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Subclinical parents assist in the detection of genetic variants in keratoconus by trio-based whole-exome sequencing

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Purpose: To explore the genetic variants of 14 keratoconus trios containing subclinical parents.
Methods: Trio-based whole-exome sequencing was performed in 14 keratoconus trios containing subclinical parents. The variants identified in candidate genes of keratoconus were analyzed by multiple bioinformatics tools.
Results: We identified 12 variants in 10 candidate genes of keratoconus (*COL5A1*, *TGFBI*, *CAST*, *MPDZ*, *WNT10A*, *MYOF*, *ERMP1*, *MAP3K19*, *COL1A1*, and *WNT16*). All variants were novel, not previously reported, and defined as uncertain significance according to the American College of Medical Genetics and Genomics guidelines. All variants were heterozygous and autosomal dominant cosegregated in keratoconus families.
Conclusions: We found that the candidate variants identified in clinically diagnosed patients and their subclinical parents may cause keratoconus through an autosomal dominant inheritance pattern, with different variable expressivity. This study indicates that genetic testing may play an important role in identifying patients with latent keratoconus and high-risk individuals for corneal ectasia after refractive surgery.

Keratoconus (KC) is a bilateral, sometimes asymmetric disease of corneal ectasia characterized by progressive thinning, bulging, and conical protrusion, leading to visual impairment [1]. The disease usually begins during puberty and progresses to varying degrees and at different speeds until the third or fourth decade of life [2]. KC is a leading cause of corneal transplantation worldwide [3], although due to timely diagnosis, many patients are currently treated with collagen cross-linking surgery. With an incidence of approximately 1 per 2,000, KC affects both sexes and different populations, but ethnic differences in KC incidence have been reported, such as higher rates in Asians and Middle Easterners [4]. Due to recent advances in diagnostic imaging, the true incidence and prevalence of keratoconus may be higher than previously reported [5].

Several strong lines of evidence suggest the role of genetics in the pathogenesis of KC, such as bilaterality, ethnic differences in incidence, familial aggregation, the consistency of identical twins [6], and a high association with other genetic diseases such as Down syndrome [7]. Keratoconus is considered a genetically heterogeneous disease because numerous genes and loci associated with keratoconus have been identified [8]. The most commonly described inheritance pattern is autosomal dominant with reduced penetrance and variable expression [9]. In our previous candidate gene study, we revealed the potential genetic cause of four KC families with dominant inheritance and variable expression. Some asymptomatic first-degree relatives carrying the variant are actually affected: they exhibit a latent subclinical KC phenotype [10]. Therefore, individuals exhibiting subclinical KC features may be "genetic carriers" in dominant inheritance but are mischaracterized as unaffected individuals in some studies due to latent phenotypes.

Here, we hypothesize that the inclusion of subclinical phenotypes could clarify the inheritance patterns in KC families and help identify genetic risk factors that segregate in these families. In this study, we recruited 14 KC trios with subclinical parents to investigate genetic variants associated with KC using whole-exome sequencing (WES).

METHODS

Ethical statement: This descriptive cross-sectional study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of The Eye Hospital of Wenzhou Medical University (2023–071-K-59). Written informed consent was obtained from all participants.

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Study participants: This study included a total of 14 trios (23 men and 19 women) of Han Chinese descent from The Eye Hospital of Wenzhou Medical University. All parents underwent a complete ophthalmologic examination consisting of manifest refraction, slit-lamp biomicroscopy examination, and corneal tomography evaluation by Pentacam HR (Oculus GmbH, Wetzlar, Germany). The Belin/Ambrosio Enhanced Ectasia Display (BAD) map was constructed from the Pentacam data, which could detect subclinical changes in KC. Each trio included a proband with KC, a parent with subclinical KC, and another parent with a normal phenotype. All participants were clinically diagnosed by the same experienced ophthalmologist and classified into KC, subclinical KC, and normal phenotype. Patients with syndromic KC and nonprimary KC were excluded.

The diagnosis of KC is based on the following criteria: (1) at least one typical clinical feature of KC (e.g., Fleischer's ring, Vogt's striae, anterior stromal scar, conical protrusion); (2) typical abnormal topographic criteria, such as asymmetric bow tie or posterior or anterior focal steepening; and (3) abnormal topographic index, such as maximum keratometry (Kmax) >47 D or inferior-superior index >1.5 [10].

The diagnosis of subclinical KC is based on the following criteria: (1) subtle corneal topographic changes within the aforementioned abnormalities of keratoconus, (2) the total deviation score of the BAD map ≥1.6, (3) corresponding anterior or posterior abnormality in the BAD map or thinnest pachymetry (TP) ≤480 μm, and (4) absence of slit-lamp or visual changes typical of keratoconus and with a best-corrected visual acuity (BCVA) of 20/20 or better [11].

Data from worse eyes in the subclinical KC group were compared with additional groups by principal component analysis (PCA). Additional data were collected from patients admitted to The Eye Hospital of Wenzhou Medical University from November 2016 to October 2017. A total of 332 eyes of 187 patients with KC and 490 eyes of 245 refractive surgery candidates with myopia were included. The control group was defined as the myopic candidates without other eye diseases. We collected 10 parameters from the BAD map for analysis, including TP, front and back elevation at the corneal thinnest location, pachymetric progression index average, Ambrosio relational thickness maximum, deviation of front elevation difference map and back elevation difference map, deviation of average pachymetric progression, deviation of minimum thickness, and deviation of the Ambrosio relational thickness maximum. PCA was performed and visualized in Spyder software (version 2.3.8) of Python 2.7.

Whole-exome sequencing: For each individual, at least 3 μg genomic DNA was fragmented to create the Illumina libraries. Whole-exome region libraries were enriched using the Twist Human Core Exome Kit (Twist Bioscience, Francisco, CA) according to the manufacturer's protocol and sequenced on Illumina NovaSeq 6000 sequencers (Illumina, San Diego, CA). WES was performed at PSI Genomics (Wenzhou, Zhejiang, China). WES yielded a mean sequencing depth of 84.5×. All sequencing reads were mapped to the human reference genome (GRCh37/hg19) using Burrows-Wheeler Aligner software (Broad Institute of MIT and Harvard, Boston, MA). Local realignments, quality control text, and genotype calling were assembled using the Genome Analysis Toolkit. Functional annotation of these variant sites was performed using the ANNOVAR software package (University of Michigan, Ann Arbor, MI). Minor allele frequency (MAF) data were retrieved from the 1000 Genomes database (1000G), the Exome Aggregation Consortium database (ExAC), and the Genome Aggregation Database (gnomAD) [12,13]. Bioinformatics analysis tools for variant pathogenicity include MutationTaster [14], SIFT [15], PolyPhen-2 [16], CADD [17], REVEL [18], and GERP++ [19]. CADD score >20 and REVEL score >0.5 were defined as pathogenic and GERP++ score >2 as conservative.

Variant filtering: The candidate variants were screened according to the analysis workflow shown in Figure 1. Based on the characteristics of the pedigree, we propose that autosomal dominant inheritance with variable expression cosegregated in KC trios may be responsible for the disease. Variant filtering included the following thresholds: (1) variants with an MAF less than 0.001 in 1000G (1000 g2015aug_all), ExAC (ExAC_all), and gnomAD (gnomAD_exome_all); (2) variants located in exons or splicing sites; (3) missense variants or loss-of-function (LoF) variants; (4) missense variants predicted to be deleterious or evolutionarily conserved up to half by 6 bioinformatics analysis tools (MutationTaster, SIFT, PolyPhen-2, CADD, REVEL, and GERP++); and (5) variants that are cosegregated in autosomal dominant inheritance. Inferred LoF variants consist of stop-gain, stop-loss, frameshift insertions/deletions, canonical splice sites, and start-loss. LoF variants were considered "damaging."

After screening the rare deleterious candidate variants, the KC-associated genes from the Online Mendelian Inheritance in Man database and the relevant references from the PubMed database were considered candidate genes for further analysis (Appendix 1). Conservation analysis and protein structure modeling for the identified variant were performed as previously described. The phosphorylation prediction score was obtained from NetPhos 3.1 [20]. According to the guidelines of the American College of Medical Genetics and Genomics (ACMG), the pathogenicity

of variants was classified by InterVar. The variants were classified into pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, and benign [21].

Additional verification of inheritance patterns: To demonstrate that subclinical phenotypes help reveal the genetic cause of KC, we experimentally removed subclinical morphologic data from all asymptomatic parents for variant screening. Based on the slit-lamp and BCVA results, all parents are defined as having a "normal" phenotype. On this basis, the genetic pattern should correspond to de novo inheritance, recessive inheritance, or complex heterozygous inheritance.

RESULTS

Clinical characteristics: Fourteen families with a total of 48 participants were included in this study. The mean age of the probands at diagnosis of keratoconus was 17.6 ± 4.9 years, including 10 men and 6 women. In each trio, one parent was diagnosed with subclinical keratoconus, and the other was diagnosed as having a normal phenotype. The mean age of the parents was 50.1 ± 6.0 years in the subclinical KC group and 51.1 ± 6.7 years in the normal phenotype group (p = 0.54).

In the subclinical KC group, there were no abnormal slit-lamp signs in either eye, and the BCVA was 20/20 or better in both eyes. For subtle topographic changes in the worse eyes, 13 parents had focal steepening in the posterior region and 1 parent had focal steepening in the anterior region. In the BAD map, all had abnormal images and parameters (Appendix 2). In the PCA plot, we observed that most eyes (12/14) of the subclinical parents happened to be in the border area (dashed line area) between the KC group and the control group (Figure 2).

Variants of candidate KC genes: We identified 12 missense variants in 10 candidate KC genes by trio-based exome

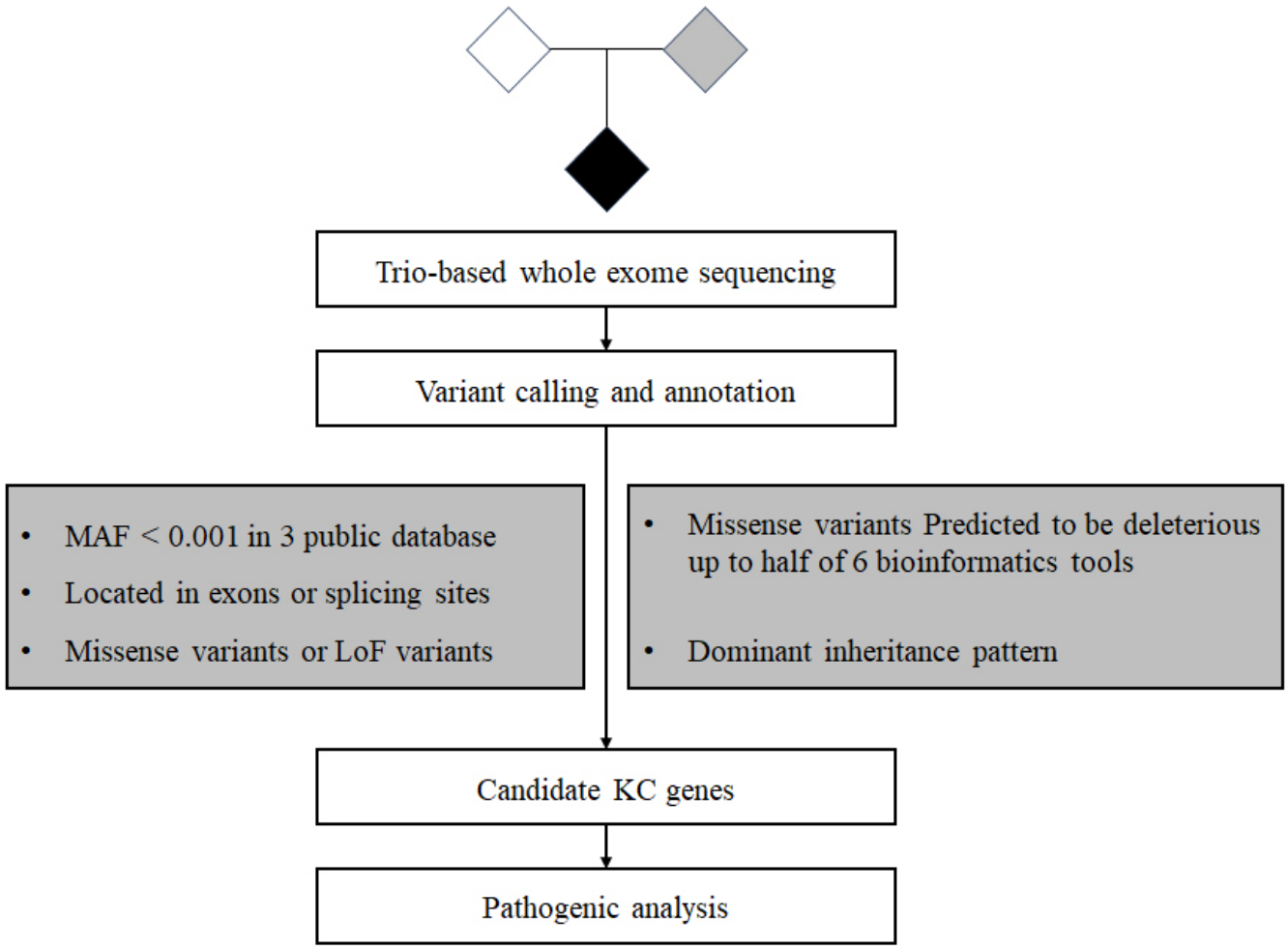

Figure 1. Analysis workflow of trio-based whole-exome sequencing data.

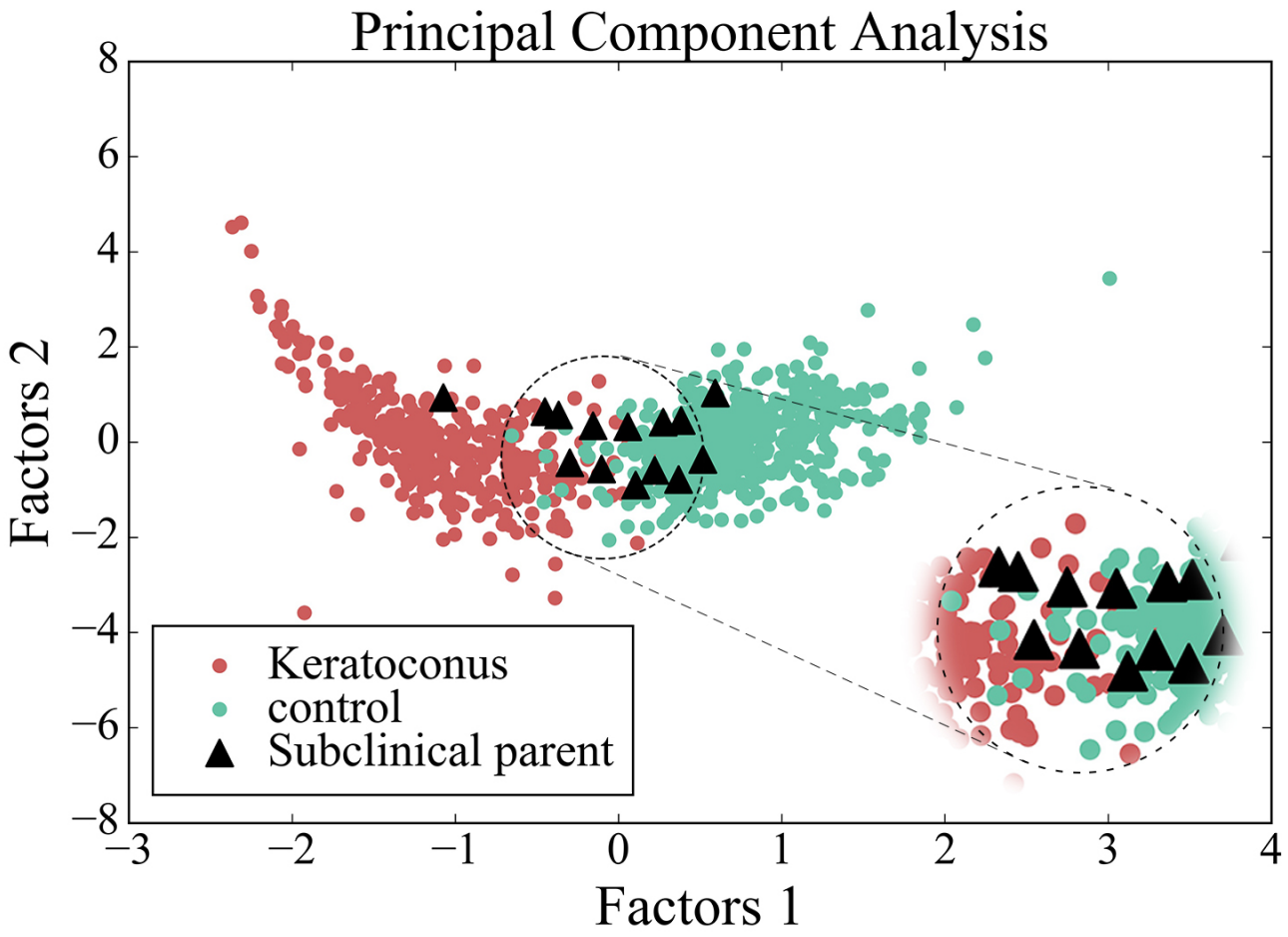

Figure 2. Scatterplot of data from Pentacam by principal component analysis. The red circle represents data from the keratoconus group, the green circle represents data from the control group, and the black triangle represents data from the subclinical parent group in this study. The dotted circle represents the critical area between the keratoconus group and the control group.

sequencing, including 1 frameshift variant and 11 missense variants (Table 1). All 12 variants were novel, not previously reported, and defined as VUS according to the ACMG standard. Variants in the *COL1A1* and *MYOF* genes were identified twice in unrelated families, and variants in the *COL5A1*, *TGFBI*, *CAST*, *MPDZ*, *WNT10A*, *ERMP1*, *MAP3K19*, and *WNT16* genes occurred only once. All 12 variants were heterozygous and autosomal dominant cosegregated in KC families (Table 1, Appendix 2).

Three of these variants in the *COL5A1* (c.C4127T:p.S1376F), *TGFBI* (c.G2006A:p.R669Q), and *WNT10A* (c.C965T:p.P322L) genes were missing from the 1000G, ExAC, and gnomAD databases, while the remaining 10 variants had a low MAF value (Table 1). Three of these variants in the *COL5A1* (c.C4127T:p.S1376F), *MPDZ* (c.5678C>T:p.A1893V), and *MYOF* (c.C1346T:p.T449I) genes were predicted in all six in silico analysis methods classified as pathogenic. Further analyses were performed for the c.C4127T:p.S1376F variant in the *COL5A1* gene, which showed high pathogenicity in MAF and bioinformatics analysis.

The variant c.C4127T:p.S1376F in the *COL5A1* gene was missing from the 1000G, ExAC, and gnomAD databases. The variant was predicted to be pathogenic in all six in silico analysis methods (Table 1) and was evolutionarily conserved across a wide range of species (Figure 3). S1376 is predicted to be phosphorylated (0.521 by NetPhos 3.1), while the F1376 variant is not. The three-dimensional structure prediction showed that loss of hydrogen bonding occurred in the mutant protein S1376F. The heterozygous variant c.C4127T was identified in the KC proband and her subclinical KC father in family F10 but not in her mother with a normal phenotype. The father carrying the variant was noted to have subclinical abnormalities in both eyes, including a paracentral focal steepening of the posterior “Refractive Elevation” map and

Table 1. Variants identified in candidate genes of keratoconus.

Family	Gene	Function	Variant	MAF[a]	In silico analysis[b]	ACMG	Inherited
F01	CAST	Missense	NM_001750.7 c.G1042T:p.A348S	0.00018	3/6	VUS	Maternal
F02	MYOF	Missense	NM_013451.4 c.C1346T:p.T449I	0.00014	6/6	VUS	Paternal
F03	MPDZ	Missense	NM_001378778.1 c.5678C>T:p.A1893V	0.00004	6/6	VUS	Maternal
F04	COL1A1	Missense	NM_000088.4 c.G3737A:p.R1246Q	0.00012	3/6	VUS	Maternal
	ERMP1	Missense	NM_024896.3 c.G604A:p.A202T	0.00006	3/6	VUS	Maternal
F05	WNT10A	Missense	NM_025216.3 c.C965T:p.P322L	0	3/6	VUS	Maternal
F06	MAP3K19	Missense	NM_001018044.2 c.C2948G:p.S983C	0.00002	5/6	VUS	Maternal
F07	TGFBI	Missense	NM_000358.3 c.G2006A:p.R669Q	0	3/6	VUS	Paternal
F08	MYOF	Missense	NM_013451.4 c.G2808C:p.Q936H	0.000008	4/6	VUS	Maternal
F09	WNT16	Frameshift	NM_016087.2 c.464dupG:p.C158Lfs*4	0.00067	NA	VUS	Maternal
F10	COL5A1	Missense	NM_000093.5 c.C4127T:p.S1376F	0	6/6	VUS	Paternal
F14	COL1A1	Missense	NM_000088.4 c.G4039A:p.D1347N	0.00003	5/6	VUS	Maternal

[a]maximum MAF in 1000G, ExAC and gnomAD;[b]The proportion of the variant predicted to be pathogenic or conserved in six tools including Mutation Taster, SIFT, Polyphen-2, CADD, REVEL and GERP++. Abbreviations: MAF, Minimum allele frequency; ACMG, American College of Medical Genetics and Genomics; VUS, Uncertain Significance.

corresponding posterior abnormalities in the "BAD Difference Elevation" map (red arrows in Figure 3D), suspicious total deviation score of the BAD map (2.05 and 2.29), suspicious TP (464 μm and 469 μm), and other abnormalities (back elevation at the corneal thinnest location, deviation of back elevation difference map, deviation of minimum thickness) in the BAD map of both eyes (Figure 3). The c.C4127T was defined as VUS according to ACMG standards. The c.4127T was cosegregated in this family in an autosomal dominant pattern with variable expression.

Additional verification of inheritance patterns: We ignored the subclinical phenotype and defined all asymptomatic parents as the "normal" phenotype for screening additional variants. Ultimately, there were no variants of candidate KC genes consistent with de novo inheritance, recessive inheritance, or complex heterozygous inheritance.

DISCUSSION

In this study, we aimed to investigate the potential candidate variants contributing to KC susceptibility by sequencing 14 KC trios with subclinical parents. Using the stringent filtering criteria, we identified 12 variants in 10 candidate KC genes, all of which were protein altered.

Among these variants, the c.C4127T:p.S1376F variant in the *COL5A1* gene exhibits strong pathogenicity in MAF and bioinformatics analysis. Extensive evidence suggests the involvement of the *COL5A1* gene, which encodes the alpha-1 chain of type V collagen in both KC and central corneal thickness (CCT) variation, such as genome-wide association studies (GWASs) and case-control WES studies. Several new related loci with genome-wide significance were identified [22]. CCT is a highly heritable endophenotype and a clinically important risk factor for KC. Rare pathogenic variants

of *COL5A1* were also identified in genetic screening of a large KC family [23]. The *COL5A1* gene is one of the most important pathogenic genes for Ehlers-Danlos syndrome (EDS), a complex connective tissue disease with joint hypermobility, high skin extensibility, and frequent bone defects. Both KC and corneal thinning have been frequently described in patients with EDS [24]. In family F10, we identified a variant c.C4127T in the *COL5A1* gene of the proband and her subclinical father but not in the mother with a normal phenotype. Notably, the subclinical father's corneal thickness was less

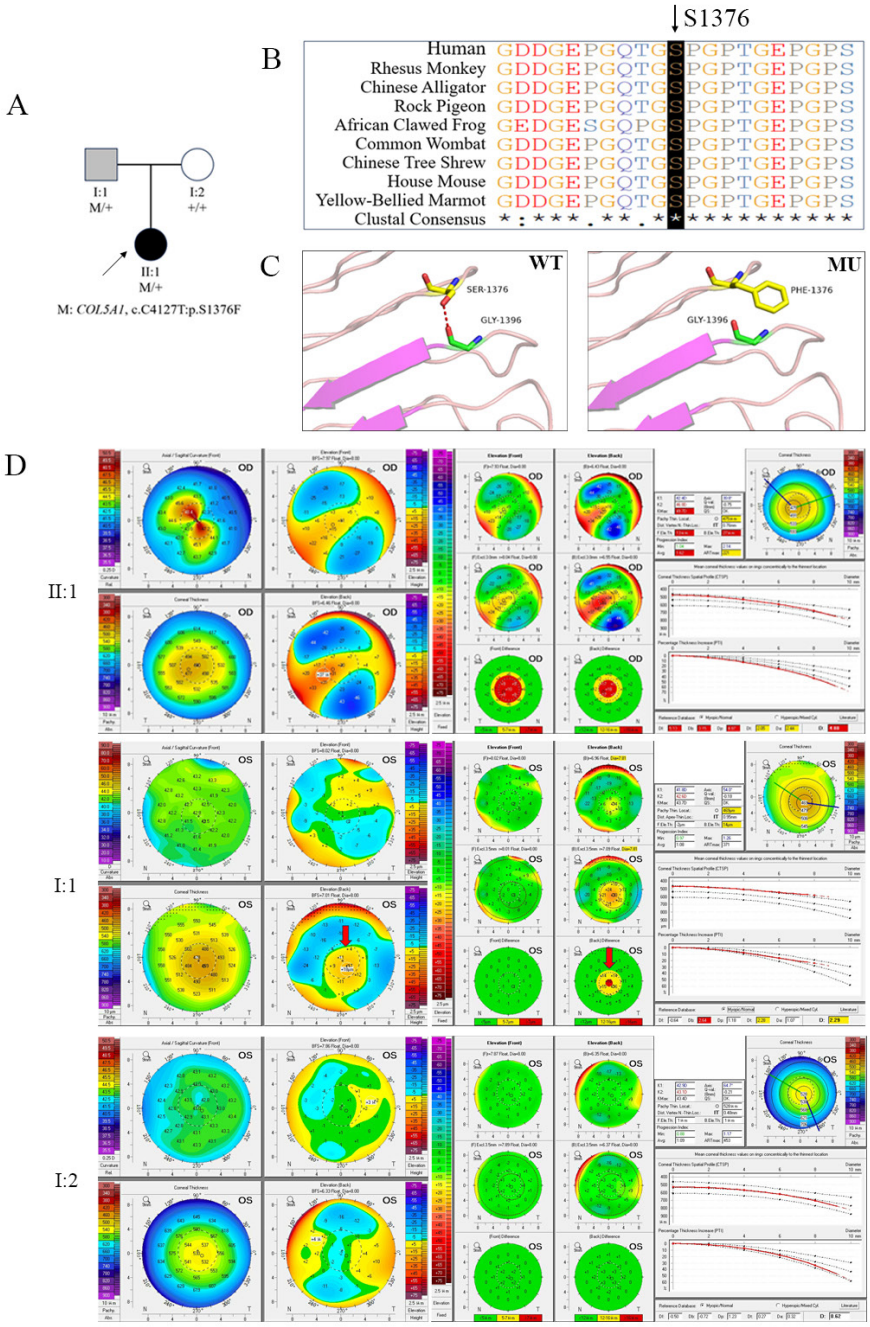

Figure 3. Analysis of the c.C4127T:p.S1376F variant in the *COL5A1* gene in family F10. **A**: Pedigree charts. The black symbol with an arrow indicates a KC proband, the white symbol indicates an unaffected parent, and the gray symbol indicates a subclinical KC parent. M, mutant allele; +, normal allele. **B**: Conservation of site S1376 for various species. C: Predicted three-dimensional structure of proteins. MU, mutant; WT, wild-type. (D) Refractive map and BAD map of the worse eye in F10 members.

than 480 μm in both eyes, indicating a suspicious anomaly that deviates from the population average. Combined with bioinformatics analysis, the *COL5A1* variant is considered a potential genetic cause through autosomal dominant inheritance patterns with variable expression.

The transforming growth factor β (TGFβ)–induced protein, encoded by the *TGFBI* gene, is an extracellular protein that mediates cell adhesion to collagen, fibronectin, and other proteins, with expression changes triggered by activation of the TGFβ signaling pathway [25]. A previous study showed that *TGFBI* expression was upregulated in KC corneas [26]. Recently, several family-based studies, including our previous study, had identified many potential variants of the *TGFBI* gene in Chinese and Greek patients with KC [10,27]. Calpastatin, encoded by the *CAST* gene, affects cellular apoptosis, proliferation, and migration. A recent study identified the potential gene–environment interactions between the *CAST* gene and eye rubbing in patients with KC [28]. Several studies have reported that *MPDZ* is involved in the genetic regulation of CCT, corneal hysteresis, and corneal resistance factor [8]. The variants in the *MPDZ* gene have been identified in several Chinese studies [29,30]. *WNT10A* encodes a specific Wnt ligand that is reduced at messenger RNA and protein levels in the KC epithelium and Bowman's layer [31]. The *WNT10A* exon variant has been reported to increase the risk of keratoconus by reducing corneal thickness [32]. A previous study found that the expression of the *MYOF* gene was downregulated in KC corneas [33]. The *MYOF* variants have been identified in the Jordanian and Chinese populations [34,35]. *ERMP1* is a zinc-binding protease that may play a role in resistance to oxidative stress and is involved in the pathogenesis of KC [36]. A previous genome-wide association study showed that *MAP3K19* is associated with KC [37]. *COL1A1* and *WNT16* are reported to be involved in the extracellular matrix signaling pathway, which has been identified to be involved in the pathogenesis of KC [38,39].

Among these genes, two candidate variants were identified in both *COL1A1* and *MYOF* genes in unrelated families, indicating a potential association with KC. We found two variants of different genes (*COL1A1* and *ERMP1*) in the F04 family. A frameshift variant c.464dupG:p.C158Lfs*4 was identified in the *WNT16* gene, but since the probability of being loss-of-function intolerant (pLi) score of the *WNT16* gene did not reach the threshold (0.9), the frameshift variant could not meet the PVS1 criteria in ACMG and was temporarily classified as a VUS variant. These variants require further functional verification to determine pathogenicity.

The prevalence of KC and the rate of subclinical corneal abnormalities in first-degree relatives of patients with keratoconus were higher than in normal controls, indicating that they were a population at high risk for developing KC. In this study, all parents carrying variants of candidate genes in the 14 families met the criteria for a subclinical KC diagnosis. In addition, most of the eyes of the parents with subclinical KC happened to be in the critical area between the patient group and the control group on the PCA plot (Figure 2), supporting the diagnosis of subclinical KC. Since reduced penetrance and variable expression of KC in combination with a low detection rate of KC pathogenic genes have been reported in some studies, we suspect that neglecting subclinical phenotypes may reduce the genetic detection rate. For example, if subclinical individuals in this study were defined not as affected individuals but as individuals with a normal phenotype, we would be missing these identified candidate variants.

This study has several limitations. First, this study included 14 trios to explore the genetic spectrum of KC, and the sample size needs to be increased in the future. Second, although the subclinical definition criteria in this study are relatively strict, there is currently no globally accepted standard for the diagnosis of subclinical KC. Finally, the functional and expression verification of these candidate genes was not investigated in the present study. Further studies are needed to investigate the association of these genes with KC.

In summary, we performed trio-based WES of 14 KC trios with subclinical parents. We found that 12 variants in candidate genes may cause KC through an autosomal dominant pattern with variable expression. This study indicates that genetic testing may play an important role in identifying patients with latent keratoconus and those at high risk of corneal ectasia after refractive surgery.

APPENDIX 1. SUPPLEMENTARY TABLE 1.

To access the data, click or select the words "Appendix 1." Candidate genes for keratoconus.

APPENDIX 2. SUPPLEMENTARY FIGURE 1.

To access the data, click or select the words "Appendix 2." Clinical data of all 14 families. (Top left) Pedigree charts. The black symbol with arrow indicates a KC proband, the white symbol indicates an unaffected parent, and the gray symbol with red dotted box indicates a subclinical KC parent. (Right and bottom left) Refractive map and BAD map of the worse eye in subclinical members.

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