Tuberous sclerosis complex (TSC) is a genetic disease affecting approximately 1 in 10,000 people. 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 the two TSC-causing genes. 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 TSC2 mutations accounting for 15% to 30% of familial cases and for 10% to 15% of sporadic cases.

Keratoconus (KC) is a bilateral progressive corneal thinning and ectasia. The exact cause of KC is unknown, with genetic factors playing a major role in the development of the disease. 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. On rare occasions TSC coincidence of TSC and KC has been reported. An isolated case of less frequent unilateral KC was described in a male TSC patient from Sri Lanka. In addition, a 4-base-pair (bp) deletion has been reported in TSC2 gene in a Caucasian female with TSC and bilateral KC.

Genetic factors have been shown to contribute to the pathogenesis of KC. A number of genomic loci have been linked with KC in family-based linkage studies and variants in several genes (LOX, COL5A1, CAST, RAB3-GAP) have been associated with the risk of KC. The role of sporadic cases is due to TSC1 mutations, with TSC2 mutations accounting for 15% to 30% of familial cases and for 10% to 15% of sporadic cases.

**Purpose.** To test candidate genes TSC1 and TSC2 in a family affected by tuberous 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, tuberous sclerosis, nonsyndromic keratoconus, mutation

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**TSC1 Mutations in Keratoconus Patients With or Without Tuberous Sclerosis**

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YB and MF contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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of variants in other genes such as VSX1,18 SOD1,19 ZNF469,20–22 TGFBI,23,24 and DOCK525 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 53-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. 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 pattern analysis 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 Gentra Puregene kits (Qiagen, Valencia, CA, USA) as described before. 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).
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 ladder 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. Potential PCR duplicate reads were removed with Picard, and variants were called with GATK, following the Broad Institute’s Best Practices Workflow. Potential PCR duplicate reads were removed with Picard, and variants were called with GATK, following the Broad Institute’s Best Practices Workflow. The called variants were annotated for their functional impact using SNPeff with annotations from Ensembl. 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 her 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 intrinsic 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.

### 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 RNA later 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.
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_5insTT (GRCh38p.7:9:132897613-132897614, 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.p7:9:132896522; 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 0.0000005962 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 GVGD,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 PolyPhen2 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
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 group\textsuperscript{[15]}), mouse, rabbit, dog, rat, and zebrafish available on the NEIBank Libraries page (https://neibank.nei.nih.gov/cgi-bin/libList.cgi?action=table&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 abnormalities\textsuperscript{[44]} 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/eyebrowse/; 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.\textsuperscript{[3]} In the reported family, mutation has been identified in a 33-year-old proband with all clinical features of the disease 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 $TSC1$ mutation D1136E exhibits all clinical features of the disease; however, none of these patients were described to exhibit symptoms of KC.\textsuperscript{[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.

Hand-picked gene expression results of eight cornea tissues are shown in Table 2. Prediction tools indicate that 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.

### Table 2. Results of In Silico Analysis of the Potential Functionality of Identified Genetic Variants in $TSC1$

<table>
<thead>
<tr>
<th>Mutation Prediction Tool</th>
<th>Score</th>
<th>Pathogenicity</th>
<th>Score</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
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<td>-0.273</td>
<td>Neutral</td>
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<td>Possibly damaging</td>
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<tr>
<td>MutPred</td>
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<td>Low confidence</td>
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<tr>
<td>Mutation Taster</td>
<td>NA</td>
<td>Disease causing</td>
<td>NA</td>
<td>Polymorphism</td>
</tr>
</tbody>
</table>

NA, not available.

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.
to the relatively high population frequency of KC (1:200045), it was present in both the ExAC and 1000G databases; however, it is entirely possible that both individuals could have diagnosed undiagnosed KC.

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 entirely possible that both individuals could have diagnosed 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 (TBC1D). 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, 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.

We used STRING online software (Search Tool for the Retrieval of Interacting Genes/Proteins) 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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