

Expression of vitamin D receptor and cathelicidin in human corneal epithelium cells during fusarium solani infection

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Abstract

• **AIM:** To observe the expression of vitamin D receptor (VDR) in human specimen and immortalized human corneal epithelium cells (HCEC) when challenged with fusarium solani. Moreover, we decided to discover the pathway of VDR expression. Also, we would like to detect the expression of cathelicidin antimicrobial peptide (CAMP) in the downstream pathway of VDR.

• **METHODS:** Immunohistochemistry was used to examine the VDR expression in HCEC from healthy and fungal keratitis patients. Real time quantitative polymerase chain reaction (qPCR) was performed to observe the messenger ribonucleic acid (mRNA) change of VDR when immortalized HCEC were challenged with fusarium solani for different hours. CAMP was detected at both mRNA and protein levels.

• **RESULTS:** We found out that the VDR expression in fusarium solani keratitis patients' specimen was much more than that in healthy people. The mRNA and protein expression of VDR increased when we stimulated HCEC with fusarium solani antigen ($P < 0.01$) and it could be inhibited by toll like receptor 2 (TLR2) monoclonal antibody. The CAMP expression was decreased because of fusarium solani antigen stimulation ($P < 0.01$).

• **CONCLUSION:** The VDR expression can be increased via TLR2/1-VDR pathway while the CAMP expression is decreased by the stimulation of fusarium solani antigen.

• **KEYWORDS:** vitamin D receptor; fusarium solani; corneal epithelium; cathelicidin

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INTRODUCTION

Fungal keratitis (FK) is one of the main causes of corneal blindness and visual disability [1]. Up to today, the treatment of FK remains a big challenge for ophthalmologists [2]. Recently, the incidence of the disease has been increased significantly [3]. Fusarium solani is known as the most common pathomycete of FK which occurs frequently among developing countries with big agriculture population [3,4], such as China and India, but relatively uncommon in developed countries. Host defense factors and fungal virulence factors play important roles in the process of FK [5-7]. The innate immune system of ocular surface is the first defensive line to fight against microbial infection. It can activate the immune defense response to identify and remove pathogens by the pattern-recognition receptors (PRRs) which are expressed by innate immune cells recognize pathogen associated molecular patterns (PAMPs) [8]. Because the lack of sensitive drugs and effective treatments, further research on the pathogenesis and mechanism of FK is of vital importance.

Vitamin D receptor (VDR) is a member of the nuclear receptor superfamily [9]. The sole human cathelicidin peptide, known as LL-37, is a free peptide part of the precursor protein hCAP18, acting as the active form of cathelicidin antimicrobial peptide (CAMP) in human. In mammals, VDR is highly expressed in metabolic tissues, such as intestine, kidney, skin, and thyroid gland, and moderately expressed in nearly all tissues [10]. Moreover, VDR is expressed in many malignant tissues [11]. The nonclassic actions are therefore potential targets for the active metabolite of vitamin D, 1,25 (OH)₂D₃. Yin *et al* [12] has discovered that the mRNA of VDR was expressed in corneas of human, rabbit and mouse. However, the change of the 1,25 (OH)₂D₃ receptor (VDR) mRNA in corneal epithelial cells during the inducing of fusarium solani are still unclear.

The aim of our study was to investigate the expression of VDR in corneal epithelium of healthy people and FK patients. We also wanted to discover the change of VDR mRNA expression when human corneal epithelium cells (HCEC) were challenged with fusarium solani antigen and to discover the specific pathway of VDR expression. Moreover, we would like to observe the expression of CAMP when stimulated with fusarium solani antigen.

MATERIALS AND METHODS

Materials The strain of *fusarium solani* (CGMCC 3.1829) was purchased from China General Microbiologic Culture Collection Center, (Beijing, China). Sabouraud culture (89579-500G-F) was purchased from Sigma company (St. Louis, USA); RNAiso Plus (Cat# 9109), PrimeScript[®] RT reagent Kit With gDNA Eraser (Perfect Real Time) (Cat# RR047A), Primers and SYBR (Cat# RR420A) were purchased from TaKaRa CO., Ltd. (Shiga, Japan). Polyclonal anti-bodies against VDR (12550, Vitamin D3 Receptor[®]) for immunohistochemistry and Western blot were purchased from Cell Signaling Technology[®] (Boston, USA); 2-step plus[®] Poly-HRP Anti-Mouse/Rabbit IgG and Diaminobenzidine (DAB) kit were purchased from ZSGB-BIO Co., Ltd. (Beijing, China); monoclonal antibody against toll like receptor 2 (TLR2; 309709, LEAF[®] Purified anti-human TLR2 antibody) as inhibitor were purchased from BioLegend (San Diego, USA); enzyme-linked immunosorbent assay (ELISA) kits for human LL-37 (HK321-01) were bought from Hycult Biotech (Pennsylvania, USA).

Preparation of Fusarium Solani Antigens *Fusarium solani* standard strain (CGMCC 3.1829) was grown on sabouraud dextrose agar in 500 mL conical flask at 37°C and 200 rpm for 14d. The spore was scraped from the culture dish and collected directly. The spore and mycelia which recovered by grinded were washed twice by sterile phosphate buffere saline (PBS) and sterilized by 75% ethanol at 4°C overnight. Inactive *fusarium solani* mycelia and spore were washed three times and added in PBS. The mycelia and spore suspensions were quantified by using a haemocytometer. The final concentration was 1×10^9 /mL for spore and 1×10^8 /mL for mycelia. Then we stored them at -20°C^[13,14] for later use.

Human Corneal Epithelial Cells Culture Immortalized HCEC were cultured in high glucose medium, 37°C, 5% CO₂. Near 90% confluence, the cells were cultured in serum free dulbecco's modified eagle medium (DMEM). Cells were used for real-time quantitative polymerase chain reaction (qPCR).

Stimulation of Fusarium Solani Antigens Immortalized HCEC were cultured with *fusarium solani* mycelia stimulation liquid of 5×10^6 /mL and spore stimulation liquid of 5×10^7 /mL after discarding the medium. The concentration of stimulation liquid was according to the previous work of our team. The expression of VDR mRNA in HCEC was detected by real-time qPCR at the stimulation of 0, 4, 8, 16 and 24h according to our earlier experiments.

Clinical Human Corneal Specimen The corneal tissue was obtained from 5 healthy donors and 5 *fusarium solani* keratitis patients. Healthy donor corneas were used as corneal graft and the rest of the peripheral corneal tissues were used for experiment. FK patients were performed with penetrating keratoplasty at the Affiliated Hospital of Qingdao University

from January 2013 to December 2013. The diagnosis of *fusarium solani* keratitis was based on the case history, clinical features, identification and cultivation of fungal elements from corneal ulcer scrapings, and the detection of corneal confocal microscope. The aims and methodology were thoroughly explained to the donors, and the samples were collected after obtaining informed consent.

Immunohistochemistry Immunohistochemistry was performed by the PV-9000 2-step plus[®] Poly-HRP Anti-Mouse/Rabbit IgG Detection System^[15]. After neutral formalin-fixed, dehydration, transparent, baptist wax and embedding processing, corneal lesions of FK and normal corneas were cut into 4 μm sections and then dewaxing and hydration, washed with distilled water for 3 times. The VDR antigen was retrieved by citric acid buffer (pH 6.0) high pressure antigen retrieval method. After getting rid of endogenous peroxidase with 3% hydrogen peroxide in microwave for 2min, slices were reacted with VDR antibody (dilution of 1:200) at room temperature for 2h. Then reacted with a Polymer Helper in room temperature for 20min, and followed by polyperoxidase-anti-mouse/rabbit IgG for 30min. Slices were developed with DAB kit. Wash the slices 3 times with PBS between each step. The reacting time was controlled under microscope. Then slices were counterstained with hematoxylin for 1min, dehydrated, and finalized with neutral balsam.

Real Time Quantitative Polymerase Chain Reaction Samples obtained at different time were extracted total RNA, determined RNA concentration, reverse transcribed and conducted real-time qPCR at the same time to minimize the experimental errors. Quantitative real-time qPCR was performed using the Eppendorf Mastercycler and SYBR green. The following primers were used (5'-3'): ATGCCATC TGCATCGTCTC (forward) and GCACCGCACAGGCTGT CCTA (reverse) for VDR (human); AAGCTGTGCTTCGTG CTATAGATGG (forward) and TGTGAAGCTCACAGGCT TTGG (reverse) for CAMP (human); TGGCACCCAGCAC AATGAA (forward) and CTAAGTCATAGTCCGCCTAGA AGCA (reverse) for β-actin (human) as housekeeping gene. Applied recommend steps in PrimeScript RT reagent Kit With gDNA Eraser as reverse transcription steps. We took 2 μL cDNA for the reaction and each sample was run in triplicates. Forty cycles were performed. Each cycle reaction ended with recording the fluorescence values. The 40 cycles formed amplification curve, and reported the number of cycles that reached the threshold fluorescence value (Ct value). Values are expressed as ratio of fold increase to mRNA levels of untreated cells. All the results were repeated at least 3 times to confirm the authenticity.

Western Blot Western blot analysis was performed under the guide of a previously reported method^[16]. Firstly, the cell lysate [50 μg per lane, measured by the Beyotime

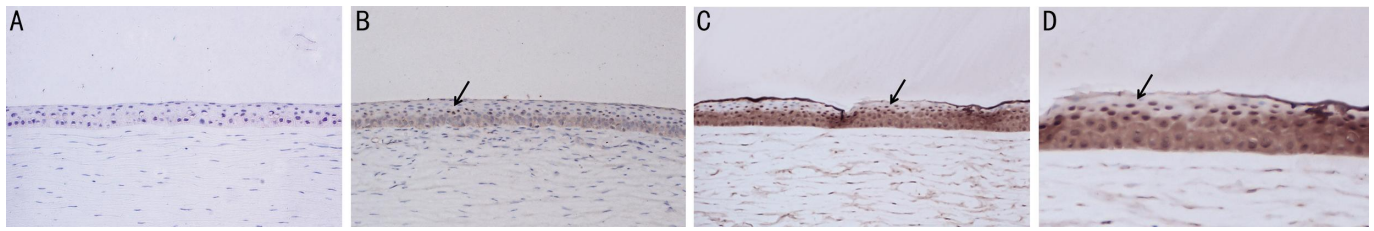


Figure 1 VDR protein expression in normal corneal epithelium by immunohistochemistry A: The negative control group of healthy cornea and IgG was used as the first antibody, 200 \times . B: The specimen of healthy cornea in 200 \times . We can see that some of the nuclei show brown yellow granules. C: The specimen of fungal keratitis cornea in 200 \times . And we could observe the expression of brownish yellow granules in figure C were much more than that in figure B. D: The specimen of fungal keratitis cornea in 400 \times .

bicinchoninic acid (BCA) protein assay kit P0012, Jiangsu Province, China] was mixed with Beyotime 6 \times SDS-PAGE Sample Loading Buffer (P0015F, Jiangsu Province, China) and then boiled for 10min before loading into the gel. The proteins were separated by molecular weight on an sodium dodecyl sulfate (SDS) polyacrylamide gel and shifted to polyvinylidene fluoride (PVDF) membranes (Millipore IPVH00010, Massachusetts, USA). Then we blocked the membranes with 5% bovine serum albumin (BSA) blocking buffer for 1h at 37 $^{\circ}$ C with gentle shaking. First treated with primary antibody against VDR overnight at 4 $^{\circ}$ C, and then treated with the secondary antibody which was horseradish peroxidase-conjugated for 1h at 37 $^{\circ}$ C. The signal bands were detected with BeyoECL Plus chemiluminescence reagent using a UVP EC3 Imaging System (Upland, CA, USA).

Enzyme-linked Immunosorbent Assay Double-sandwich ELISA for human LL-37 was performed according to the manufacturer's protocol, to detect the concentration of LL-37 protein in conditioned media from different treatments. Coated plate with 100 μ L diluted capture antibody incubate overnight at 4 $^{\circ}$ C. After adding the diluted standard and samples for 60min at room temperature, we treated the standard and samples with 100 μ L biotinylated tracer antibody for 60min. Then the plate was added with 100 μ L streptavidin-peroxidase conjugate for 60min before reacting with tetramethylbenzidine (TMB) solution for 30min. At last, we stopped the reaction by using stop solution (2N H₂SO₄). Absorbance was read at 450 nm with a reference wavelength of 570 nm by a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis All the experiments were repeated at least 3 times. Each sample was at least run in triplicates and all the data were presented as mean \pm SD from independent experiments. The data were analyzed using SPSS19.0 statistical package. One-way ANOVA test was used to compare among three or more groups, and Fisher's least significant difference (LSD) was used to identify between each two groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Expression of Vitamin D Receptor Protein in the

Corneal Epithelium of Healthy Donors and Fusarium Solani Keratitis Patients Immunohistochemistry staining on paraffin sections of normal human and fusarium solani keratitis patients could display the existence and different expression of VDR protein in the nuclei of the epithelium cells in both 200 \times (Figure 1B, 1C) and 400 \times (Figure 1D). We could see the brownish yellow granules indicating VDR displayed in the nuclei of most epithelium cells both in Figure 1B and Figure 1C. The VDR expression in the nuclei of corneal epithelium of healthy people accounted for about 22%, however, about 81% in the corneal epithelium of fusarium solani keratitis patients. The expression of VDR in FK patients was much more than that in healthy people. For the positive expression, IgG was used as the first antibody for negative control (Figure 1A).

Vitamin D Receptor mRNA Expression Increased by Fusarium Solani Inflammation While the HCEC were challenged with fusarium solani mycelia, the fold change of VDR mRNA increased obviously in 2h (1.842 \pm 0.862, $P < 0.05$), 4h (2.907 \pm 1.208, $P < 0.01$) and 8h (2.310 \pm 0.853, $P < 0.01$), and the peak appeared at 4h (Figure 2A). While challenged with fusarium solani spore, the VDR mRNA expression increased from 1h (1.667 \pm 0.603, $P < 0.05$) and reached the peak at 2h (2.773 \pm 0.673, $P < 0.01$) (Figure 2B).

Vitamin D Receptor mRNA Expression Inhibited by Toll Like Receptor 2 Monoclonal Antibody We used the TLR2 monoclonal antibody as the TLR2/1 inhibitor to investigate the connection between TLR2/1 and VDR mRNA expression. When we treated the HCEC with TLR2 monoclonal antibody for half an hour before challenging HCEC with fusarium solani mycelia, the increase of mRNA expression of VDR was inhibited apparently in 2h (control: 1.956 \pm 0.472; TLR2 antibody treated: 1.217 \pm 0.463, $P < 0.05$), 4h (control: 2.897 \pm 0.374; TLR2 antibody treated: 1.189 \pm 0.168, $P < 0.01$) and 8h (control: 2.353 \pm 0.457, TLR2 antibody treated: 1.223 \pm 0.327, $P < 0.01$) (Figure 2C). Because dectin-1 is the member of PRRs just like TLR2, so we also used the dectin-1 inhibitor laminarin to treat HCEC before challenge to verify if dectin-1 could also play a role in the expression of VDR. However, the result came out to be negative ($P > 0.05$) (Figure 2D).

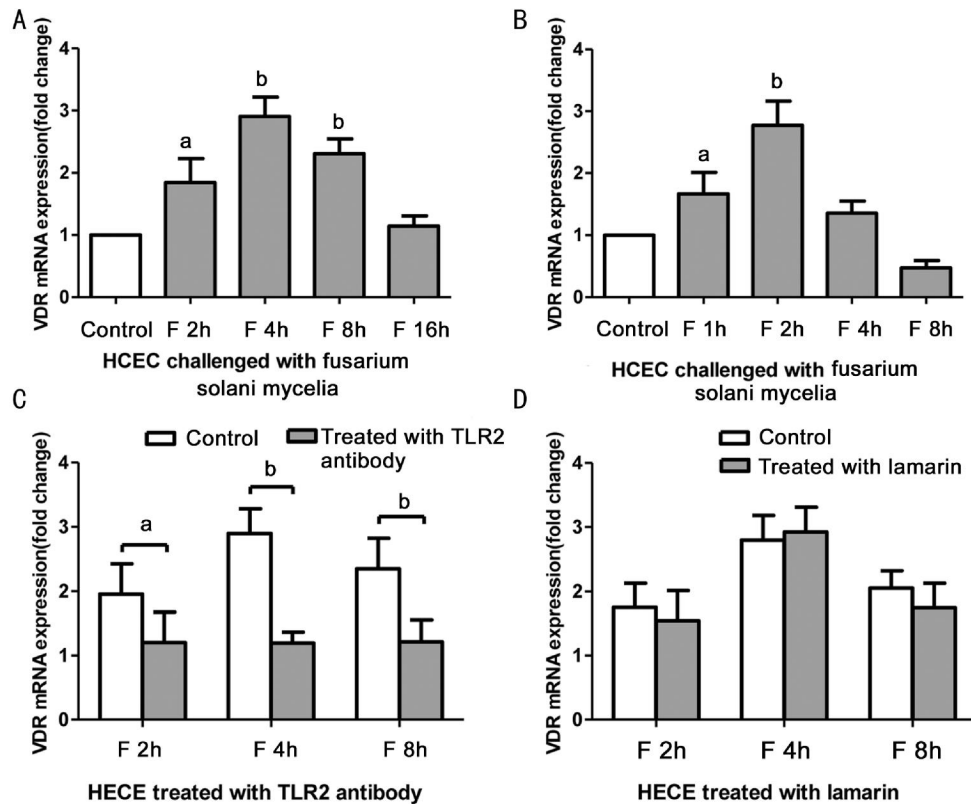


Figure 2 The change of VDR mRNA expression when HCEC were challenged with fusarium solani antigen by realtime qPCR A showed VDR mRNA expression when HCEC were challenged with 5×10^6 fusarium solani mycelia for different hours. The peak appeared in 4h. B was the result of HCEC which was stimulated with 5×10^7 fusarium solani spore for different hours. The peak appeared in 2h. C showed the groups treated with TLR2 monoclonal antibody of 2 $\mu\text{g}/\text{mL}$, the VDR mRNA expression declined when compared with the groups only challenged with fusarium solani mycelia. D was treated with dectin-1 specific inhibitor laminarin of 0.3 mg/mL and the result was negative (^a $P < 0.05$; ^b $P < 0.01$).

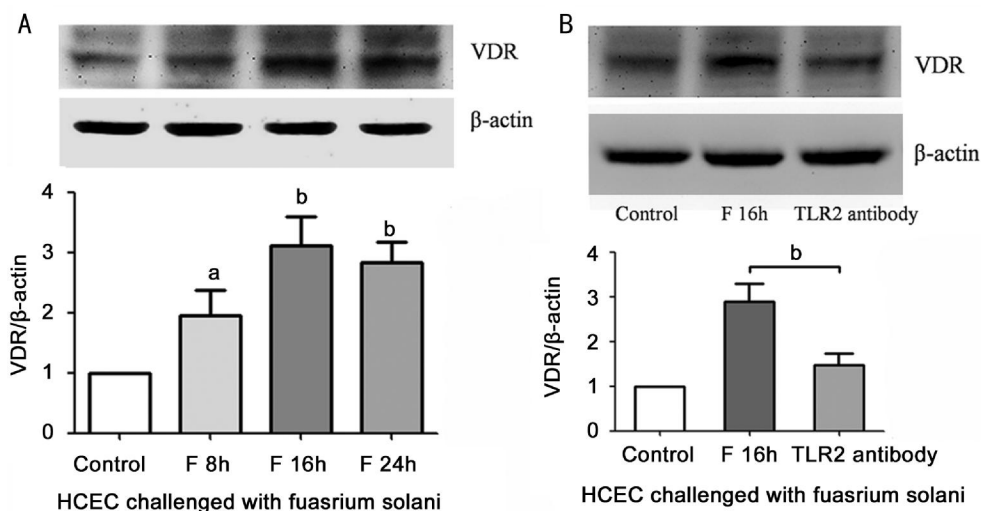


Figure 3 The change of VDR expression when HCEC were challenged with fusarium solani mycelia by Western Blot A showed the VDR expression of HCEC when challenged with 5×10^6 fusarium solani mycelia for different hours. The peak appeared after 16h. B showed the HCEC which was treated with TLR2 monoclonal antibody of 2 $\mu\text{g}/\text{mL}$, the VDR expression was inhibited when compared with the groups only challenged with fusarium solani mycelia (^a $P < 0.05$; ^b $P < 0.01$).

Correlation Between Vitamin D Receptor Expression and Toll Like Receptor 2 Monoclonal Antibody Western blot was taken to discover the expression of VDR protein. The result came out to show that the VDR expression increased when HCEC was stimulated by fusarium solani mycelia for different time (Figure 3A). When we pretreated

HCEC with TLR2 monoclonal antibody for half an hour, the VDR expression was inhibited obviously (Figure 3B).

Expression of Cathelicidin Antimicrobial Peptide Down-regulated During Fusarium Solani Mycelia Inflammation We detected that the mRNA expression of CAMP decreased obviously after fusarium solani mycelia challenged for 8h

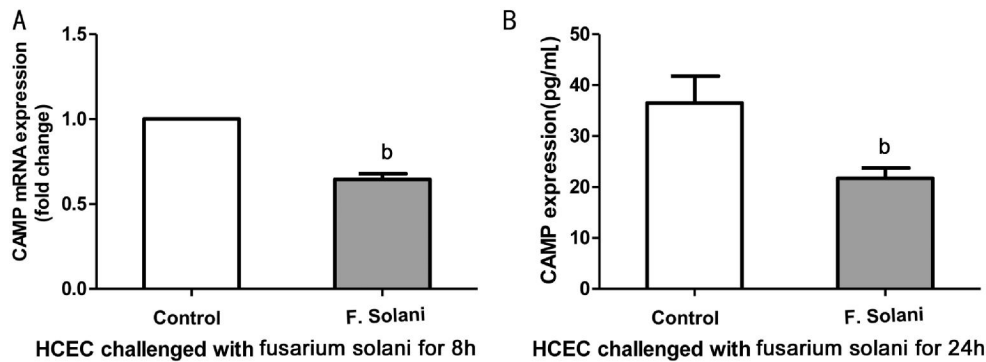


Figure 4 The mRNA and protein expression of CAMP were inhibited by fusarium solani mycelia by realtime qPCR and ELISA. We stimulated the HCEC with fusarium solani mycelia. The CAMP mRNA expression was detected after fusarium solani mycelia challenged for 8h. The CAMP protein expression in cell culture supernatant was detected after fusarium solani mycelia challenged for 24h. It could be observed that the CAMP expression was inhibited (^b $P < 0.01$).

(0.6438 ± 0.133 , $P < 0.01$). The decrease of protein expression was detected after fusarium solani mycelia challenged for 24h (control: 36.467 ± 9.201 , F. Solani: 21.693 ± 3.495 , $P < 0.01$). The expression of CAMP in both mRNA and protein level were inhibited (Figure 4A, 4B).

DISCUSSION

Despite the immunomodulatory effects of vitamin D and VDR in innate immune have been discovered gradually, the role of VDR response to fusarium solani in corneal epithelium cells has not been reported. According to our preliminary experiments we came out with a hypothesis that the VDR and CAMP expression could be affected by the fungal pathogen. Therefore, we decided to explore the pathway and specific function of VDR in corneal epithelium cells against fusarium solani in order to find a new way to prevent and treat FK.

The expression of VDR mRNA has been confirmed in cultured corneal epithelium cells from mouse, rabbit, and human by Yin *et al* [12]. In our study, we confirmed that the corneal epithelium cells of fusarium solani keratitis patients expressed more VDR in the nucleus than that in normal human by Immunohistochemistry. Furthermore, we decided to stimulate the HCEC by fusarium solani antigen *in vitro* in order to detect the mRNA level quantitatively.

According to the research, Aspergillus fumigatus antigen could up-regulate the expression of TLR2, TLR4 and Dectin-1 in corneal epithelium. In human monocytes, the activation of TLR2/1 could induce the vitamin D dependent antimicrobial activity against mycobacteria [17]. Meanwhile, the activation of VDR and other signalings could be induced by activated TLR2/1. In our study, with the stimulation of fusarium solani antigen, the expression of VDR increased obviously in HCEC. Also, we discovered that the effect of mycelia was much better than spore. Afterwards, we treated the HCEC with TLR2 monoclonal antibody to block TLR2 pathway in order to verify the pathway of VDR expression in corneal epithelium. The result showed that the increase of VDR expression was inhibited by TLR2 monoclonal

antibody. So we could conclude that the VDR express *via* TLR2/1-VDR pathway in corneal epithelium as well. Nevertheless, we didn't find the change of VDR expression in groups treated with dectin-1 specific inhibitor laminarin. So that dectin-1 doesn't take part in the VDR expression.

CAMP is derived from human cationic antimicrobial protein-18. It has been discovered to mediate many host responses, including bactericidal action, angiogenesis, epithelial wound repair, and activation of chemokine secretion [18]. Human CAMP has been well known to have antimicrobial and immunomodulatory multifunctions against fungal infection [19]. Adams *et al*'s [20] study indicated that the mRNA expression of CAMP could be decreased during the stimulation of lipopeptide and lipopolysaccharide. In our study, we found out that the expression of CAMP was down-regulated by fusarium solani mycelia. The corneal epithelium, not like the other tissue of human body, is lack of blood capillary. So, even the VDR expression increased by the stimulation of fusarium solani, there was not enough vitamin D to combine with. As we know, vitamin D can promote the expression of CAMP. The mechanism of CAMP expression remains unclear. We still need to do some further research.

In conclusion, VDR expression of human corneal epithelium increased during the fusarium solani infection *via* TLR2/1-VDR pathway. The CAMP expression is beneficial for the repair of corneal epithelium infection. It could show us a new target spot to discover and treat FK. Maybe the findings could help us diagnose FK in clinical application. However, it is difficult to be used into clinic for now. We still need to do some more research to solve the problems and roll them out into clinical practice.

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