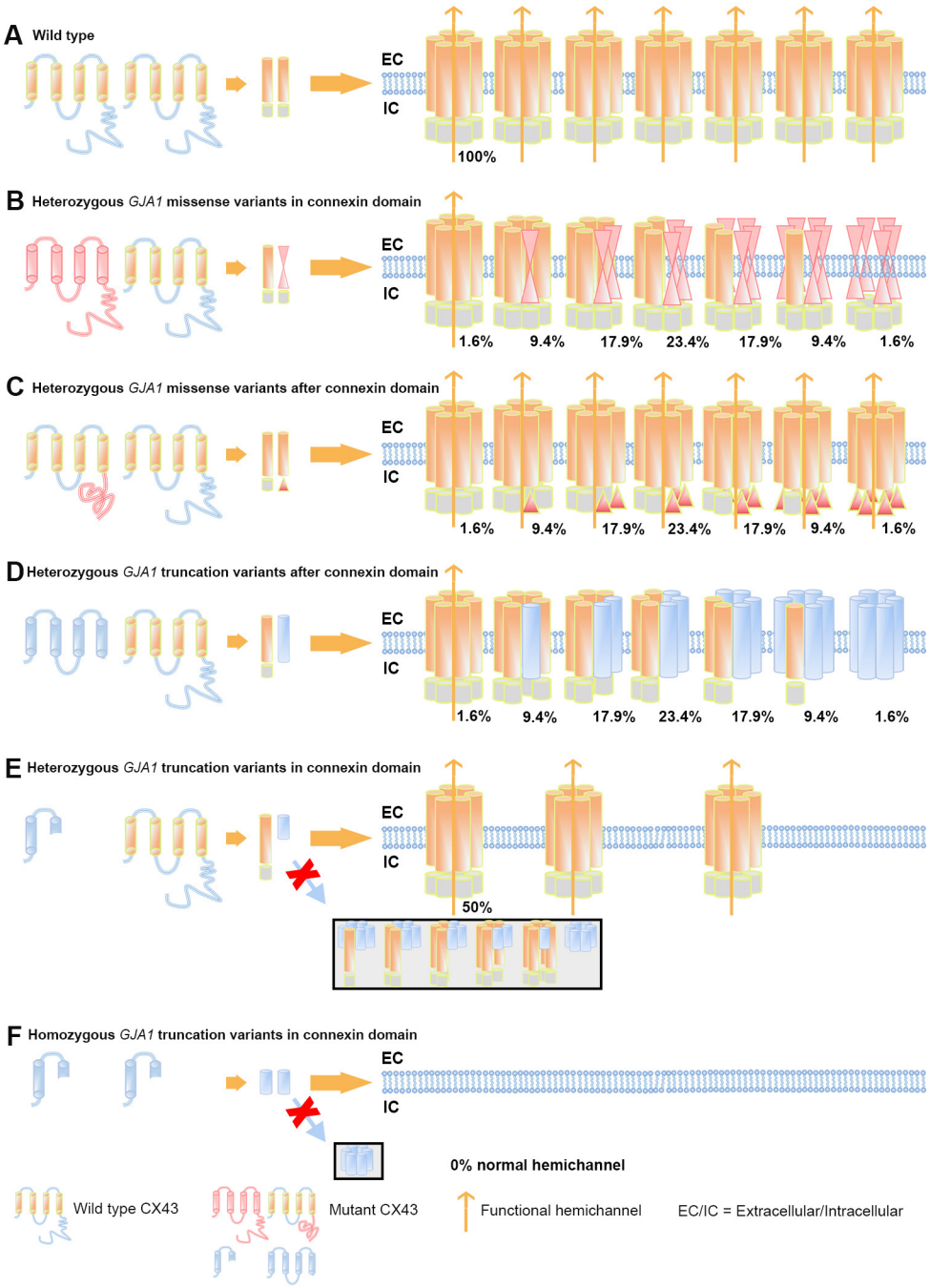

Figure 4. Outline of the pathogenicity of different *GJA1* variants.

autosomal dominant diseases. Recognizing this phenomenon is critical in the era of the widespread application of clinical gene tests and subsequent genetic counseling, because loss-of-function mutations are considered pathogenic if such variants have been previously reported to be disease-causing, even in the ACMG guidelines [16].

Connexin gap junctions, formed as a hexamer of connexin subunits, play a critical role in intercellular communication [75]. Mutations in at least ten connexin genes, including *GJA1*, have been reported to cause human diseases [65,67]. The mechanisms underlying the causative mutations in these genes can be briefly classified into two major groups: loss of hemichannel function or gain of hemichannel function [60,65,67]. The position- and type-dependent pathogenicity of mutations in *GJA1* may be well explained based on these two mechanisms, as illustrated in Figure 4. The hexameric connexin subunits of CX43 encoded by *GJA1* form gap junction channels connecting the two cytoplasmic compartments [10,76]. CX43 bearing missense PPVs might interfere with the assembly process of the hexameric subunits, and mutations in the domain region would have more severe effects than outside the domain. Heterozygous truncated CX43 in the domain is unable to incorporate into the hexameric complex, such that CX43 encoded by the normal allele can form structurally normal channels with reduced amounts, while heterozygous CX43 truncated outside the domain is able to incorporate into most hexameric complexes but is functionally inactive.

Most abnormalities in patients with mutations in *GJA1* are present since birth and stationary. Therefore, attention should be paid to the severe complications of ocular anomalies, such as glaucoma. The development of secondary glaucoma later in life may lead to irreversible blindness [77]. In our previous study, a large family with microcornea and glaucoma illustrated the high risk of glaucoma in patients with *GJA1* PPVs [22]. A review of the clinical data for patients with mutations in *GJA1* revealed that at least 26.7% of patients had glaucoma, and the incidence of glaucoma increased with age, reaching 38.7% at approximately 30 years of age and probably increasing thereafter. Recognizing such severe complications is important as glaucoma is preventable and treatable with good outcomes if it is detected and managed at the early stage.

In summary, the characteristics of PPVs in *GJA1* associated with ocular phenotypes are defined as follows: The most common heterozygous *GJA1* PPVs are missense variants inside the connexin domain and truncation variants downstream of the connexin domain. Further studies on *GJA1* are essential to validate this feature as well as its molecular mechanism. Severe eye abnormalities are frequently associated with *GJA1* PPVs, including a high risk of glaucoma in adulthood. Early preventative treatment is important for preventing irreversible blindness.

APPENDIX 1. *GJA1* VARIANTS DETECTED IN THE PRESENT STUDY.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. HETEROZYGOUS *GJA1* VARIANTS ASSOCIATED WITH DIFFERENT PHENOTYPES.

To access the data, click or select the words "Appendix 2."

APPENDIX 3. BIALLELIC *GJA1* VARIANTS ASSOCIATED WITH HUMAN CONDITIONS.

To access the data, click or select the words "Appendix 3."

APPENDIX 4. THE DISTRIBUTION AND ALLELE FREQUENCY OF MISSENSE VARIANTS FROM OUR COHORT AND HGMD.

To access the data, click or select the words "Appendix 4." (**A**) Missense variants with MAF <0.01 identified in the gnomAD database. (**B**) Missense variants within the connexin domain and predicted as damaging ones through at least four in-silico tools from the gnomAD database (above), HGMD and our cohort (below). (**C**) Missense variants within the connexin domain. (**D**) Missense variants predicted as damaging ones through at least four in-silico tools. (**E**) Missense variants from inhouse data and HGMD related (above) and irrelevant (below) to the ocular signs.

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