• Basic Research •

Activation of autophagy in the retina after optic nerve crush injury in rats

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Abstract

• AIM: To investigate the activation of autophagy in rat retina after optic nerve crush (ONC) and evaluate its relationship with apoptosis of retinal ganglion cells (RGCs).

• METHODS: The ONC model was established. Western blots were performed to investigate expression of p62, LC3 and Beclin-1. Transmission electron microscopy was performed to discover the autophagosomes in the retina after ONC. Immunohistochemistry was used to confirm the distribution of LC3. TUNEL was performed to confirm the relationship between autophagy and RGC apoptosis.

• RESULTS: p62/Beclin-1 ratio was declined shortly after ONC until to day 7 after ONC and then restored to a normal level at day 21. There was an opposite change in the LC3-II/LC3I ratio in the retina compared to the p62/Beclin-1 ratio. Increased autophagosomes were found after ONC using transmission electron microscopy, and most of the LC3-stained cells were colocalized with RGCs and Müller cells. More LC3-immunoreactive cells and apoptotic RGCs were found on day 7 following ONC.

• CONCLUSION: Possible activation of autophagy in RGCs after ONC; autophagy mainly occurred in RGCs and Müller cells, and the apoptosis of RGCs after ONC may be partly associated with autophagic activation.

• KEYWORDS: autophagy; optic nerve crush; apoptosis; retinal ganglion cells; rat

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INTRODUCTION

The neurodegenerative process after optic nerve crush (ONC) is similar to the pathological process of glaucomatous optic neuropathy[1]. The pathological basis of ONC is progressive retinal ganglion cells (RGCs) loss in retina and optic nerve fibers loss, leading to irreversible changes in visual function[2]. RGCs are one of the three major retinal neurons in the retina. Their axons form the optic nerve and send visual information to higher brain[3]. RGCs degeneration is often modeled using ONC, which can better simulate the secondary apoptosis of RGCs[4-6]. The mechanism of optic nerve injury and the repair of RGCs after injury[7] has become one of the hot topics in ophthalmology. It is also an urgent problem for ophthalmology.

Autophagy in cell death is characterized by a large aggregation of autophagic vesicles and no nuclear condensation. Autophagy refers to some degradable components, such as protein and organelles, being encapsulated and transported for lysosomal degradation. The amino acids and other small molecules produced by autophagic degradation can be reused or can generate energy[8-9]. Under normal conditions, autophagy occurs at a fairly low level in many cells. Autophagy is a controllable defense. When cells are exposed in physiological stress stimuli (*e.g.*, starvation, high temperature, external stimulation, mutated protein aggregation or microbial invasion), autophagy can be activated[10-11]. Previous studies showed that autophagy, a cellular homeostasis-maintaining process, plays an important role in response to many environments, but excessive autophagy can directly lead to programmed cell death[12].

Autophagy can play a protective or harmful role in different stages of pathological processes. Many pathological processes have been found to be related to autophagy, such as cancer, neurodegenerative diseases, and central nervous system injury[13-14]. Autophagy has been found to play a critical role in neuronal survival, the clearance of senescent organelles and misfolded proteins in nervous system, and a protective role in neurons[15-17]. However, autophagy can be one of modes of nerve cell death[18]. In visual system, autophagy participates in the pathological changes in RGCs after optic nerve injury[19-23]. In animal models of optic nerve transection and intraocular hypertension, autophagy was formed in RGCs, and high expression of autophagy-related genes/protein indicated that autophagy was activated in RGCs after optic nerve injury[19-22].

Although autophagy has been demonstrated in photoreceptors in mouse and fly models[24-25], few studies have explored the activation of autophagy in rats *in vivo*[26-27]. Our aim is to investigate the activation of autophagy in the rat retina after ONC and to evaluate its relationship to RGC apoptosis *in vivo*. In this study, we demonstrated that autophagy could be activated after ONC in rats and has a relationship with apoptotic RGCs. By using an ONC rat model, we investigated that the apoptosis of RGC might be partially associated with the activation of autophagy *in vivo*. Thus, autophagy modulation might provide a potential therapeutic target for the amelioration of RGC degeneration in ONC.

MATERIALS AND METHODS

Ethical Approval Adult Sprague Dawley rats (200-250 g) of either sex were bought from the animal facility of Nantong University in line with the National Medical Standards for animal use of clean grade and provided by the Animal Experimental Center. All animal experiments were approved by the Jiangsu Branch of the Chinese National Committee for the Use of Experimental Animals for Medical Purposes and followed the guidelines of the Care and Use of Laboratory Animals (National Research Council, 1996, USA).

The animals were randomly divided into seven groups (n=12 each): Sham, 1, 3, 5, 7, 14, and 21d after injury. The retinas from 6 of them at the indicated time were used for westem blots (WB). The retinas from 3 of them were used for immunohistofluorescence (IHF), and the others were used for transmission electron microscopy.

The Establishment of Optic Nerve Crush The rats were deeply anesthetized with an intraperitoneal injection of a local anesthetic (10% chloral hydrate). Analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg; Schering-Plough, Madrid, Spain). During and after surgery, the eyes were covered with an ointment containing tobramycin (Tobrex; Alcon, S. A., Barcelona, Spain) to avoid corneal desiccation. Under a binocular surgical microscope, a lateral canthotomy was used in the eye. An incision was performed in the skin overlying the superior orbital rim, the supero-external orbital contents were dissected, and the extraocular were sectioned. The nerve was crushed 2 mm behind the eyeball with a micromaterial clip vertical to the long axis for 10s. The contralateral eye did not crush the optic nerve.

Western Blot Protein pyrolysis and WB were performed for individual retinas at different time points postoperatively, as previously described[28]. Proteins were electrophoresed on 10% SDS-polyacrylamide gel and were transferred to polyvinylidene difluoride filters. After overnight incubation with antibodies against Beclin-1 (1:800; Cell Signaling Technology, USA), LC3B (1:800; Sigma, USA), p62 (1:5000; Abcam, USA) and GAPDH (1:2000; Santa Cruz Biotechnology, USA) overnight at 4°C. Primary antibody binding was re-incubated using an HRP-conjugated secondary antibody at 37°C for 1h. Proteins were detected using enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Transmission Electron Microscopy Blocks of 1 mm^3 of retinal tissue were fixed for 4h at 4°C in 4% glutaraldehyde (v/v) buffer, washed with 0.01 mol/L phosphate buffer solution (PBS, pH 7.0), fixed again for 2h at 4°C in aqueous 1% (w/v) osmium tetroxide, and then embedded in Epon. Observation and photography were carried out by a JEOL JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

Immunohistofluorescence Retinal isolation and IHF were detected as previously described[28]. The sections were blocked and incubated with antibodies against LC3B (Sigma, 1:100), Brn3a (Abcam, 1:100), and GS (Santa Cruz, 1:100) overnight at 4°C. The sections were stained with Alexa Fluor® 568 goat anti-rabbit IgG (1:200, Jackson Laboratory) and 488 goat anti-mouse IgG (1:200, Jackson Laboratory) for 2h at 4°C. The images were taken with fluorescence microscope (Leica, Germany).

TUNEL Staining TUNEL staining of fragmented DNA was detected on whole retinal sections according to previous methods using an *in situ* Cell Death Detection kit, POD (Roche Applied Science) and following the manufacturer's instructions. The retinal sections were fixed with 4% paraformaldehyde for 1h and washed with 0.01 mol/L PBS (pH 7.0). Then, the slides were incubated with permeabilization solution for 8min on ice and subsequently added to citrate buffer for microwave irradiation for 3min, followed by incubated with LC3B (Sigma, 1:100) for 4h on ice. The TUNEL reaction mixture and 568 goat anti-rabbit IgG (1:200, Jackson Laboratory) were incubated to the slides for 1h at 37°C in a wet box in the dark. After being double-stained with LC3B and TUNEL, the cell nuclei were labeled with DAPI (1:5000; Life Technologies), and the sections were taken with fluorescence microscope (Leica).

Statistical Analysis The data were expressed as the mean±SD and analyzed using the SPSS software (version 17.0, SPSS Inc, IL, USA). Differences among the groups were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison tests. P values of <0.05 were considered statistically significant.

RESULTS

The Expression of Autophagy-Related Proteins LC3, p62/Beclin-1 in the Retina After ONC in Rats To investigate the precise dynamics of RGC autophagic activation after ONC, the expression levels of p62, Beclin-1 and LC3 in retinas were analyzed at different time points after the injury using WB. p62 and p62-bound polyubiquitinated proteins are incorporated into autophagosomes and degraded in autolysosomes, thus serving

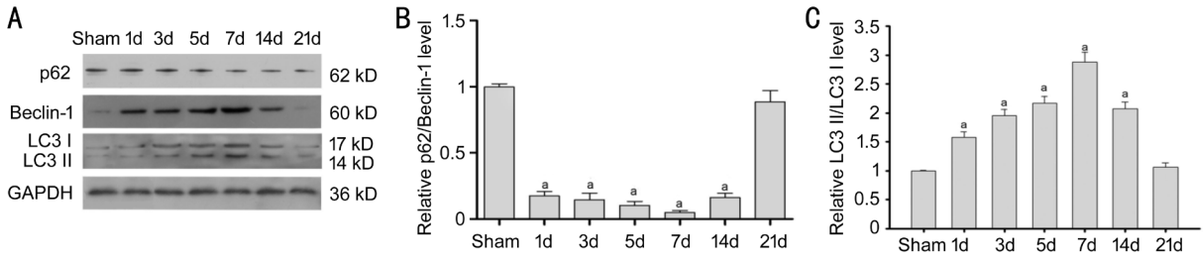

Figure 1 The expression of LC3, Beclin-1 and p62 in retinas with or without ONC The level of GAPDH protein was performed as the internal control. Data are presented as the mean±SD of 3 independent experiments. $^{a}P<0.05$ *vs* sham group.

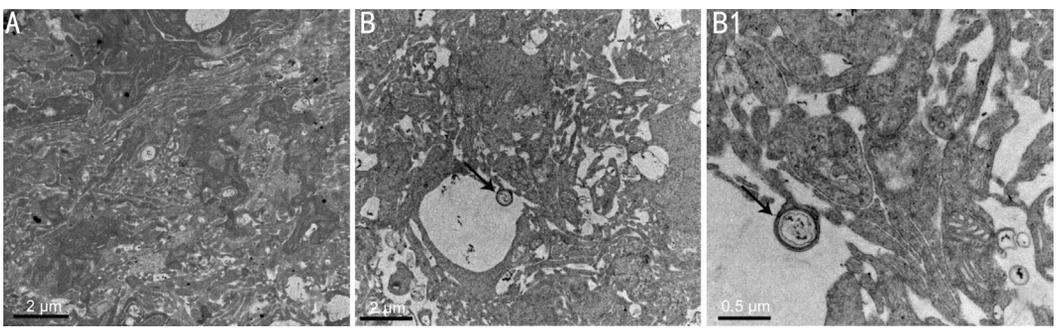

Figure 2 Electron microscopy analysis of representative RGCs from the corresponded 7 day-sham and 7 day-injured retinas A: Normal retinal ultrastructure; B: The ultrastructure of the retina 7d after ONC. Bar=2 μm. B1: Indicate the enlargement of autophagosomes in diagram B. Bar=0.5 μm.

as an index of autophagic degradation[29]. Beclin-1, as a key regulator in autophagy, regulates autophagosome formation[30]. The p62/Beclin-1 ratio are used as a readout of autophagy[31]. High basal levels of the p62/Beclin-1 protein level ratio were found in the normal adult rat retinas but were greatly decreased after ONC (Figure 1). At 7d after ONC, a slight decrease in the retinal p62/Beclin-1 ratio was lower than that of the basal expression in the sham retinas. At 21d after ONC, no aberrant changes in p62/Beclin-1 ratio were detected when compared to the retinas from untreated nerves. LC3 is also a marker of autophagy. When autophagy is formed, cytoplasmic LC3 (LC3-I) will hydrolyze a small polypeptide and turn it into (autophagy) membrane type (LC3-II), and LC3-II/LC3I ratio can be used as an index for measuring the level of autophagy. There was an opposite change in the LC3-II/LC3I ratio in the retina compared to the p62/Beclin-1 ratio (Figure 1). The level of LC3 was found to be lower in the normal adult rat retinas than in the ONC retinas. LC3 was slightly increased in the retinas 7d after ONC, and no significant changes were observed 21d after ONC compared to the basal expression in the sham retinas.

The Observation of Autophagosomes in the Retina After ONC Using Transmission Electron Microscopy Under transmission electron microscope, we observed that there was little or no bilayer membrane autophagosomes in the sham retinas. However, the number of autophagosomes increased in the retinal tissue after ONC (Figure 2), indicating that retinal autophagy was activated after ONC.

The Distribution of LC3 in the Retina After ONC To investigate the distribution of LC3 in retina and to clarify its relationship to retinal cells, we used the IHF method, labeled Müller cells with GS, labeled RGCs with Brn3a, and detected whether there was a co-localization between the two cells and the LC3 cells. Both in the sham and ONC rat retinas, double-stained for LC3 and RGCs were detected. In addition, the co-localization of LC3 and RGCs increased significantly at 7d (Figure 3). In addition, we obtained a similar result with LC3 (green fluorescent signal) and GS-marked Müller cells (red fluorescent signal), as they exhibited good overlap in ganglion cell layer (GCL) of the retinas (Figure 4), suggesting that LC3 was mainly expressed in the RGCs and Müller cells.

The Relationship Between Autophagy and RGC Apoptosis After ONC To further confirm the relationship between autophagy and RGC apoptosis after ONC, double-staining of LC3B and TUNEL were performed. There were few LC3+ cells or TUNEL+ cells in the ganglion cell layer (GCL) of the sham group (Figure 5). In addition, the co-localization of LC3+ and TUNEL+ cells increased markedly at 7d. Notably, not all the TUNEL+ cells overlapped with the LC3+ cells, and some TUNEL+ cells had no LC3+ signals. This suggested that part of RGC apoptosis after ONC might be related to the activation of autophagy.

DISCUSSION

Optic nerve injury is a critical cause of visual impairment in the world. Here, we addressed the role of autophagy in retinal neurodegeneration after optic nerve injury *in vivo*. First, by

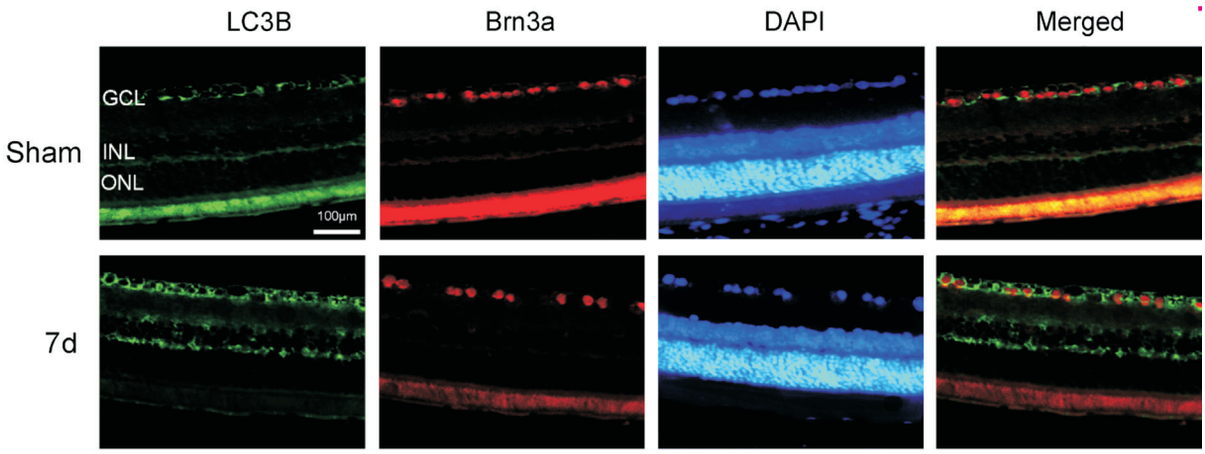

Figure 3 Immunofluorescence analysis of the association between LC3 and the RGC marker, Brn3a, was conducted in corresponded 7 day-sham and 7 day-injured retinas GCL: Ganglion cell layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; Bar=100 μm.

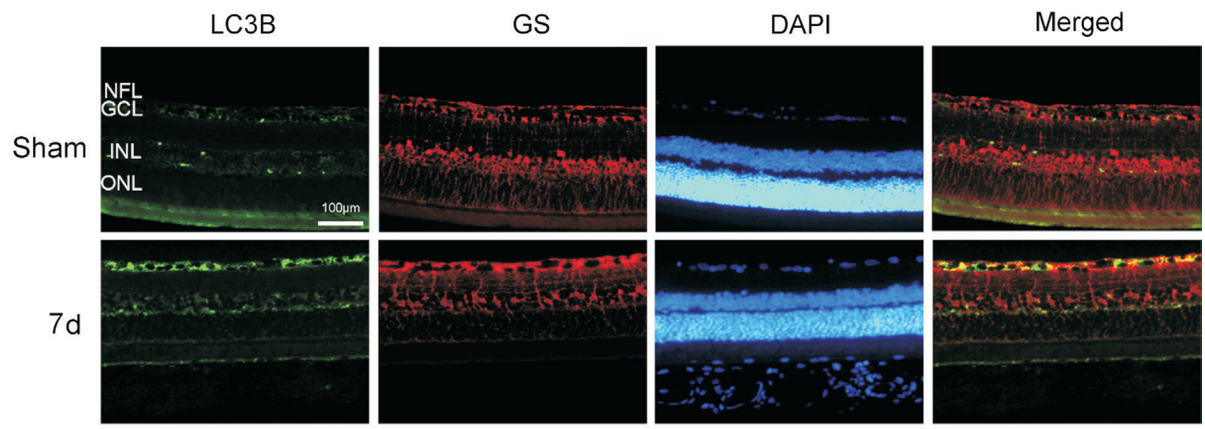

Figure 4 Immunofluorescence analysis of the association between LC3 and the Müller cell marker, GS, was conducted in corresponded 7 day-sham and 7 day-injured retinas NFL: Nerve fiber layer; GCL: Ganglion cell layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; Bar=100 μm.

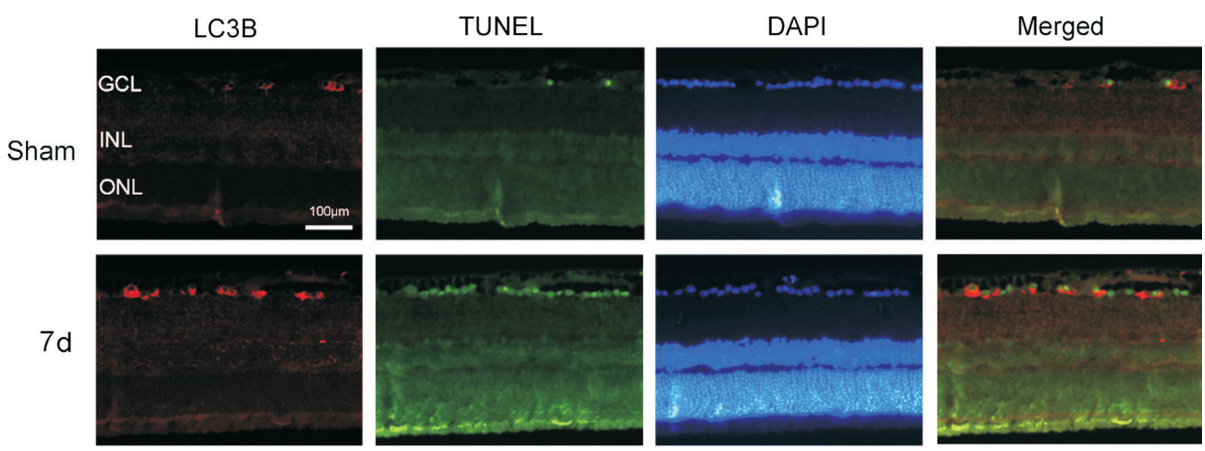

Figure 5 Immunofluorescence and TUNEL staining analysis of the association between autophagy and RGC apoptosis GCL: Ganglion cell layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; Bar=100 μm.

using the model of ONC in rats that induces RGC apoptosis and by testing autophagic proteins and autophagosomes *in vivo*, we found that autophagy was rapidly activated in the retina after ONC. Further, we demonstrated that autophagic activation might be related to the regulation of RGC apoptosis. Altogether, these data showed autophagy in retinal cells after ONC in rats.

Many studies have found that retinal or optic nerve injury derived from various causes can induce autophagic activation in mice and Ganges River monkeys[32]. In the optic nerve transection injury model, the number of green fluorescent-labeled GFP-LC3 positive cells in the retinal GCL increased,

and the level of autophagy-related gene Atg5 mRNA increased significantly, indicating that autophagy was activated rapidly[19]. In the mouse model of retinal ischemia-reperfusion injury caused by an acute increase in intraocular pressure (IOP), the autophagy at 12h and 24h in retinal neurons was obviously activated after the injury[20]. In the Ganges River monkey chronically high IOP model, the expression levels of LC3B II/LC3B I and Beclin-1 protein were detected. Meanwhile, autophagic bodies were observed using transmission electron microscope. The results showed that the autophagic flow increased after the increase in chronic IOP[21]. In rat models of chronic ocular hypertension, autophagy was activated after the increase in IOP, and IOP increased early. Autophagic bodies were detected in the plexiform layer (IPL). After that, autophagic bodies decreased in the IPL and increased in GCL, indicating that autophagy in the RGCs was activated after optic nerve injury[22]. Our study showed that the retinal autophagy-related protein LC3 increased significantly 1d after ONC in rats and peaked at 7d, and the p62/Beclin-1 ratio decreased, a finding that was consistent with previous finding[33]. In addition, we used immunofluorescence and observed LC3 co-localization with RGCs in the GCL after ONC and co-localization with Müller cells in GCL, indicating that autophagy was activated in RGCs and Müller cells in retina after optic nerve injury. The autophagic activation of Müller cells after optic nerve injury has not been reported, but in central nervous system injuries, such as spinal cord injury models and traumatic brain injury models, the autophagy-related gene Beclin-1 is expressed in neurons, astrocytes, and oligodendrocytes, which was consistent with our results.

There was still controversy regarding the activation of autophagy in order to promote neuronal survival or apoptosis after nerve injury in the central nervous system. In the rat model of persistent middle cerebral artery occlusion, autophagy was found to be significantly activated in the ischemic area, there was a reduction in the obstruction area after autophagic inhibition, and there was alleviation of cerebral edema and neurological symptoms[34]. However, in two studies of brain trauma, autophagy was found to be activated, and autophagy exerted opposite effects. One found that autophagy protected neurons in the early stage of the injury[35], and the other found that autophagy could lead to the death of neurons[36]. Thus, autophagic activation after nerve injury has different effects on different researchers and different models of neurons, which may be related to the time and extent of the injury. Some scholars believe that mild to moderate activation of autophagy plays a protective role in ischemic and hypoxic neurons. Over-activation of autophagy may destroy the dynamic balance of cell metabolism and lead to neuronal death[35-37].

There have been few studies on the effects of autophagy in the fate of RGCs in retinal optic nerve injury. In the model of retinal ischemia-reperfusion injury induced by ocular hypertension, autophagy was activated, and the apoptosis of RGCs was increased. The death of RGCs was reduced in responses to autophagic inhibitors[20]. Autophagy has been shown to activate and induce RGC death in chronic ocular hypertension[22], whereas inhibition of autophagy decreases RGC apoptosis[20], suggesting that autophagy may promote loss of retinal neurons, consistent with our results. However, some scholars have also reported that in serum-free culture conditions, the application of drugs to inhibit autophagy will reduce the viability of RGCs[23]. In this study, the results of the TUNEL assay showed that autophagic activation in RGCs was accompanied by apoptosis of RGCs after ONC, indicating that autophagic activation may be related to RGC apoptosis in this model.

In light of the fact that autophagic activation might change the fate of RGCs after ON injury, we hope to offer greater protection of the optic nerve through the study of autophagy regulation in future research.

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Conflicts of Interest: Kang LH, None; **Zhang S,** None; **Jiang S,** None; **Hu N,** None.

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