

# The expression of MAPK signaling pathways in conjunctivochalasis

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## Abstract

• This study investigated the potential role of MAPK signaling pathways in conjunctivochalasis (CCH). Twenty loose conjunctival biopsy samples from 20 CCH and 15 conjunctival biopsy samples from 15 normal controls (CON) were collected. The conjunctival fibroblasts were cultured *in vitro*. Immunofluorescence, ELISA, Western blot and reverse transcription-polymerase chain reaction (RT-PCR) were used. Our results showed that the expression of p-ERK, p-JNK, and p-p38 in CCH conjunctiva was significantly higher than that in CON group. The expression of p38 MAPK, JNK, and ERK proteins in CCH fibroblasts was significantly higher than that in CON group. The total expression of MAPK mRNA in CCH fibroblasts was significantly higher than that in CON group. The activated forms of p38 MAPK, JNK, and ERK proteins and mRNAs might up-regulate the expression of MMPs in CCH loose conjunctival tissue and fibroblasts, causing the degradation of collagen fibers and elastic fibers and promoting the occurrence of CCH. Our results deepen the understanding of CCH pathological mechanism.

• **KEYWORDS:** ERK; JNK; p38 MAPK; conjunctivochalasis; phosphorylation level

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## INTRODUCTION

Conjunctivochalasis (CCH) is one of the most common clinic eye diseases in elderly people. Epidemiological

studies in China have indicated a 44.8% prevalence of CCH in people older than 60y. The incidence increases with age, reaching 89% in people older than 70y. CCH induces dry eye, epiphora, and other uncomfortable symptoms, thus severely affecting patients' life quality<sup>[1-2]</sup>. Traditional treatment mainly comprises surgical therapies, including resection of crescent shaped conjunctiva, conjunctival limbal trapezoid excision, conjunctiva suture fixation and so on. However, the operation outcomes are not ideal with recurrence rate<sup>[3-4]</sup>. The main pathological changes in CCH are the decreasing of elastic fibers and dissolution of collagen fibers, which lead to excessive degradation of the stroma and Tenon's capsule in the conjunctiva<sup>[5-7]</sup>. Recent studies have shown that the main reason for the dissolution of collagen fibers is over-expression of matrix metalloproteinases-1 (MMP-1), MMP-3, and MMP-9 in conjunctival fibroblasts, thus resulting in imbalanced expression of MMPs and their tissue inhibitor of metalloproteinases (TIMPs)<sup>[8-9]</sup>.

In recent years, the mitogen activated protein kinase (MAPK) signaling pathways have been found to up-regulate the expression of MMPs<sup>[10]</sup>. Members of the MAPK family play vital roles in many cellular processes, including proliferation, apoptosis, differentiation, metabolism, senescence and survival. In mammals, the MAPK signaling pathways divide into four groups: ERKs (extracellular signal-regulated kinases), JNKs (c-Jun N-terminal kinases), p38 MAPKs ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ), and ERK5. They are activated by diverse extracellular stimuli, such as growth factors, cytokines, mitogens, hormones, and other various cellular stresses including oxidative stress, heat shock, hypoxia, ischemia, ultraviolet irradiation, and DNA-damaging agents<sup>[11]</sup>.

Although the MAPK signaling pathways have been found to up-regulate the expression of MMPs in many cells, it has not been demonstrated to have the same function in CCH. Therefore, we hypothesized that ERK, JNK, and p38 MAPK might accelerate the occurrence of CCH through up-regulating the expression of MMPs.

## SUBJECTS AND METHODS

**Ethical Approval** The sample collection protocol was approved by the ethics committee of Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine. Informed consents were obtained from all the CCH patients and control individuals before participation.

**Specimen Collection** Loose conjunctival tissue from 20 CCH patients (9 males, 11 females; mean age:  $70.43 \pm 8.89$ y; CCH group) were collected between October 2016 and December 2017 in the Department of Ophthalmology, Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine. Normal conjunctival tissues from 15 patients (7 males and 8 females, mean age:  $69.50 \pm 8.97$ y) as normal control group (CON group) scheduled to undergo cataract phacoemulsification surgery, through a protocol performed and described in a previous study<sup>[12]</sup>. All CCH patients and CON individuals were examined by the same optometrist. Slit lamp were used to examine the presence and location of CCH. CCH patients had absent tear-lake, replacing the cul-de-sac with loose conjunctiva. The loose conjunctival folds (nasal, middle or temporal) upon the lower eyelid were show in Figure 1.

**Cell Culture** Conjunctival fibroblasts were obtained from primary cell culture. Primary and passaged cultures of fibroblast cells were grown under Xiang *et al*'s<sup>[8]</sup> fibroblasts culturing methods. The specimens were rinsed with 0.9% saline three times to remove blood, then were cut into 0.5-1 mm<sup>2</sup> pieces and placed in sterile saline-containing vials.

Then, each conjunctival tissue sample was placed at the bottom of a 75-cm<sup>2</sup> cell culture flask. The 3 mL Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS), 1% double antibody, 1% fibroblast growth supplement (FGS), 1% penicillin, and 1% streptomycin was then slowly added to avoid tissue floating, and the flasks were placed in a humidified atmosphere of 5% CO<sub>2</sub>. When the cells overflowed from the conjunctival tissue and 80% confluency was reached under inverted microscope, the cells were routinely subcultured.

**Immunofluorescence Staining** The conjunctival tissues were fixed in 10% formalin for 48h, then rinsed with water, dehydrated with different concentrations of ethanol, and made transparent with ethanol and xylene. The tissues were then dipped in wax, then embedded and sliced to a thickness of 4-7 μm. After dewaxing, the slices were repaired with 0.01 mol/L sodium citrate buffer for 15min, and then incubated with primary antibody (dilution, 1:150) at 4°C overnight. Then, the slices were incubated with FITC-conjugated secondary antibody (dilution, 1:300) for 2h at room temperature and sealed with anti-quenching and 4', 6-diamidino-2-phenylindole (DAPI; dilution, 1:500). The slices were kept in a -20°C refrigerator until being photographed under a fluorescence microscope.

**Enzyme-linked Immunosorbent Assay** Cells at generation 3-6 were selected for the logarithmic growth period. After the cells grew to 90% in the culture medium, they were washed with PBS two times, and 500 μL lysate liquid was added. After centrifugation, the supernatant was placed in an aseptic



**Figure 1** Slit lamp photograph showing CCH.

EP tube. The relative concentration was detected with ELISA kits (R&D, Minneapolis, MN, USA). The enzyme labeling was detected at a wavelength of 450 nm. The absorbance (A450) OD value of each well was measured according to the manufacturer's protocol.

**Western Blotting Analysis** Total protein was isolated from the cells lysed with cell lysis reagent (Cell Signaling Technology, US). Protein quantification was performed using BCA assays. Twenty micrograms of total protein were subjected to 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride membranes (PVDF; EMD Millipore, Bedford, MA, USA). The PVDF membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) and washed three times with Tris-buffered saline containing Tween (TBST; Beyotime Institute of Biotechnology). The membranes were then incubated with specific primary antibodies anti-human p38 MAPK, p-p38 MAP, ERK, p-ERK, JNK, p-JNK, and rabbit monoclonal anti-human GAPDH antibodies (Santa Cruz Biotechnology) overnight at 4°C, then washed three times with TBST and then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) for 2h at room temperature. Finally, immunoreactive bands were detected with a Chemiluminescent HRP Substrate ECL kit (EMD Millipore, Bedford, MA, USA).

**RT-PCR** Total RNA was extracted from the fibroblasts of the normal control conjunctiva and CCH samples with TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific). RNA was then converted to cDNA with a Prime Script RT Master Kit (Invitrogen; TaKaRa, Tokyo, Japan). RT-PCR was performed with SYBR Premix Ex Taq II (Invitrogen; TaKaRa) on an ABI Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). The relative expression levels of each cell line in each group were measured using the 2<sup>-ΔΔCt</sup> methods.

**Statistical Analysis** All statistical analyses were performed using the Statistical Package of Social Sciences software (version 23.0; SPSS, Chicago, IL, USA). The data were

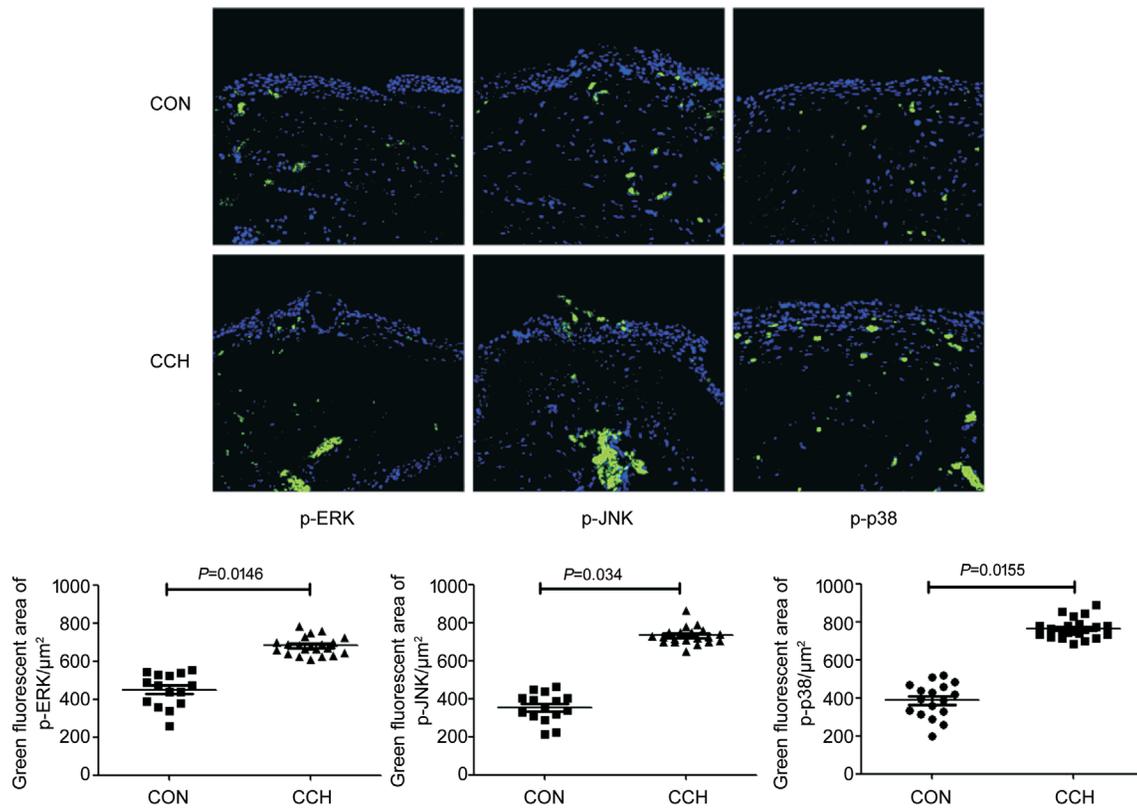


Figure 2 Immunofluorescence staining of conjunctival tissue.

Table 1 OD values of MAPKs and their phosphorylation levels in fibroblasts

Group	n	p38	p-p38	JNK	p-JNK	ERK	p-ERK
CON	6	2.51±1.21	0.09±0.02	1.44±0.44	0.21±0.06	1.56±0.51	1.02±1.04
CCH	6	3.98±0.99 <sup>a</sup>	0.15±0.04 <sup>a</sup>	1.95±0.22 <sup>a</sup>	0.33±0.07 <sup>a</sup>	2.40±0.76 <sup>a</sup>	2.87±0.89 <sup>a</sup>
Z		-1.984	-2.402	-1.984	-2.611	-1.984	-2.193
P		0.047	0.016	0.047	0.009	0.047	0.028

CCH vs CON group, <sup>a</sup>P<0.05.

presented as means±standard deviations (SD) for all patients. Groups were tested for normal distribution with the Shapiro-Wilk test and for variance homogeneity with Levene's test. Differences between two paired data sets were compared with a two-sample *t*-test. All statistical significance were considered a value of *P*<0.05.

## RESULTS

The green fluorescence area of p-ERK in CCH group (677.70±49.97 µm<sup>2</sup>) was significantly larger than that in CON group (448.47±86.97 µm<sup>2</sup>; *P*=0.0146). The green fluorescence area of p-JNK in CCH group (730.85±45.16 µm<sup>2</sup>) was significantly larger than that in CON group (353.73±76.54 µm<sup>2</sup>; *P*=0.0340). The green fluorescence area of p-p38 in CCH group (768.25±50.55 µm<sup>2</sup>) was significantly larger than that in CON group (375.53±90.15 µm<sup>2</sup>; *P*=0.0155). The expression of p-ERK, p-JNK, and p-p38 in CCH, on the basis of fluorescence, was significantly higher than that in CON (Figure 2).

To investigate the expression of MAPKs protein in the conjunctival fibroblasts, we used ELISA to detect the protein levels. The OD values of p38 MAPK, p-p38 MAPK, JNK, p-JNK, ERK, and p-ERK in the conjunctival fibroblasts were significantly higher in CCH group than those in CON group (p38 MAPK: *P*=0.047, p-p38 MAPK: *P*=0.016, JNK: *P*=0.047, p-JNK: *P*=0.009, ERK: *P*=0.047, p-ERK: *P*=0.028; Table 1). The expression of p-p38 MAPK, p-JNK, and p-ERK protein was significantly higher in fibroblasts of CCH group than that of CON group (p-p38 MAPK: *t*=-2.809, *P*=0.048; p-JNK: *t*=-5.470, *P*=0.005; p-ERK: *t*=-2.891, *P*=0.045). The expression of p38 MAPK, JNK, and ERK proteins in fibroblasts was higher of CCH group than that of CON group without statistically significance (p38 MAPK: *t*=-1.321, *P*=0.257; JNK: *t*=-1.582, *P*=0.189; ERK: *t*=-1.481, *P*=0.213; Figure 3). The total expression of MAPKs mRNA in fibroblasts of CCH group was significantly higher than that of CON group (p38 MAPK: *P*=0.019; JNK: *P*=0.010; ERK: *P*=0.028; Table 2).

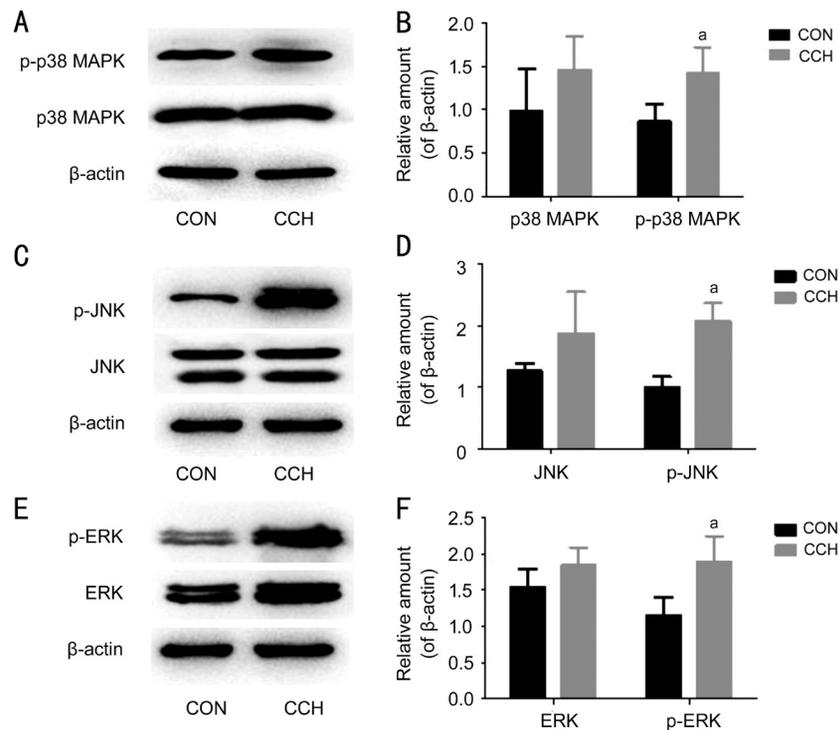


Figure 3 Expression of MAPKs protein in fibroblasts CCH vs CON group, <sup>a</sup>*P*<0.05.

Table 2 Expression of MAPKs mRNA in fibroblasts

		mean±SD, normalized to GAPDH		
Group	<i>n</i>	p38 MAPK	JNK	ERK
CON	6	1.00±0.00	1.00±0.00	1.00±0.00
CCH	6	7.41±3.80 <sup>a</sup>	1.98±0.47 <sup>a</sup>	4.17±2.11 <sup>a</sup>
<i>t</i>		-3.806	-4.632	-3.358
<i>P</i>		0.019	0.010	0.028

CCH vs CON group, <sup>a</sup>*P*<0.05.

**DISCUSSION**

CCH is an ocular surface disease accompanied by redness, dryness, irritation, epiphora and blurry vision<sup>[13]</sup>. The tear microenvironment then changes, owing to excessive conjunctival relaxation with or without high tension of the lower eyelid<sup>[14]</sup>. CCH is generally considered a condition affecting the older population with CCH severity increasing with age<sup>[15]</sup>. Mimura *et al*<sup>[16]</sup> have reported a higher prevalence of 75.5% in a hospital-based Japanese population. CCH is most often located in the nasal and temporal regions of the inferior conjunctiva<sup>[17]</sup>. Although CCH will not cause blindness, it strongly affects the vision-related quality of life. So it is necessary to clarify the mechanism of CCH and improve treatment methods.

Recent studies have shown that redundant conjunctiva results in the instability of tear film and dysfunction of tear meniscus, which cause disrupted tear flow, delayed tear clearance and increased tear osmotic pressure. All the above factors can cause ocular surface inflammation, leading to dry eye<sup>[18]</sup>. Despite the high prevalence of CCH, particularly among the elderly<sup>[1-2,16]</sup>, the exact mechanisms involved in CCH pathogenesis have

remained unknown. The gradual dissolution of Tenon’s capsule is widely expected to lead to the loss of subconjunctival adhesion and conjunctival thinning and stretching, which is the main pathological mechanism of CCH<sup>[6-7]</sup>. Moreover, the degradation of collagen fibers and fibroblast apoptosis, which cause decreased collagen synthesis, lead to thinning of the conjunctival tissue together. Zhang *et al*<sup>[7]</sup> have reported decreased elastic fiber and chronic inflammation lead to CCH. MMPs play a critical role in wound healing, tissue remodeling, and many diseases, including ocular surface diseases. Acera *et al*<sup>[19]</sup> have reported that pro-MMP-9 levels are significantly higher in CCH and then decrease significantly after resection of loose conjunctiva. MMP-1, MMP-3, and MMP-9 were thought to participate in the CCH pathogenesis. Li *et al*<sup>[9]</sup> have reported up-regulation of MMP-1 and MMP-3 in cultured CCH fibroblasts and imbalance between MMPs and TIMPs leading to the degeneration of collagen fibers and elastic fibers and excessive degradation of the conjunctival matrix in CCH. Our previous study has shown that the over-expression of MMP-1 and MMP-3 in conjunctival tissue and fibroblasts might play an important role in the pathogenesis and development of CCH<sup>[8]</sup>. However, the regulatory mechanism has not been fully clarified.

Recently MAPKs have been reported to increase the levels of MMPs in some diseases<sup>[20]</sup>. ERKs function is to control cell division, whereas JNKs are key regulators of transcription. The p38 MAPKs are activated by inflammatory cytokines and environmental stresses and involved in cell apoptosis and senescence<sup>[21]</sup>. Yang *et al*<sup>[22]</sup> have reported that activation

of the ERKs/JNKs signaling pathways contributes to the up-regulation of MMP-9. Moreover, MMP-9 may be one of the most important molecules in cancer cell metastasis. Aroui *et al*<sup>[23]</sup> have shown that naringin could attenuate the MAPK signaling pathways, such as the ERKs, JNKs and p38 MAPKs, decrease the expression and enzymatic activities of MMP-2, MMP-9, thus inhibiting the metastasis of U87 cells. Simon *et al*<sup>[24]</sup> have found that p38 and MKK-6 isoform mutants decrease the MMP-9 levels *in vitro* in UM-SCC-1 cells.

However, previous studies have not elucidated the MAPKs expression and the relationship between MAPKs and MMPs in CCH. Our study showed that the expression of p-ERK, p-JNK, and p-p38 of conjunctival tissue in CCH group was apparently higher than that in CON group. In cultured conjunctival fibroblasts, the OD values of p38 MAPK, JNK, ERK, and their phosphorylation in CCH group were apparently higher than those in CON group ( $P<0.05$ ). The protein expression of phosphorylated p38 MAPK, JNK, and ERK in CCH group was apparently higher than that in CON group ( $P<0.05$ ). The total expression of p38 MAPK, JNK, and ERK mRNA of the fibroblasts in CCH group was significantly higher than that in CON group ( $P<0.05$ ). Thus, the MAPKs expression of conjunctival tissue and human conjunctival fibroblasts in CCH group were higher than those in CON group. These differences might also cause up-regulation of MMPs expression. The MAPK signaling pathways regulate the abundance and activity of MMP-9 by activating transcription factors such as NF- $\kappa$ B and AP-1 in various target cells<sup>[25]</sup>. Heat shock protein 27 (HSP-27) activates the ERK and JNK signaling pathways, thus leading to over-expression of MMP-1 and MMP-3<sup>[26]</sup>. The expression of MMPs acting at the transcriptional and translational levels was imbalanced: the synthesis and activity of degrading enzymes increased, leading to extracellular matrix degradation. The tear excretion is delayed, the tears accumulate, which cause the inflammatory cytokines in the tears accumulate and concentrate, and the tear osmotic pressure rises. High osmotic pressure would then also activate the MAPKs signaling pathway, induce high expression of pro-inflammatory cytokines and chemokines<sup>[27]</sup>, and ultimately lead to CCH.

In conclusion, we found the up-regulation of p38 MAPK, JNK, ERK proteins and mRNA in CCH loose conjunctival tissue and fibroblasts, which would also activate the expression of MMPs. This up-regulation might cause the degradation of collagen fibers and elastic fibers and promote CCH. Our results improve the understanding of the pathological mechanism underlying CCH. But this study also has some limitations, and further experiments are needed to establish a direct link between MAPKs and MMPs in CCH. In addition, a larger sample size may be needed to adequately detect differences in the future.

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**Conflicts of Interest:** Jia YL, None; Liu XJ, None; Wen H, None; Zhan YP, None; Xiang MH, None.

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