

TSC1 Mutations in Keratoconus Patients With or Without Tuberos Sclerosis

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PURPOSE. To test candidate genes *TSC1* and *TSC2* in a family affected by tuberos sclerosis complex (TSC) where proband was also diagnosed with bilateral keratoconus (KC) and to test the hypothesis that defects in the same gene may lead to a nonsyndromic KC.

METHODS. Next-generation sequencing of *TSC1* and *TSC2* genes was performed in a proband affected by TSC and KC. Identified mutation was confirmed by Sanger DNA sequencing. Whole exome sequencing (WES) was performed in patients with nonsyndromic KC. Sanger DNA sequencing was used to confirm WES results and to screen additional patients. RT-PCR was used to investigate *TSC1* expression in seven normal human corneas and eight corneas from patients with KC. Various in silico tools were employed to model functional consequences of identified mutations.

RESULTS. A heterozygous nonsense *TSC1* mutation g.132902703C>T (c.2293C>T, p.Gln765Ter) was identified in a patient with TSC and KC. Two heterozygous missense *TSC1* variants g.132896322A>T (c.3408A>T, p.Asp1136Glu) and g.132896452G>A (c.3278G>A, p.Arg1093Gln) were identified in three patients with nonsyndromic KC. Two mutations were not present in The Genome Aggregation (GnomAD), The Exome Aggregation (ExAC), and 1000 Genomes (1000G) databases, while the third one was present in GnomAD and 1000G with minor allele frequencies (MAF) of 0.00001 and 0.0002, respectively. We found *TSC1* expressed in normal corneas and KC corneas, albeit with various levels.

CONCLUSIONS. Here for the first time we found *TSC1* gene to be involved in bilateral KC and TSC as well as with nonsyndromic KC, supporting the hypothesis that diverse germline mutations of the same gene can cause genetic disorders with overlapping clinical features.

Keywords: bilateral keratoconus, tuberos sclerosis, nonsyndromic keratoconus, mutation

Tuberos sclerosis complex (TSC) is a genetic disease affecting approximately 1 in 10,000 people.¹ The classical TSC affects the brain, skin, eyes, kidneys, heart, and lungs. TSC alters cellular proliferation and differentiation, resulting in hamartomas—benign tumors of various organs, consisting of a mixture of cell types native to the tumor's host tissue but abnormal in number, organization, and morphology. TSC is an autosomal dominant disorder caused by mutations in either of two genes: *TSC1* on chromosome 9q34 or *TSC2* on chromosome 16p13, encoding hamartin and tuberin, respectively.² Approximately 60% of patients have no family history of the disease and are thought to represent sporadic mutations in one of the two TSC-causing genes. Approximately 10% to 30% of all TSC cases are due to *TSC1* mutations, with *TSC1* mutations accounting for 15% to 30% of familial cases and for 10% to 15% of sporadic cases.³

Keratoconus (KC) is a bilateral progressive corneal thinning and ectasia.⁴ The exact cause of KC is unknown, with genetic factors playing a major role in the development of the disease.⁵ KC is most commonly seen in its nonsyndromic form; however, it has also been identified in patients with multisystemic diseases such as Down syndrome and Ehlers-Danlos syndrome, among others.⁴ On rare occasions TSC coincidence of TSC and KC phenotypes has been reported. An isolated case of less frequent unilateral KC was described in a male TSC patient from Sri Lanka.⁶ In addition, a 4-base-pair (bp) deletion has been reported in *TSC2* gene in a Caucasian female with TSC and bilateral KC.⁷

Genetic factors have been shown to contribute to the pathogenesis of KC.⁸ A number of genomic loci have been linked with KC in family-based linkage studies,^{9,10} and variants in several genes (*LOX*,¹¹⁻¹³ *COL5A1*,¹⁴ *CAST*,¹⁵ *RAB3-GAPI*^{16,17}) have been associated with the risk of KC. The role

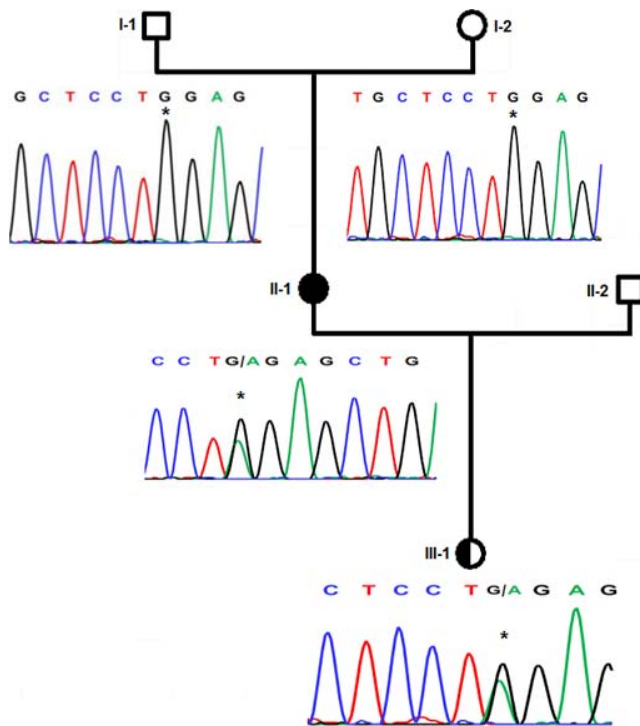


FIGURE 1. Sanger sequencing confirms *TSC1* gene mutation (Q765X) in an Iranian family with TSC and KC. Fully shaded circle (individual II-1) indicates simultaneous presentation of TSC and KC. Half-shaded circle (individual III-1) indicates presence of TSC only.

of variants in other genes such as *VSX1*,¹⁸ *SOD1*,¹⁹ *ZNF469*,^{20–22} *TGFBI*,^{23,24} and *DOCK9*²⁵ in the pathogenesis of KC remains to be determined (also all recently reviewed in Ref. 5). Variants in *COL5A1* gene associated with KC have been independently associated with population variation in KC-related trait central corneal thickness¹⁴ and are implicated in Ehlers-Danlos syndrome.²⁶ Several genes implicated in KC have also been found to be mutated in patients with various types of corneal dystrophies (reviewed in Ref. 5). These findings suggest that different genetic disorders with overlapping clinical features may have the same genetic determinants. Currently, next-generation sequencing (NGS) technologies including whole exome and genome sequencing are being integrated into the genetic research on complex genetic disorders such as KC. In this study, we successfully applied these novel technologies to uncover *TSC1* gene mutations in familial patients with concurrent presentation of TSC and bilateral KC as well as patients with nonsyndromic KC.

MATERIALS AND METHODS

Subjects

Patients With TSC and KC. Here, we report a family that includes a 33-year-old female patient (Proband, Fig. 1, individual II-1) presenting with both bilateral KC and TSC; her parents and her spouse, who are not affected with these conditions; and her 4-year-old daughter, who has been recently diagnosed with TSC only. Informed consent was obtained per requirements of the ethics committee in Shiraz University of Medical Sciences.

Patients With Nonsyndromic KC. Clinically affected KC patients were recruited at the Cornea Genetic Eye Institute, Cedars-Sinai Health Center, and at the Duke University Eye Center. Written informed consent was obtained from all

subjects. The study was conducted in accordance with the provisions of the Declaration of Helsinki and with Institutional Review Board (IRB) approval from the Cedars-Sinai Medical Center and Duke University Medical Center.

Clinical Diagnosis

The diagnosis of TSC with KC was based on complete medical history and a physical examination undertaken by a group of specialists. In a proband (Fig. 1, individual II-1), clinical signs of TSC included two tumors of moderate size in left atrium (rhabdomyoma or myxoma); a moderately sized echo dense mass in left ventricular (LV) mitral valve base without malfunction; two episodes of reflux of gastric content distal to esophagus in 8 minutes' examination, which was noted in ultrasound of abdomen and pelvis; focal epilepsy and generalized epileptiform activity in brain the encephalogram. In addition, chest computed axial tomography (CT) scan incidentally identified two low attenuated and low enhanced areas of 16 and 12 mm within spleen. These could be due to cystic lesions or focal ischemic changes. Diagnosis of KC in the proband with TSC was based on the clinical examination and videokeratography pattern analysis as described in detail below. Proband's 4-year-old daughter has been diagnosed with TSC based on the clinical signs described above. At the age of 2 she was examined by an ophthalmologist and found to have no symptoms of KC.

The diagnosis of KC was performed by a cornea fellowship-trained ophthalmologist based on clinical examination and videokeratography pattern analysis. Clinical examination has included slit-lamp biomicroscopy, cycloplegic retinoscopy, and fundus evaluations. Slit-lamp biomicroscopy was used to identify stromal corneal thinning, Vogt's striae, or a Fleischer ring. Retinoscopy examination was performed with a fully dilated pupil to determine the presence or absence of retro-illumination signs of KC, such as the oil droplet sign and scissoring of the red reflex 20 minutes after phenylephrine 2.5% and cyclopentolate 1% drops had been instilled in the eye. Videokeratography evaluation was performed on each eye using the Topographic Modeling System (Computed Anatomy, New York, NY, USA). Patients were considered as having KC if they had at least one clinical sign of KC and a confirmatory videokeratography map with an asymmetric bowtie with skewed radial axis above and below the horizontal meridian (AB/SRAX) pattern.

DNA Isolation

Patients With TSC and KC. Five-milliliter blood samples were collected from the proband, her parents, her spouse, and a child. Genomic DNA was then extracted from peripheral blood leukocytes by CinnaPure DNA extraction kit (CinnaGen, Tehran, Iran) according to manufacturer's instructions.

Patients With Nonsyndromic KC. Genomic DNA was extracted using NucleoSpin Tissue kit (Macherey-Nagel, Inc., Bethlehem, PA, USA) from lymphoblastoid cell lines of KC patients according to the manufacturer's protocol or using Genra Puregene kits (Qiagen, Valencia, CA, USA) as described before.²⁷ The concentration of all DNA samples was determined by DNA-specific fluorescence binding assay (PicoGreen, Thermo Fisher Scientific, Grand Island, NY, USA) with a standard curve using a microplate reader.

Next-Generation Sequencing

NGS of *TSC1* gene in the proband with TSC and KC was performed using a custom designed NimbleGen chip capturing the *TSC1* (NM_000368, exon 1-23) and *TSC2* (NM_000548, exon 1-42) genes followed by NGS (BGI-Clinical Laboratories, Shenzhen, China).

TABLE 1. Mutations and Variants in *TSC1* Gene Identified in Family With TSC and KC and in Patients With Nonsyndromic KC

Location, GRCh38.p7	Identifier	Classification	Nucleotide Change	Amino Acid Change	MAF, ExAC	MAF, 1000G	Clinical		Patient Sex	Patient Phenotype	Other Syndromes, ClinVar
							Significance, ClinVar	Ethnicity			
9:13,290,2703	rs118203673	Stopgain	NM_000368.4 c.2293 C>T	p.Gln765Ter, Q765X	NA	NA	Not provided	Middle Eastern	Female	TSC and bilateral KC	TSC
9:13,289,6322	rs751398082	Nonsyn SNV	NM_000368.4 c.3408 A>T	p.Asp1136Glu, D1136E	NA	NA	Unknown significance	Middle Eastern Caucasian Caucasian Caucasian	Female Female Female	KC NA KC	Hereditary cancer- predisposing syndrome NA
9:13,289,6452	rs550526986	Nonsyn SNV	NM_000368.4 c.3278 G>A	p.Arg1093Gln, R1093Q	0.00001	0.0002	Unknown significance	Hispanic	Male	KC	NA
Chr9: 13,289,7613 -13,289,7614	rs5901000	Insertion	NM_000368.4 c.2626-4_2626-3insTT	Intron variant	0.43929	NS	Benign	Middle Eastern	Female	TSC	NA

SNV, single nucleotide variation; NA, not available; NS, not specified. ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>; in the public domain.

Whole exome sequencing (WES) in KC patients was performed using Roche NimbleGen SeqCap EZ Exome Library v3.0 (Roche, Pleasanton, CA, USA). The quality of DNA was evaluated with the ratio of OD260/OD280, the ratio of OD260/OD230, and 2% agarose gel electrophoresis with high-mass DNA ladders to select only high-quality DNA samples greater than 10 KB in size with no or little degradation. In addition, we included a step of RNase treatment during DNA extraction to remove any RNA contamination. Only high-quality DNA samples were enriched with Roche NimbleGen SeqCap EZ Exome Library v3.0 with a 64-Mb sequence capture for WES with 100-bp paired-end sequencing with Illumina HiSeq sequencers at Duke Center for Human Genome Variation (Duke University, Durham, NC, USA). For the bioinformatic analysis, all DNA sequencing data underwent strict quality control with the TrimGalore package (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/; in the public domain) by removing any Illumina (San Diego, CA, USA) adapter sequences or low-quality base calls from the 3'-end of the reads. Sequencing reads aligned to human reference genome NCBI Build 37 with the BWA algorithm.²⁸ Potential PCR duplicate reads were removed with Picard, and variants were called with GATK, following the Broad Institute's Best Practices Workflow.²⁹⁻³¹ The called variants were annotated for their functional impact using SnpEff with annotations from Ensembl.³² All the samples were sequenced with at least 75× average coverage. To identify potential KC mutations, we used software SVS 8.0 (SNP & Variation Suite; Golden Helix, Bozeman, MT, USA) to filter and prioritize all the variants.

Sanger Sequencing

To confirm and assess segregation pattern of the *TSC1* mutation Q765X identified in a proband with TSC and KC, we used DNA samples of the proband, her parents, her spouse, and daughter to perform Sanger sequencing. We used the Primer3 program (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>; in the public domain) to design intronic PCR primers to amplify corresponding exon 18. Primers were evaluated by Oligo Analyzer (<https://www.idtdna.com/calc/analyzer>, IDT, Coralville, IA, USA) and National Center for Biotechnology Information BLAST (Bethesda, MD, USA). PCR products were sequenced using Sanger DNA sequencing kit (Bioneer, Daejeon, Korea). Mutation nomenclature was based on the *TSC1* cDNA sequence of NM_000368.4 (<http://www.ncbi.nlm.nih.gov>; in the public domain).

To confirm *TSC1* variants R1093Q and D1136E in KC patients, we designed PCR primers for overlapping sequence of exon 23 using the Primer3 program. Standard PCR was performed to amplify the genomic DNA followed by BigDye-based Sanger sequencing (Thermo Fisher Scientific). Variants were confirmed with both forward and reverse primers.

All primer sequences are included in Supplementary Materials.

Tissue Collection and Processing

Corneal buttons from eight patients with KC and one with corneal scarring secondary to radial keratotomy surgery were obtained during penetrating keratoplasty. Tissue was kept on ice and was generally processed within 3 hours of surgical removal, but no later than within 24 hours. Corneal buttons were stored in a -80 freezer or in RNAlater Stabilization Solution (Thermo Fisher Scientific) reagent in a -20 freezer. Corneal buttons of seven normal controls were purchased from Minnesota Lions Eye Bank (Saint Paul, MN, USA) and North Carolina Eye Bank (Winston-Salem, NC, USA). They were shipped on ice and received within 24 hours of removal.

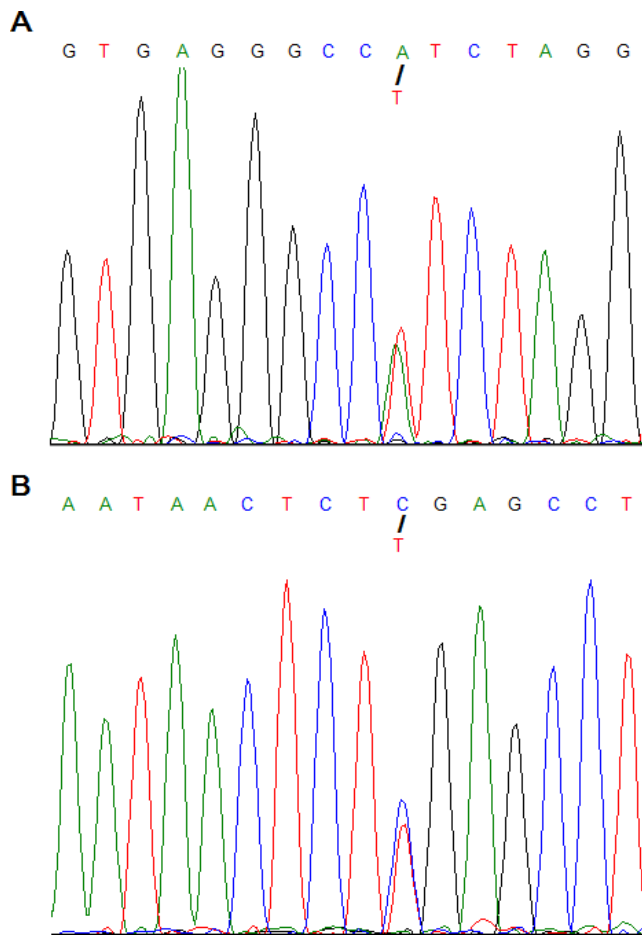


FIGURE 2. Sanger sequencing confirms *TSC1* gene variants in patients with nonsyndromic KC. (A) Variant D1136E in a male patient from Saudi Arabia. (B) Variant R1093Q in a male patient of Hispanic descent residing in the United States.

RNA Extraction and cDNA Preparation

Corneal tissue was homogenized in Precellys24 high-throughput tissue homogenizer (Bertin Corp., Rockville, MD, USA) using three 30-second cycles of homogenization at 5500 rpm with 2.8-mm ceramic beads. Total RNA was extracted using NucleoSpin RNA/Protein isolation kit (Macherey-Nagel, Inc., Bethlehem, PA, USA). Between 1 and 4 μ g high-quality RNA was extracted from approximately half a button as a starting material as measured by NanoDrop 2000c (Thermo Scientific, Wilmington, DE, USA). Extracted RNA underwent quality assessment on Agilent 2100 Bioanalyzer (Thermo Scientific) by visual examination of ribosomal bands and RIN (RNA Integrity Number) calculation. RNA was reverse transcribed to cDNA using RT² First Strand Kit (SABiosciences, Qiagen, Valencia, CA, USA).

Reverse Transcription–Polymerase Chain Reaction

Primers for gene-specific RT-PCR amplification were designed using Primer3. We amplified cDNA with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) using the designed primers for *TSC1*. Betaine solution (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the PCR reaction mixture to improve the amplification of DNA. Products were analyzed via agarose gel electrophoresis.

RESULTS

Sequencing in Family With TSC and KC

A pathogenic heterozygous nonsense mutation *TSC1*:c.2293C>T g.132902703C>T (*TSC1*:c.2293C>T, GRCh38.p7 9:132902703, p.Gln765Ter, Q765X, rs118203673) in *TSC1* gene was identified in the proband with bilateral KC and TSC. Location of the mutation was confirmed by Sanger sequencing (Fig. 1, individual II-1; Table 1). The proband's young daughter (Fig. 1, individual III-1) was also found to be a carrier of this mutation. This nonsense mutation causes early termination of transcription and is expected to affect the protein's function. Additionally, we have detected in the proband a heterozygous single nucleotide polymorphism (SNP) c.2626-4_3insTT (GRCh38.p7: 9:132897613-132897614, rs5901000), which was found to be a common benign intronic copy number variant (CNV) with minor allele frequency (MAF) of 0.4393 in the ExAC database.

Sequencing in Patients With Nonsyndromic KC

Targeted analysis of *TSC1* gene in the WES data of 75 KC patients of Caucasian, African American, or Hispanic ethnicity has identified pathogenic heterozygous missense variant g.132896322A>T (c.3408A>T, GRCh38.p7 9:132896322; p.Asp1136Glu, D1136E, rs751398082) in a Caucasian male KC patient (Fig. 2A; Table 1). Further analysis of high-throughput sequencing data revealed that this change is present in the mother of this patient. This potential mutation is not present in the Exome Aggregation Consortium (ExAC) database containing exomes of 60,706 unrelated humans,³³ the Genome Aggregation Consortium (GnomAD) database, which contains 126,216 exomes and 15,137 genomes from unrelated individuals, and 1000 Genomes (1000G) database.³⁴

Further screening for the presence of this change in 500 Caucasian and Hispanic KC cases using Sanger sequencing has identified a female Caucasian KC patient to be a heterozygous carrier of D1136E mutation (Table 1). In addition, we have found another potentially pathogenic heterozygous rare polymorphism g.132896452G>A (c.3278G>A, GRCh38.p7 9:132896452; p.Arg1093Gln, R1093Q, rs550526986) in the *TSC1* gene in a male Hispanic KC patient (Fig. 2B; Table 1). This variant is present in a single individual in ExAC and a single individual in 1000G databases with resulting MAF of 0.000003962 and 0.0002, respectively. Both individuals are of Asian descent.

In Silico Functional Assessment of Identified Variants

We performed functional and evolutionary analysis of two missense variants using several publicly available bioinformatic tools: PROVEAN (Protein Variation Effect Analyzer),³⁵ PolyPhen-2 (Polymorphism Phenotyping v.2),³⁶ MutationAssessor,³⁷ Panther,³⁸ Fathmm,³⁹ Align GVD,⁴⁰ MutPred,⁴¹ and Mutation Taster.⁴² Although some of these tools have predicted both mutations to have moderate pathogenic potential, others found more evidence for functional significance of R1093Q missense variant and assigned it into the damaging/potentially damaging category (Table 2). The most significant damaging potential is identified for R1093Q by PolyPhen-2 with a high score of 0.999 (1.0 being the highest possible prediction). Similarly, a highly suggestive damaging low score of -2.59 for R1093Q is suggested by Fathmm, representing very low overall tolerance of the *TSC1* protein to this particular mutation. MutPred was relatively confident about the potential deleterious effect of R1093Q variant with a g score of 0.434, and

TABLE 2. Results of In Silico Analysis of the Potential Functionality of Identified Genetic Variants in *TSC1*

Mutation	R1093Q		D1136E		
	Prediction Tool	Score	Pathogenicity	Score	Pathogenicity
PROVEAN		-1.168	Neutral	-0.273	Neutral
PolyPhen-2		0.999	Damaging	0.016	Benign
Align GVGD		42.81/Class C35	Intermediate	44.60/Class C35	Intermediate
MutationAssessor		2.38	Medium	2.665	Medium
Panther		361	Possibly damaging	324	Possibly damaging
Fathmm		-2.59	Damaging	-1.54	Damaging
MutPred		0.434	Potentially actionable hypothesis	0.201	Low confidence
Mutation Taster		NA	Disease causing	NA	Polymorphism

NA, not available.

potential structural changes of gain of ubiquitination at K1091 ($P = 0.0171$) and loss of Molecular Recognition Features (MoRF) binding ($P = 0.0189$), which may affect protein-protein interaction. Mutation Taster unequivocally predicted R1093Q to be disease causing. Overall less confident scores were calculated for D1136E variant.

Expression of *TSC1* Gene in Normal and KC Corneas

Initial bioinformatic testing of gene expression databases showed that none of the eight cornea libraries from human (including the KC corneal library established by our group⁴³), mouse, rabbit, dog, rat, and zebrafish available on the NEIBank Libraries page (<https://neibank.nei.nih.gov/cgi-bin/libList.cgi? tissue=cornea>; in the public domain) organized by the National Eye Institute contained *TSC1* transcripts. However, an unambiguous high level of *TSC1* expression signal was identified in RNA sequencing data from corneas of KC patients and patients with other corneal abnormalities⁴⁴ available in the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>; in the public domain). In addition, analysis of the EyeBrowse database (<https://hpcwebapps.cit.nih.gov/eye/browse/>; in the public domain) revealed presence of *TSC1* transcript in several fetal and adult ocular tissues including iris, lens (multiple), pterygium, retina, eyeball, and mixed eye tissue.

To experimentally test for the presence of *TSC1* transcript in seven normal (obtained post mortem) and diseased (eight from patients with KC and one from patient with corneal scarring, obtained during keratoplasty) corneas, we performed

RT-PCR testing. RT-PCR showed a band of the expected length (792 bp) corresponding to the *TSC1* transcript in all tested tissues (Fig. 3). However, intensity of the signal corresponding to the amount of transcript varied between different samples. Lower band intensity was observed in tissues obtained from patients with corneal scarring (Fig. 3B, lane 1) and KC (Fig. 3B, lanes 3, 12). In addition, faint superfluous bands of approximately 700, 500, and 400 bp were observed in a patient with corneal scarring (Fig. 3B, lane 1) and possibly in KC patients (Fig. 3B, lanes 9, 10), indicating potential splice variants.

DISCUSSION

TSC is a genetically heterogeneous disorder underlined by numerous mutations in *TSC1* and *TSC2* genes. Protein truncating *TSC1* mutation Q765X, identified in our family, has been previously identified in a screen of patients with TSC; however, none of these patients were described to exhibit symptoms of KC.³ In the reported family, mutation has been identified in a 33-year-old proband with all clinical features of TSC as well as clinical KC, and in her 4-year-old daughter, who has been diagnosed with TSC but currently does not yet exhibit any symptoms of KC. This represents the first finding of a *TSC1* mutation manifested by both KC and TSC.

Rare *TSC1* variants unidentified in patients with non-syndromic KC vary in their penetrance. A male Caucasian KC patient with *TSC1* variant D1136E exhibits all clinical signs of the disease; however, the mother of this patient, who also is a heterozygous carrier, has not been diagnosed with KC. A female Caucasian KC patient with *TSC1* mutation D1136E was diagnosed with bilateral KC very early at the age of 15.

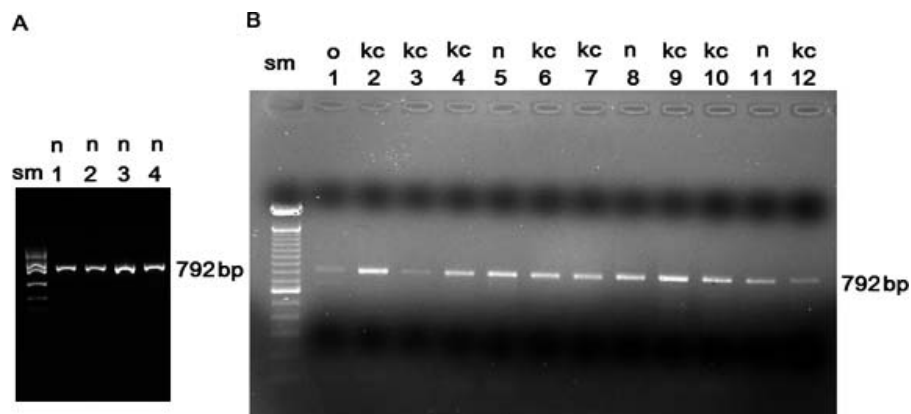


FIGURE 3. Expression of *TSC1* gene in 16 normal, abnormal, and KC corneas. Main PCR product of expected 792 bp is indicated. Additional PCR products are indicated by arrows. (A) n, normal cornea; sm, size marker. (B) n, normal cornea; o, cornea from patient with corneal scarring; kc, cornea from patient with KC.

TABLE 3. Protein Interactors of TSC1 Protein as Identified by STRING

Protein Interactor	Name	Function	Expression in Corneal Libraries
AKT1, AKT2, AKT3	AKT serine/threonine kinases	Three closely related kinases regulate many processes including metabolism, proliferation, cell survival, growth, and angiogenesis. AKT mediates insulin-stimulated protein synthesis by phosphorylating TSC2, thereby activating mTORC1 signaling.	Yes, AKT2
CDK1	Cyclin-dependent kinase 1	Is a member of the Ser/Thr protein kinase family and is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle.	No
IKBKB	Inhibitor of κ light polypeptide gene enhancer in B-cells, kinase beta	Serine kinase that plays an essential role in the nuclear factor- κ B signaling pathway, which is activated by multiple stimuli such as inflammatory cytokines, bacterial or viral products, DNA damages, or other cellular stresses.	No
MTOR	Mechanistic target of rapamycin	Belongs to a family of phosphatidylinositol kinase-related kinases that mediate cellular responses to stresses such as DNA damage and nutrient deprivation. It also acts as the target for the cell cycle arrest and immunosuppressive effects of the FKBP12-rapamycin complex.	Yes
RHEB	Ras homologue enriched in brain	A member of the small GTPase superfamily and encodes a cell membrane protein with five repeats of the RAS-related GTP-binding region. Because of its role in the insulin/TOR/S6K signaling pathway, it is essential for regulation of growth and cell cycle progression. Disorders caused by impaired RHEB include tuberous sclerosis and anisakiasis.	No
RICTOR	RPTOR independent companion of MTOR, complex 2	Subunit of mTORC2, which regulates cell growth and survival in response to hormonal signals. mTORC2 seems to function upstream of Rho GTPases to regulate the actin cytoskeleton, probably by activating one or more Rho-type guanine nucleotide exchange factors.	No
RPS6KA1	Ribosomal protein S6 kinase, polypeptide 1	A Ser/Thr protein kinase that acts downstream of ERK (MAPK1/ERK2 and MAPK3/ERK1) signaling and is involved in the mTOR nutrient-sensing pathway by phosphorylating TSC2, which inhibits TSC2 ability to suppress mTOR signaling.	Yes
TSC2	Tuberous sclerosis 2	In complex with TSC1, inhibits the nutrient-mediated or growth factor-stimulated phosphorylation of S6K1 and EIF4EBP1 by negatively regulating mTORC1 signaling. Acts as a GTPase-activating protein (GAP) for the small GTPase RHEB, a direct activator of the protein kinase activity of mTORC1.	No

However, KC severity varied from mild in the right eye to moderate/advanced in the left eye. She underwent the surgical procedure of Intacs implantation in the left eye during her first year after diagnosis.

A male Hispanic KC patient with *TSC1* variant R1093Q was diagnosed with bilateral KC at the age of 32 and was subjected to keratoplasty of the left eye within the first year of treatment, indicating advanced and severe disease. Interestingly, R1093Q is present in both the ExAC and 1000G databases; however, it is extremely rare, appearing in a heterozygous form in 1 out of 60,706 ExAC samples and 1 out of 2504 1000G samples. Due to the relatively high population frequency of KC (1:2000⁴⁵), it is entirely possible that both individuals could have diagnosed or undiagnosed KC.

To evaluate the disease-causing potential of the two missense variants, we performed extensive *in silico* functionality testing using several different publicly available tools. While both variants D1136E and R1093Q showed moderate to high evidence of their damaging nature to the structure and function of the TSC1 protein with somewhat stronger evidence of R1093Q variant's damaging potential, *in vitro* and *in vivo* testing is required to confirm these predictions.

Our limited expression testing of 16 cornea samples derived from patients with KC, corneal scarring, and normal controls confirms the unequivocal expression of *TSC1* gene in both normal and diseased human corneas. There is also some preliminary evidence to suggest a possibility of splicing defect. However, further studies correlating genetic variation in *TSC1*

to its expression in both normal and diseased corneal tissue are needed.

TSC1 protein in complex with TSC2 protein (hamartin and tuberin, respectively) forms a heterodimeric complex that is stabilized by a third protein partner (TBC17D).⁴⁶ This complex negatively regulates cell growth and proliferation through a canonical signaling pathway involving Ras homologue enriched in brain (Rheb) and the mammalian target of rapamycin complex 1 (mTORC1). A germline mouse model of *TSC1* defect leads to widespread tumor development and embryonic lethality,⁴⁷ while targeted disruption of *TSC1* in retinal precursors recapitulate many of the neuropathologic hallmarks of TSC, such as hamartoma-like lesions with retinal detachment, eye enlargement, and loss of retinal architecture.⁴⁸

We used STRING online software (Search Tool for the Retrieval of Interacting Genes/Proteins)⁴⁹ to identify potential protein interactors of *TSC1* protein. Ten functional partners were identified with a high degree of confidence based on the presence of text mining, database, coexpression, and experimental evidence from several curated sources (Table 3). We checked these functional partners for evidence of expression in the eye using EyeBrowse and NEIBank databases and found three of them (*AKT2*, *MTOR*, and *RPS6KA1*) to be expressed in one or more ocular tissues. These findings reveal the important function of *TSC1* in the eye and can support the hypothesis that a disrupted interaction with essential proteins expressed in the eye may be responsible for the KC pathogenesis in TSC patients.

In conclusion, we suggest that specific pathogenic variants in *TSC1* gene can affect TSC1 protein function and lead to different clinical manifestations affecting different tissues including the eye. We also hypothesize that the pathway leading to KC may involve disruption of essential interactions with important interactors of TSC1 expressed in ocular tissues, including cornea. Identification of *TSC1* as a potential novel gene for syndromic and nonsyndromic KC provides further evidence for the developing understanding of phenotypic spectrum and molecular diversity of germline mutations, which can lead to multifactorial genetic disorders with overlapping clinical features.

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