• Basic Research •

CCPG1 involved in corneal *Aspergillus fumigatus* infection

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Abstract

● **AIM:** To investigate whether non-canonical autophagy transport receptor cell cycle progression 1 (CCPG1) is involved in the corneal antifungal immune response.

● **METHODS:** Human corneal epithelial cells (HCECs) and human myeloid leukemia mononuclear cells (THP-1) macrophages stimulated by *Aspergillus fumigatus* (*A. fumigatus*) were used as cell models. The expression of CCPG1 mRNA was detected by qRT-PCR. Western blot was used to determine the protein expression of CCPG1 and interleukin-1β (IL-1β). The dectin-1 neutralizing antibody was used to detect the association between dectin-1 and CCPG1. Immunofluorescence was used to observe the colocalization of CCPG1 and C-type lectin-like receptor-1 (CLEC-1) in THP-1 macrophages.

● **RESULTS:** The expression of CCPG1 started to increase at 4h after infection and increased in a time-dependent manner in HCECs and THP-1 macrophages. With dectin-1 neutralizing antibody pretreatment, the expression of IL-1β was down-regulated. CCPG1 up-regulation in response to *A. fumigatus* infection was independent of dectin-1. Immunofluorescence showed the colocalization of CCPG1 and CLEC-1 in THP-1 macrophages.

● **CONCLUSION:** As a specific autophagy protein of non-canonical autophagy pathway, CCPG1 is involved in corneal infection with *A. fumigatus*.

● **KEYWORDS:** cell cycle progression 1; fungal keratitis; *Aspergillus fumigatus*; C-type lectin-like receptor-1

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INTRODUCTION

Corneal diseases, especially infectious corneal diseases, are one of the leading causes of blindness worldwide[1-3]. Its importance in the prevention and treatment of eye diseases is second only to cataracts[4]. Fungal keratitis (FK), as a kind of blinding keratopathy, has been paid more attention globally[5]. In developing countries and tropical and subtropical regions, FK usually occurs after vegetative trauma[6]. In developed countries, improper wearing of contact lenses is the main reason for FK[7]. Due to the lack of effective drugs and surgical treatments, the cure of FK is still challenging.

Recent studies have shown that autophagy plays an immunomodulatory role in innate and adaptive immune responses by selectively targeting signal transduction molecules. Autophagy is a highly conserved cellular process in eukaryotes. Its function of maintaining cell homeostasis is achieved by supporting cell survival and regulating inflammation. Autophagy can degrade unnecessary or functionally abnormal intracellular components, such as abnormal proteins, old organelles[8] and pathogens[9], and has been extensively studied in various immune cells including T cells[10], B cells[11], and macrophages[12]. More and more evidences show that autophagy plays an important role in the host's defense against microbial infections and inflammation. For example, it can inhibit the activation of inflammasomes in macrophages[13-14], and may eliminate active inflammasomes through p62 ubiquitination[15].

Bernales *et al*[16] and Schuck *et al*[17] found that in yeast, the endoplasmic reticulum provided a membrane for autophagosomes and was also a target of autophagy. As a key intracellular

organelle, the endoplasmic reticulum is responsible for overseeing the synthesis of transmembrane proteins and secreted proteins. The unfolded protein response (UPR) occurs when the endoplasmic reticulum is under stress. UPR is a response that resolves the defects of endoplasmic reticulum cavity protein inhibition through transcriptional activation. When a protein misfolds and accumulates in the endoplasmic reticulum, some signaling pathways are activated, and then a cascade reaction is triggered to inhibit translation[18].

Cell cycle progression 1 (CCPG1) is an endoplasmic reticulum protein induced by endoplasmic reticulum stress. Its expression is up-regulated under the induction of UPR. It can interact with GABARAP and LC3 family proteins[19].

The role of CCPG1 in UPR is still under further study. Smith and Wilkinson[20], by inhibiting the expression of CCPG1, found that the expansion of endoplasmic reticulum (ER) and the destruction of cell distribution in mouse retinal acinar cells were aggravated; at the same time, molecular markers of ER stress also increased. That is to say, it is related to the loss of UPR-regulated ER phagocytosis above the maintenance of the ER.

This type of selective autophagy promotes the targeted elimination of specific organelles or cargo through the action of specific autophagy receptors. These substances are recruited and isolated into autophagosomes[21].

This experiment predicts whether CCPG1 can participate in the current harmful FK, which can prevent excessive inflammation, protect the host from collateral damage and increase the treatment of FK.

MATERIALS AND METHODS

Preparation of *Aspergillus Fumigatus* *Aspergillus fumigatus* (*A. fumigatus*) strain 3.0772 was purchased from the China General Microbial Culture Collection Center (Beijing, China) and was cultured for 3-4d. Remove impurities, filter with sterile cotton gauze to obtain a pure conidia suspension, and adjust the concentration to 5×10^7 cfu/mL for retention.

***In Vitro* Experiments** Human corneal epithelial cells (HCECs) and THP-1 macrophages were seeded into 6-well plates and 12-well plates, respectively, and then grown to 80% confluence in an incubator (37℃, 5% CO_2). After stimulation with *A. fumigatus* conidia for 4, 8, and 16h later, the cells were collected and lysed, and then qRT-PCR and Western blot tests were performed.

THP-1 macrophages were pretreated with dectin-1 neutralizing antibody (R&D) and IgG neutralizing antibody (R&D) for 2h. After 16h of stimulation with *A. fumigatus* conidia, the cells were collected for qRT-PCR and Western blot.

Quantitative Polymerase Chain Reaction Use RNAiso plus reagent (Takara) to extract total RNA. PrimeScript RT kit (Takara) was used for reverse transcription of total RNA (2 μg). SYBR Green was used for real-time PCR. The sequences of all oligonucleotide primers used can be as follows: hβ-actinF-GCTCCTCCTGAGCGCAAG and R-CATCTGCTGGAAGGTGGACA, hCCPG1 F-GTCACACTTTTTCCCCTCCA and R-CTCAGTGGCC ATAAAGCACA.

Western Blot The HCECs and THP-1 were collected and placed in RIPA buffer (Solarbio, China): PMSF (Solarbio, China): phosphatase inhibitor cocktail I (MedChemExpress, USA) at a ratio of 98:1:1 and lysed on ice. A BCA kit (Solarbio, China) was used to determine the protein concentration.

Then used 8%-16% SDS-PAGE gel (GenScript, China) for total protein electrophoresis, and transfferd to PVDF membrane. The PVDF membrane was blocked in blocking buffer (Beyotime, China) at 37℃ for 2h, and with anti-interleukin-1β (IL-1β) primary antibody incubated (R&D, USA) or anti-β-actin primary antibody (CST, USA) or anti-CCPG1 primary antibody (Santa Cruz Biotechnology, USA) overnight at 4℃. The membrane was then incubated with HRP-labeled secondary antibody. Western ECL blotting substrate (Bio-Rad, USA) was added to the PVDF membrane to develop blots. Digital images were analyzed using a Vilber Solo 4S chemiluminescence imaging system.

Immunofluorescence Staining The slides with THP-1 cells were soaked in phosphate buffer saline (PBS) for 3 times, and fixed with 4% paraformaldehyde. Then the slides were permeated with 0.5% Triton X-100 (prepared in PBS) for 20min at room temperature, and immersed in PBS 3 times. Next, normal goat serum was added to the slides, blocking for 30min at 37℃; then the slides were incubated overnight with the following antibodies: anti-CCPG1 (Santa Cruz Biotechnology, USA), anti-CLEC1 (Novus, USA).

Fluorescent secondary antibody (ProteinTech) was added in the dark for 1h, and then the slides were soaked with PBST 3 times. Next, DAPI solution (Solarbio, China) was added dropwise and incubated for 5min in the dark, and then the specimens were stained with nuclei and immersed in PBST 3 times. Finally, a fluorescence micrograph was taken by Zeiss Axiovert microscope.

Statistical Analysis The statistical significance of each score and qRT-PCR was determined by *t*-test. The data are expressed as mean±standard deviation (SD) and analyzed by GraphPad 5.0 software. When $P<0.05$, the data is considered significant.

RESULTS

CCPG1 Expression Increased in HCECs Stimulated by *Aspergillus Fumigatus* *A. fumigatus* stimulated HCECs to establish fungal infection cell models. qRT-PCR results showed that the mRNA expression of CCPG1 increased after 4h, and the CCPG1 mRNA levels increased significantly with time (Figure 1A). Western blot experiments showed that

after 4h infection, the protein expression of CCPG1 increased significantly with time (Figure 1B). The above results show that CCPG1 participated in the antifungal immune response of HCECs.

CCPG1 Expression Increased in THP-1 Stimulated by *Aspergillus Fumigatus* Similarly, *A. fumigatus* stimulated THP-1 to establish cell models. The mRNA expression of CCPG1 did not change significantly after 4h, and increased significantly after 8 and 16h (Figure 2A). Through the Western blot experiment, differently, we observed that the protein level of CCPG1 began to increase after 4h stimulation, and increased significantly after 8 and 16h (Figure 2B). Accordingly, CCPG1 also participated in the THP-1 antifungal immune response.

The Effects of Using Dectin-1 Neutralizing Antibody on the Expression of CCPG1 After stimulation by *A. fumigatus*, the mRNA expression of CCPG1 increased indistinctively in THP-1 pretreated with dectin-1 neutralizing antibody (Figure 3A). But compared with the control group, there was no statistical difference.

The protein expression level of IL-1β was significantly decreased compared with the control group. However, treatment with dectin-1 neutralizing antibody did not affect the protein expression of CCPG1 (Figure 3B). Although all regulate the immune response by participating in non-canonical autophages, there was no necessary connection between dectin-1 and CCPG1.

Co-Expression of CCPG1 and CLEC-1 in Macrophages According to STRING Interaction Network Preview (Figure 4A), we predicted that C-type lectin-like receptor-1 (CLEC-1) and CCPG1 might be interacting proteins. Immunofluorescence results showed that CCPG1 and CLEC1 proteins were distributed in macrophages, and there was obvious intracytoplasmic co-localization (Figure 4B).

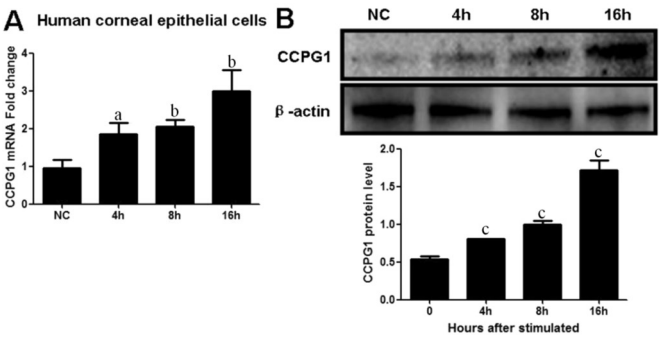

Figure 1 CCPG1 expression increased in HCECs stimulated by *A. fumigatus* The mRNA (A) and protein (B) expression of CCPG1 at 4, 8, 16h after infection. [a]$P \leq 0.05$, [b]$P \leq 0.01$, [c]$P \leq 0.001$.

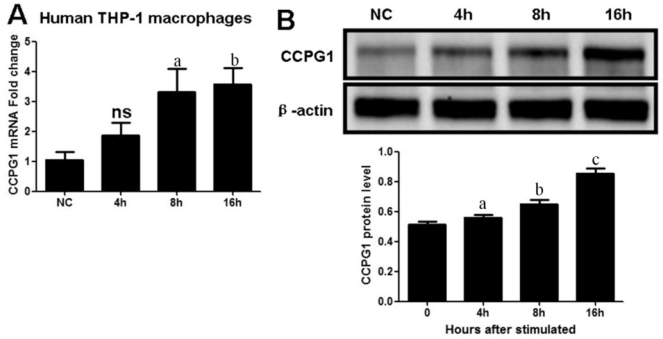

Figure 2 CCPG1 expression increased in THP-1 stimulated by *A. fumigatus* The mRNA (A) and protein (B) expression of CCPG1 at 4, 8, 16h after infection. [a]$P \leq 0.05$, [b]$P \leq 0.01$, [c]$P \leq 0.001$, ns means $P > 0.05$.

DISCUSSION

Autophagy was originally described as a process of self-decomposition, but now it is known that it plays a key role in the face of many aspects such as bacteria, viruses and parasitic pathogens[9]. Recent mechanism studies have shown that autophagy played an immunomodulatory role in innate and adaptive immune responses by selectively supplementing signal molecules[22]. In the past decade, another form of autophagy had emerged, called LC3- (microtubule-associated protein 1A/1b light chain 3-)-associated phagocytosis (LAP) or non-canonical autophagy. LAP is a unique pathway that participates in cell surface receptor signaling by recruiting the LC3-phosphatidylethanolamine (PE) binding system during phagocytosis[23].

LAP has recently become a major anti-inflammatory pathway, playing an important role in intracellular and physiological[24]. Jostins *et al*[25] found that LAP was associated with Crohn's disease, a common inflammatory bowel disease. This non-canonical autophagy pathway can prevent inflammation during the process of removing dead cells and protect autoimmunity and inflammatory bowel disease[25].

The ER is the largest membrane-bound organelle in eukaryotic cells. Its complex morphology, including flakes, tubules and impurities[26-28], reflects its different roles in various physiological processes including autophagosomes[8,29-30]. Studies have confirmed that the four receptors RETREG1, SEC62, CCPG1, and RTN3 helped to isolate the ER separation products into autophagosomes. Among them, CCGP1 and RTN3 were mainly responsible for the conversion of ER tubules[31].

When the non-canonical autophagy pathway is activated, UPR induction leads to an increase in the ER CCPG1 protein. When CCPG1 has FIR and LIR motifs, it can interact with autophagy proteins of the RB1CC1/FIP200 and ATG8 families, respectively. It helps to isolate part of the ER to the phagosome, thereby restoring the ER replacement[20].

Both corneal epithelial cells and macrophages were important cells involved in the antifungal infection of the cornea. When HCECs and THP-1 stimulated by *A. fumigatus*, the expression of CCPG1 increased significantly. Our experiment proved that CCPG1, as an alternative autophagy protein for non-canonical

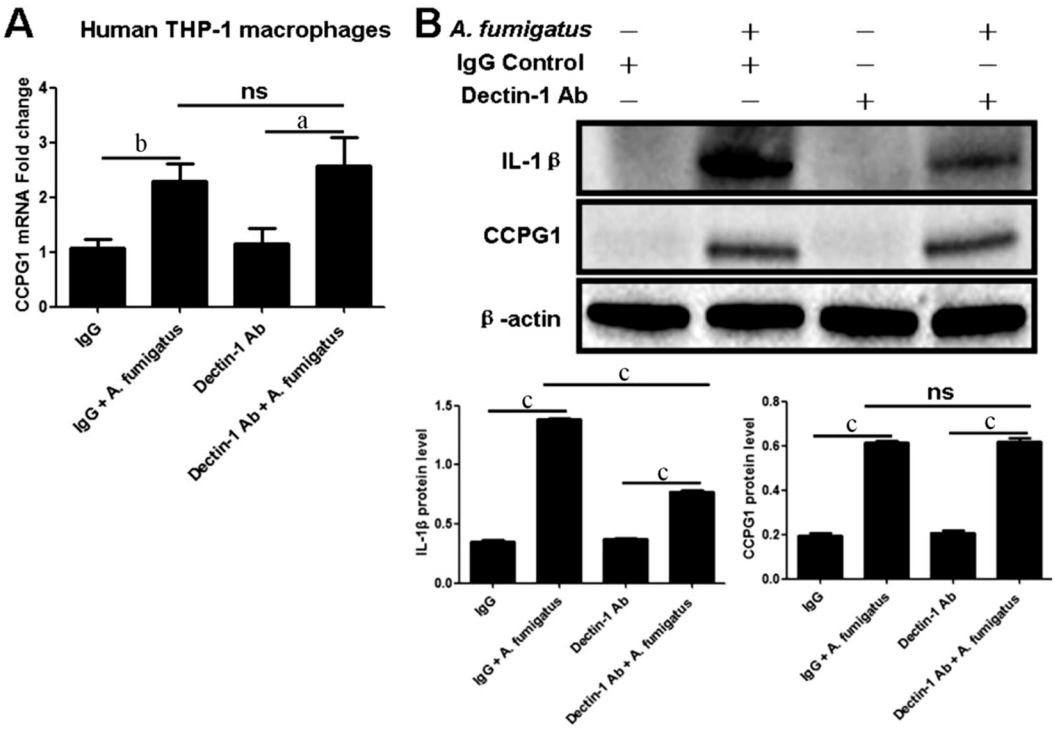

Figure 3 Dectin-1 neutralizing antibody does not affect the expression of CCPG1 Pretreated with dectin-1 and IgG neutralizing antibody, THP-1 was stimulated by *A. fumigatus*. A: The mRNA expression of CCPG1; B: The protein expression of CCPG1and IL-1β. $^{a}P\leq0.05$, $^{b}P\leq0.01$, $^{c}P\leq0.001$, ns means $P>0.05$.

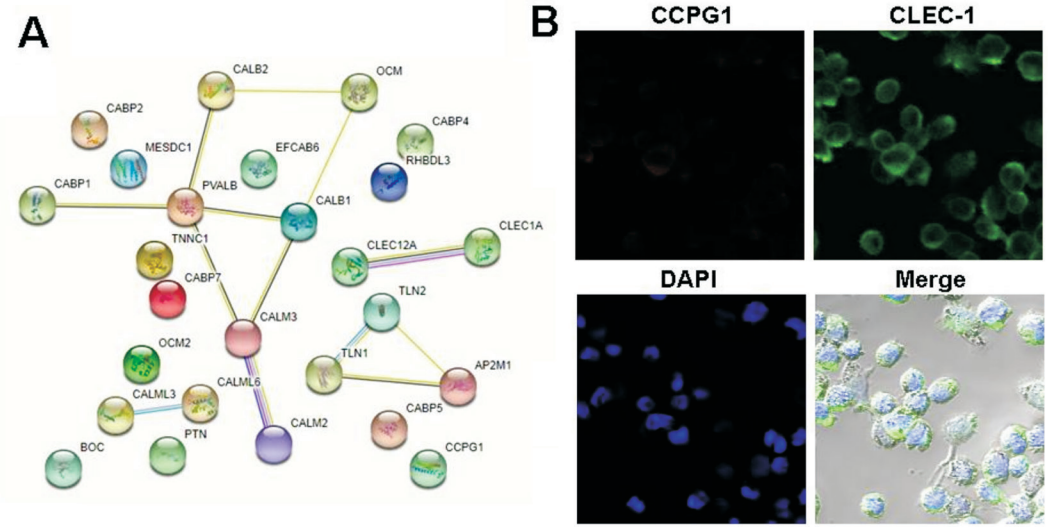

Figure 4 CCPG1 is co-expressed with CLEC-1 in macrophage A: STRING Interaction Network Preview; B: In THP-1, red: CCPG1 staining; green: CLEC-1 staining; blue: nuclear staining (DAPI); yellow: CCPG1 and CLEC-1 were co-expressed.

autophagy pathways, participated in corneal antifungal immunity. Since THP-1 cell line is the most common in FK, follow-up experiments use THP-1 cells as the cell model.

Previous experiments[32-33] have found that dectin-1 was one of the most important host anti-fungal pattern recognition receptors. The members of the dectin-1 cluster include CLEC-1 receptors[34-35]. We found that using dectin-1 neutralizing antibody to inhibit the expression of dectin-1 down-regulated the expression of IL-1β, indicating that the neutralization was effective. Surprisingly, there was no significant change in the expression of CCPG1. This experiment proved that there was no necessary connection between dectin-1 and the generation of CCPG1.

According to the STRING Interaction Network Preview, we predicted that CLEC-1 and CCPG1 might interact with proteins. We verified the expression positions of the two were basically coincident by immunofluorescence experiments: CCPG1 and CLEC-1 were co-expressed in macrophages. At

present, there are few studies on CLEC-1 and CCPG1, so it is difficult to buy commercial antibodies, and there is no way to do further research such as co-IP.

This study emphasizes that CCPG1 is involved in corneal antifungal immunity, but more in-depth research is needed. Future research directions will continue to explore the mechanism of CCPG1 in the corneal research response. In summary, CCPG1 as a non-canonical autophagy cargo receptor is involved in corneal antifungal immunity.

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