• Clinical Research •

Differential expression of antimicrobial peptides in human fungal keratitis

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Abstract

● **AIM:** To analyze a series of antimicrobial peptides (AMPs) in corneal tissue from individuals with fungal keratitis (FK) during the active phase of the fungus infection and after healing.

● **METHODS:** Patients undergone lamellar keratoplasty for the treatment of severe FK or corneal scar had their corneal buttons sampled. Quantitative real-time polymerase chain reaction (PCR) was used to ascertain the gene expression of human beta-defensin (HBD)-1, -2, -3, -9, S100A7, 8, 9, and LL-37.

● **RESULTS:** All AMPs' messenger ribonucleic acid (mRNA) expression was considerably elevated in all samples (n=12). In contrast to controls, where HBD-2, -3, and S100A7 mRNAs were expressed at very low levels, it was discovered that HBD-1, -9, S100A8, S100A9, and LL-37 were constitutively expressed in all healed samples (n=4). HBD-1, -2 -3, S100A7, and LL-37 mRNAs were significantly increased in all active FK samples (n=8). The levels of HBD-9, S100A8, and S100A9 mRNAs were moderately upregulated in all active FK samples. Subgroup comparison showed that HBD-2 was significantly increased in *Fusarium* keratitis samples (n=5), and LL-37 mRNAs were significantly enhanced in *Aspergillus* keratitis samples (n=3). Whereas there was not significantly increased of HBD-1, -3, -9, S100A7, 8, 9 mRNA in *Aspergillus* keratitis samples compared with *Fusarium* keratitis samples.

● **CONCLUSION:** AMPs expression is increased in active FK, but not all AMPs are equally expressed. HBD-2 and LL-37 expression levels are the highest, showing some specificity of AMP expression related to FK. Human AMPs, particularly HBD-2 may play a significant role in *Fusarium* keratitis and LL-37 might be the key player in *Aspergillus* keratitis.

● **KEYWORDS:** antimicrobial peptides; fungal keratitis; *Aspergillus*; *Fusarium*

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INTRODUCTION

Fungal keratitis (FK) is a serious infection of the cornea that can lead to irreversible vision loss, with approximately 100 000 eyeballs were removed worldwide per year[1-5]. The current challenges are poor treatment response due to a number of factors, such as difficulty in microbiological identification, inadequate efficacy and permeability of antifungal drugs, and extremely wide drug sensitivity of existing drugs[6-9]. Therefore, the main alternative therapy is to study the human immune response to fungal infection.

Antimicrobial peptides (AMPs) are host defense protein peptides secreted naturally by epithelial cells and immune cells, playing an important role in the primitive immune system and adaptive immune system, and having a wide range of activities against pathogens such as viruses, bacteria, and fungi[10-13]. Several articles have described the presence of AMPs, such as human beta-defensin (HBD) and LL-37 on the surface of the human ocular surface (OS) and their expression in corneal infections[14-17].

In recent years, a large number of studies have confirmed the antifungal activity of AMPs in animals[18-21], in corneal epithelial cells[22-23] and in humans[24-25]. However, the expression of AMPs was divergent, and the response profile of human AMPs to fungal infections has not been completely elucidated. We therefore analyzed gene expression of human AMPs common in corneal specimens during active fungal infection and after healing cases. At the same time, we compared the gene expression of AMPs in *Fusarium* keratitis and *Aspergillus* keratitis, two of the most common strains.

Table 1 Demographic data, diagnosis and laboratory results

No.	Age/sex	Diagnosis	Microbiological culture	Size of lesion (mm^2)	Depth of lesion (μm)	CCT (μm)	Diameter of sample (mm)
1	76/F	Corneal scar	NA	6×5	215	552	7
2	66/M	Fungal keratitis	*Aspergillus spp.*	7.2×6	306	628	7.5
3	73/F	Fungal keratitis	*Fusarium spp.*	7×7	299	596	7.25
4	59/M	Corneal scar	NA	6.5×6	233	546	7
5	58/M	Fungal keratitis	*Fusarium spp.*	7×6	287	724	7.25
6	67/F	Fungal keratitis	*Fusarium spp.*	7.5×6.5	318	651	7.75
7	49/M	Fungal keratitis	*Aspergillus spp.*	6×5.5	236	560	6.25
8	54/M	Fungal keratitis	*Fusarium spp.*	5×5	210	632	5.25
9	52/F	Corneal scar	NA	5×5.5	285	525	7.5
10	65/M	Corneal scar	NA	6×5	196	492	6.25
11	64/M	Fungal keratitis	*Aspergillus spp.*	7.5×6	312	687	7.75
12	56/F	Fungal keratitis	*Fusarium spp.*	8×7	330	819	8.25

M/F: Male/female; NA: Not applicable; CCT: Central corneal thickness.

SUBJECTS AND METHODS

Ethical Approval In accordance with the principles of the Declaration of Helsinki, this comparative and retrospective study was approved by the Ethics Committee of Union Hospital, Tongji Medical College and Huazhong University of Science and Technology (UHCT230035). All patients received informed consent prior to corneal collection.

Inclusion criteria were: 1) active FK with typical clinical symptoms and positive fungal culture; 2) active FK that did not respond to treatment or did not receive any treatment; 3) corneal scar was FK ulcer healing after antifungal treatment. Exclusion criteria were as follows: 1) mixed keratitis or other types of keratitis; 2) eye inflammation not caused by infectious agents; 3) systemic or local steroid therapy; 4) those with known immunosuppression or undergoing immunosuppression therapy.

The experimental group received deep anterior lamellar keratoplasty (DALK) for corneal buttons (CB) in patients with active FK, and the control group received DALK for corneal scar.

Isolation and Reverse Transcription of Total Ribonucleic Acid Corneal tissue was homogenized in an RLT buffer using TissueRuptor (Qiagen, Germany), iced for 60s, total ribonucleic acid (RNA) was isolated and reverse-transcribed. RNeasy mini kit (catalog number 74104; Total RNA was isolated from CB samples using Qiagen, Germany), following the instructions provided by the manufacturer, including the optional DNase step. The isolated RNA was quantified using a biological spectrophotometer (Eppendorf, Germany). Totally 200 nanograms of total RNA was reverse-transcribed (RT) into complementary deoxyribonucleic acid (cDNA) using the Eurogentec reverse transcription core kit (RT-RTCK-03, Eurogentec, Belgium). In addition, no RT-enzyme control samples were available.

Real-Time Quantitative Polymerase Chain Reaction TaqMan probe chemistry was used to analyze the selected AMPs by real-time quantitative polymerase chain reaction (qPCR). The decision to select only AMPs was influenced by the small amounts of RNA that could be extracted from tiny tissue samples. TaqMan tests were repeated for AMPs, subxanthine-guanine phosphate ribose transferase and appropriate controls on a 96-well plate equipped with an Mx3005p real-time PCR instrument (Agilent technologies, Milton Keynes, UK). Simply put, the cDNA template is first diluted 1:5 in nuclease-free water. According to the instructions for TaqMan gene expression Master Mixture (Applied Biosystems, Waltham, MA, USA), 20 mL of reaction mixture was prepared per well. The 10 mL of 2 master stock, 1 mL of 20 TaqMan assay, 5 mL of diluted cDNA, and 4 mL of nuclease-free water were added to each reaction mixture. This study used only one template per probe. However, appropriate non-RT controls also include probes for each gene to exclude any genome amplification.

Statistical Analysis All aggregated data were expressed as mean±standard deviation (SD). Statistical analysis of qPCR data was performed by GraphPad Prism 8.1 software (GraphPad software, San Diego, CA, USA), and the alpha value was set as $P\leq0.05$. Due to differences in group size, we used the unpaired Welch unequal variance *t*-test.

RESULTS

Demographics A total of 12 samples were collected, as shown in Table 1. We examined n=4 CB specimens from the healed group and n=8 in active FK group. Final diagnosis was based on the positive growth of fungal in cultures from corneal scrapings. In active FK cases, all samples showed well growth on culture, *Fusarium solani* (5 of 8, 62.5%) and *Aspergillus flavus* (3 of 8, 37.5%) were identified, respectively.

Differential Expression of AMPs Between Active FK and Corneal Scar Here, we reported the expression of several

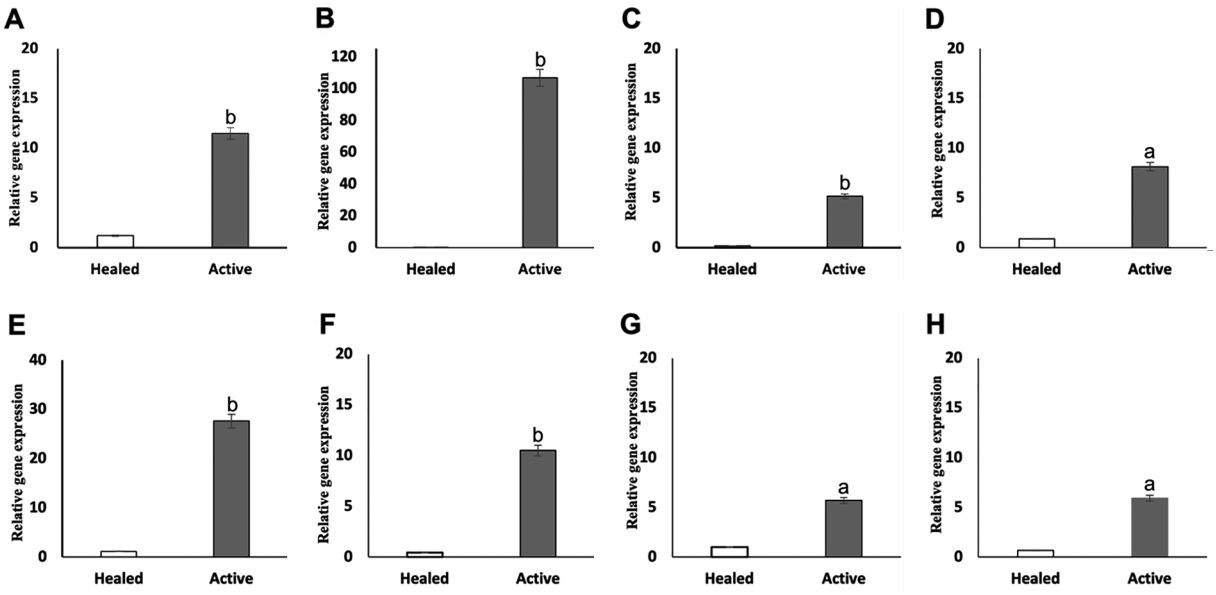

Figure 1 Gene expression of AMPs in corneal tissue from patients with FK Relative fold change of HBD-1 (A), HBD-2 (B), HBD-3 (C), HBD-9 (D), LL-37 (E), S100A7 (F), S100A8 (G), S100A9 (H) in active FK and healed groups. Student's *t*-test with Welch's correction was performed to compare active FK versus healed with $P \leq 0.05$ denoting statistical significance. $^{a}P \leq 0.05$, $^{b}P \leq 0.01$. AMPs: Antimicrobial peptides; FK: Fungal keratitis.

AMPs in patients with FK as determined by qPCR. As shown in Figure 1, AMPs were differentially expressed in patients with FK. HBD-1, -9, S100A8, 9 and LL-37 were shown to be constitutively expressed in all healed samples, whereas mRNAs for HBD-2, -3, S100A7 were expressed at a very low level.

As shown in Figure 1, all AMPs were increased in active FK. HBD-1, -2 -3, S100A7 and LL-37 mRNAs were significantly increased in all active FK samples. HBD-1 was upregulated by 11.49±0.56-fold (P=0.00033), HBD-2 was elevated by 106.78±11.12-fold (P=0.0036), HBD-3 was increased by 5.16±0.56-fold (P=0.0038), S100A7 was increased by 10.50±1.01-fold (P=0.0039) and LL-37 was upregulated by 27.63±3.14-fold (P=0.0046) in active FK compared with controls.

The levels of HBD-9, S100A8 and S100A9 mRNAs were moderately upregulated in all active FK samples. HBD-9 was increased by 8.15±1.18-fold (P=0.011), S100A8 was elevated by 5.70±1.03-fold (P=0.025) and S100A9 mRNA expression was shown to be increased by 5.93±1.44-fold (P=0.027) in active FK samples compared with controls.

Variable Expression of AMPs Between *Fusarium* and *Aspergillus* Keratitis As shown in Figure 2, subgroup comparison shown that HBD-2 mRNAs were significantly elevated in *Fusarium* keratitis, while LL-37 mRNAs were significantly increased in *Aspergillus* keratitis samples. HBD-2 was upregulated by 111.79±6.25-fold in *Fusarium* keratitis compared with 95.91±2.72-fold in *Aspergillus* keratitis samples (P=0.041). LL-37 was elevated by 29.77±1.28-fold in *Aspergillus* keratitis compared with 23.38±2.72-fold in *Fusarium* keratitis samples (P=0.028).

Whereas, there were no significantly increased of HBD-1, -3, -9, S100A7, 8, 9 (P>0.05) mRNA in *Aspergillus* keratitis compared with *Fusarium* keratitis samples.

DISCUSSION

FK is a serious public health problem affecting the agricultural poor and therefore requires special attention. High rates of corneal perforation have been reported in cases of FK, although the progression of FK is slower compared to bacterial keratitis[3-5]. Due to the limited available treatments, there is an urgent need to study the mechanism of host immune response to fungal infections in order to find new treatments. Compared with existing antibacterial agents, AMPs have unique mode of action, so they have received special attention as a potential treatment for microbial infections[26]. As numerous studies over the past two decades have shown, human AMPs already play a crucial role in microbial keratitis[11,15-17,24-26].

In this study, we observed an increased pattern of AMPs (HBD-1, -2, -3, -9, S100A7, 8, 9, and LL-37) expression in corneal specimen during the active phase of infection and healed corneal scar. Notably, all AMPs' mRNA was found to be at a basal level in healed specimens. The increased levels of AMPs varied in active FK specimens. HBD-1, -2, -3, S100A7, and LL-37 mRNAs were significantly increased, while the levels of HBD-9, S100A8, and S100A9 mRNAs were moderately upregulated in all active FK. Among them, HBD-1, -2, and LL-37 increased in the 3 most significant proportions. Subgroup comparison between the two most

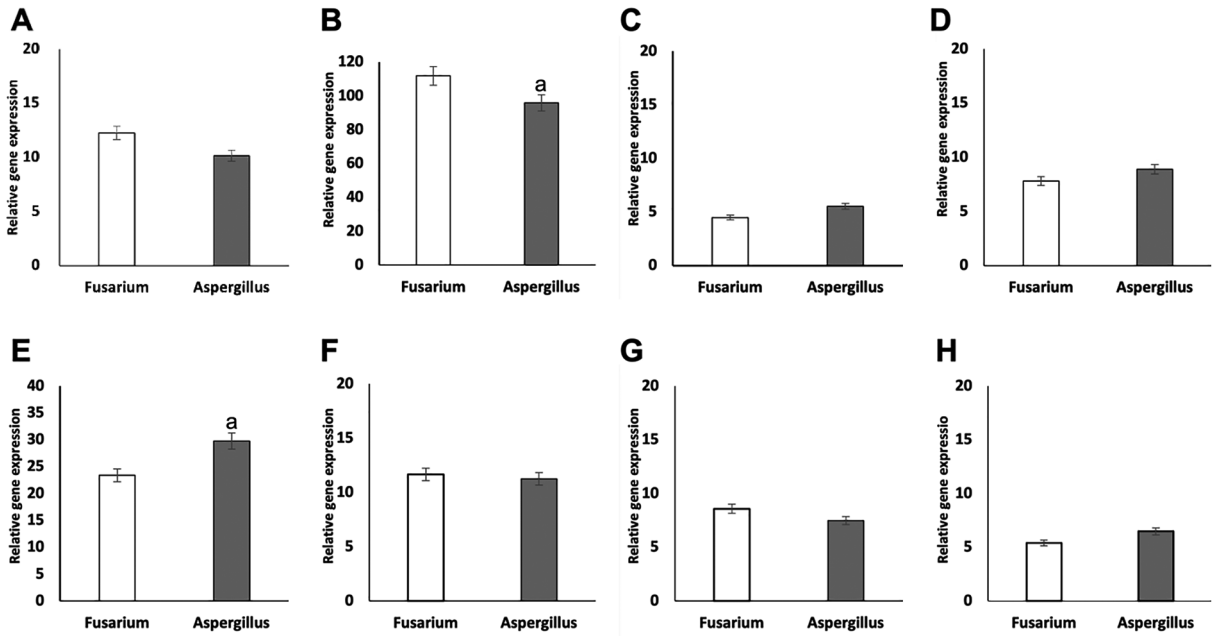

Figure 2 Gene expression of AMPs in corneal tissue between patients with *Fusarium* keratitis samples and *Aspergillus* keratitis samples Relative fold change of HBD-1 (A), HBD-2 (B), HBD-3 (C), HBD-9 (D), LL-37 (E), S100A7 (F), S100A8 (G), S100A9 (H) in *Fusarium* keratitis samples and *Aspergillus* keratitis samples. Student's *t*-test with Welch's correction was performed to compare *Fusarium* keratitis samples versus *Aspergillus* keratitis samples with $P \leq 0.05$ denoting statistical significance. [a]$P \leq 0.05$. AMPs: Antimicrobial peptides; FK: Fungal keratitis.

common FK, HBD-2 was significantly increased in *Fusarium* keratitis samples and LL-37 was significantly increased in *Aspergillus* keratitis samples. The other AMPs were not significantly increased in *Aspergillus* keratitis compared with *Fusarium* keratitis. As a result, active FK samples showed an elevated pattern of CAMP and β-defensins expression in the current study. This raised the possibility that the human AMPs, particularly HBD-1, -2, and LL-37, might be key players in FK. Our study, similar to previous studies, confirmed that mRNA levels of the HBD family, class S100A, and LL-37 in human OS specimens were significantly higher during active FK than after healing[24]. In response to *Fusarium spp.*[22] and *C. albicans*[23], corneal epithelial cells also expressed LL-37, hBD-2, and hBD-3 more frequently. In addition, cathelicidin related antimicrobial peptides (CRAMP) and β-defensins have shown variable expression at the onset of disease in an animal model of *C. albicans* keratitis, but they returned to their normal levels by healing on day 7 after infection[21]. In a mouse *Fusarium* keratitis model, the expressions of endogenous mBD-3, -4, -14, and CRAMP were significantly increased on day 3 after infection and returned to baseline levels after ulcer healing[20]. Nevertheless, Mallela *et al*[25] found that β-defensin expression was decreased and LL-37, S100A12, and RNase 7 expression was significantly increased in corneal scrapes in patients with *Aspergillus* keratitis. However, the consistency of the results would be affected by the diversity of AMPs, various materials and different samples. We could speculate that the human AMPs, particularly β-defensins might play a significant role in *Fusarium* keratitis and LL-37 might be the key player in *Aspergillus* keratitis.

AMPs have been shown to induce adaptive immunity and is an effective chemical attractant[26]. Thus, in addition to directly killing microorganisms, increased level of AMPs during FK may potentially increase neutrophil infiltration and other unknown antifungal mechanisms. LL-37 and β-defensins have fungicidal activity against *Candida in vitro* by binding to cell wall β-1, 3-exoglucase *via* secreted glycosylated exodomain protease Msb2[27] and reducing their ability to infect the host. The yeast cell membrane is penetrated by human LL-37 and β-defensin, resulting in fungal death[28]. Psoriasis protein (S100A7) in linear peptide-reducing form kills *Aspergillus spp.* and induces fungal apoptosis by chelating zinc metals[11]. Calprotectin (S100A8/A9) inhibited the growth of *Aspergillus spp.*, inhibited hyphal growth, and exhibited antifungal activity. In a mouse model of FK[29], zinc and manganese transport were prevented by fungi.

The limitation of this study was that it only involved assessment of gene expression of AMPs in active and healed groups. A subsequent study should focus on neutrophil infiltration and understanding the mechanisms of immune activation of AMPs during FK.

In conclusion, our results corroborate that AMPs expression increased in active FK. HBD-2 and LL-37 expression levels were the highest, showing some specificity of AMP expression related to FK. Subgroup comparison showed that HBD-2 mRNAs were significantly elevated in *Fusarium*

keratitis, while LL-37 mRNAs were significantly increased in *Aspergillus* keratitis samples. We could speculate that the human AMPs, particularly HBD-2 might play a significant role in *Fusarium* keratitis and LL-37 might be the key player in *Aspergillus* keratitis. This further implicates a potential pivotal role for AMPs in OS defense against fungal pathogens.

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