Insufficient Dose of ERCC8 Protein Caused by a Frameshift Mutation Is Associated With Keratoconus With Congenital Cataracts

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Purpose. The purpose of this study was to identify a new candidate gene for keratoconus and congenital cataracts and further investigate its underlying pathogenic mechanisms.

Methods. This study, using a Chinese family with keratoconus and congenital cataracts, 262 patients with sporadic keratoconus, and 20 patients with sporadic congenital cataract as subjects, used clinical and genetic analysis and in vitro cell experiments to detect genetic mutations and further investigate the underlying pathogenic mechanisms.

Results. We found that a novel frameshift mutation of *ERCC8* (NM_000082.3: c.394-398del, p. L132Nfs*6) is responsible for familial keratoconus with congenital cataracts. This mutation showed co-segregation with the phenotype in the family. This was revealed in another patient with sporadic keratoconus, absent in the 210 unrelated health controls, and considered to be "disease-causing." *ERCC8* was expressed both in the cornea and lens. Through an in vitro cell experiment, we further demonstrated that the mutant proteins of *ERCC8* were degraded and could lead to an insufficient dose of the ERCC8 protein. An insufficient dose reduced the DNA damage repair ability of human corneal fibroblast (HTK) and lens epithelial cells (HLEC) treated with hydrogen peroxide, leading to both cells showing higher DNA damage levels. In addition, it decreased cell viability, resulting in decreased collagen expression in HTK and increased apoptosis in HLEC via aberrant activation of the unfolded protein response. All these results suggested that *ERCC8* plays an important role in the normal function of corneal stromal and lens epithelial cells.

Conclusions. Our study showed that *ERCC8* is a new gene associated with keratoconus and congenital cataracts.

Keywords: keratoconus, mutation, *ERCC8*, DNA damage repair, insufficient dose

背景. 圆锥角膜(KC)是一种与遗传相关的进行性角膜扩张性疾病。由于该病的遗传异质性, 很多病人的遗传病因仍不明确。

方法. 本研究以一个圆锥角膜伴先天性白内障的家系、20例散发性先天性白内障患者及262例散发性圆锥角膜患者为研究对象, 采用临床和遗传学分析检测KC及先天性白内障新的候选基因突变, 并通过体外细胞实验对其进行功能研究, 进一步探讨候选基因突变的潜在发病机制。

结果. 我们发现ERCC8基因的一个新的移码突变(NM_000082.3:c.394-398del, p.L132Nfs*6)与家族性圆锥角膜伴先天性白内障有关。该突变与该家族的表型共分离, 且在210名无关健康对照中未发现该突变。据预测, 该突变会改变在不同物种中都高度保守的氨基酸, 功能预测被认为是"致病的"。262名散发KC患者的筛查显示, 该突变在中国KC患者中的突变频率为0.4%(1/262)。ERCC8在角膜中高表达, 提示其在角膜中的重要作用。体外细胞实验表明, ERCC8 p.L132Nfs*6突变蛋白被降解, 这可能导致ERCC8蛋白剂量不足。ERCC8剂量不足会降低经H_2O_2处理的人角膜成纤维细胞DNA损伤修复能力, 导致细胞活力降低, 和胶原蛋白表达降低, DNA损伤和炎症水平升高, 从而影响角膜基质细胞的正常功能。这些变化可能在KC角膜的基质变薄中起重要作用。ERCC8剂量不足同样会降低经H_2O_2处理的人晶状体上皮细胞DNA损伤修复能力, 导致细胞活力降低, 并通过非折叠蛋白反应的异常激活增加晶状体上皮细胞的凋亡水平。所有这些结果表明, ERCC8在角膜基质和晶状体上皮细胞的正常功能中起着重要作用。

结论. 我们的研究表明ERCC8是一个与KC及白内障都相关的新基因, 功能实验支持了该蛋白在人类角膜及晶状体中具有额外功能的证据, 为圆锥角膜及先天性白内障的诊断和治疗了提供新的靶点和依据。

Keratoconus (KC) is a progressive corneal ectatic disorder characterized by corneal ectasia and thinning, resulting in reduced vision, irregular astigmatism, and corneal scarring, eventually necessitating corneal transplantation.[1] The worldwide prevalence of KC is approximately 1:2000.[2] The onset of KC usually occurs during adolescence, then progresses with age and has a lifelong effect on patients.[3] KC has a clear genetic tendency, and genetic factors play a critical role in its pathogenesis.[4,5] Recently, the use of high-throughput omics techniques has promoted the advancement of KC genetics. Until now, more than 80 KC-associated genes or regions have been identified by linkage analysis, genomewide association studies (GWAS), whole exome or genome sequencing (WES or WGS), or candidate gene association studies.[6] However, owing to the genetic heterogeneity among patients with KC in different populations, the genetic etiology of most cases has not been effectively identified, and new candidate genes need to be explored.

High-quality families represent extremely good material for genetic disease research, can often bring very valuable insights to genetic etiology research, and can lead to a breakthrough in the identification of pathogenic genes in many human genetic diseases.[7–9] A high-quality KC pedigree is very rare, and most patients with KC are sporadic.[2,4,5] Nevertheless, some inherited diseases are complicated by KC symptoms, such as brittle cornea syndrome,[10] Wolfram syndrome,[11] Leber's congenital amaurosis,[12] and early-onset anterior polar cataracts,[13] among others. Some well-known KC candidate genes, such as *ZNF469*, *miR-184*, *TGFBI*, and *ZEB1*, have been identified in families with these related diseases in the past, bringing valuable insights into KC genetic etiology.[6,13–18]

This study collected a pedigree with reports of KC made complicated by congenital cataracts, which provided valuable samples for the study of the genetic mechanism of KC. The WES analysis of patients is an effective strategy for identifying candidate pathogenic gene mutations. The screening of candidate gene mutations in more than 200 patients with KC and healthy controls was conducted to further determine the pathogenicity of candidate gene mutations. In vitro cell experiments were performed to study the functional changes and pathogenic mechanisms of candidate gene mutations. Finally, the findings of this study identified a new disease gene (*ERCC8*) underlying KC and congenital cataracts and provided insight into the insufficient dose of the ERCC8 protein in human corneal degeneration and lens cell apoptosis. These results will contribute to making accurate diagnoses and will provide new targets for the treatment of KC and congenital cataracts.

Methods

Subject Recruitment and Clinical Examination

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Shandong Eye Hospital (Jinan, China). A Chinese family with KC with congenital cataracts, 262 additional patients with sporadic KC, 20 patients with sporadic congenital cataract, and 210 unrelated healthy controls were recruited from this hospital. Written informed consent was obtained from all participants (or guardians). A Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract genomic DNA.

WES and Data Analysis

We conducted WES in the proband (I-2) of the family and her son (II-1) to identify the causal gene. Agilent SureSelect Human All Exon V6 (Agilent, Santa Clara, CA, USA) was used for exome capture. The IlluminaHiseq 2500 platform (Illumina Inc., San Diego, CA, USA) was used for sequencing. We then conducted data analysis and filtering according to the filtering strategy described in a previous study.[19] The variants were classified according to the American College of Medical Genetics and Genomics (ACMG) variant-classification guidelines. Finally, the splice, non-synonymy, termination, and frameshift variants occurring in exons or located in canonical splicing sites, with a minor allele frequency <1% in database-single-nucleotide polymorphism (dbSNP), HapMap, and the 1000 Genomes Project databases and damaging functional prediction, which were classified as pathogenic or likely pathogenic and co-segregated with the phenotype in this family, were considered to be candidate causal variations. The variants in the genes reported to be associated with KC or cataracts have the priority of verification.

ERCC8 Sequencing, Genotyping, and Protein Structure Prediction

The novel frameshift mutation of *ERCC8* was genotyped in 262 additional patients with sporadic KC and 210 unrelated healthy controls using high-resolution melt (HRM) analysis, as described in a previous study.[19] Sanger sequencing of *ERCC8* was performed to confirm the results obtained using WES and HRM. Sanger sequencing of *ERCC8* was also performed in 20 unrelated patients with congenital cataract for further cataract-related *ERCC8* variant detection. The primers used for sequencing and genotyping the mutation of *ERCC8* are shown in Supplementary Table S1.

Furthermore, MEGA software was used to perform multiple protein sequence alignments among various species.[20] MutationTaster (http://www.mutationtaster.org/) was applied to test the possible effect of amino acid substitution on protein function.[21] The tertiary structure of mutant proteins was predicted using HOPE online software (http://www.cmbi.umcn.nl/hope).[22]

ERCC8 Expression Assays in Mouse Eyes

ERCC8 protein immunofluorescence staining was performed on mouse eyeballs, as described in prior research,[23] and then

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examined under a Nikon DS-Ri2 microscope (Nikon, Tokyo, Japan). Total RNA was extracted from the heart, liver, lungs, cornea, lens, conjunctiva, and retina tissues of a mouse. PrimeScript RT Reagent Kit (Perfect Real Time) was used to synthesize cDNA from RNA. The expression of the *ERCC8* gene was measured by quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Mutant *ERCC8* Constructs and Expression in Human Embryonic Kidney 293 Cells

The human embryonic kidney 293 (HEK293) cell line was cultured in a DMEM medium (Corning, Corning, NY, USA) containing 10% fetal bovine serum (FBS; ExCell Bio, China) at 37°C with 5% CO2. The wild type and c.394-398del (p.L132Nfs*6) CDS sequences of the *ERCC8* gene were synthesized, subcloned into the pIRES2-EGFP expression vector, and then transfected in 6-well plates using Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. Transfection efficiency was judged by the fluorescence intensity of green fluorescent protein (GFP) after transfection for 48 hours. The cells were then collected to measure the mRNA and protein expression levels of *ERCC8* and genes related to transcription-coupled DNA repair (TCR; *CSB*, *XPA*, *RPA,* and *XPG*), respectively.

Knockdown of *ERCC8* in Human Corneal Fibroblast and Lens Epithelial Cells

The human corneal fibroblast cell line (HTK) and human lens epithelial cells (HLECs) were cultured, as described before.[19,24] Specific small interfering RNA (*ERCC8* siRNA) was designed and synthesized based on the *ERCC8* CDS sequence; it was then transfected in 6-well plates using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. After 48 hours of transfection, the cells were collected to measure the mRNA and protein expression levels of *ERCC8*, TCR, and matrix metalloproteinase, or collagen IV, SOD1, respectively.

To explore the effect of insufficient ERCC8 dosing on the DNA damage repairs of HTK and HLECs, we knocked down *ERCC8* in DNA damage cells induced by hydrogen peroxide (H_2O_2). *ERCC8* siRNA was transfected in 6-well or 96-well plates, as described. After 24 hours of transfection, the cells were treated with H_2O_2 (200 µmol/L) and then collected at 48 hours for subsequent cell viability, apoptosis, and DNA damage and repair-related mRNA and protein level tests.

Cell Viability, Apoptosis, and DNA Damage Assays

After treatment with H_2O_2 for 24 hours, cell viability assays of the cells transfected with *ERCC8* siRNA and negative controls (NCs) were performed using a Cell Counting Kit-8 (Beyotime, China) according to the manufacturer's protocol. The Hoechst Staining Kit (Beyotime, China) was used to stain the cell nuclear morphism. An Annexin V-FITC Apoptosis Detection Kit (Yeasen, China) was used to detect cell apoptosis levels. The unfolded protein response (UPR)-related genes' (*HSPA5*, *DDIT3*, and *ERN1*) mRNA expression levels were measured by qRT-PCR. The expression levels of γ- H2AX (a DNA damage marker) were measured with a Western blot.

mRNA and Protein Expression Levels Measured

Related gene mRNA expression levels in the cell samples were measured with qRT-PCR, as described previously. The primer sequences of the genes used for qRT-PCR are shown in Supplementary Table S1. Related protein expression levels in cell samples were detected by Western blot analyses.[19] Primary antibodies included those of collagen IV (ab6586; Abcam), ERCC8 (sc-376981; Santa Cruz), SOD1 (MABC684; Millipore), γ-H2AX (AP0099; Abclonal), NF-kB p65 (phosphor S536, ab76302; Abcam), MMP1 (ab52631; Abcam), Caspase 3 (D320074; Sangon), and GAPDH (KC-5G5; Kangchen, Shanghai, China).

Results

Clinical Features

The clinical features of the patients in the family are shown in Table 1. The proband (I-2), aged 26 years old, had been diagnosed with congenital cataracts since childhood and had undergone binocular cataract extraction 20 years previously. Seven years ago, her right eye (OD) showed progressive protrusion. She came to the hospital for medical treatment and was diagnosed with KC. Her right eye had nystagmus, corneal ectasia in the central cornea, thinning, scarring in the superficial medium matrix, and a cone-shaped protrusion with Fleischer's ring. The vision of the proband right eye (OD) was 0.05 and could not be corrected. The central corneal thickness of the right eye was 196 µm, and the corneal curvature was 82.75, which were typical KC characteristics (see Table 1).

The clinical examination of her family members showed that her son (II-1) was also a congenital patient with cataract with KC. Lens opacity had been seen in both eyes since birth, and he was diagnosed with congenital cataracts. At the age of 9 years, both of the II-1's eyes showed abnormal corneal curvature (Ks >49), astigmatism, and uncorrected poor vision, showing the subclinical manifestation of KC (see Table 1).

Table 1. Clinical Features of the Patients in the Family With Keratoconus and Congenital Cataract

								Cornea			
Sample	**Gender**	**Age***	**Eyes**	**Lens**	**IOP**	**SPH**	**CDVA**	**CYL**	**Kf**	**Ks**	**TCT**
I-2	F	26	OD	Congenital cataract	20 mm Hg	ND	0.05	ND	>87.25 D	>87.25 D	196 µm
			OS	Congenital cataract	19 mm Hg	ND	0.10	ND	45.75 D	49.50 D	ND
II-1	M	9	OD	Congenital cataract	16 mm Hg	−1.00	0.15	−3.00	47.27 D	49.93 D	ND
			OS	Congenital cataract	16 mm Hg	+1.75	0.30	−2.75	46.55 D	49.63 D	ND

Notes: IOP, intraocular pressure; SPH, spherical equivalent; CDVA, corrected distance visual acuity; CYL, corneal astigmatism; Kf, flat keratometry; Ks, steep keratometry; TCT, thinnest corneal thickness; ND, no data.

*Age at admission.

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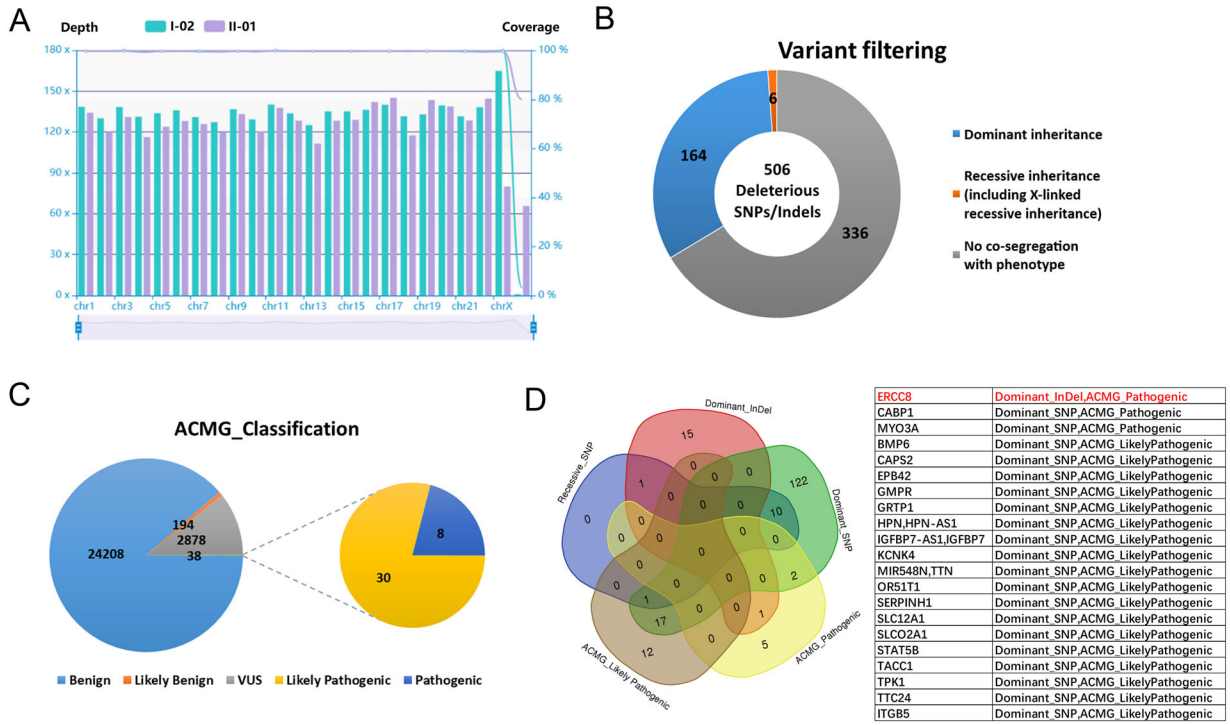

ERCC8	Dominant_InDel,ACMG_Pathogenic
CABP1	Dominant_SNP,ACMG_Pathogenic
MYO3A	Dominant_SNP,ACMG_Pathogenic
BMP6	Dominant_SNP,ACMG_LikelyPathogenic
CAPS2	Dominant_SNP,ACMG_LikelyPathogenic
EPB42	Dominant_SNP,ACMG_LikelyPathogenic
GMPR	Dominant_SNP,ACMG_LikelyPathogenic
GRTP1	Dominant_SNP,ACMG_LikelyPathogenic
HPN,HPN-AS1	Dominant_SNP,ACMG_LikelyPathogenic
IGFBP7-AS1,IGFBP7	Dominant_SNP,ACMG_LikelyPathogenic
KCNK4	Dominant_SNP,ACMG_LikelyPathogenic
MIR548N,TTN	Dominant_SNP,ACMG_LikelyPathogenic
OR51T1	Dominant_SNP,ACMG_LikelyPathogenic
SERPINH1	Dominant_SNP,ACMG_LikelyPathogenic
SLC12A1	Dominant_SNP,ACMG_LikelyPathogenic
SLCO2A1	Dominant_SNP,ACMG_LikelyPathogenic
STAT5B	Dominant_SNP,ACMG_LikelyPathogenic
TACC1	Dominant_SNP,ACMG_LikelyPathogenic
TPK1	Dominant_SNP,ACMG_LikelyPathogenic
TTC24	Dominant_SNP,ACMG_LikelyPathogenic
ITGB5	Dominant_SNP,ACMG_LikelyPathogenic

Figure 1. Whole exome sequencing (WES) analysis results of patients in a family with keratoconus and congenital cataracts. (**A**) The sequencing depth and coverage of patients. (**B**) Variant filtering results. (**C**) American College of Medical Genetics and Genomics (ACMG) variant-classification results. (**D**) Twenty-one pathogenic or likely pathogenic variants co-segregated with the phenotype.

WES Analysis Detects a Frameshift Variant in *ERCC8*

We conducted a WES analysis of the proband and her son to find candidate gene mutations in this family. The average sequencing depth of the WES was 131.00X, and the average coverage was 99.55% (Fig. 1A). With the data analysis and filtering strategy described in the Materials and Methods section, 170 genes with deleterious SNPs/InDels co-segregated with the phenotype of this family were identified, in which 164 genes conform to dominant inheritance, and 6 genes conform to recessive inheritance (Fig. 1B). According to ACMG variant classification guidelines, 38 variants were classified as pathogenic or likely pathogenic (Fig. 1C). Finally, 21 pathogenic or likely pathogenic variants co-segregated with the phenotype in this family were identified (Fig. 1D). Specifically, the *ERCC8* gene with the dominant pathogenic frameshift variant has been reported to be associated with congenital cataracts complicated by Cockayne syndrome before.[25,26] No pathogenic or likely pathogenic mutations of other reported candidate genes for cataracts[27,28] and KC[6] were detected.

The heterozygous frameshift variant in *ERCC8* (NM_000082.3: c.394-398del, p. L132Nfs*6) was confirmed with Sanger sequencing. The results showed that the affected members had the heterozygous frameshift variant of *ERCC8*, whereas the normal member did not (Figs. 2A, 2B). It showed co-segregation with the phenotype of this family. This variant was predicted to be disease-causing by the MutationTaster and was absent from the 1000 Genomes Project databases. The p. L132Nfs*6 change was located in a highly conserved domain (Figs. 2C, 2D) and led to a truncated protein with many protein feature losses (Fig. 2E), which is very likely to affect the normal function of *ERCC8*. These findings suggested that it was a candidate mutation.

Additional Patients With Sporadic KC With c.394-398del Mutation

To further verify its pathogenicity and examine the frequency of this frameshift mutation in KC and congenital cataracts, it was also genotyped in 262 additional patients with sporadic KC, 20 unrelated congenital patients with cataract, and 210 unrelated healthy controls; one additional patient with KC (KC5) with p. L132Nfs*6 was identified (see Fig. 2B). It was absent in 210 unrelated healthy controls (Table 2). In addition, another disease-causing rare variation (NM_000082.4:c.1080T>C) of *ERCC8* was detected in 6 unrelated patients with congenital cataract (see Table 2). These results supported the finding that p. L132Nfs*6 of *ERCC8* is associated with KC and congenital cataracts.

ERCC8 Was Expressed Both in the Corneal Stroma and Lens

To determine whether *ERCC8* was expressed in the cornea, lens, or other tissues of the eye, we performed ERCC8 protein immunofluorescence staining of mouse eyeballs. The results showed that *ERCC8* was highly expressed in the mouse corneal stroma (Fig. 3A). The qRT-PCR results of various tissues of the mouse confirmed that *ERCC8* was expressed the most in the cornea, followed by the conjunctiva, lens, lungs, retina, liver, and heart (Fig. 3B). These results suggested that *ERCC8* is involved in the normal function of the cornea and lens, and supported the finding that *ERCC8* is a disease-causing gene in this family.

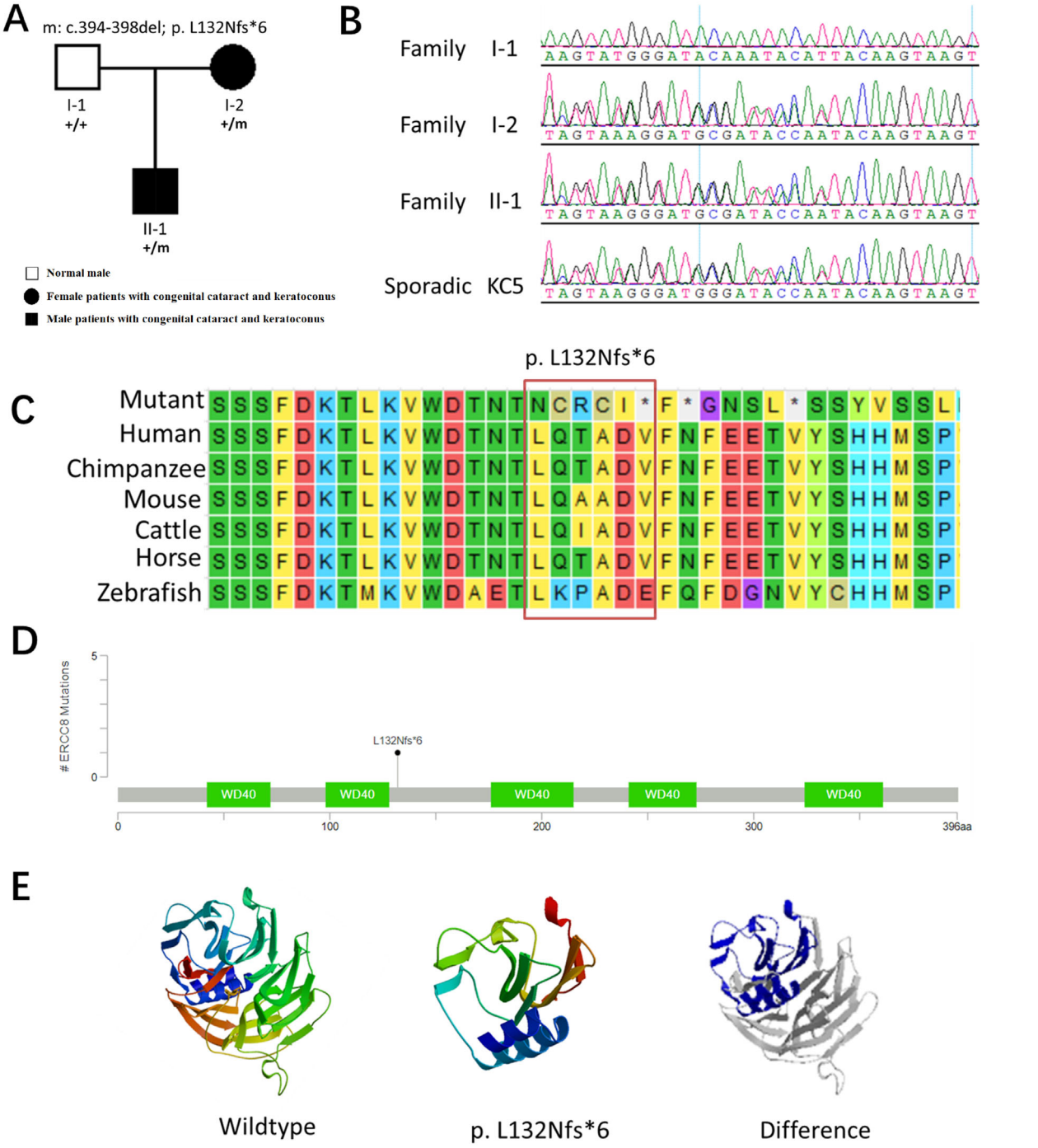

Figure 2. Pedigree and genetic mutation of the family with keratoconus and congenital cataracts. (**A**) Pedigree. (**B**) Sanger sequencing confirms the c.394-398del (p. L132Nfs*6) mutation identified by WES (I-2 and II-1) and HRM (KC5). (**C**) Cross-species comparison of the region of *ERCC8* indicates that the identified mutation affects highly conserved residues. (**D**) Domain analysis of the p. L132Nfs*6 mutation. (**E**) The 3D structures of mutant and wild type protein.

The c.394-398del (p. L132Nfs*6) Mutation Leads to an Insufficient Dose of ERCC8 Protein

To examine whether the identified frameshift mutation affected the expression levels of *ERCC8*, we constructed plasmids with the c.394-398del (p. L132Nfs*6) mutation expressed in HEK293 cells. Immunofluorescence results showed a similar transfection efficiency of the wild type and c.394-398del plasmids (Fig. 4A). Further, qRT-PCR results revealed that c.394-398del (p. L132Nfs*6)-expressing cells showed significantly reduced relative mRNA expression levels of *ERCC8* compared with wild type expressing cells (Fig. 4B). The Western blot (Figs. 4C, 4D) also revealed that wild type expressing cells detected significantly higher ERCC8 protein expressions, whereas c.394-398del (p. L132Nfs*6)-expressing cells showed a very weak signal of ERCC8, which is similar to that of cells that are not overexpressed. All these results suggested that the c.394-

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Table 2. Pathogenicity Assessment of ERCC8 Mutation

Nucleotide Change	Amino Acid Change	Predictions of Pathogenicity (Mutation Taster)	Frequency in KC Patient Alleles (Methods)	Frequency in Congenital Cataract Patient Alleles (Sanger Sequencing)	Frequency in Control Alleles (Methods)
NM_000082.4: c.394-398del	p. L132Nfs*6	Disease causing	2/263 (WES sequencing, Sanger sequencing and HRM)	0/20	0/200 (HRM)
NM_000082.4:c.1080T>C	p.Ala360=	Disease causing	ND	6/20	66/1008 (1000G- East Asian)

Notes: NA, not application; HRM, high-resolution melting analysis; 1000G, 1000 Genomes; ND, no data.

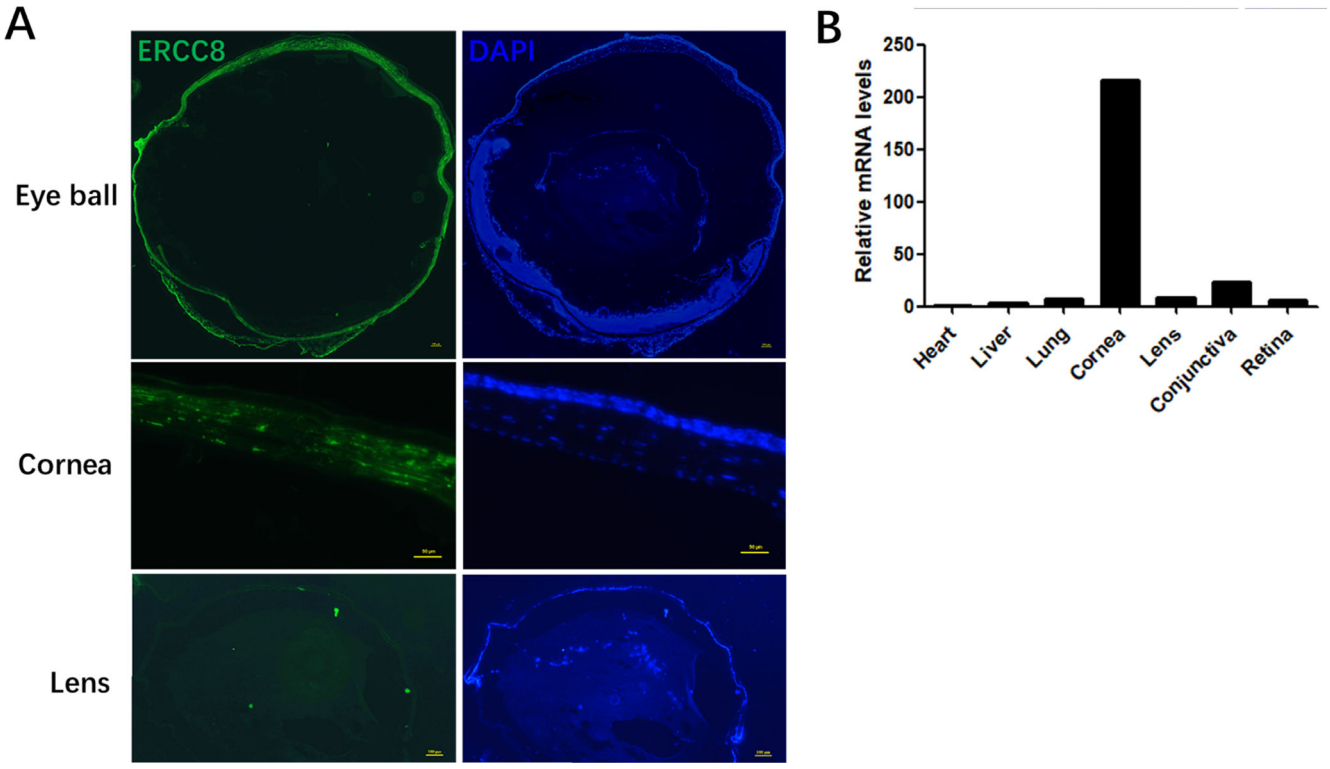

Figure 3. Expression of *ERCC8* in different mouse tissues. (**A**) Representative figures of immunofluorescence staining of ERCC8 in the mouse eye (the upper was a panoramic image, and the lower were local images). (**B**) The mRNA expression levels of *ERCC8* in different mouse tissues.

398del (p. L132Nfs*6) mutation leads to reduced mRNA expression levels and an insufficient dose of ERCC8 protein.

Insufficient Dose of ERCC8 Protein Leads to Abnormal Expressions of TCR Genes and Collagen IV in HTK Cells

To examine whether an insufficient dose of ERCC8 protein affected the expression of TCR-related genes and collagen protein in human corneal fibroblast cells (HTK), *ERCC8* siRNA was constructed and transfected into the cells. The results showed that *ERCC8* siRNA could significantly reduce the mRNA (Fig. 5A) and protein (Figs. 5C, 5D) levels of ERCC8. The *ERCC8* siRNA-transfected cells showed significantly decreased expression levels of *CSB* and *XPG* and increased expression levels of *XPA* (see Fig. 5B) compared with the NC cells, which suggested that the insufficient dose of ERCC8 protein caused by *ERCC8* siRNA affected transcription-coupled DNA repair in HTK. In addition, the collagen IV protein level decreased significantly after *ERCC8* siRNA transfer (see Figs. 5C, 5D), which suggested an effect of an insufficient dose of ERCC8 protein on the extracellular matrix. There was no significant difference in the expression of SOD1 between *ERCC8* siRNA transferred cells and NC cells (see Figs. 5C, 5D).

Insufficient Dose of ERCC8 Protein Results in the Reduced DNA Damage Repair Ability of HTK Treated by H_2O_2

To better simulate the DNA damage repair of HTK cells, we induced DNA damage by H_2O_2 in HTK cells after *ERCC8* siRNA transfection. The results showed that *ERCC8* siRNA-transfected cells showed significantly decreased cell viabil-

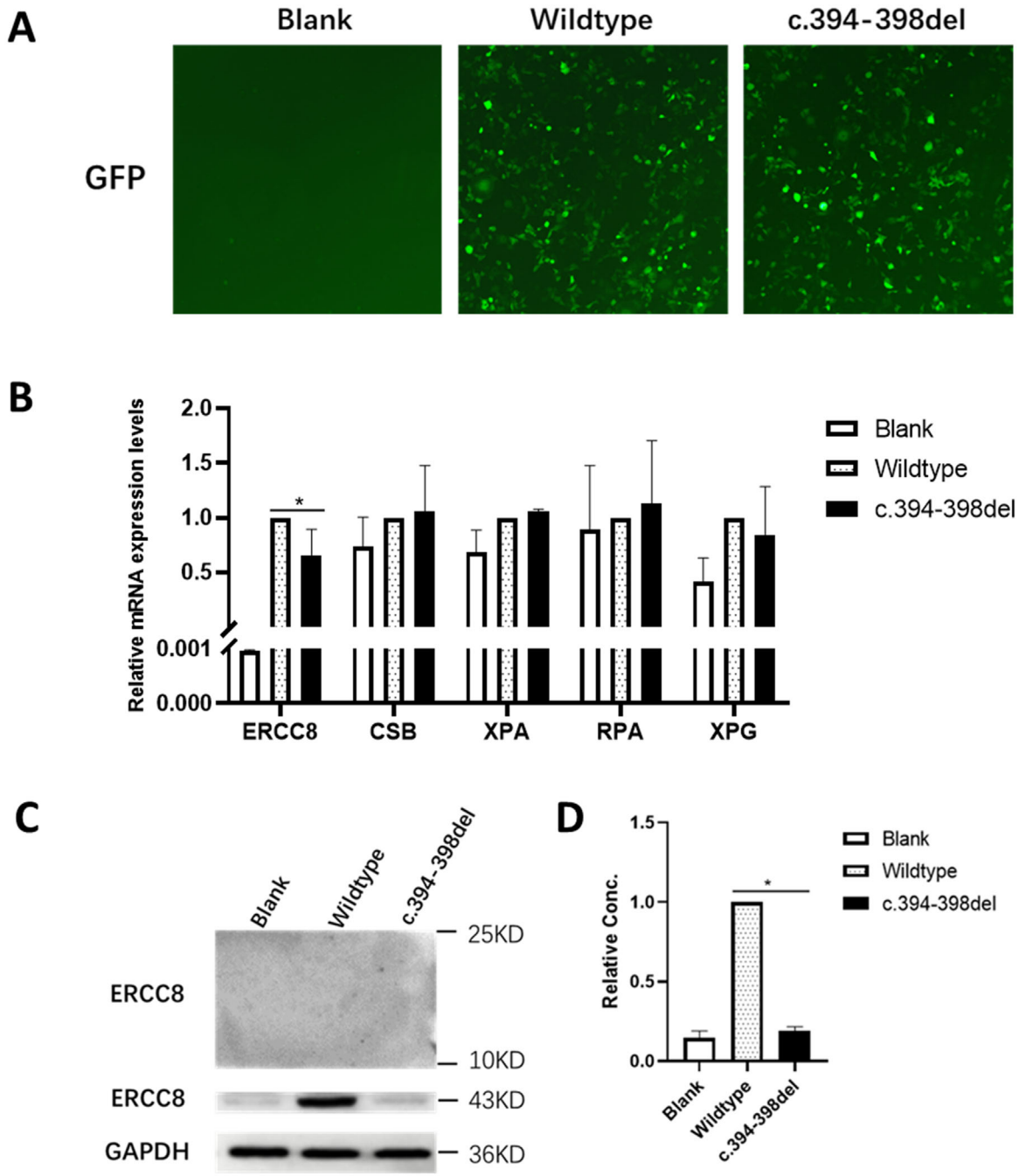

Figure 4. Mutant *ERCC8* constructs and expression in human embryonic kidney 293 cells. (**A**) The fluorescence intensity of GFP after transfection for 48 hours. (**B**) mRNA expression levels of *ERCC8* and TCR-related genes (*CSB*, *XPA*, *RPA*, and *XPG*) in overexpressed wild type and mutant cells. (**C**) Western blot results of ERCC8 protein in overexpressed wild type and mutant cells. (**D**) Statistical results of the Western blot; $N = 3$, *, $P < 0.05$.

ity after being treated with H_2O_2, compared with NC cells (Fig. 6A). The *ERCC8* siRNA-transfected cells showed more cells with a rupture in the nuclear membrane and content overflow, indicating more serious damage caused by H_2O_2 (Fig. 6B). The Western blot (Figs. 6C, 6D) also revealed that the expression levels of γ- H2AX (DNA damage marker), phosphor-p65 (inflammatory marker), and MMP1 (matrix metalloproteinase) were significantly higher in the *ERCC8* siRNA-transfected cells compared with the NC cells after H_2O_2 treatment, which suggested more DNA damage and higher cell inflammatory and matrix metalloproteinase levels in cells with an insufficient dose of ERCC8 protein.

Insufficient Dose of ERCC8 Protein Results in Reduced DNA Damage Repair Ability and Increased Apoptosis of HLECs

ERCC8 siRNA-transfected HLECs showed significantly decreased cell viability (Fig. 7A), abnormal TCR-related gene

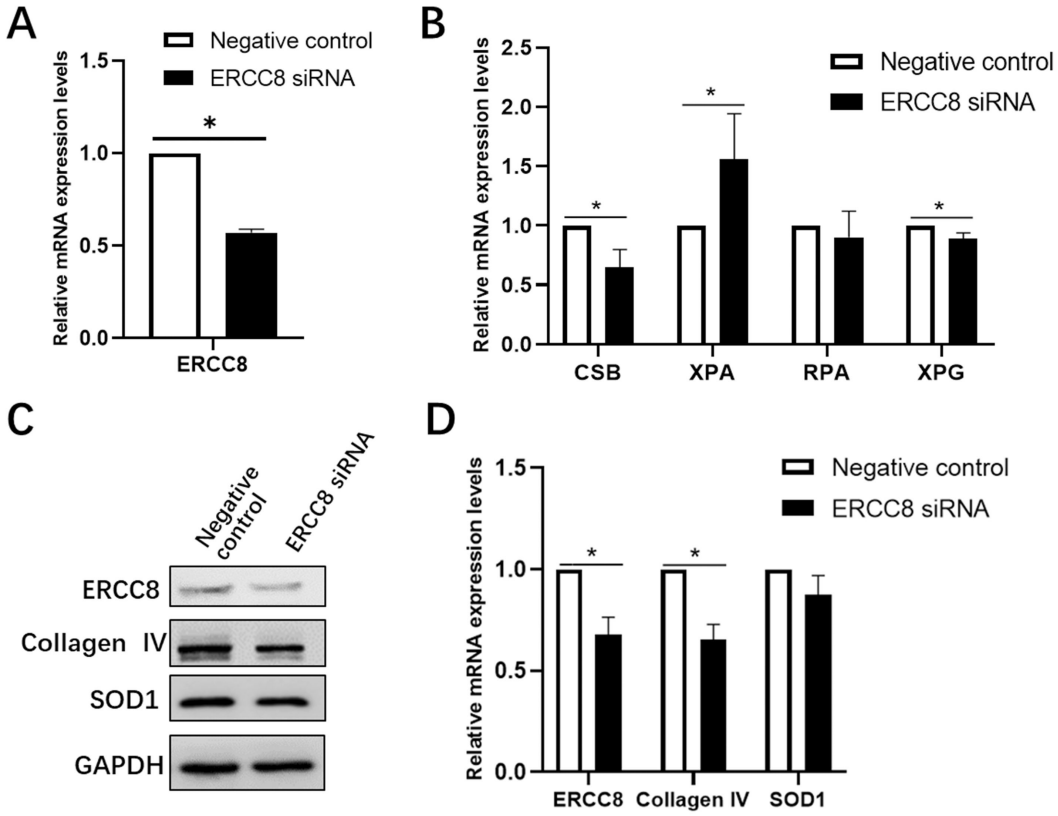

Figure 5. Knockdown of *ERCC8* in human corneal fibroblast cells. (**A**) The mRNA expression levels of *ERCC8* after being transfected by *ERCC8* siRNA. (**B**) The mRNA expression levels of TCR-related genes (*CSB*, *XPA*, *RPA*, and *XPG*) after being transfected by *ERCC8* siRNA. (**C**) Western blot results of ERCC8, collagen IV, and SOD1 proteins after being transfected by *ERCC8* siRNA. (**D**) Statistical results of the Western blot; $N = 3$, *, $P < 0.05$.

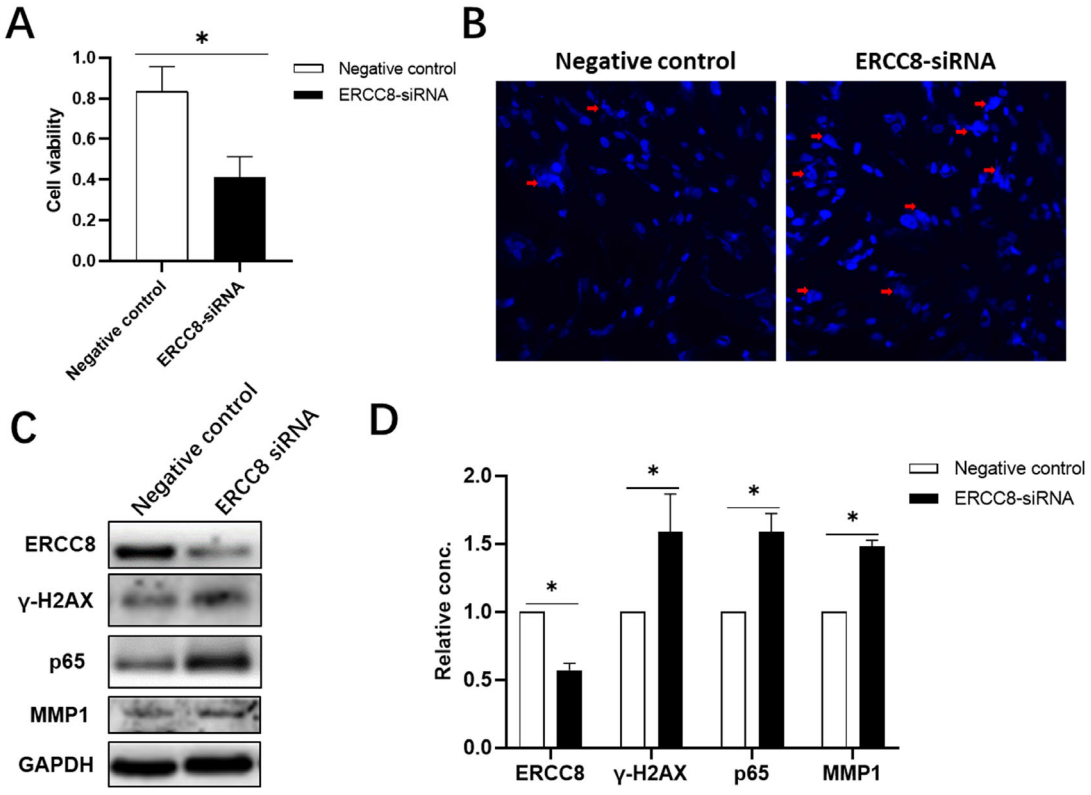

Figure 6. Cell viability and DNA damage assays of HTK treated by H_2O_2 after *ERCC8* siRNA transfection. (**A**) Cell viability assay results. (**B**) Hoechst staining results. (**C**) Western blot results of ERCC8, γ- H2AX, and phospho p65 protein. (**D**) Statistical results of the Western blot; $N = 3$, *, $P < 0.05$.

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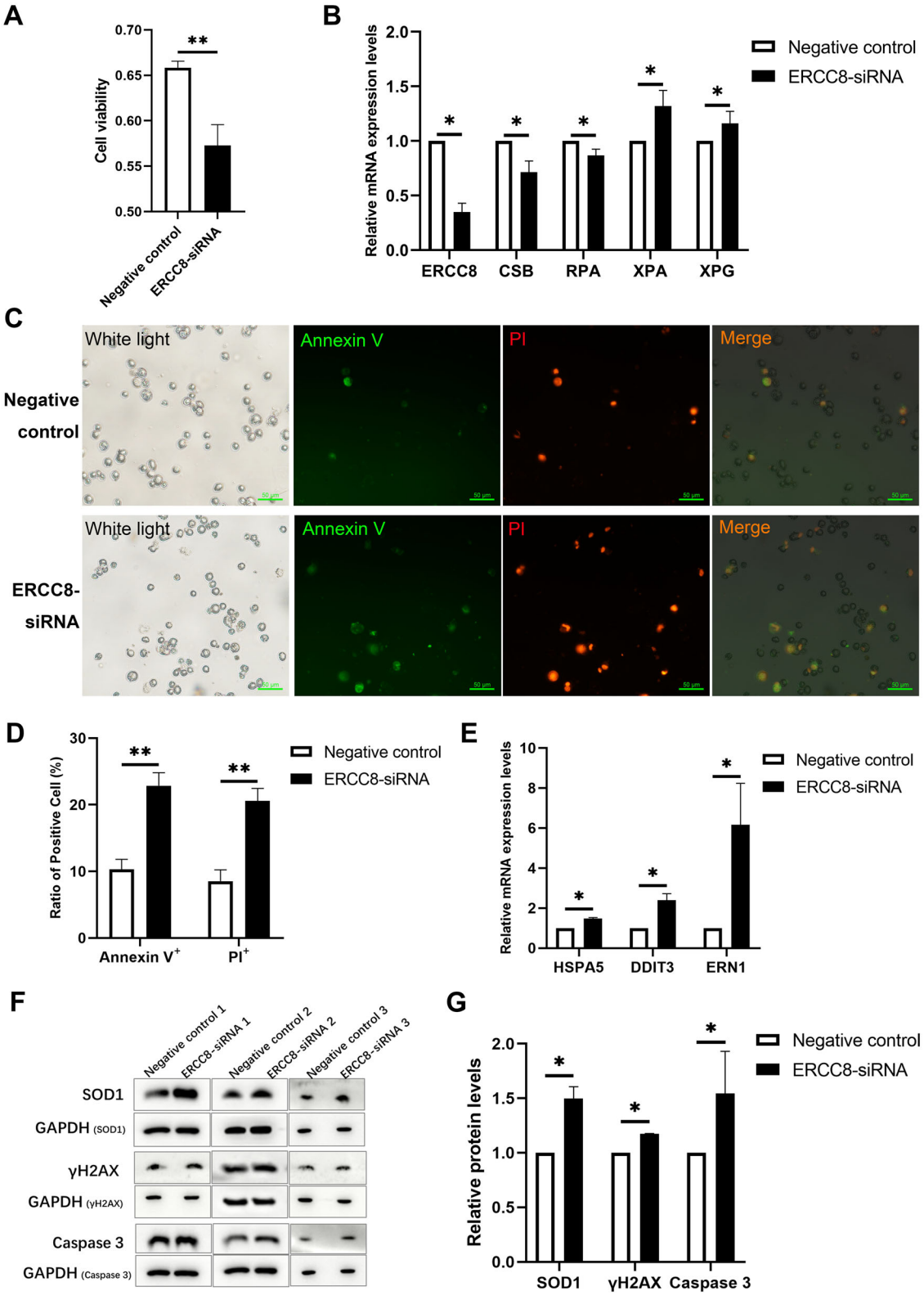

Figure 7. Cell viability, apoptosis, and DNA damage assays of HLECs treated by H_2O_2 after *ERCC8* siRNA transfection. (**A**) Cell viability assay results. (**B**) The mRNA expression levels of TCR-related genes. (**C**) Representative figures of immunofluorescence staining of cell apoptosis. (**D**) Statistical results of the cell apoptosis analysis. (**E**) The mRNA expression levels of UPR-related genes. (**F**) Western blot results of SOD1, γ- H2AX, and caspase 3 protein. (**D**) Statistical results of the Western blot; $N = 3$, *, $P < 0.05$.

expression (Fig. 7B), and significantly high cell apoptosis levels (Figs. 7C, 7D) after being treated with H_2O_2, compared with NC cells. The URP-related genes (*HSPA5*, *DDIT3*, and *ERN1*) mRNA expression levels (Fig. 7E) and caspase 3 (apoptotic protease) protein levels (Figs. 7F, 7G) were significantly increased in the *ERCC8* siRNA-transfected HLECs, indicating increased UPR-induced apoptosis caused by H_2O_2. The expression levels of SOD1, γ- H2AX were significantly higher in *ERCC8* siRNA-transfected cells, which suggested greater oxidation pressure and DNA damage levels in cells with an insufficient dose of ERCC8 protein.

Discussion

In this study, using whole exome sequencing of familial cataracts congenital with KC, and screening 262 unrelated KC and 20 unrelated patients with congenital cataract, we identified *ERCC8* as a novel gene linked to KC and congenital cataract. A novel frameshift mutation of *ERCC8* (c.394-398del, p. L132Nfs*6) was identified in the proband patient and her son from this family. This mutation showed cosegregation with phenotype in this family. It was not found in the 210 unrelated healthy controls and was predicted to alter highly conserved amino acids across the different species and considered to be disease-causing by a function prediction. A screening performed on 262 additional unrelated patients with KC revealed it in one patient. These findings support the idea that p. L132Nfs*6 of *ERCC8* is a disease-causing gene mutation in KC. In vitro functional studies provide initial evidence of its role in the pathogenesis of KC and congenital cataracts.

ERCC8 is located on 5q12.1 and encodes the DNA excision repair protein ERCC-8, a substrate recognition component of the CSA complex that is involved in transcription-coupled nucleotide excision repair.[29] This gene is expressed both in the cornea and lens, especially highly in the cornea, as shown in the immunofluorescence staining and qRT-PCR analysis results, which suggest that ERCC8 might play an important role in the maintenance of corneal and lens structure and function via DNA damage repair. The cornea directly exposed to air and light is more susceptible to exogenous oxidizing UV and blue light, which are common DNA damage inducers.[30] Previous studies observed increased mitochondrial DNA (mtDNA) damage and double-stranded DNA breaks in KC corneas compared with normal corneas.[31–33] DNA damage could induce a cell inflammatory response.[34] The accumulation of these DNA damage may lead to decreased cell activity and increased cell inflammation levels in the cornea and may result in the loss of normal corneal structure and function. Therefore, DNA damage repair is very important for the cornea.

The mutant overexpression experiments in this study showed that the c.394-398del (p. L132Nfs*6) mutation of *ERCC8* leads to reduced mRNA expression levels and an insufficient dose of the ERCC8 protein. Functional experiments further confirmed that an insufficient dose of the ERCC8 protein leads to decreased expression levels of CSB and XPG and increased expression levels of XPA in HTK. *CSB* interacts with several transcription and excision repair proteins and may promote complex formation at DNA repair sites.[35] XPG is a single-strand-specific DNA endonuclease that is responsible for making the 3′ incision in DNA excision repair.[36] XPA is a DNA damage recognition and repair factor and acts as a scaffold to assemble the nucleotide excision repair incision complex at sites of DNA damage.[37] The abnormal expression of these TCR genes might lead to the reduced DNA damage repair ability of cells.

The subsequent experimental results of the HTK DNA damage model again confirmed our inference. The results showed that there was more serious DNA damage in *ERCC8* siRNA-transfected HTK cells than in control cells after H_2O_2 treatment. The insufficient dose of ERCC8 protein results in a reduced DNA damage repair ability of HTK and then leads to decreased cell viability and increased cell inflammation levels of HTK. Inflammation is known to be involved in the pathogenesis of KC, and several studies have reported significantly increased levels of inflammatory markers in KC cells, such as NF-κB, IL-1α, and TNF-α.[38,39] Moreover, an insufficient dose of the ERCC8 protein also leads to abnormal expressions of collagens and matrix metalloproteinase, which suggests an effect of an insufficient dose of the ERCC8 protein on the extracellular matrix. Collagens are the main component of the corneal stroma, and matrix metalloproteinases are responsible for proteolytic phenomena. These changes might play an important role in stromal thinning, which is characteristic of KC corneas. Therefore, these functional and cellular changes may affect the normal structure and physiological function of the cornea and play an important role in the pathogenesis of KC.

At present, there are no reports of KC or other corneal diseases related to *ERCC8*. However, some patients with congenital cataract condition is complicated by Cockayne syndrome, and they have been reported to be associated with *ERCC8* mutations.[25,26] *ERCC8*, also known as *CSA*, is a well-known candidate gene for Cockayne syndrome (OMIM: 216400) and UV-sensitive syndrome 2 (OMIM: 614621) with autosomal recessive inheritance. Some patients with Cockayne syndrome had ocular manifestations of congenital cataract.[25,26] Our familial patients, only harboring heterozygous mutations of *ERCC8*, did not show other known symptoms of Cockayne syndrome except for congenital cataracts. Identification of another disease-causing rare variation (c.1080T>C) of *ERCC8* in 6 unrelated patients with congenital cataract supports that *ERCC8* is associated with congenital cataracts. The experimental results of the DNA damage model also showed that the insufficient dose of ERCC8 protein results in the reduced DNA damage repair ability of HLECs. This then leads to decreased cell viability and increased cell apoptosis levels in HLECs via aberrant activation of the unfolded protein response. An unfolded protein response is known to be involved in the pathogenesis of cataract, and several studies have reported that it is involved in cataract formation.[40–43] Cockayne syndrome-associated CSA mutations were reported to impair protein folding before.[26] All these findings support the idea that these functional and cellular changes caused by an insufficient dose of ERCC8 protein play an important role in the pathogenesis of cataracts.

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