Original Article

Expressions of Antimicrobial Peptides LL-37, Human Beta Defensin-2 and -3 in the Lesions of Cutaneous Tuberculosis and Tuberculids

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Abstract

Background: Antimicrobial peptides, including cathelicidin LL-37, human beta defensin (HBD)-2, and HBD-3, are important elements of the innate immune response and involved in modulation of the adaptive immunity, and they also play an important role in cutaneous defense against *Mycobacterium tuberculosis*.

Methods: The fresh skin tissues and paraffin-embedded biopsy samples from three cutaneous tuberculosis, two tuberculids, and ten healthy individuals were collected. The expressions of LL-37, HBD-2, and HBD-3 mRNA in the lesions of three cutaneous tuberculosis and two tuberculids were detected by quantitative real-time polymerase chain reaction; the protein expressions were detected by immunohistochemistry and Western blotting methods.

Results: The expressions of LL-37 mRNA and protein in the lesions of cutaneous tuberculosis and tuberculids were similar to that of normal skin. The expression of HBD-2 mRNA had an increasing trend in the lesions of cutaneous tuberculosis and tuberculids compared with that of normal skin; however, the expression of HBD-2 protein in the lesions of cutaneous tuberculosis had a decreasing trend compared with that of normal skin, and the expression of HBD-2 protein in the lesions of tuberculids was similar to that of normal skin. The expressions of HBD-3 mRNA and protein in lesions of cutaneous tuberculosis and tuberculids were similar to that of normal skin.

Conclusions: Our study indicated that the expression of HBD-2 and HBD-3 mRNA and protein in lesions of cutaneous tuberculosis may be not consistent with that of tuberculids. However, an inherent limitation of the present study was that the sample size was small, and the roles and regulation mechanisms of LL-37, HBD-2, and HBD-3 in cutaneous tuberculosis and tuberculids need to be further investigated.

Key words: Antimicrobial Peptides; Cutaneous Tuberculosis; Expression; Lesions; Tuberculids

Introduction

Cutaneous tuberculosis which is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) complex group shows different clinical manifestations and development processes because of mechanisms of disease acquisition and host immunity. Cutaneous tuberculosis is not common; however, the incidence has been increasing in recent years due to the epidemic of the human immunodeficiency virus (HIV) and the application of immunosuppressants.[1]

There are four major categories of cutaneous tuberculosis: (1) inoculation from an exogenous source (primary inoculation tuberculosis, tuberculosis verrucosa cutis); (2) endogenous cutaneous spread contiguously or by auto-inoculation (scrofuloderma, tuberculosis cutis oroficialis); (3) hematogenous spread to the skin (lupus vulgaris, acute miliary tuberculosis, and tuberculosis ulcer, gumma, or abscess); (4) tuberculids (erythema induratum [Bazin's disease], papulonecrotic tuberculids, and lichen scrofulosorum).

Antimicrobial peptides (AMPs), the important elements of the innate immune system and involved in modulation

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of the adaptive immunity, are small polypeptides found in plants, animals, and microorganisms.[2] AMPs include cathelicidins and defensins: Cathelicidin LL-37, human beta defensin (HBD)-2, and HBD-3 play important roles in cutaneous defense against *M. tuberculosis*. The expression and the role of AMPs have not been studied clearly in cutaneous tuberculosis and tuberculids. Here, we selected LL-37, HBD-2, and HBD-3, which have a close relationship with tuberculosis infection, and we investigated the expressions in lesions with cutaneous tuberculosis and tuberculids and explored the preliminary role of LL-37, HBD-2, and HBD-3 in cutaneous tuberculosis and tuberculids.

Methods

Patients

Three patients with cutaneous tuberculosis and two patients with tuberculids were included in this study; these patients visited our hospital from February 2014 to February 2015. The five patients had typical clinical symptoms presenting from 2 to 12 years and were proved cutaneous tuberculosis or tuberculids based on the clinical, histological features, and laboratorial examinations [Table 1].

The fresh skin tissues and paraffin-embedded biopsy samples from three cutaneous tuberculosis, two tuberculids, and ten healthy individuals were collected. This research was approved by the Medical Ethics Committee of Peking University People's Hospital. All patients were informed and agreed to participate in this study.

Quantitative real-time polymerase chain reaction

Skin biopsies preserved in RNA later were put into 300 μl of buffer RLT (RNeasy Fibrous Tissue Mini Kit, Qiagen GmbH, Hilden, Germany) and homogenized. Total RNA was then extracted according to the manufacturer's protocol in the presence of DNase I (Qiagen). A total of 0.8 μg RNA was reverse-transcribed into 20 μl cDNA by using SuperScript III First-Strand system (Invitrogen, Carlsbad, CA, USA) containing Oligo (dT) and SuperScript III enzyme (Invitrogen).

The primers used in the quantitative polymerase chain reaction (qPCR) of LL-37, HBD-2, HBD-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from previous references.[3,4] LL-37: forward, 5'-CTGATGCCTCTTCCAGGTGT-3', and reverse, 5'-GAGGGAGCCCTTTCTGAATC-3'; HBD-2: forward, 5'-ATCTCCTCTTCTCGTTCCTC-3', and reverse, 5'-ACCTTCTAGGGCAAAAGACT-3'; HBD-3: forward, 5'-AGCCTAGCAGCTATGAGGATC-3', and reverse, 5'-CTTCGGCAGCATTTTGCGCCA-3'; GAPDH: forward, 5'-CCACCCATGGCAAATTCCATGGCA-3', and reverse, 5'- GGTGCTGCTTGTTAGGAGGTCAAGT AAAGGGC-3'.

Each qPCR reaction system (20 μl) contained 6 μl cDNA, 10 μl THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan), 0.6 μl forward and reverse primers, respectively, and 2.8 μl diethylpyrocarbonate water. qPCR was performed on Opticon 2 (BioRad, Mississauga, ON, Canada), followed by activation (denaturation) at 95°C for 1 min. A 40 cycles of amplification was then performed at 95°C for 15 s, at 60°C for 15 s, and at 72°C for 45 s. The relative expression ratio was calculated using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry

Formalin-fixed, paraffin-embedded skin tissues (3 samples of tuberculosis, 2 samples of tuberculids, and 10 samples of normal skins) were used for the immunohistochemical studies. For immunoperoxidase staining, sectioned tissues were treated for endogenous peroxidase inactivation (3% hydrogen peroxide). Then, the tissues were blocked. Specimens were then incubated overnight with primary antibody. Anti-HBD-2 antibody (Abcam, UK) was used at 1:100 dilution, anti-HBD-3 antibody (Novus Biological, USA) at 1:50 dilution, and anti-LL-37 antibody (Abcam, UK)

Table 1: Clinical data of three cases of cutaneous tuberculosis and two cases of tuberculids

Items	Case 1	Case 2	Case 3	Case 4	Case 5
Sex	Male	Female	Female	Male	Female
Age (years)	37	67	50	29	33
Course of disease (years)	3	2	2	10	12
Precipitating factor	Trauma	Trauma	None	None	None
Distribution of lesions	Wrist, arms and abdomen	Wrist	Face, trunk, and extremities	Trunk and extremities	Both lower extremities
Clinical manifestations	Plaque, nodule	Plaque	Nodule	Papule, pustule, varioliformis scarring	Erythema, ulcer, atrophic scar
Acid-fast staining	–	–	–	–	–
T-SPOT test	+	+	+	+	+
PPD test	ND	ND	+++	+++	+++
Chest X-ray examination	ND	ND	ND	–	ND
Previous history	None	None	Laryngeal tuberculosis and thyroid carcinoma	None	None
Diagnosis	Primary inoculation tuberculosis	Primary inoculation tuberculosis	Miliary tuberculosis	Papulonecrotic tuberculids	Erythema induratum

T-SPOT: An interferon gamma-released assay; PPD: Purified protein derivative of tuberculin; ND: Nondetected.

was diluted to 1:500. Then, secondary antibody (anti-mouse/anti-rabbit antibody) was added sequentially for 30 min, followed by 3-amino-9-ethylcarbazole/hematoxylin color spectrum analysis. The result was observed by the use of DC 300F microscopic image analysis system (Leica Microsystems GmbH, Wetzlar, Germany), and the mean optical density values of three distinct groups were measured.

Western blotting

Skin tissues were homogenized in radioimmunoprecipitation assay buffer with phenylmethanesulfonyl fluoride on ice for 30 min, vibrated, and centrifuged. Total protein of the skin tissue lysates was evaluated by BCA protein assay (Fish Scientific, Fair Lawn, NJ, USA). Skin lysates containing 30 μg of crude skin tissue lysate protein were analyzed by NuPAGE 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and was transferred to polyvinylidene fluoride membranes (Invitrogen). The membranes were incubated overnight with the polyclonal rabbit anti-HBD-2 antibody (Abcam), anti-HBD-3 antibody (Novus Biological, USA), or anti-LL-37 antibody (Abcam) at 1:1000 dilution, respectively. The analysis was performed with chemiluminescence reagents. Protein expression was normalized to the quantity of beta-actin. The signal and grayscale values were visualized and analyzed by using ImageJ software (GE Healthcare Piscataway, NJ, USA), and grayscale value ratios were calculated.

Results

Expression of LL-37, human beta defensin-2, and human beta defensin-3 mRNA in the lesions of cutaneous tuberculosis and tuberculids

The expressions of LL-37 and HBD-3 mRNA in the lesions of cutaneous tuberculosis and tuberculids were similar to that of normal skin. The expression of HBD-2 mRNA in the lesions of cutaneous tuberculosis and tuberculids patients had an increasing trend compared with that of normal skin [Table 2].

Expression of LL-37, human beta defensin-2, and human beta defensin-3 protein in lesions of cutaneous tuberculosis and tuberculids

Immunohistochemistry results showed that the expression of LL-37 was moderate in the lesions of cutaneous tuberculosis and tuberculids, and it was similar to that of normal skin [Figure 1]. The expression of HBD-2 had a decreasing trend in the lesions of cutaneous tuberculosis compared with that of normal skin. The expression of HBD-2 was moderate in the lesions of tuberculids, and it was similar to that of normal skin [Figure 2]. The expression of HBD-3 was moderate in the lesions of cutaneous tuberculosis and tuberculids, and it was similar to that of normal skin [Figure 3 and Table 3].

Western blotting results showed that the grayscale value ratios of LL-37, HBD-2, and HBD-3 in the lesions of acute miliary tuberculosis (case 3) were 0.48, 0.05, and 0.28, respectively. These results showed that the expressions of LL-37, HBD-2, and HBD-3 had a decreasing trend in the lesions of acute miliary tuberculosis compared with that of normal skin. The grayscale values ratio of LL-37, HBD-2, and HBD-3 in the lesions of papulonecrotic tuberculids (case 4) were 4.11, 1.39, and 1.35, respectively; the grayscale value ratios of LL-37, HBD-2, and HBD-3 in the lesions of erythema induratum (case 5) were 5.12, 1.15, and 1.66, respectively; which showed the expressions of LL-37, HBD-2, and HBD-3 in tuberculids had an increasing trend compared with that of normal skin [Figure 4].

Table 2: Expression of LL-37, HBD-2, and HBD-3 mRNA in the lesions of cutaneous tuberculosis and tuberculids by the real-time polymerase chain reaction

Items	Case 1	Case 2	Case 3	Case 4	Case 5
LL-37	0.42	8.28	0.42	0.33	16.29
HBD-2	35.99	630.13	81.54	12.38	10.82
HBD-3	2.65	15.00	2.00	2.78	1.79

Data are represented as the relative expression ratio compared with the normal skin. LL-37: Cathelicidin LL-37; HBD: Human beta defensin.

Table 3: Expression of LL-37, HBD-2, and HBD-3 protein in the lesions of cutaneous tuberculosis and tuberculids by the immunohistochemistry

Items	Case 1	Case 2	Case 3	Case 4	Case 5	Control
LL-37	0.25	0.25	0.30	0.25	0.27	0.26
HBD-2	0.22	0.24	0.23	0.27	0.33	0.29
HBD-3	0.26	0.29	0.26	0.29	0.26	0.28

Data are represented as the mean optical density values. LL-37: Cathelicidin LL-37; HBD: Human beta defensin.

Discussion

LL-37 that forms the first host immunity defense with other antimicrobial substances together against microbe's infection is composited and expressed after induction in human epidermal cells. Rivas-Santiago *et al.*[5] confirmed that human cells can produce a large number of LL-37 in the early stage of infection with *M. tuberculosis*, and it plays an important role in resistance to *M. tuberculosis* infection.

The expression of LL-37 mRNA was increased in A549 epithelial cells by infection with *M. bovis* bacillus Calmette–Guérin (BCG), and it was dependent on dose and time.[5] Research confirmed that the 200 μg/ml of LL-37 can reduce 75.7% growth of *M. tuberculosis* and 20 μg/ml can reduce 52.4%, which prompted that antibacterial peptide LL-37 was involved in the growth controlling of *M. tuberculosis*.[6] Rivas-Santiago *et al.*[7] also reported that LL-37 has higher expression in alveolar epithelial cells by infection with *M. tuberculosis*. However, it was pointed out that LL-37 may only play a role in the early stage of infection.

In our study, the expression of LL-37 mRNA and protein in the lesions of cutaneous tuberculosis had no obvious difference compared with that of normal skin, and the

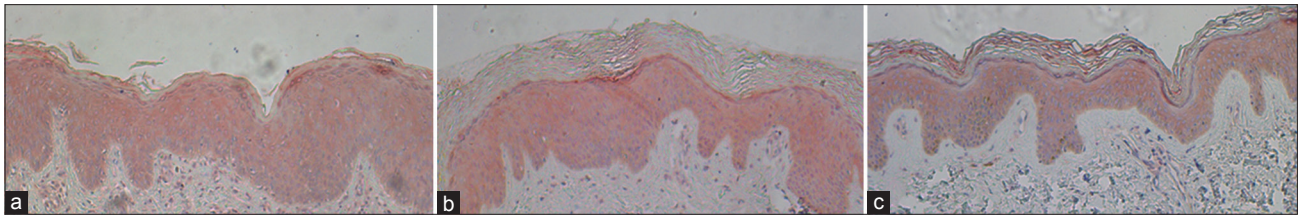

Figure 1: The expression of LL-37 in the lesions of cutaneous tuberculosis, tuberculids, and normal skin (3-amino-9-ethylcarbazole immunohistochemistry staining, original magnification ×200). (a) Cutaneous tuberculosis: Moderate expression in full-thickness; (b) tuberculids: Moderate expression in full-thickness; (c) normal skin: Moderate expression in full-thickness.

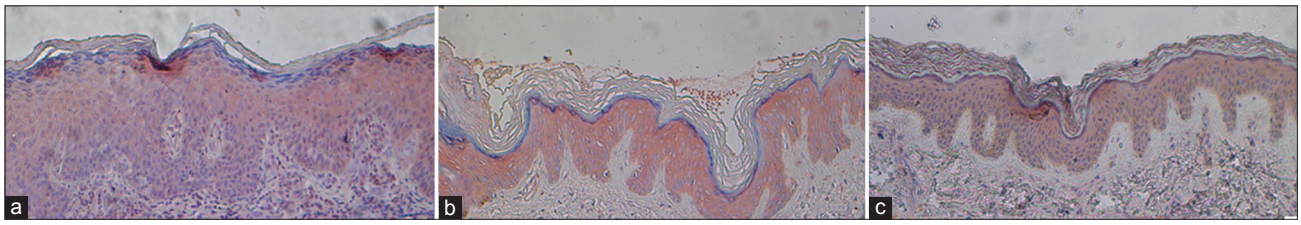

Figure 2: The expression of human beta defensin-2 in the lesions of cutaneous tuberculosis, tuberculids, and normal skin (3-amino-9-ethylcarbazole immunohistochemistry staining, original magnification ×200). (a) Cutaneous tuberculosis: Lower expression in full-thickness; (b) tuberculids: Moderate expression in full-thickness; (c) normal skin: Moderate expression in full-thickness.

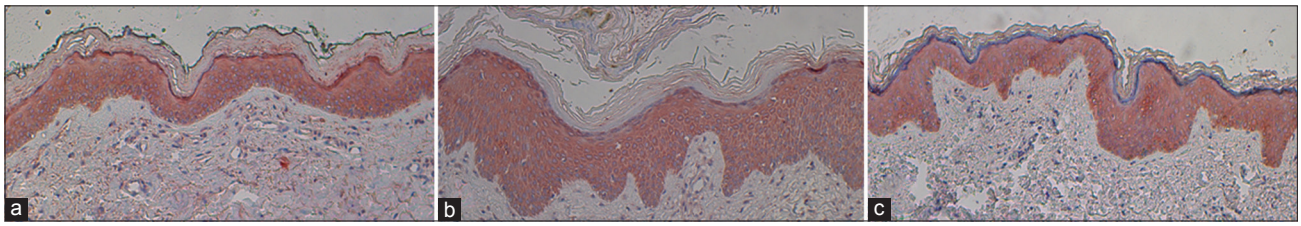

Figure 3: The expression of human beta defensin-3 in the lesions of cutaneous tuberculosis, tuberculids, and normal skin (3-amino-9-ethylcarbazole immunohistochemistry staining, original magnification ×200). (a) Cutaneous tuberculosis: Moderate expression in full-thickness; (b) tuberculids: Moderate expression in full-thickness; (c) normal skin: Moderate expression in full-thickness.

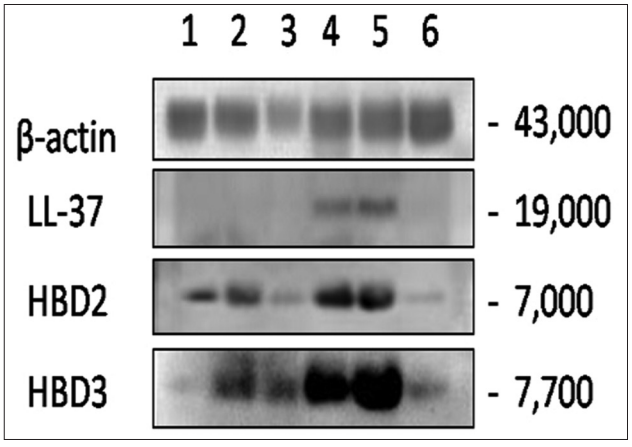

Figure 4: The expression of LL-37, human beta defensin-2, and human beta defensin-3 in lesions with acute miliary tuberculosis, tuberculids, and normal skin (Western blotting). Lanes 1–3: Normal skin; lane 4: Papulonecrotic tuberculid; lane 5: Erythema induratum; lane 6: Acute miliary tuberculosis. LL-37: Cathelicidin LL-37; HBD: Human beta defensin.

reason was considered that the chronic and long (2–3 years) course of disease may lead the expression of LL-37 to be not up-regulation. The increased expression of LL-37 protein (Western blotting) in two patients of tuberculids compared with that of normal skin tissue, which was considered that the patients of tuberculids had good immunity and the potential nidus can cause blood spread infection contiguously. Our results indicated that LL-37 mainly plays a role in the early stage of infection as a protective antibacterial peptide.

HBD-2 and HBD-3 were considered to be two important immune molecules of beta-defense elements against *M. tuberculosis*. It was been confirmed that HBD-2 gene expression was induced in the A549 epithelial cells by infection with *M. tuberculosis*.[8] In previous *in vitro* studies, mononuclear cells were transfected through the coding HBD-2 gene, which inhibited the growth of *M. tuberculosis*; and transfected mononuclear cell was better than that of nontransfected mononuclear cell, which suggested that HBD-2 can effectively control the growth of *M. tuberculosis*.[9] However, the expressions of HBD-2 mRNA and protein were not reported in the lesions of tuberculids at present.

In our study, the expression of HBD-2 mRNA was detected to be an increasing trend in the lesions of cutaneous tuberculosis and tuberculids. The expression of HBD-2 protein also had an increasing trend in the lesions of tuberculids compared

with that of normal skin. Although the expression HBD-2 protein had a decreasing trend in cutaneous tuberculosis, the expression of HBD-2 protein was higher in the lesions of cutaneous tuberculosis in our previous study (data not shown). However, the results of this study may be limited by the small sample number.

HBD-3 could participate in killing *M. tuberculosis* infection.[10] A study confirmed that the expression of HBD-3 and HBD-4 can be induced by L-isoleucine, and HBD-3 and HBD-4 could decrease bacterial loads and tissue destruction.[11] HBD-3 also plays an important role in immune regulation; it can improve the phagocytosis of phagocytes, mononuclear cells, dendritic cells, and T-cells. The research found that HBD-3 interacted with toll-like receptor (TLR) 1 and TLR2, which activated and induced dendritic cells directly, and strengthen the acquired immune response.[12]

Similar to HBD-2, the expression of HBD-3 can also be induced increasingly by cytokines interleukin (IL)-1, IL-17, tumor necrosis factor-α, interferon-γ, *Candida albicans*, Gram-positive and Gram-negative bacteria stimulus.[13-16] Research showed that the release of IL-17 and IL-23 were down-regulated by an individual immune decline in the HIV patients combined with tuberculosis infection, which can injury the protect immune and make the bacterial multiply.[17] However, the expressions of HBD-3 mRNA and protein were not reported in the lesions of tuberculids at present.

In this study, the expression of HBD-3 protein was normal or showed an increasing trend in cutaneous tuberculosis and tuberculids except disseminated miliary tuberculosis. The results were especially obvious in the lesions of tuberculids, it is possible that the patients of tuberculids had relatively good immunity, and the secretion and expression of HBD-3 can be increased by the stimulus of different inflammatory factor and bacteria, which can strengthen the acquired immune reaction, and control against the infection of *M. tuberculosis*.

The expression of HBD-2 and HBD-3 mRNA had an increasing trend in the lesions of disseminated miliary tuberculosis, and the expression of protein had a decreasing trend compared with that of normal skin tissues. The separation phenomenon of mRNA and protein expression can also be showed in other studies.[18] The time and locus of the eukaryotic cell gene transcription and translation are different, and research showed that the small RNA as a regulatory factor can participate in many important bacteria (including *M. tuberculosis*) regulation and play a role in the activity of the protein molecules through complementary pairing with mRNA.[19] In addition, the immunity of this patient was considered to be weak, which induced the deficiency of HBD-2 and HBD-3 secretion.

In conclusion, the expressions of LL-37, HBD-2 and HBD-3 mRNA, and proteins were detected in the lesions of cutaneous tuberculosis and tuberculids. The expression of HBD-2 and HBD-3 was not consistent with that of tuberculids. These findings may be helpful for the diagnosis of these diseases. However, an inherent limitation of the present study was that the sample size was small, and the roles and regulation mechanisms of LL-37, HBD-2, and HBD-3 in cutaneous tuberculosis and tuberculids need to be further investigated.

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Conflicts of interest

There are no conflicts of interest.

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