Molecular Vision 2013; 19:2501-2507 <http://www.molvis.org/molvis/v19/2501>
Received 17 July 2012 | Accepted 6 December 2013 | Published 8 December 2013

Analysis of superoxide dismutase 1, dual-specificity phosphatase 1, and transforming growth factor, beta 1 genes expression in keratoconic and non-keratoconic corneas

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Purpose: To quantitatively assess the superoxide dismutase 1 (*SOD1*), transforming growth factor, beta 1 (*TGF-β1*), and dual-specificity phosphatase 1 (*DUSP1*) messenger ribonucleic acid (mRNA) expression levels as the main intracellular reactive oxygen species neutralizers, wound healing mediators, and immunomodulators (respectively) in keratoconic (KCN) and non-KCN corneas.
Methods: Total RNA was extracted from normal and keratoconic cultured corneal stromal fibroblasts. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to measure the relative expression levels of mRNAs of the *SOD1*, *TGF-β1*, and *DUSP1* genes.
Results: The mRNA expression of *TGF-β1* and *DUSP1* was augmented in the KCN corneas (three- and fivefold, respectively; both $p<0.05$). The KCN and non-KCN samples showed no difference in comparative *SOD1* mRNA levels.
Conclusions: This study demonstrated a higher level of *DUSP1* and *TGF-β1* expression as known molecules in the inflammatory process. These results may provide new insight into the complex molecular pathways underlying KCN for investigating other inflammatory molecules.

Keratoconus (KCN) is the most common cause of non-inflammatory corneal thinning that leads to progressive myopia, irregular astigmatism, and cornea curvature changes [1]. KCN has an incidence rate of between 50 and 230 and a prevalence of 8.8 to 54.4 people per 100,000 [2]. KCN occurs in the second decade of life, and the rate of progression is heterogeneous. There is no significant difference in gender and race preponderance [3,4]. KCN is one of the leading causes of penetrating keratoplasty in developed countries. The etiology of KCN has not been fully clarified; however, KCN is categorized in three major classes: KCN associated with genetic disorders and syndromes (such as Down syndrome, nail-patella syndrome, neurofibromatosis, Ehlers-Danlos syndrome, Marfan syndrome, etc.); KCN in the setting of commonly reported associations (contact lens wear, eye rubbing, atopy, allergy, Leber congenital amaurosis, mitral valve prolapse, and positive family history); and isolated KCN with no associations [2].

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KCN usually occurs as an isolated disease, but sometimes there is a positive family history [2]. In such cases, autosomal recessive and dominant patterns of inheritance have been reported [5,6]. Histologically, KCN is characterized by breakage in the Bowman membrane and subepithelial scarring. The affected areas have marked alterations in extracellular matrix (ECM) components because of the disturbance in enzyme activities and apoptosis, which leads to thinning of the corneal stroma [7-14].

The human eye is vulnerable to oxidative damage because of light exposure, high metabolic activity, and oxygen tension that lead to reactive oxygen species (ROS) production. As primary antioxidant systems, superoxide dismutase *(SOD*) isoenzymes catalyze the dismutation of the superoxide radicals to generate hydrogen peroxide. Since the superoxide anion radical exerts its effects locally and the anion poorly penetrates the membranes, different kinds of *SODs* (*SOD1*, *SOD2*, and *SOD3*) compartmentalize in the cytosol, mitochondrial matrix, and extracellular space [15]. It is suggested the predominance of a variant *SOD1* transcript with exon 2-skipping or exon 2- and 3-skipping deletions may lead to a decrease in enzyme activities in the KCN cornea [16,17]; thus, in this study, *SOD1* was investigated as an intracellular isoenzyme in KCN and non-KCN corneas.

Dual-specificity phosphatases (*DUSP*s) or mitogen-activated protein kinase phosphatases (MKPs) are a subset of proteins, many of which dephosphorylate threonine and tyrosine residues on mitogen-activated protein kinases. For immune cells, *DUSPs* regulate responses in positive and negative ways and are key regulators of immune responses [18,19]. One of the external stimuli that activate *DUSP1* gene expression is oxidative stress [20]. To better understand the phenomenon, we characterized the expression of the *DUSP1* gene in KCN and non-KCN corneas. Transforming growth factor beta 1 (*TGF-β1*), which is associated with various corneal dystrophies, is involved in regulating keratocyte activation, myofibroblast transformation and proliferation, chemotaxis, and wound healing [21]. In this study, we investigated the mRNA expression of *DUSP1*, *TGF-β*, and *SOD1* genes in the stromal primary cell culture in KCN and non-KCN corneas.

METHODS

This study was approved by the Institutional Review Board (IRB) of Tehran University of Medical Sciences, Iran, and informed consent was obtained from all family members wishing to participate in this study before corneal transplantation. Non-KCN cornea-derived fibroblasts belonging to sclerocorneal rims were obtained from healthy donor subjects within 24 h of death, and the KCN corneas were removed at the time of penetrating keratoplasty. All patients had advanced KCN (Noor Eye Hospital, Tehran, Iran). After the epithelium and the endothelium were removed, stromal explants were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Auckland, New Zealand) supplemented with 20% fetal bovine serum (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen).

The medium was changed twice a week. When the cells reached the appropriate confluency (70%), the culture medium was completely removed, and the cells were washed with PBS (Invitrogen; 1.05 mM KH_2PO_4, 155.17 mM NaCl, 2.96 mM Na_2HPO_4-$7H_2O$ and a pH 7.4) and detached enzymatically with 0.25% trypsin–0.02% EDTA (Invitrogen). For genetic analysis, cultured corneal stromal fibroblasts of the third passage were harvested and maintained at −70 °C.

Total ribonucleic acid isolation: Total RNA was extracted using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany), from the cultured stromal fibroblasts of KCN and non-KCN corneas according to the manufacturer's instructions. Briefly, the lysis buffer was added to the cell pellets and homogenized by pipetting. After mixing well, the solution was applied to the filter tube and centrifuged. After DNase I treatment, total RNA was eluted in TE buffer (Tris-HCl 10 mM, EDTA 1 mM). The quality and quantity of the total RNA were determined with electrophoresis and ultraviolet-spectrophotometry with Nano-Drop ND-2000 (Thermo Scientific, Wilmington, NC), respectively.

Semiquantitative reverse transcriptase–polymerase chain reaction: Two microgram of total extracted RNA from cultured corneal stromal fibroblasts was reverse trancribed using QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) according to protocols supplied by the manufacturers. The expression of the genes was investigated with reverse transcription–polymerase chain reaction (RT–PCR) using gene-specific primers (see Table 1 for the primer sequences). The PCR reaction was performed with a final reaction volume of 25 µl containing 50 ng of first-strand cDNA, 0.6 µl of primer F (10 µM), 0.6 µl of primer R (10 µM), and 10 µl of 2× Taq Polymerase Master Mix (Ampliqon, Copenhagen, Denmark) together with the beta 2 microglobulin (*β2M*) gene used as the internal control. Then, PCR was performed with a thermalcycler (ABI 2720, Applied Biosystems, Foster City, CA) under the following conditions: an initial denaturating step (94 °C for 3 min), a cycling step (denaturation at 94 °C for 30 s), annealing (58 °C for 30 s), extension (72 °C for 20 s), and final extension (72 °C for 5 min). The amplified PCR products were separated in 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. No template and RT-minus controls were run to detect contamination, dimer formation, and presence of genomic DNA.

For semiquantitative RT–PCR and exponential phase determination, PCR was performed with *β2M* primers for all samples. PCR products of different cycles (21, 24, 27, and 30) were loaded on 2% agarose gel. Based on band intensity, cycle 24 was the best representative of the exponential phase.

According to the *β2M* results, semiquantitative RT–PCR was performed to measure gene expression for *SOD1*, *DUSP1*, and *TGF-β1* in ten KCN samples and non-KCN samples. The RT–PCR conditions were the same as discussed previously. Normal corneal samples were pooled for reducing tissue preparation errors. Amplicons were quantified using system image analysis performed with a GS-800 Calibrated Imaging Densitometer (BioRad Laboratories, Hercules, CA), and the bands were standardized to the *β2M* levels.

Statistical analysis: The independent sample Student *t* test was used to compare *SOD1, DUSP1*, and *TGF-β1* expression between the KCN and non-KCN samples. We considered 0.05 the level of statistical significance.

Table 1. RT–PCR Primers Used for *B2M*, *TGFB1*, *DUSP1* and *SOD1*

Target gene	Primer sequences	PCR product size
B2M (NM_004048.2)	F: 5′-TATCCAGCGTACTCCAAAGA-3′ R: 5′-GACAAGTCTGAATGCTCCAC-3′	191
TGFB1 (NM_000358)	F: 5′-ATCACCAACAACATCCAGCA-3′ R: 5′-CCGTTACCTTCAAGCATCGT-3′	165
DUSP1 (NM_004417)	F: 5′-GAGGGTCACTACCAGTACAAGAGC-3′ R: 5′-GCCTGGCAGTGGACAAACA-3′	137
SOD1 (NM_000454)	F: 5′-CAGTGCAGGGCATCATCAAT-3′ R: 5′-CATTGCCCAAGTCTCCAACA-3′	222

All primers are from 5′ to 3′. *β2M*, beta-2-microglobulin; *SOD1*, superoxide dismutase1; *DUSP1*, Dual-specificity phosphatases; *TGF-β1*, Transforming growth factor beta1

RESULT

Cell culture: Seven days after tissue cultivation, the cells migrated out of the tissue pieces. When the colonies were visible (it took about 2 weeks), the tissue pieces drained out of the plates (Figure 1). After they achieved confluence (70%), they were transferred to tissue culture flasks. The second and third subcultures were used for total RNA extraction. The disease stage, gender, and concentration of the RNAs obtained from the KCN samples are depicted in Table 2.

Semiquantitative reverse transcriptase–polymerase chain reaction: Based on band intensity, cycle 24 was the best representative of exponential phase (Figure 2). Total extracted RNAs were subjected to cDNA synthesis followed by semi-quantitative RT–PCR. Results of gel electrophoresis in the 24th cycle are shown in Figure 3.

Image analysis: Amplicons were quantified using system image analysis performed with a GS-800 Calibrated Imaging Densitometer (BioRad Laboratories). The results of the target genes image densitometry were normalized with the housekeeping gene (*β2M*) as shown in Figure 4. As demonstrated in Figure 4, the average mRNA level of *SOD1* in KCN and non-KCN showed no significant difference (0.353 versus 0.36), but statistical analysis showed overexpression of *TGF-β1* and *DUSP1* mRNA levels in the KCN samples (three- and fivefold; both $p<0.05$), respectively.

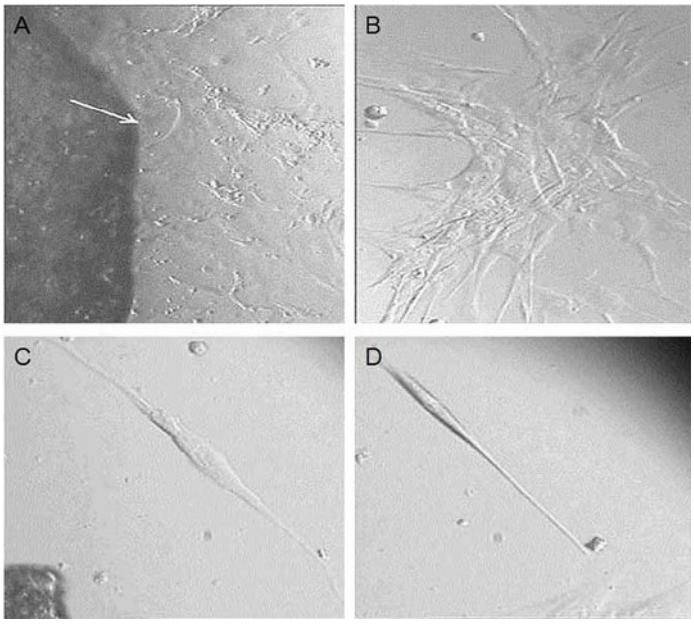

Figure 1. Primary corneal tissue culture. This figure represents primary tissue culture from sample no. 4 under light microscopy. **A**: The migration of cells out of the tissue started on day 7 after seeding (white arrow shows margin of tissue, 10X magnification). **B**: One of the cell colonies was performed after 15 days post-seeding (20X magnification). **C**, **D**: After cell culture expansion, the cultivated cells were starved for 24 h to compare the morphological difference between fibroblasts and keratocytes (**C**, **D**, respectively).

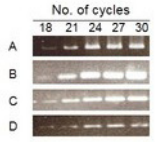

Figure 2. Determination of the exponential cycle of amplification. Semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) mixtures were taken for five alternating cycles starting at cycles 18, 21, 24, 27, and 30 for **A**: beta 2 microglobulin (*B2M*), **B**: superoxide dismutase 1 (*SOD1*), **C**: dual-specificity phosphatase 1 (*DUSP1*), and **D**: transforming growth factor, beta 1 (*TGF-B1*). Cycle 24 was selected as an exponential cycle.

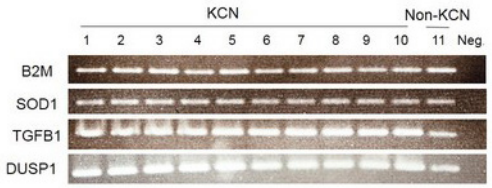

Figure 3. Differential messenger ribonucleic acid expression of the target genes via semiquantitative reverse transcription–polymerase chain reaction (RT-PCR; 24th cycle). This figure shows the results of the RT–PCR products in 2% agarose gel electrophoresis, stained with ethidium bromide. Lanes 1 to 10: Keratoconus (KCN) corneas; lane 11: pooled normal corneas and lane 12: negative control without RT. The fluorescent intensity of the bands was quantified with a GS-800 densitometer. The signal intensities were normalized to values obtained for beta 2 microglobulin (*B2M*). The messenger ribonucleic acid (mRNA) expression of transforming growth factor, beta 1 (*TGF-β1*), and dual-specificity phosphatase 1 (*DUSP1*) was increased in the KCN corneas (three- and fivefold, respectively; both $p<0.05$). The comparative superoxide dismutase 1 (*SOD1*) mRNA level showed no difference between the KCN and non-KCN samples.

DISCUSSION

KCN is a corneal disorder with heterogeneity and variability in symptoms. The pathogenesis of KCN is partially known. Many hypotheses have been suggested regarding its etiology [22].

In the late 1990s, oxidative stress and damaging environmental factors were suggested as key players in changing corneal tissue integrity [23]. This view is supported by the observation that ROS such as superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide increase in KCN samples compared with normal samples [24-26]. Although epithelial damage due to corneal surface exposure to exterior stimuli (ultraviolet radiation, trauma, atopy, allergy, pathogens, and contact lenses) predisposes the corneal tissue to KCN development [22,27], cytokines and growth factors with chemotactic effects on other inflammatory cells induce epithelial damage processes [27-29].

These observations challenge the pathophysiological concept of KCN. Today, we know that other concomitant factors such as oxidative stress and many genes play an important role; therefore, in this study, the expression of three genes in hypothesized pathways with focus on oxidative stress and the immune response mechanism was evaluated.

Dual-specificity phosphatase 1: DUSP1/MKP1, the first member of the heterogeneous family of dual-specificity phosphatases, is located on 5q34 [30]. DUSP1 dephosphorylates mitogen-activated protein kinases, which are evolutionary conserved enzymes that play an important role in orchestrating various cellular processes, including proliferation, differentiation, and apoptosis. This mediator has a regulatory role in innate and adaptive immune responses by inactivating p38 and c-Jun N-terminal kinase [31,32]. Some stimuli such as lipopolysaccharides, hypoxia, heat shock proteins, and oxidative stress can activate *DUSP1* gene expression [33]. Our results showed a significant increase in the *DUSP1* mRNA levels in almost all the KCN samples compared with

Table 2. Characteristics of KCN and normal corneas

Sample no.	Gender	Disease stage	RNA concentration (ng/ul)
1	M	Advance	585
2	M	Advance	434
3	M	Advance	443
4	F	Advance	646
5	F	Advance	590
6	M	Advance	660
7	F	Advance	731
8	F	Advance	234
9	M	Advance	758
10	M	Advance	327

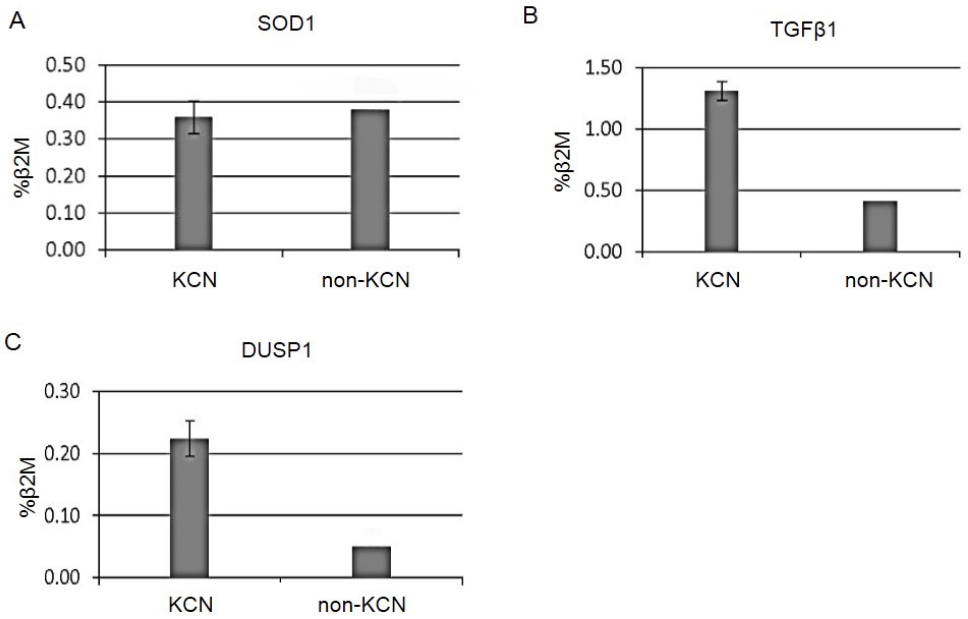

Figure 4. The comparative mRNA expression of target genes in ten keratoconus (KCN) and pooled normal corneal tissues. This graph indicates the comparison of transcript band intensities for genes encoding superoxide dismutase 1 (*SOD1*), transforming growth factor, beta 1 (*TGF-β1*), and dual-specificity phosphatase 1 (*DUSP1*) between the KCN and non-KCN samples. The values were calculated relative to the beta 2 microglobulin (*B2M*) levels (as normalizer) and reported as mean percentages. Note that the KCN corneas showed a three- and fivefold increase in *TGF-B1* and *DUSP1* when compared to the pooled normal corneas. The RNA levels for *SOD1* were similar for the KCN and normal corneas ($p<0.05$). Normal corneal samples were pooled to reduce tissue preparation errors. The error bars represent the standard deviation.

the pooled non-KCN samples. These findings may indicate an ongoing stress- or inflammation-related reaction in KCN fibroblasts and are consistent with data that propose that environmental stresses are the main causes of KCN pathophysiology [7,26,29]. There is no evidence for investigating *DUSP1* mRNA expression in corneal cells.

Superoxide dismutase 1: Superoxide dismutase gene maps to chromosome 21q22 [34,35]. This gene encodes three major mammalian enzymes (*SOD1, SOD2*, and *SOD3*) that have been identified in the cytosol, mitochondrial matrix, and its secretory form in tissue interstitium, respectively. They catalyze superoxide to hydrogen peroxide and molecular oxygen [34,35]. One of the main factors in the oxidative stress pathway is environmental stimuli such as ultraviolet exposure and epithelial trauma that damage corneal cells and may lead to destruction of the cell vital sites such as the nucleus and mitochondria. Loss of mitochondrial membrane integrity causes ROS efflux. This efflux can trigger apoptosis in corneal stromal cells as the first frontline of exposure.

Although oxidative stress has been nominated as the main cause of cell damage in KCN and a decrease in *SOD1* expression has previously been demonstrated in KC corneas [16,36], the mRNA expression level of *SOD1* was similar in the KCN and non-KCN samples in our study. Our results confirm other studies that have shown similar RNA levels for *SOD1* in KCN and normal corneas [37]. According to our data, stromal cell ROS accumulation and mitochondrial damage might not be the only source of cellular damage and apoptosis.

Transforming growth factor, beta 1: The *TGF-β1* gene, which is located on 19q13.1 [38], is a persuasive divergent growth factor and participates in embryogenesis, development, immune, and inflammatory processes. The gene's ligands and receptors are expressed in various types of cells [39-42]. Previous studies have reported *TGF-β1* as a known mediator in corneal fibrosis and wound healing [43]. *TGF-β1* regulates cell cycle progression by binding to transforming growth factor, beta receptor II (*TGFβR2*) and activating *TGF-βR1*. The *TGF-βR1* receptor can be inactivated in many cases of human tumor cells refractory to *TGF-β*-mediated cell cycle arrest [44]. In this study, we investigated the *TGF-β1* mRNA level in KCN and non-KCN fibroblasts. Our results showed a significant increase in *TGF-β1* expression levels in KCN samples.

Engler et al. provided evidence that *TGF-β* signaling pathway components were upregulated in advanced KCN [39]. This pathway is one of the main cascades that modulate ECM elements and cells through its ligands and receptors.

According to the literature, KCN is defined as the non-inflammatory thinning of the cornea, and ROS are considered the main players in the pathogenesis of KCN; however, few studies have focused on the function of the inflammatory cells and mediators in KCN. In addition, our data indicated

high expression of *TGF-β1* as a wound healing mediator in KCN samples. It would be useful to further investigate the role of inflammation in correlation with different components of the ECM separately and in the context of tissue. Based on our body of data and definition of KCN, these new findings would create better insight into the mechanisms of KCN to find ways to prevent disease progression or to discover new treatment options.

ACKNOWLEDGMENTS

This work was supported by a grant number NORC900415 from Noor ophthalmology Reasearch Center, Noor Eye Hospital. The authors are grateful to Fatemeh Jafari and Mr. Olaad-daar for their kind collaboration.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 8 December 2013. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.