Investigation of Mitophagy Biomarkers in Corneal Epithelium of Keratoconus Patients

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Running Title: Mitophagy in Keratoconic Corneal Epithelium

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Purpose: The pathological mechanisms of keratoconus (KC) has not been elucidated yet. Mitophagy is an important mechanism that eliminates damaged mitochondria under oxidative stress, and it could be one of the leading pathological causes of keratoconus. This study aimed to find out the role of mitophagy in the keratoconic corneal epithelium.

Methods: The corneal epithelia were collected from the 103 progressive KC patients and the 46 control subjects. The real-time quantitative PCR was performed for PTEN-putative kinase 1 (PINK1), PARKIN, p62, and BNIP3 gene expressions in 31 keratoconus and 9 control subjects. Western blot analyses were performed to investigate the protein expressions of PINK1, PARKIN, LC3B, ATG5, and BECLIN in the remaining 109 corneal epithelium samples from 72 patients and 37 control subjects.

Results: mRNA and protein expressions of PINK1 decreased significantly in the corneal epithelium of KC patients compared to the control subjects. No significant change was found in mRNA levels of PARKIN, p62, and BNIP3 in KC patients. The protein expression of PARKIN, LC3B, ATG5, and Beclin did not significantly differ between KC patients and control subjects. Gene expression levels of mitophagy biomarkers were not affected by the keratoconus grade.

Conclusions: PINK1/PARKIN-dependent mitophagy is affected in the keratoconic corneal epithelium. We found significant decreases in both mRNA and protein expressions of PINK1 in the keratoconic corneal epithelium. However, we did not observe any other significant change in mitophagy markers. Mitochondrial stress-related mitophagy pathways could be interrupted by the decreased levels of PINK1 in the keratoconic corneal epithelium, but solely PINK1 dysregulation is not likely to induce keratoconus pathogenesis.

Keywords: corneal epithelium; keratoconus; mitochondrial dysfunction; mitophagy; PINK1

Introduction

Keratoconus (KC) is characterized by corneal thinning due to degeneration in the corneal stromal tissue and biochemical changes that occur in the corneal stromal tissue[1]. It is one of the most common corneal diseases affecting 55 out of 100.000 people in the white populations and up to 229 per 100.000 individuals in the Asian populations for both genders[2]. The disease is caused by the weakening of stromal collagen fibers, which leads to the formation of the thinner and steeper cornea. It is reported as the most common indication for corneal transplantation in developed countries[3]. Although genetic mechanisms, tear biomarkers, and inflammatory factors are related to KC, the exact molecular mechanism associated with KC pathogenesis is not yet fully understood[4,5].

The cornea is the most exposed part of the body to UV radiation and atmospheric oxygen, leading to free radicals and reactive oxygen species[6]. Toprak et al. demonstrated the effect of systemic oxidative stress in the progression of keratoconus by showing higher serum oxidant status in KC patients compared to the controls[7]. In healthy corneas, antioxidant enzymes, including superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase, protect other ocular structures against reactive oxygen species (ROS)[8]. Previous reports demonstrated that antioxidant response decreased in the corneal buttons, tears, and serum from KC patients[9–11]. Under physiological conditions, ROS formation and ROS clearance are balanced by antioxidative defense mechanisms and free-radical scavengers[12]. ROS are produced as side products of cellular energy metabolism, and the mitochondrial electron transfer chain is one of

the primary sources of ROS generation[13]. The corneal antioxidative enzymes include matrix metalloproteinases, lysyl oxidase, superoxide dismutase, aldehyde dehydrogenase, catalase, cathepsin, glutathione reductase, and transferase, are affected by keratoconus[10,14–17]. An increase of the oxidative stress biomarkers and the decrease of antioxidant defense mechanisms create cytotoxic products, which accumulate in corneal epithelium and corneal stroma[9].

Autophagy is a primary cellular degradation mechanism initiated by double-membrane autophagic vesicles, namely autophagosomes engulfing intracellular cargos, including long-lived proteins, aggregates, and organelles such as mitochondria[18]. Fusion of autophagosomes with lysosomes allows degradation and recycling of those macromolecules[19]. The selective degradation to clear damaged mitochondria by autophagy pathways is called mitophagy[20]. It is reported that ROS not only triggers mitochondrial DNA damages but also activates the mitophagy pathways, which are upregulated to eliminate impaired mitochondria in response to oxidative stress[21]. The cumulative effect of the increased ROS production and ROS removal defect initiate mitophagy cascades due to oxidative stress-induced mitochondrial depolarization[22]. Multiple signaling pathways are involved in this process, such as Phosphatase and tensin homolog-induced putative kinase protein-1 (PINK1)/PARKIN pathway and BNIP3/NIX pathway[23]. PINK1 and PARKIN are the well-known mitophagy regulators in mitochondrial clearance. Under normal conditions, PINK1 is translocated to mitochondria from cytoplasm through the translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM) complexes. When PINK1 is translocated, it becomes post-translationally modified within mitochondria by mitochondrial proteases to maintain mitochondrial homeostasis, and PARKIN stays inactive[24]. Under oxidative stress conditions, PINK1 translocation to mitochondria is arrested. The mitochondrial depolarization leads to the

accumulation of PINK1 on the outer mitochondrial membrane, which then recruits PARKIN accompanied by ubiquitin phosphorylation[25]. The stimulation of PARKIN and these polyubiquitinated proteins initiates the recruitment of autophagic machinery that involves the autophagosome formation induced by the LC3 family and its subsequent fusion with lysosomes. As a result, the engulfed mitochondrial cargo is degraded

by lysosomal hydrolases in order to maintain mitochondrial health via PINK1/PARKIN-mediated mitophagy[26]. In this way, the PINK1/PARKIN-mediated mitophagy pathway leads the mitochondria to the common autophagy pathway and autophagosome-lysosome assembly. While Beclin (BECN1) and ATG5 proteins induce autophagosome formation, LC3B plays an essential role in autophagosome-lysosome conjugation during the late stage of mitophagy[27,28].

We hypothesized that accumulated reactive oxygen species in keratoconus might be associated with dysregulated mitophagy in corneal epithelial cells. Hence, the present study aimed to investigate the potential role of mitophagy in KC pathogenesis. We investigated the expression of both mitophagy and autophagy markers in KC patients' corneal epithelium. For this investigation, we examined gene expression levels of PINK1, PARKIN, p62, and BNIP3 proteins and protein expression levels of BECN1, ATG5, LC3B, PINK1, and PARKIN proteins in corneal epithelia of both control subjects and KC patients. As BECN1, ATG5, and LC3B are critical proteins of the autophagy pathways, and their protein levels significantly change with post-translational modifications. They are required for autophagosome nucleation, formation, expansion, and maturation in late mitophagy steps[29–31].

Methods

Clinical Evaluation and Collection of Corneal Epithelia

103 progressive keratoconus patients and 46 control subjects who had undergone photorefractive keratectomy for myopia correction were included in this study. The study was approved by the local medical ethics committee and followed the tenets of the Declaration of Helsinki. Both written and verbal consents were obtained from the patients for sample collection as per the ethical guidelines. Briefly, patient selection for the study was made by detailed ophthalmologic examination, including best-corrected visual acuity, slit-lamp biomicroscopy, fundoscopy, and corneal topography Pentacam HR (Oculus, Wetzlar, Germany). Keratoconus patients without any previous ocular surgery or other active ocular disease were examined in detail, and keratoconus patients with evident clinical characteristics were included in the study. Skewed corneal axes, stromal thinning, conical protrusion, Fleischer ring, Vogt striae, anterior stromal scar on slit-lamp examination, and asymmetric bowtie pattern on corneal topography measurement considered as clinical characteristics of keratoconus. Keratoconus progression analysis was made based on Amsler-Krumeich classification[32]. This classification based on mean keratometry readings on anterior curvature sagittal map, thickness at the thinnest location, and the refractive error of the keratoconus patients was used. The exclusion criteria for KC patients were followed reported previously in detail[33,34].

Corneal epithelium from progressive KC patients (n = 103) who had undergone corneal crosslinking (CXL) was collected. The corneal epithelium of control subjects (n= 46) was collected from those who underwent photorefractive keratectomy (PRK) for myopia correction. The subjects were separated into age- and sex-matched experimental groups for gene and protein expression analyses in both keratoconus and control populations. Briefly, the corneal epithelium was scraped with a mechanical scraper before CXL and PRK treatment. No alcohol was used during the collection of corneal epithelia. The collected corneal epithelium was immediately

placed in the 1.5 ml tubes with RNAlater (QIAGEN, Hilden, Germany), and each sample from individual subjects was separated for western blot and quantitative real-time PCR analyses. Clinical characteristics of the study subjects are shown in **Table 1**.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from 31 keratoconic and 9 control cornea epithelial tissues using the QuickRNA Microprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol and quantified using the spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA). The RNA quality was confirmed by both the ratio of A260/280 and A260/230 and gel electrophoresis. The RNA extracted from was reverse transcribed to complementary DNA (cDNA). RNA-free H2O, random primers (Thermo Fisher Scientific, MA, USA), and dNTPs (Thermo Fisher Scientific, MA, USA) were added to the sample RNA, and the sample was heated to 65°C for 5 min. The solution was left on ice for 1 min, after which 5X first-strand buffer (Invitrogen, CA, USA) and 0.1 M DTT (Invitrogen, CA, USA) was added, mixed, and left at RT for 2 min. Subsequently, M-MLV-RT enzyme (Invitrogen, CA, USA) was added, after which the sample was left at RT for 10 min, heated to 42°C for 50 min, and then heated to 70°C for 15 min. Quantitative real-time PCR was performed for PINK1, PARKIN, p62, and BNIP3 mRNA quantification by using the Light Cycler 480 SYBR Green I Master 2X (Roche Diagnostics GmbH, Mannheim, Germany) and 7500 Real-Time PCR System (Applied Biosystems, CA, USA) according to the manufacturer's instructions. An initial 10 minutes cycle of 95°C was performed to activate the SYBR green, followed by PCR reaction 40 cycles of 95°C for 15 sec and 59.3 °C 60 sec. The target genes' relative mRNA expression levels are calculated using the 2-ΔΔCt method using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA as an internal control. Primers used during the study are indicated in **Table 2**. All quantitative PCR

analyses were performed in triplicate to confirm the accuracy and reliability of measured mRNA expression levels.

Protein Isolation and Western Blot Analysis

In separated samples for western blotting, RNAlater was washed with DPBS (PAN-BioTech, Aidenbach, Germany) from the corneal epithelial tissues of 72 keratoconus patients and 37 control subjects. After that, each tissue sample was centrifuged at +4°C 10000 g for 5 minutes to eradicate RNAlater. After removing DPBS, RIPA buffer (Thermo Fisher Scientific, MA, USA) containing protease and phosphatase inhibitors (PhosSTOP and cOmplete Mini, Roche, Basel, Switzerland) was used to lyse the corneal epithelial tissues. Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). Equal amounts of protein (20 µg) were loaded and run on 4-15% SDS PAGE (MiniProtean TGX Gels, Bio-Rad, CA, USA), blotted onto Immuno-Blot® PVDF membrane (Bio-Rad, Hercules, CA, USA). After that, the membrane was blocked with 5% non-fat dry milk powder in TBST followed by overnight incubation with primary antibodies of ATG5 (#12994, Cell Signaling Technology, Massachusetts, United States), Beclin (#3495, Cell Signaling Technology), PINK1 (ab23707, Abcam, Cambridge, UK), PARKIN (ab77924, Abcam), LC3B ([EPR18709], Abcam), and Vinculin (ab130007, Abcam). The secondary antibodies (1:1000; Anti-rabbit IgG [7074], Anti-mouse IgG [7076], Sigma-Aldrich, MO, USA) were conjugated with horseradish peroxidase, and a chemiluminescence substrate was used to visualize the band with the Pierce™ ECL Western Blotting Substrate and detected by the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). All western blot analyses were duplicated for each subject to validate the results.

Image Analysis and Statistics

During image analysis of western blot results, lanes were detected, and background subtraction was adjusted. Bands in the chemiluminescent blot were detected, and lane profiles were quantitatively measured. All measurements and analyses were performed in ImageLab software (Bio-Rad, Hercules, CA, USA). Adjusted volumes of the target protein bands were normalized according to adjusted volumes of Vinculin bands. Relative expression values were calculated by using normalized volumes.

The statistical significance of the differences between the control and patient groups for real-time PCR and western blot results were analyzed by student t-test and two-way ANOVA by using Prism 8 software (GraphPad, CA, USA). Results were considered statistically significant when the p-value was less than 0.05.

Results

Demographic and Clinical Findings

We have included the study, 103 (40 female, 63 male) KC patients and 46 (19 female, 27 male) control subjects. The mean age for keratoconus patients was 24.7±1.6 years, and the mean age for control subjects was 24.5±0.8 years. There were no differences in age and sex distribution between KC patients and control subjects (p=0.568 and p=1.00). All pachymetry and refraction measurements in keratoconus patients differed significantly from control subjects (**Table 1**). The average flat curvature power (K1) was 48.1±2.6, average steep curvature power (K2) was 52.5±1.9 D, the average spherical error was -2.12±0.90 D, and average cylindrical error was 3.21±1.57 D in keratoconus patients.

Quantitative Real-Time PCR Results

The mRNA levels of PARKIN, p62, and BNIP3 in the KC epithelium were compared to the control epithelium. Only PINK1 mRNA levels showed a statistically significant difference in keratoconus patients in quantitative real-time PCR analysis compared to controls ($p<0.05$). Other genes did not show a statistically significant difference between groups ($p=0.866$ and $p=0.536$). mRNA levels of PINK1 were downregulated significantly in the corneal epithelium of KC patients compared to the control epithelium (1.000±0.092 vs. 0.712±0.034, $p=0.001$) (**Figure 1a**). When mRNA transcription levels of PINK1 were analyzed in the corneal epithelium with different grades of KC, gene expression of PINK1 showed a significant decrease in all KC grades without any statistical difference between grades (**Figure 1b**).

Western Blot Results

We examined protein expression levels of the BECN1, ATG5, LC3B, PINK1, and PARKIN in cornea epithelia of both control subjects and KC patients. Protein expression of PINK1 is also decreased in corneal epithelia of KC patients (1.639±0.170 vs. 0.765±0.075, $p<0.001$) (**Figure 2**). However, the protein expressions of PARKIN, LC3B, BECN1, and ATG5 did not show any statistically significant changes compared to the control epithelium (**Figure 3 and Supplementary Figure 1**). No significant association was found between the patient demographics and the grade of keratoconus with PINK1 protein expressions.

Discussion

Autophagy is the primary cellular degradation mechanism in cells, and its proper function enables the recycling of cellular contents. Mitophagy is defined as the selective removal of defective mitochondria by autophagosome engulfment and lysosomal degradation. Different studies showed that both upregulations of ROS production and dysregulations of autophagic

degradation are found in KC patients[35,36]. In the present study, mitophagy-related genes and proteins were analyzed in corneal epithelium samples of control subjects and KC patients for the first time. We investigated the mitophagy biomarkers in KC patients' epithelium and compared them with the mitophagy biomarkers of the control subjects. We found that both PINK1 gene and protein expression levels were lower in KC patients compared to the control subjects.

However, PINK1 mediated mitophagy pathway is not the only regulator of oxidative stress in the epithelium. Other intracellular mechanisms such as Wnt/β-catenin and alpha-enolase pathways are also important determinants of cell behavior under oxidative stress[37]. Oxidative stress-related dysregulation in the Wnt/β-catenin pathway could lead to chronic inflammation in the cell[38]. These disruptions in Wnt/β-catenin pathway-related genes in keratoconic corneas are also demonstrated by various research groups[39–41]. Amit et al. show that keratoconus-related defects in the Wnt/β-catenin pathway could lead to the loss of tight junctions between the corneal epithelium and basal membrane, which will induce corneal stromal changes in KC pathogenesis[42]. Also, it has been observed that alpha-enolase, which involves mitochondrial membrane stabilization, causes an increase in the amount of ROS by silencing[43,44]. Keratoconus-related decrease in alpha-enolase is demonstrated in high amounts at the re-epithelialization stage and interacts with adhesion proteins in the pericyte matrix, possibly related to corneal thinning pathogenesis[45]. In addition to Wnt/β-catenin and alpha-enolase pathways, impairments associated with Hedgehog and Notch pathways have been shown in the KC epithelium[41]. An increase in these proteolytic enzymes because of the epithelial thinning possibly leads to damage to the underlying tissue, and this increase is directly proportional to the severity of keratoconus[16]. Therefore, the focus of our study was the keratoconic corneal epithelium, which is affected by oxidative stress via disruptions of various intracellular pathways.

The keratoconus pathogenesis is still not fully understood. In addition to genetic predisposition, environmental factors such as eye rubbing, contact lens wear, and UV light exposure leads to oxidative stress on the ocular surface[36]. In the result of the proteomic studies, three hypotheses are emphasized for the pathological changes on the corneal extracellular matrix: cytokine dysregulation, oxidative stress, and changes in TGF-β levels[46]. Exposure of the cornea to various environmental factors, including oxidative stress, enhances levels of ROS and causes corneal damage and dysfunction[47]. The accumulation of ROS in KC corneal epithelium and stroma shows that ROS has a significant role in the pathogenesis of this disease[48]. ROS triggers a signaling mechanism to induce mitophagy as a vital cell survival strategy. Mitophagy will be triggered if the cell cannot recover from damage via mitochondrial fission and fusion mechanisms because of the loss in mitochondrial polarization[20]. As a result of loss in mitochondrial polarization, PINK1 will phosphorylate the PARKIN at Ser65, an inducer protein for mitophagy cascades. The authors illustrated the detailed map of PARKIN-dependent and independent mitophagy pathways in **Figure 4**. After that, phosphorylated PARKIN ubiquitinates the substrates on the outer mitochondrial membrane through E3 ligase activity, and damaged mitochondria will be labeled for recycling[49]. PARKIN translocation is an essential step for the mitochondrial clearance process. Our data suggest that reduced levels of PINK1 obstruct the translocation of PARKIN protein in the inner membrane of mitochondria. Due to that, mitophagy could not be activated effectively through the PINK1-PARKIN pathway, and this causes the accumulation of ROS in KC patients' corneal epithelium. Although the underlying mechanism is still unclear, it has been reported that PINK1 deficiency is closely associated with ROS production[50]. While downregulation of PINK1 is also related to epithelial cell death, upregulation of the PINK1 gene could show a significant protective role in the diseased

epithelium[51,52]. These findings suggest that mitophagy, as an anti-apoptotic mechanism, reduces pro-apoptotic cytokine release by removing damaged mitochondria.

Damaged or depolarized mitochondria could be eliminated by a different mechanism other than PINK1 dependent pathway[37]. While ubiquitin-dependent mitophagy occurs via PINK1, ubiquitin-independent mitophagy is mediated by BNIP3[53]. In our study, no statistically significant difference was observed in the gene expression of BNIP3 in KC patients compared to the control group.

In addition to mitophagy pathway biomarkers, we also investigated autophagy pathway markers in keratoconus and the control corneal epithelia. We found no significant difference in p62 gene expression and LC3B protein expression between KC patients with different grades and controls. We would like to answer why LC3B protein expression did not change, although the mitophagy pathway was dysregulated. In the autophagy pathways, Beclin induces autophagy cascades and ATG5 helps elongate and close the autophagosomal membranes[18]. Because of that, we checked the expression levels of autophagy-related proteins, Beclin and ATG5. No difference was found in the expressions of ATG5 and Beclin proteins between the control and keratoconus groups.

One of the most important limitations of our study is the analysis of corneal epithelium without division to cone and periphery regions. The cone and periphery regions of the cornea are morphologically different from each other, and they show different molecular expression profiles in keratoconus patients[54]. One of the previous studies shows that while BCL2 (B-cell lymphoma-2 gene) levels decreased, BAX (Bcl-2 associated X protein gene) levels increased in the cone region compared to the periphery region, which shows higher levels of apoptosis in the cone region of keratoconic epithelium[35]. Contrary to the study of Shetty et al., we could not find any significant difference in p62 gene expression and autophagy-related protein (LC3B, ATG5, and

Beclin) expressions between KC patients with different grades and controls because of lack of separation for cone and peripheral epithelium of the cornea. Also, cytokeratin-3/cytokeratin-12 positive differentiated epithelial cells were significantly reduced in the cone region compared to the peripheral region in keratoconus[55]. Because of that, other autophagy-related proteins, such as lysosome-associated membrane protein-2 (LAMP-2), the BCL2 Interacting protein-3 like (BNIP3L/NIX), and mitofusin2 (MFN2), and various differentiation markers, such as cytokeratin proteins, should be investigated in further studies on keratoconus[45,56–58].

Altogether, dysregulated mitophagy pathways due to oxidative damage in the cornea might be involved in the pathogenesis and progression of keratoconus. Upon activation, mitophagy can protect corneal epithelium against oxidative stress and pro-apoptotic proteins by removing damaged mitochondria to promote epithelial cell survival. However, since autophagy is a dynamic process and changes depending on tissue's physiological condition, we could eliminate the limitations in our interpretation by developing new techniques. Because of that, investigation of mitophagy in keratoconus requires further validation and a detailed assay of intracellular pathways in the cornea epithelium.

Conclusions

Mitophagy-related pathways, except PINK1, were not affected by keratoconus. However, dysregulation of PINK1 expression on mitochondria could result from increased oxidative stress and mitochondrial damage at keratoconic corneal epithelia. Mitophagy pathways should be investigated in realistic, dynamic in vitro corneal cell culture models to understand better the molecular mechanisms behind these findings of the study. The results of in vitro corneal cell

culture models also should be validated with consistent clinical investigations, such as mitophagy biomarker levels in tears of keratoconus patients.

Gene	Sequence	

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Declaration of Interest

The authors report that there are no competing interests to declare.

Table and Figure Legends

Table 1. The pachymetry data from Pentacam and refraction measurements on corneal thickness. K1, K2: Readings of corneal curvature by keratometry. The ANOVA p-value column shows group statistics.

Clinical details	**Control**	**Grade 1**	**Grade 2**	**Grade 3**	**p-value**
Age	24.5±0.8	22.1±2.2	26.3±1.3	25.7±1.4	>0.05
K1	41.9±0.8	44.7±1.4	48.3±4.3	51.1±2.2	<0.05
K2	44.1±0.6	48.4±1.8	53.3±2.1	55.7±1.8	<0.05
Spherical error	-2.23±0.47	-0.13±0.43	-2.52±0.55	-3.71±1.74	<0.05
Cylindrical error	-0.79±0.34	-1.93±1.15	-3.11±1.54	-4.57±2.04	<0.05

Table 2. The primers of genes used in the qRT-PCR analysis.

GAPDH	F	5’-ACAACTTTGGTATCGTGGAAGG-3’
	R	5’-GCCATCACGCCACAGTTTC-3’
PINK1	F	5’-GGAGGAGTATCTGATAGGGCAG-3’
	R	5’-AACCCGGTGCTCTTTGTCAC-3’
PARKIN	F	5’-GTGTTTGTCAGGTTCAACTCCA-3’
	R	5’-GAAAATCACACGCAACTGGTC-3’
p62	F	5’-GGAACAGCGACTCTTGCTTC-3’
	R	5’-GGTGCTCGATATGGCATTAGTG-3’
BNIP3	F	5’-TGAGTCTGGACGGAGTAGCTC-3’
	R	5’-CCCTGTTGGTATCTTGTGGTGT-3’

Figure 1. The mRNA expression analysis of mitophagy-related genes in the corneal epithelium of KC patients (a) PINK1, PARKIN, p62 and BNIP3 gene expressions in the corneal epithelium of KC patients and control subjects. (b) PINK1 gene expression compared to grades of KC. The qPCR results were normalized with GAPDH. Asterisks * denote a significance with p-values <0.05.

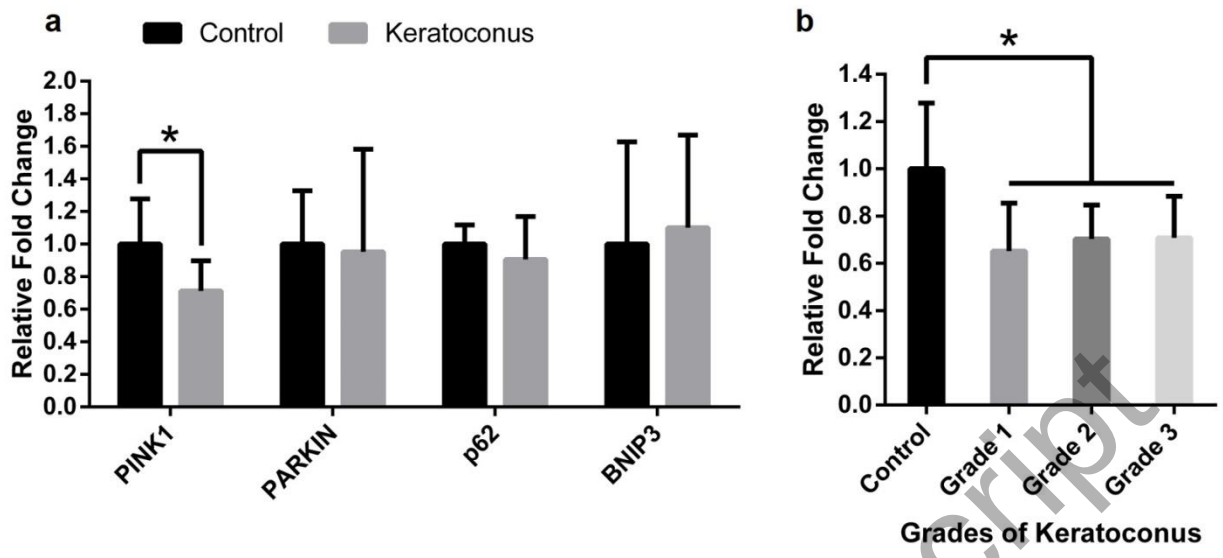


Figure 2. (a) PINK1 protein expression compared between control and keratoconus groups. (b) Western blot images of the protein levels of PINK1 and Vinculin compared to control and keratoconus groups. Vinculin was used as a loading control, and western blot results were relatively measured to Vinculin. Data were shown as mean ± SD of independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

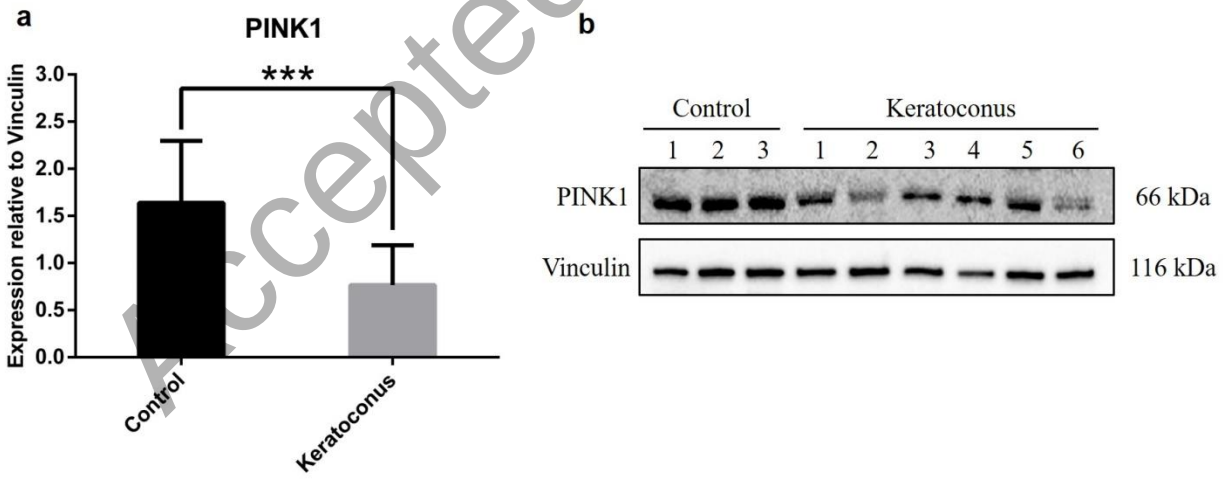


Figure 3. The protein expressions of mitophagy pathway-related genes in the corneal epithelium of KC patients. (a) PARKIN, (b) LC3B, (c) Beclin, and (d) ATG5 protein expressions in the corneal epithelium of keratoconus patients and control subjects. Vinculin was used as a loading

control, and western blot results were relatively measured to Vinculin. Data were shown as mean ± SD of independent experiments.

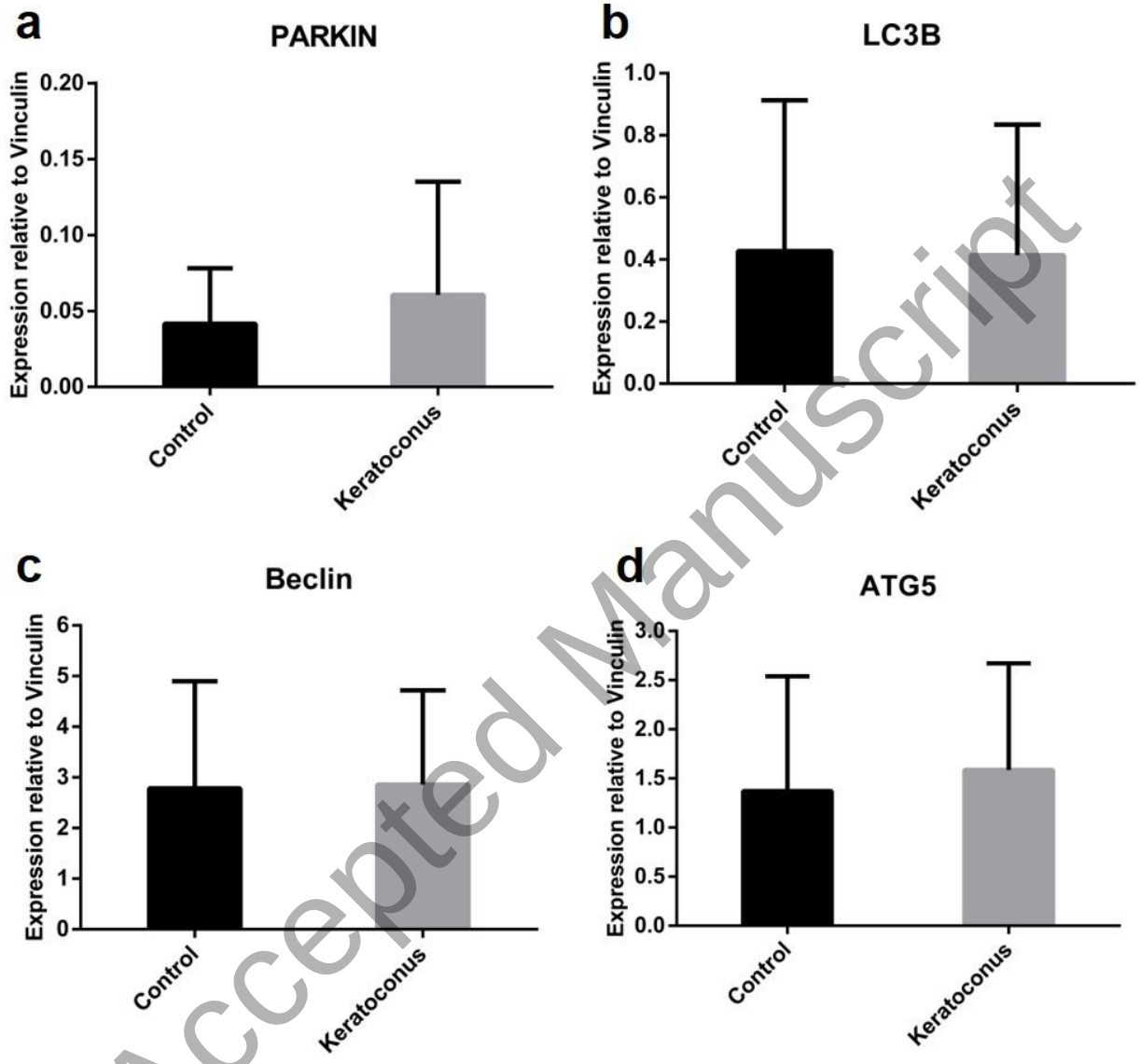
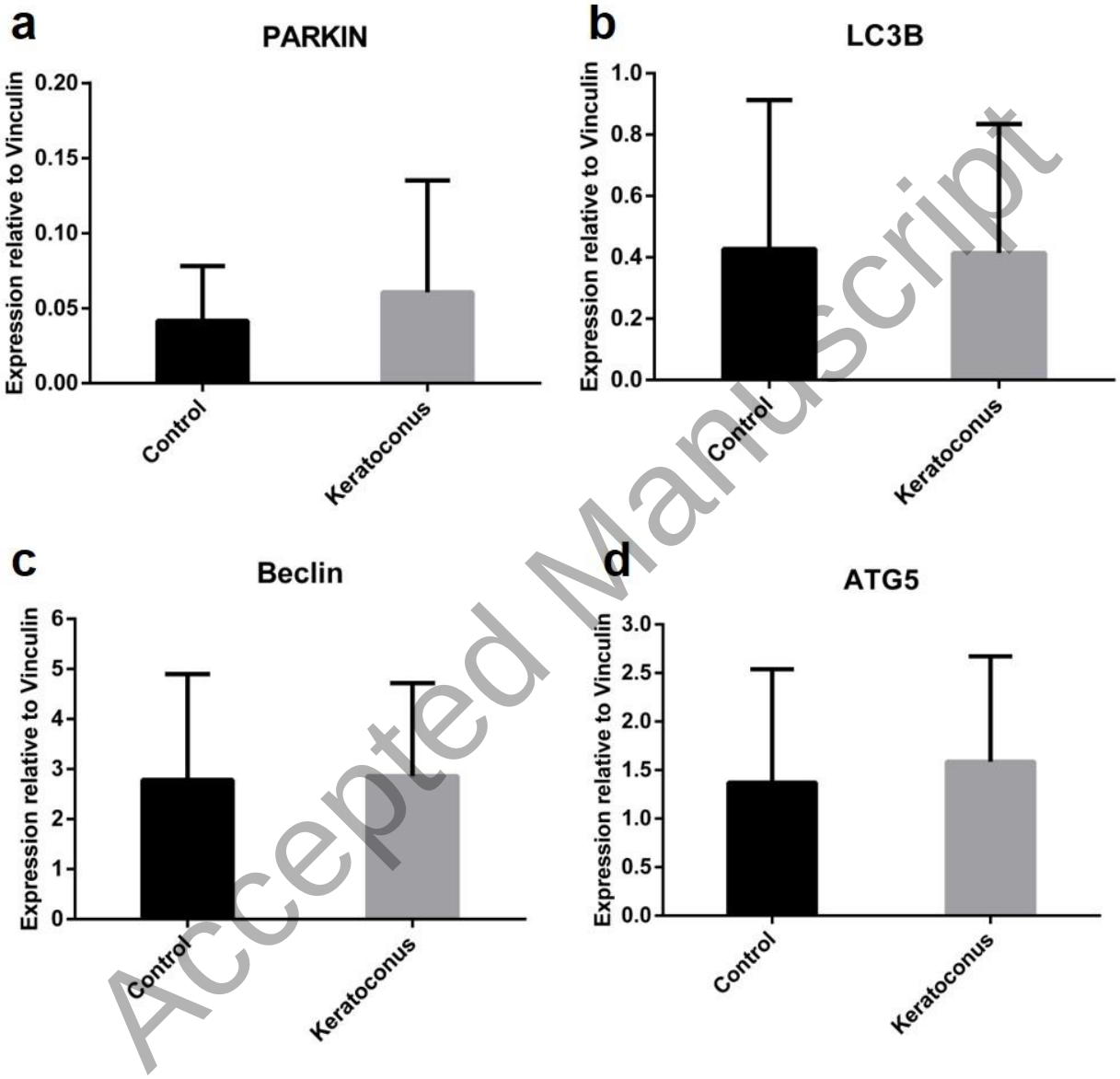


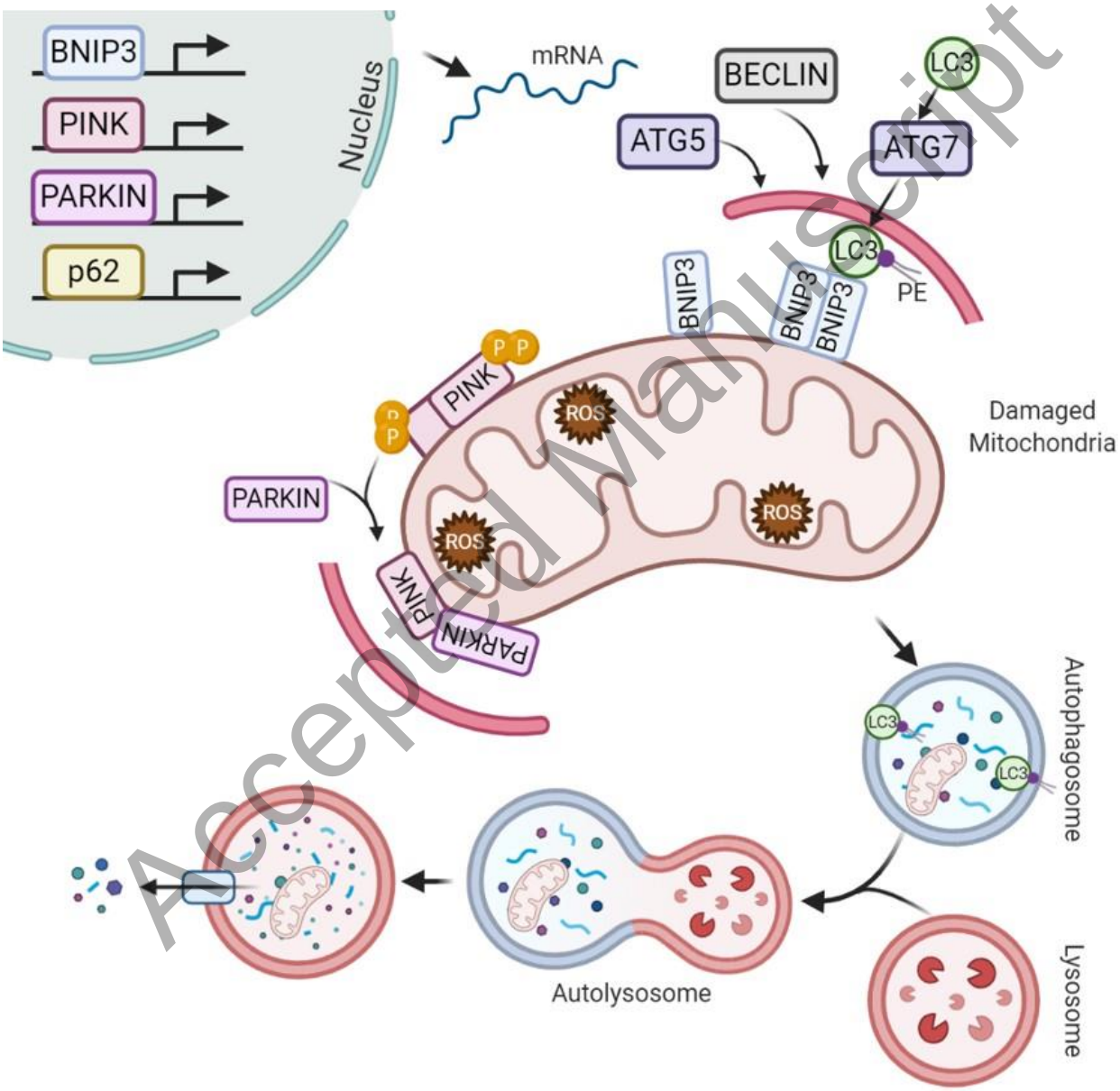
Figure 4. Intracellular mechanisms of PARKIN dependent and independent mitophagy pathways. In the PARKIN-dependent pathway, mitochondria enter the mitophagy via PINK1-phosphorylation and PARKIN attachment to the mitochondrial membrane. In PARKIN independent mitophagy pathway, BNIP3 or BNIP3-like proteins induce LC3 attachment, which leads to autophagosome formation around the mitochondria. As a common late stage of both

PARKIN dependent and independent mitophagy pathways, autophagosomes are created around mitochondria with the help of ATG5, ATG7, Beclin, and LC3B leads to autophagosome-lysosome conjugation and mitochondrial degradation.



Supplementary Material

Supplementary Figure 1. (a) Western blot images of the protein levels of LC3B, PARKIN, and Vinculin compared to control and keratoconus groups. (b) Western blot images of the protein levels of ATG5, Beclin, and Vinculin compared to control and keratoconus groups. Vinculin was used as a loading control, and all western blot results were relatively measured to Vinculin.



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