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Insight into genes responsible for cornea plana, megalocornea, keratoconus and brittle cornea syndrome

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Purpose: Inherited diseases characterized by abnormal corneal morphology include cornea plana, megalocornea, keratoconus and brittle cornea syndrome. This study aims to investigate genes responsible for these diseases.
Methods: Variants in genes responsible for cornea plana, megalocornea, keratoconus and brittle cornea syndrome were analyzed and characterized by multistep bioinformatics approach based on three large data sets, including our in-house exome sequencing database from patients with inherited eye diseases, literature review, and gnomAD database. Additionally, the phenotypes of patients carrying these variants were collected.
Results: 125 variants in six genes, namely *KERA* (Keratocan; OMIM: 603288), *CHRDL1* (Chordin-like 1; OMIM: 300350), *VSX1* (Visual system homeobox 1; OMIM: 605020), *TUBA3D* (Tubulin, alpha-3d; OMIM: 603288), *ZNF469* (Zinc finger protein 469; OMIM: 612078), and *PRDM5* (PR domain-containing protein 5; OMIM: 614161), have been reported in 244 families by literature review, of which 78 with cornea plana, 38 with megalocornea, 67 with keratoconus, and 62 with brittle cornea syndrome. Three variants in *KERA* were identified in 2 families with cornea plana in our cohort. Moreover, all reported variants in *VSX1* were reclassified as likely benign or benign based on several major evidence, including high allelic frequency in gnomAD, presence in unaffected individuals in in-house data set, and relatively tolerated by multiple computational prediction tools. Misinterpreted variants in *VSX1* has been detected in up to 3.13% of the general population.
Conclusions: This study delineates the genetic and clinical landscape of cornea plana, megalocornea, keratoconus and brittle cornea syndrome for the first time. The pathogenicity of *VSX1* variants could not be confirmed, making *VSX1* an unlikely candidate gene for keratoconus. Correct classification of genes like *VSX1* is critical in the era of genomic medicine.

Diseases characterized by abnormal corneal morphology and inherited as Mendelian traits currently include cornea plana, megalocornea, keratoconus and brittle cornea syndrome. Characterized by a reduced curvature of the cornea, cornea plana is a rare disease that often results in hyperopia, a cloudy corneal limbus, and early arcus lipoides [1]. It is inherited either as a mild autosomal dominant (Cornea plana 1, CNA1, OMIM 121400) or a more severe recessive trait (Cornea plana 2, CNA2, OMIM 217300) [2,3]. Variants in *KERA* (Keratocan; OMIM: 603288), which encodes a small leucine-rich proteoglycan has been widely reported to cause CNA2, but not CNA1 [4,5]. Megalocornea (MGC1, OMIM 309300), an X-linked recessive disorder, is characterized by bilateral corneal enlargement (>13 mm in diameter) without elevated intraocular pressure [6]. Hemizygous pathogenic variants in *CHRDL1* have been reported to cause MGC1 [7]. Keratoconus is a bilateral, noninflammatory progressive ectatic corneal disease with complex risk factors like eye rubbing, environmental and genetic factors [8]. Variants in *VSX1* have been reported to contributed to keratoconus [9]. However, there has always been controversy surrounding the role of *VSX1* in keratoconus pathogenesis [10-16]. *TUBA3D* was also identified as a new disease gene underlying keratoconus [17]. Brittle cornea syndrome (BCS), an autosomal recessive disease resulting from pathogenic variants in *ZNF469* [18] or *PRDM5* [19], is identified by blue sclera, keratoglobus or keratoconus, corneal rupture following minor trauma, joint laxity, and hyperelastic skin [20].

All severe forms of these diseases can lead to significant visual morbidity, with a corneal transplant considered to be the ultimate treatment. 40% of deep anterior lamellar keratoplasties and 18% of penetrating keratoplasties have been reported to be performed as treatments for severe keratoconus [21,22]. However, keratoplasty for these patients is regularly challenged by a scarcity of donor corneas as well as a high

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incidence of graft failure. The key challenge in managing BCS is avoiding ocular rupture [23], which requires early detection for preventive steps. For megalocornea, early diagnosis to prevent confusion with primary congenital glaucoma, which carries a high risk of vision loss and requires prompt treatment, is crucial [24]. Genetic testing is crucial for diagnosis since it is challenging to make a definitive diagnosis based solely on clinical symptoms. The next-generation sequencing allows for the identification of pathogenic variants in genes related to monogenic diseases, which supports precise genetic diagnosis and counseling, providing an alternative way to disease management for at-risk family members. For precise clinical genetic testing, it is important to identify the characteristics of pathogenic variants in these genes. Despite numerous reports on variants in these genes, a detailed summary with comparative analysis of extensive data sets is lacking. Using large databases (in-house and/or online), to cross-reference variant information has identified that non-pathogenic variants and genes are not infrequently described as pathogenic [25]. In the same way, our earlier research has shown that these analyses provide insight into pathogenic variants and have also revealed benign variants misinterpreted as causative in some genes [26-28]. The frequency of these issues has risen with the advent of advanced sequencing technologies, which make it easier to detect and report variants.

In this study, variants in the *KERA*, *CHRDL1*, *VSX1*, *TUBA3D*, *ZNF469* and *PRDM5* were analyzed in our cohort of 10,530 families with inherited eye diseases. Variants of six genes were comparatively analyzed using large data sets, including our own data, gnomAD, HGMD, and some newly reported literature. This research aims to investigate the landscape of genes that contribute to these diseases, with the goal of enhancing the identification of clinical and genetic features of pathogenic variants in these genes, ultimately improving the effectiveness of clinical genetic testing. The findings from our research not only illustrate a genetic and clinical spectrum of pathogenic variants in the *KERA*, *CHRDL1*, *ZNF469* and *PRDM5* but also reveal that *VSX1* is probably not a candidate gene for keratoconus. These findings are important for clinical gene testing, which now commonly conduct as a routine process.

METHODS

Subjects: Patients with inherited eye diseases were enrolled from Pediatric and Genetic Clinic at the Zhongshan Ophthalmic Centre, Guangzhou, China with ethics approval from the Institutional Review Board of the Zhongshan Ophthalmic Center, Sun Yat-sen University (2011KYNL012). Before collecting clinical data and peripheral venous blood samples, informed and written consent was obtained from patients or their guardians and available family members, in accordance with the Declaration of Helsinki. Genomic DNA was isolated from leukocytes in peripheral venous blood samples.

Review of genes and variants: The search was first conducted on OMIM with the terms “cornea plana,” “megalocornea,” “keratoconus” and “Brittle corneal syndrome.” Based on the evidence in the “Molecular Genetics” section on OMIM, 6 genes: *KERA* (OMIM 603288), *CHRDL1* (OMIM 300350), *VSX1* (OMIM 605020), *TUBA3D* (OMIM 617878), *ZNF469* (OMIM 612078) and *PRDM5* (OMIM 614161) were found. The gene names and the terms “cornea plana,” “megalocornea,” “keratoconus” and “brittle cornea syndrome” were then applied as search terms in PubMed (accessed on 2 January 2024), with pertinent English references up to January 2024 being included. We also used the HGMD database (accessed on 2 January 2024) and consulted the literature cited therein to verify the relationships between reported gene variants and their associated phenotypes, leading to the selection of these six genes for our study. Variants of the 6 genes were compiled, following the Human Genome Variation Society's guidelines (HGVS, accessed on 2 January 2024) for naming. Acorrding to the mutalyzer website (accessed on 12 March 2024), the variant record was finally unified into a single NM identifier for readability (*KERA*: NM_007035.4, *CHRDL1*: NM_001143981.2, *VSX1*: NM_014588.6, *TUBA3D*: NM_080386.4, *ZNF469*: NM_001367624.2 and *PRDM5*: NM_018699.4). Variants were documented and categorized into non-coding variants, in-frame variants, synonymous variants, missense variants, frameshift variants, nonsense variants, and splicing variants. To assess the variants, multiple bioinformatics analyses were performed. The potential effects of missense variants were estimated with five online tools, which include Rare exome variant ensemble learner (REVEL, accessed on 24 March 2024) [29], combined annotation dependent depletion (CADD, accessed on 24 March 2024) [30], sorting intolerant from tolerant (SIFT;, accessed on 24 March 2024) [31], polymorphism phe-notyping v2 (PolyPhen-2, accessed on 24 March 2024) [32], and protein variation effect analyzer (PROVEAN, accessed on 24 March 2024) [33], while varSEAK database (accessed on 24 March 2024), BDGP (accessed on 24 March 2024), and HSF (accessed on 24 March 2024) were used to predict the possible splicing effect of the synonymous variants and splicing variants. Additionally, we compared reported variants’ allelic frequency to that in genome aggregation database (gnomAD, v2.11, accessed on 24 March 2024) and our exome database. Whether a variant has undergone

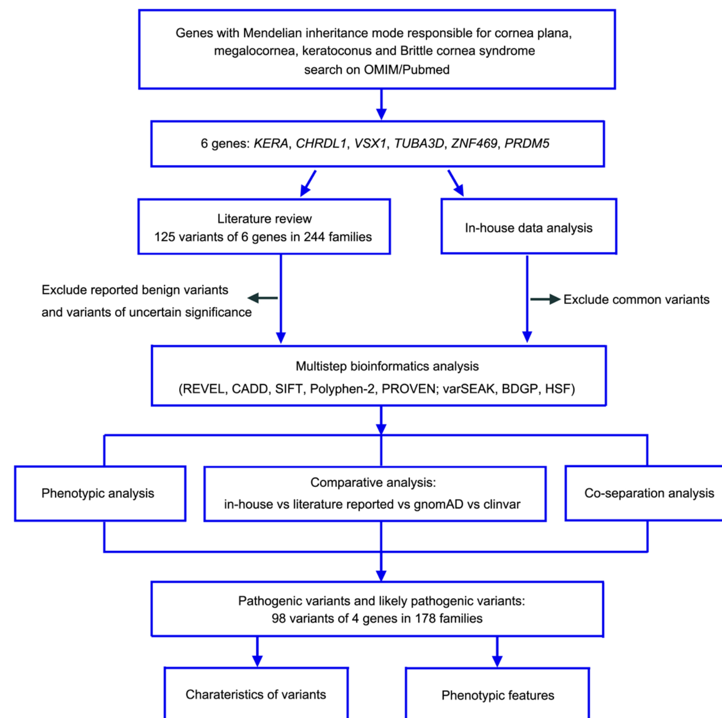

Figure 1. Flow diagram of this study.

functional studies and consistent with cosegregation were also taken into consideration. Afterwards, the variants were categorized based on American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) classification criteria [34] (Figure 1). ClinVar (accessed on 2 January 2024) and HGMD database, which contain pathogenicity annotations of variants, were also used to compare with the ACMG classifications.

Genes and variants of In-house database: In our cohort of 10,530 unrelated families collected from 1997 to 2024, exome sequencing data of the six genes were obtained, which included two families with cornea plana and 10,528 families with other inherited eye diseases except cornea plana, megalocornea, keratoconus or brittle cornea syndrome. As described in our previous research [35,36], the data of exome sequencing consisted of whole-exome sequencing (WES) and targeted exome sequencing (TES). After a multistep bioinformatic analysis, pathogenic variants of corneal plana were found in our cohort. Initially, we removed variants with low sequencing quality that had coverage below 5, as well as common variants which have an allelic frequency over 0.01 in gnomAD. Subsequently, we used previously mentioned online tools to predict the potential impact of these variants. Additionally, the allelic frequencies of the variants were compared to those in gnomAD. After that, we performed further assessments of the selected variants through Sanger sequencing following the method described in the previous study [16] and co-segregation analysis. Ultimately, the selected variants were categorized based on ACMG/AMP classification criteria (Figure 1). Variants in the 6 genes were evaluated through a comparative analysis among the 3 groups in this study: (1) the patients reported in literature and our cohort with detailed genotypic and phenotypic information; (2) the in-house control group consisting of probands with variable ocular conditions; and (3) the general population from the gnomAD database.

Analysis of clinical information: Clinical data was collected from patients with variants in the *KERA*, *CHRDL1*, *VSX1*, *TUBA3D*, *ZNF469* or *PRDM5* and their family members reported in literature and in our cohort. The clinical data recorded comprised age at diagnosis, sex, and ophthalmic features. For BCS, the age of corneal rupture and extraocular features were also recorded. The clinical statistics excluded those families and patients who did not have clinical data available. Based on the ethnicities of patients provided in the literature, the families of probands were divided into three ethnic groups. When ethnicity was not specified, the authors' country was used instead. The clinical information

was analyzed as thoroughly as possible, and the genotype–phenotype correlations are summarized. Moreover, according to the World Health Organization's age classification, onset age groups were categorized as follows: Childhood (ages less than 10 years), Adolescence (ages 10 to 19 years), and Adulthood (ages 20 years and above). Comprehensive and stratified analyses were conducted based on these classifications and are documented in Appendix 1.

Protein structure prediction: The 3D structures of both the wild-type and mutant proteins were predicted using SWISS-MODEL [37], while PyMOL software (https://pymol.org/) was employed for molecular visualization of the protein structures. A comparative analysis was conducted based on the 3D structures of the wild-type and mutant proteins.

Statistical analysis: For statistical analysis, IBM SPSS Statistic 26.0 was used. Numbers and percentages were used for categorical parameters, while median and quartiles was used to describe continuous parameters. When decimals were involved, one decimal place was used. Chi-square test or Fisher's exact test was used to analyze inter-group differences. Monte Carlo simulations with 10,000 iterations and a 95% confidence level were conducted to ensure result stability. Value of Cramer's V was used to quantify effect sizes, and Bootstrap resampling with 1,000 replicates was used to estimate 95% confidence intervals. The Kruskal–Wallis test was employed to assess the differences in median values across groups, and value of ε^2 was calculated to quantify effect sizes. Bonferroni correction was applied to adjust for multiple comparisons. Differences were considered statistically significant when $p<0.05$.

RESULTS

Variant spectrum of genes responsible for cornea plana, megalocornea, keratoconus and brittle cornea syndrome: In total, 125 variants in 6 genes were reported in 244 families with cornea plana, megalocornea, keratoconus or BCS. Seventeen variants of *KERA* in 78 families and 33 variants of *CHRDL1* in 38 families were reported to cause cornea plana and megalocornea, respectively. Twenty-four variants of *VSX1* in 64 families and 3 variants of *TUBA3D* in 3 families were reported to cause keratoconus. Thirty two variants of *ZNF469* in 37 families and 16 variants of *PRDM5* in 25 families were reported to cause BCS, accounting for 59.7% and 40.3% of BCS cases respectively (Table 1 and Appendix 2).

Result of multistep bioinformatic analysis was list in Appendix 2. Among these genes, the predominant variant types varied, and pathogenic variants in each gene has its own characteristics. The most highly reported variants in *KERA* are missense (52.9%), followed by truncation (41.2%), and inframe small indels (5.9%). The reported missense variants are all distributed within the NEL domain with highly conserve leucine-rich motif (Figure 2) and both homozygous or compound heterozygous forms of either missense or truncating variants can lead to cornea plana (Appendix 3). Most reported hemizygous variants in *CHRDL1* were truncation including nonsense, splicing, and frameshift (78.8%), followed by missense (41.2%) variants in von Willebrand factor type C (VWC) or CHRDL_1_2_C domain, with high predicted pathogenicity and low population frequency in both gnomAD and our cohort, that contributed to megalocornea. Truncating variants in 2 genes: *ZNF469* (90.6%) and *PRDM5* (81.3%) inherited as autosomal recessive traits were also the most common, while missense variants in PR-SET_PRDM5 domain can be pathogenic (Figure 2).

Misinterpreted variants in keratoconus: Because there has been ongoing controversy regarding the pathogenicity of variants in genes responsible for monogenic keratoconus, this study included a reevaluation of these variants. The global prevalence of keratoconus was 138/100,000 in 2020 [38] and generally 14% of patients with keratoconus reported a family history [39]. Therefore, the prevalence of genetically related keratoconus is a minimum of approximately 1.93/100,00. For *VSX1*, the reported population frequency of all 24 proposed causative variants in gnomAD is 5318/212768, or 2,499/100,000, which is far higher than the prevalence of genetically related keratoconus and even that of keratoconus as a whole. For individual reported variants in *VSX1*, 37.5% have a population frequency higher than the prevalence of genetically related keratoconus, 66.7% have low pathogenicity as predicted by the online tools including REVEL, CADD, SIFT, Polyphen-2, and PROVEAN, and 37.5% are present in unaffected individuals in our cohort (Figure 1, Appendix 4). Comparing all the variants in gnomAD, our cohort, and those pathogenic variants reported in the literature, we found no significant difference among the distribution of the variants, which do not cluster in any of the identified functional domains in the protein, so did the pathogenicity of varinats predicted by online tools (Figure 3A). Missense variants make up the majority of variants in this gene, followed by truncation variants. However, there is no significant difference in the distribution of types of variants across the gnomAD, our cohort and reported studies ($p=0.575$, Cramer's $V=0.011$, 95% confidence interval=[0.005, 0.034]; Figure 3B). According to the three-dimensional protein prediction results from SWISS-MODEL, the only truncation variant, c.758_765delTCAACTCC/p.(L253RX), demonstrates a significant difference compared to the wild-type, as its predicted protein structure indicates a premature termination of the peptide chain. In contrast, the

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Table 1. Literature review of genes responsible for cornea plana, megalocornea, keratoconus and Brittle cornea syndrome.

Gene	HGNC ID	Gene MIM number	location	Inheritance	Phenotype	Disorder MIM number	Reported Families
KERA	HGNC:6309	603,288	12q21.33	AR	CNA2	217,300	78
CHRDL1	HGNC:29861	300,350	Xq23	XLR	MGC1	309,300	38
VSX1	HGNC:12723	605,020	20p11.21	AD	Keratoconus 1	148,300	64
TUBA3D	HGNC:24071	617,878	2q21.1	AD	Keratoconus 9	617,928	3
ZNF469	HGNC:23216	612,078	16q24.2	AR	BCS1	229,200	37
PRDM5	HGNC:9349	614,161	4q27	AR	BCS2	614,170	24

Notes: Abbreviations: AR: autosomal recessive; XLR: X-Linked recessive; AD: autosomal dominant; CNA2: autosomal recessive cornea plana-2; MGC1: Megalocornea 1, X-linked; BCS: Brittle cornea syndrome.

other reported missense variants in *VSX1* show no obvious changes in their protein secondary structures (Appendix 5). However, the variant c.758_765delTCAACTCC/ p.(L253RX) was only reported in one keratoconus patient but identified in 11 unaffected individuals within our cohort and exhibits a population frequency (37/183872 in gnomAD) that exceeds the prevalence of genetically related keratoconus, thereby raising questions about its pathogenicity. Furthermore, reevaluation of the reported variants in *VSX1* according to the ACMG/AMP categories resulted in significant changes in their estimated pathogenicity (Appendix 4). From HGMD to ACMG, the evaluation of 2 variants changed from damaging mutation (DM) to Benign (B), 6 variants changed from DM to Likely Benign (LB), 3 variants changed from DM? to B, and 9 variants changed from DM? to LB. Except 4 benign variants whose classification remained unchanged, the remaining variants all shift toward lower pathogenic classification from ClinVar to ACMG (Figure 3C). According to the ACMG classification, all the reported variants in *VSX1* were reclassified as benign or likely benign variants, supporting the evaluation that the identified variants in *VSX1* were unlikely to be causative of keratoconus in a highly penetrant monogenic mode. Therefore, misinterpreted variants in *VSX1* has been detected in up to 3.13% of the general population.

For *TUBA3D*, only 2 truncation variants have been found in a pair of twins as well as an isolated patient, and a non-coding variant has been identified in an independent patient so far. Control populations in both gnomAD and our database include a large number of truncating and non-coding

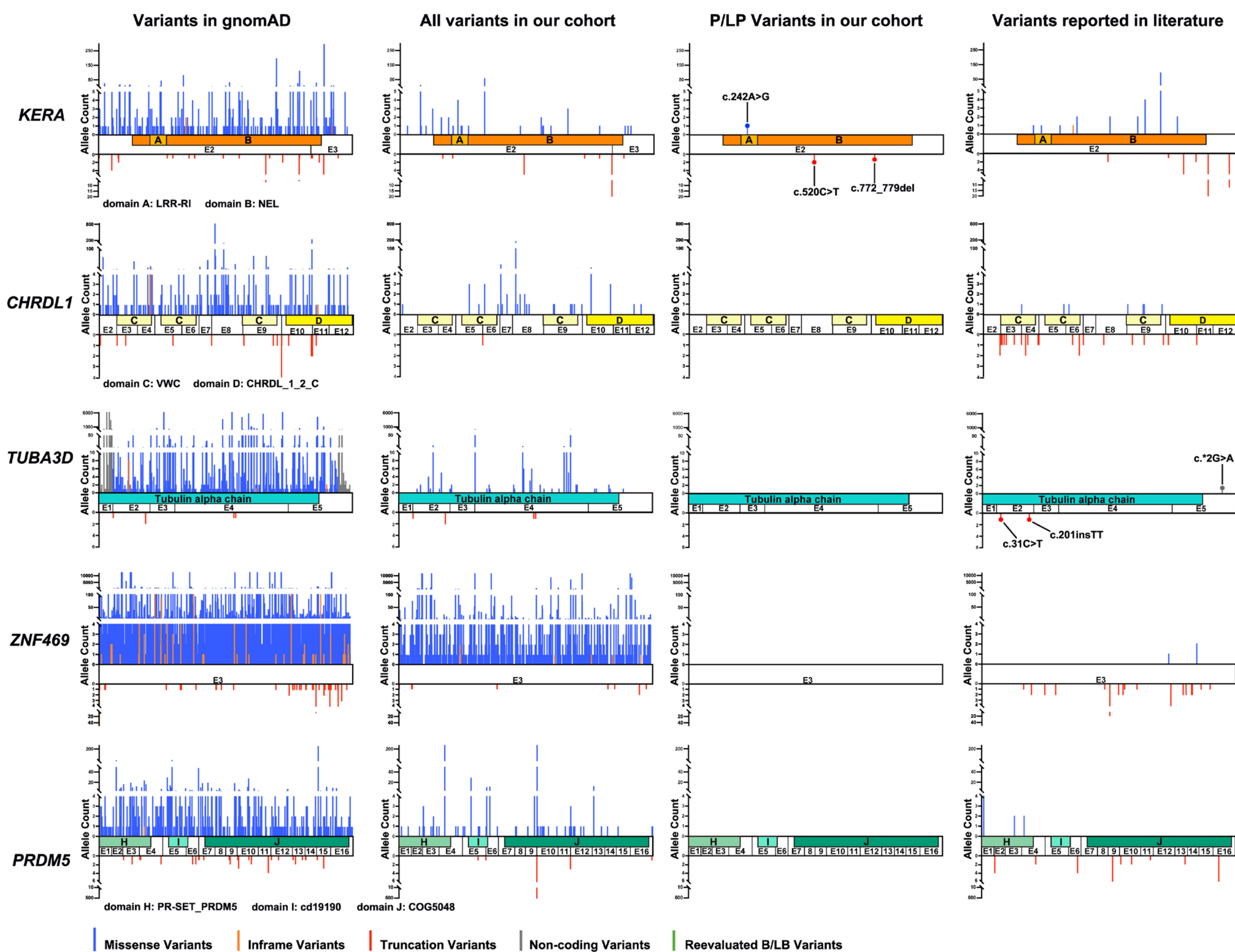

Figure 2. Distribution and frequency of variants in *KERA*, *CHRDL1*, *VSX1*, *TUBA3D*, *ZNF469* and *PRDM5* in our gnomAD and our cohort, pathogenic/likely pathogenic (P/LP) variants in our cohort, and variants reported in literature. The green font indicates benign/likely benign (B/LB) variants. Truncation variants include splicing variants, frameshift variants, and nonsense variants.

variants, no pathogenic characteristics of reported variants can be identified, and no functional studies in animals have been reported. Therefore, further research is needed to confirm the pathogenicity of variants in *TUBA3D* as a cause of keratoconus.

Clinical characteristics of genes responsible for cornea plana, megalocornea and brittle cornea syndrome: All reported variants in *CHRDL1* cause megalocornea and all reported variants in *ZNF469* and *PRDM5* cause BCS. Nearly all *KERA* variants led to cornea plana, with the exception of one family with homozygous c.835C>T, p.(Arg279*), presenting both cornea plana and pellucid marginal degeneration [40] and a sporadic instance with homozygous c.937C>T, p.(Arg313*), presenting anterior segment dysgenesis [41] (Appendix 3). Although the names of cornea plana and megalocornea appear to refer only to abnormalities in corneal morphology, they both are associated with various intraocular abnormalities beyond the cornea. Additionally, BCS involves not only corneal abnormalities but also extraocular anomalies with joint laxity and hearing defects being the two most frequent manifestations (Figure 4A). Based on statistical data from the literature, this study confirms that there is no significant difference between BCS caused by *ZNF469* or *PRDM5* in terms of intraocular manifestations, extraocular manifestations, or even the timing of corneal rupture (Figure 4B).

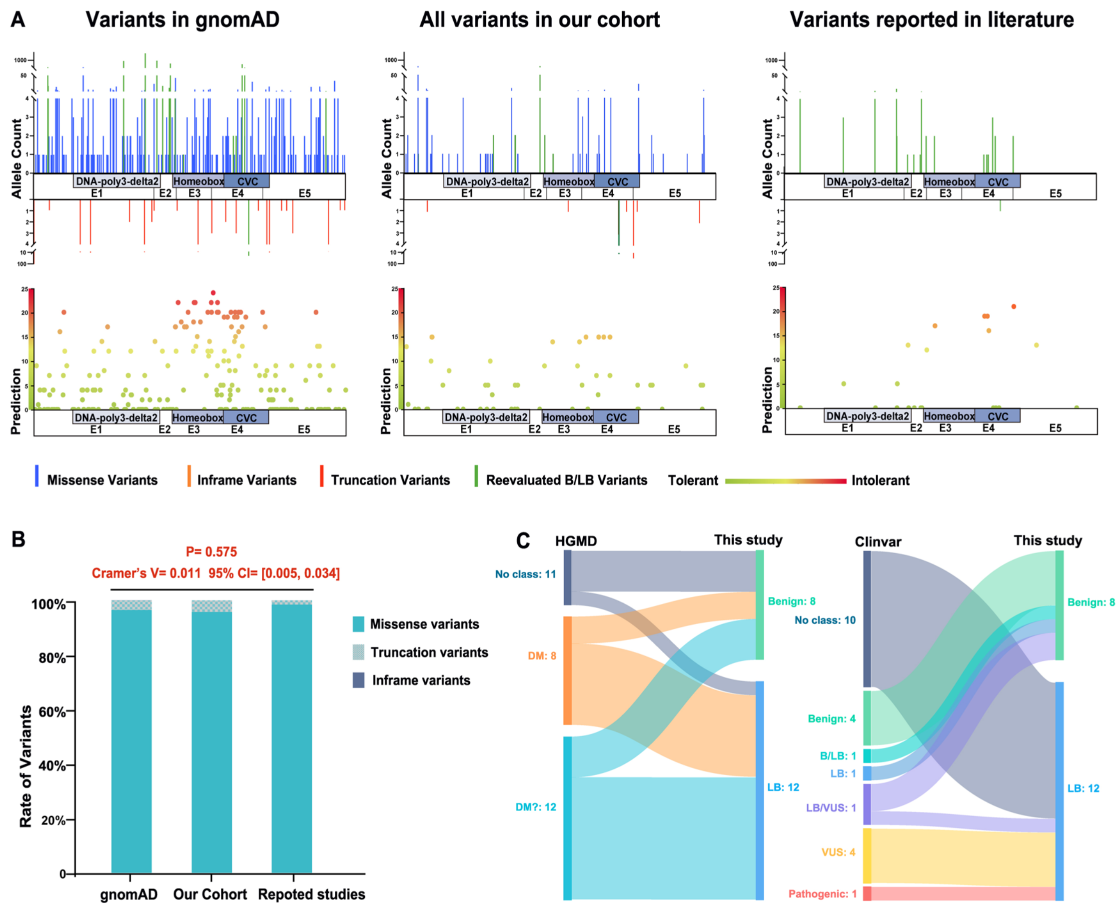

Figure 3. Comparision and reevaluation of variants in *VSX1*. **A**: Distribution and frequency of variants in *VSX1* in gnomAD and our cohort, and variants reported in literature. The scoring criteria of prediction are as follows: the total score is 25 points. For SIFT, a rating of D scores 5 points, and T scores 0 points. For PolyPhen-2, a rating of D, PD and T scores 5, 3 and 0 points, respectively. For PROVEAN, D scores 5 points and N scores 0 points. For REVEL/ CADD, a score above the 95% cutoff scores 5 points, between the 75% and 95% cutoffs scores 3 points, and below the 75% cutoff scores 0 points. In addition, each occurrence of gnomAD Allele count deducts 1 point. **B**: Rate of types of variants in *VSX1* in gnomAD, our cohort and reported studies. CI=Confidence interval. (C) The sankey diagram of reevaluation of reported variants in *VSX1* from HGMD or Clinvar to this study.

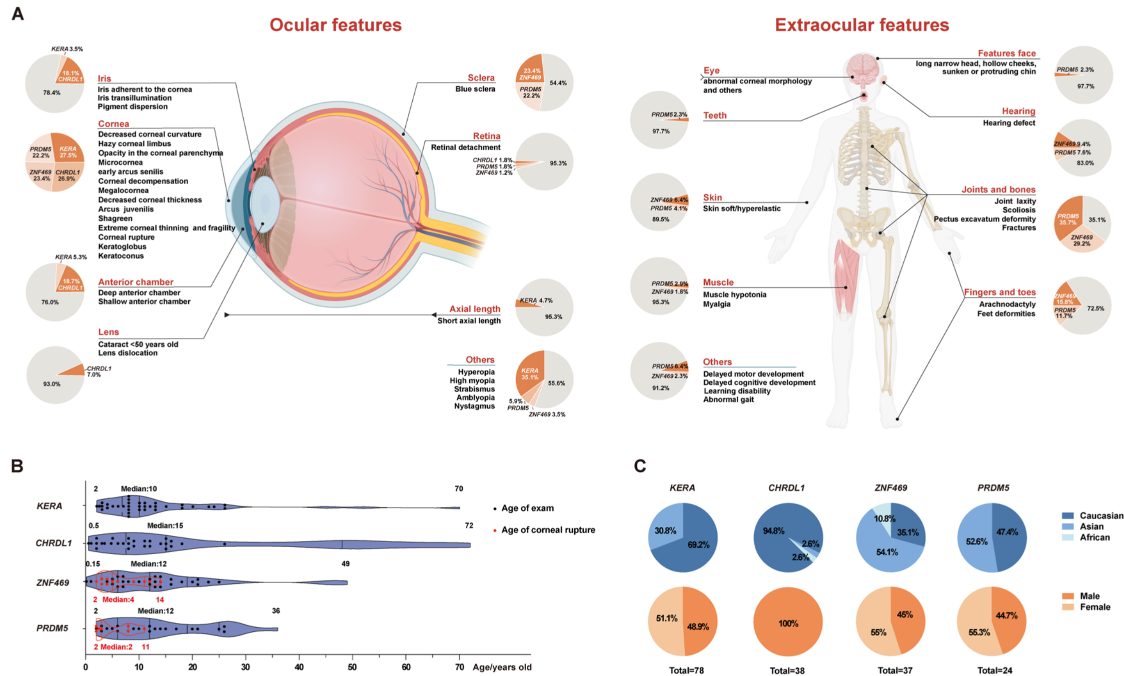

Figure 4. Clinical features across four genes. **A**: Clinical features of genes responsible for cornea plana, megalocornea and brittle cornea syndrome; In the percentage pie chart, the gray section represents the proportion of families carrying pathogenic variants in *KERA*, *CHRDL1*, *ZNF469* and *PRDM5* that do not exhibit the phenotype, relative to all families carrying pathogenic variants in *KERA*, *CHRDL1*, *ZNF469* and *PRDM5*. The image was created by Biorender and Adobe illustrator. **B**: Age of exam and age of corneal rupture of *KERA*, *CHRDL1*, *ZNF469* and *PRDM5*; (**C**) Race and sex of *KERA*, *CHRDL1*, *ZNF469* and *PRDM5*.

The stratified analysis reveals that a substantial proportion of patients exhibited the disease during childhood or adolescence (81.0% in *KERA*, 60.5% in *CHRDL1*, 75.7% in *ZNF469*, and 66.7% in *PRDM5*). No significant phenotypic differences were detected among the various age-of-onset cohorts (Appendix 1). Furthermore, the analysis of the male-to-female ratio and best-corrected visual acuity demonstrated no statistically significant differences across the different age-of-onset groups (Appendix 6).

Variants in *KERA* (69.2%) and *CHRDL1* (94.8%) have most frequently been reported as causes of cornea plana and megalocornea in Caucasian patients, while variants in *ZNF469* (54.1%) have more commonly been reported as a cause of BCS in Asian patients. Half of the families with BCS caused by variants in *PRDM5* were Caucasian and the other half Asian. In addition, a small number of families with BCS caused by variants in *ZNF469* were African (10.8%) and a small number of families with megalocornea caused by variants in *CHRDL1* were African (2.6%; Figure 4C).

Two newly reported familes of cornea plana in our cohort: In our cohort, three pathogenic/ likely pathogenic variants (based on ACMG/AMP standards) in *KERA* were identified in two families with cornea plana, including c.242A>G, p.(Asn81Ser; likely pathogenic) and c.772 c.772_779del, p.(Gly258Cysfs*30; pathogenic) in Family 1; and c.520C>T, p.(Gln174*; pathogenic) in Family 2. The patient in Family 1 is a 26 year old female with congenital decreased corneal curvature of the right eye (20.1D;not available for left eye), hazy corneal limbus, hyperopia (+12DS; +14DS), microphthalmia, microcornea, opacity in the corneal parenchyma, shallow anterior chamber (1.36mm; 1.04mm), short axial length (20.8mm; 22.3mm), and anterior iris adhesion of both eyes. She had poor vision (0.2; 0.3) since childhood, with worsening vision blurriness and photophobia over the past year. Due to peripheral anterior chamber slit appearance, pupil displacement, and pinhead-sized iris, a left eye iris optical iridectomy and anterior iris adhesion release surgery were performed. The second patient, in Family 2 is a 5 year old boy carrying a homozygous c.520C>T, p.(Gln174*) variant. He presented congenital poor vision (counting fingers for both eyes), decreased corneal curvature, hazy corneal limbus, hyperopia (+10 DS; +10 DS), microphthalmia, microcornea (8 mm; 8 mm), shallow anterior chamber (1.72 mm; 1.49 mm), and short axial length (22.66 mm; 22.63 mm).

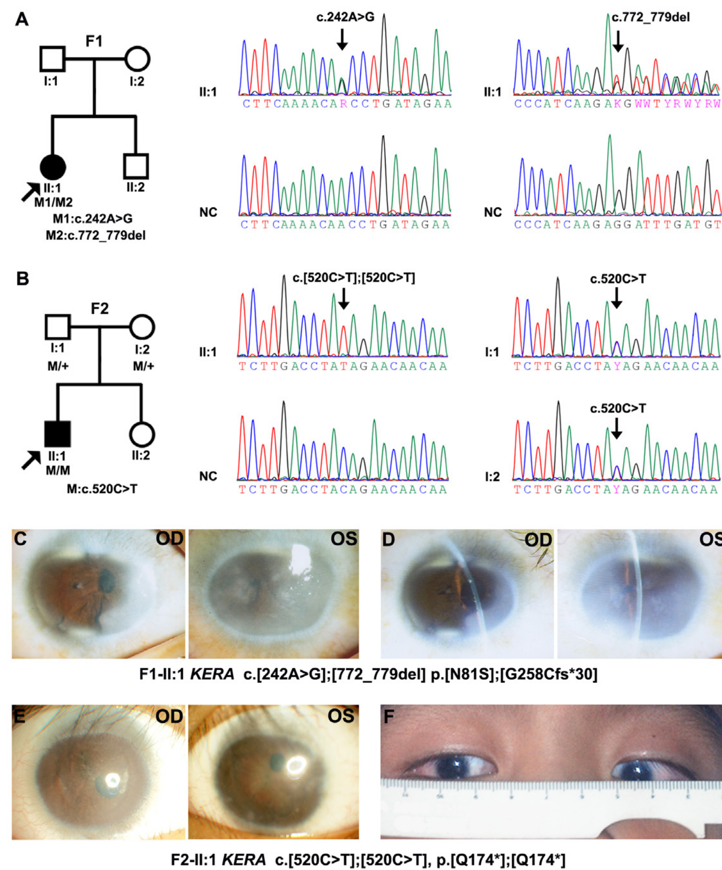

Figure 5. Pedigree diagram and phenotypic photographs. **A-B**: Pedigree of the families with sequencing of pathogenic variants in *KERA*. **C-F**: Slit-lamp photographs and photograph of diameter of cornea of patients with pathogenic variants of *KERA* identified in our cohort.

Both his parents are carriers for the variant and have a normal phenotype.These variants were confirmed using Sanger sequencing (Figure 5).

DISCUSSION

This report summarizes the cases of corneal abnormalities in previous literature and adds those identified in the current study: 98 variants of 4 genes in 178 families, including 17 variants in *KERA* in 78 families with cornea plana; 33 variants in *CHRDL1* in 38 families; 32 variants in *ZNF469* in 37 families and 16 variants in *PRDM5* in 25 families with BCS. In addition, the genetic landscapes of *KERA*, *CHRDL1*, *ZNF469* and *PRDM5* were defined through an extensive comparative study using gnomAD database, HGMD data, literature reports and our in-house data. Finally, probably benign variants of *VSX1* misinterpreted as pathogenic were also highlighted and it was suggested that currently, there is insufficient evidence to consider *VSX1* to be a candidate gene for keratoconus. Additional research is necessary to elucidate the variants in question and the role this gene has in corneal disease, as now there are only relatively rare reports.

Only 3 variants in *KERA* were indentified in our cohort. The limited number of cases and the infrequency of the variants may have contributed to the scarcity of the other genes' variants, reflecting the rarity of these diseases. According to our combined data, variants in each gene are responsible for varying fractions of corneal diseases in the different ethnic groups. The detection of various intraocular abnormalities beyond the cornea in cornea plana, megalocornea, and BCS as well as extraocular abnormalities associated with BCS suggests that close attention to the variable phenotypes is necessary, and certain features beyond the cornea may aid in early diagnosis and treatment.

Most importantly, comparative analysis in this study revealed that uncertain and perhaps erroneous interpretation of variants in the genes of keratoconus is a significant concern. A lack of attention to this problem might cause significant repercussions in clinical practice, especially in the

current era of genome medicine with its widespread application of clinical gene tests.

Most studies identify variants that are present in cases but not in controls, or that show notable differences in allele frequencies. Numerous studies have been published with fewer than 100 patients and/or controls, and they often did not thoroughly screen the controls for rare variants, opting instead to only check for variants found in the case group. This resulted in an underestimation of the actual diversity of candidate genes among the general population. These problems might have arisen from the genetics community's insufficient acknowledgment of the large number of harmless rare missense variants even in healthy people. Moreover, there have been limited follow-up studies to assess whether the specific variants identified truly contribute to keratoconus susceptibility or have biologic significance, and none have connected the functional effect to a disease mechanism. *VSX1*, known as a transcription factor, was initially considered a candidate gene for posterior polymorphous corneal dystrophy (PPCD). In the landmark paper in 2002 [9], the authors analyzed the *VSX1* gene in a cohort of 63 keratoconus patients due to the simultaneous occurrence of PPCD and keratoconus in some cases and discovered four missense variants, two of which were not present in the control group. They concluded that the missense variants in *VSX1* are responsible for keratoconus in at least 4.7% of the cases. Subsequently, *VSX1* was excluded as candidate gene for PPCD in 2005 [42]. Five frequently occurring variants in *VSX1* across different cohorts were analyzed, revealing no association with keratoconus in a meta-analysis in 2017 [14]. In addition, there is a lack of evidence supporting any functional impact of *VSX1* variants on the cornea. A prior study has confirmed that the p.P247R mutation in *VSX1* does not result in pathogenic changes in the corneas of either heterozygous or homozygous mice, offering convincing evidence that at least one previously reported variant in *VSX1* is non-pathogenic [15]. In summary, substantial evidence from previous studies indicates that *VSX1* is not associated with keratoconus.

In the current study, 24 variants of *VSX1* in 64 families were found to be unlikely to cause keratoconus in a highly penetrant monogenic mode, due to higher allele frequencies than keratoconus prevalence in general population, unrelatedness with keratoconus in our cohort, and the prediction by online programs that most of the variants are benign (Appendix 4). If these misinterpreted variants are not corrected in a timely manner, 3.13% of the general population will be considered to have keratoconus through genetic diagnosis.

As the process of detecting, describing, analyzing, and reporting variants moves from highly academic settings to general medical practice, the issue of misinterpreted variants seems likely to grow over time. This tendency is likely to be exacerbated due to the surge in small-scale commercial companies offering sequencing of the exome, clinicians not formally trained in medical genetics, and journals without critical review criteria, among other trends. The issue could be significantly minimized if ACMG/AMP standards and guidelines are properly implemented in describing disease-related variants. Furthermore, comparing extensive data sets can clarify misinterpreted variants, even those with multiple prior reports.

APPENDIX 1. SUPPLEMENTARY TABLE S4.

To access the data, click or select the words “Appendix 1.” Clinical feature of *KERA*, *CHRDL1*, *ZNF469* and *PRDM5* (Ovearall and Age stratification).

APPENDIX 2. SUPPLEMENTARY TABLE S1.

To access the data, click or select the words “Appendix 2.” Summary of variants of genes responsible for cornea plana, megalocornea, keratoconus and Brittle cornea syndrome.

APPENDIX 3. SUPPLEMENTARY TABLE S2.

To access the data, click or select the words “Appendix 3.” Detailed information of literature review.

APPENDIX 4. SUPPLEMENTARY TABLE S3.

To access the data, click or select the words “Appendix 4.” Benign variants and likely benign variants in *VSX1* reported to cause keratoconus.

APPENDIX 5. SUPPLEMENTARY FIGURE S1.

To access the data, click or select the words “Appendix 5.” The three-dimensional protein structures of the reported variants in *VSX1*. In each group, the left side illustrates the wild-type protein structure, while the right side displays the mutated protein structure. Amino acid alterations at the corresponding sites are highlighted using purple patterns. Variants that induce alterations of protein's secondary structure are emphasized in red.

APPENDIX 6. SUPPLEMENTARY FIGURE S2.

To access the data, click or select the words “Appendix 6.” Clinical feature of *KERA*, *CHRDL1*, *ZNF469* and *PRDM5* (Age stratification). (A) Male versus female ratio for three genes by onset age (*CHRDL1*: X-Linked recessive and no

female patients reported). (B) BCVA for four genes by onset age. BCVA=best-corrected visual acuity; CI=confidence interval; $p<0.05$: statistically significant differences; In Fisher's exact test, Cramer's V:0 (no association) to 1 (perfect association); In Kruskal–Wallis test, H (number of groups −1) rises as group differences become more distinct; ε^2: 0 (no difference) to 1 (complete difference); * represents insufficient data for statistical analysis.

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