

Expression of Antimicrobial Peptides according to Changes of Transepidermal Water Loss Levels in Patients with Atopic Dermatitis

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Background: The innate immune system of human skin contains antimicrobial peptides(AMPs) known as cathelicidins(LL-37) and human beta-defensins(hBD)-2. These peptides immediately respond to intruded microbes for prevention of further invasion. In normal skin these peptides are negligible, but they are accumulated in the skin affected by inflammatory diseases such as psoriasis. Although atopic dermatitis(AD) is recurrent inflammatory skin disease, it has been known that the expressions of AMPs in AD are decreased compared to that of psoriasis.

Objective: Current research was to identify the expressions of AMPs according to changes transepidermal water loss(TEWL) levels in AD.

Methods: The involved and uninvolved sites of patients diagnosed as having AD were evaluated using TEWL. And the expression of LL-37 and hBD-2 in skin biopsies specimens from involved and uninvolved site of these patients were determined by RT-PCR, Western blotting and immunohistochemical staining(IHC).

Results: The TEWL levels increased in both uninvolved and involved sites, more increased in involved sites. It implicated that permeability barrier function was more disrupted in involved sites of atopic dermatitis. In RT-PCR, the expression levels of hBD-2 and LL-37 mRNA were down-regulated in both uninvolved and involved sites, more decreased in involved sites. In Western blotting of hBD-2 and LL-37 proteins, the levels of hBD-2, LL-37 protein expressions of uninvolved sites were determined to be more intense than those observed in the involved sites. These findings were also confirmed by IHC.

Conclusion: Our results demonstrate that expression of antimicrobial peptides downregulated according to increasing TEWL levels in atopic dermatitis lesions. Therefore, this deficiency may account for the susceptibility of patients with atopic dermatitis to skin infection, also implicate that localized skin barrier disruption play a role for decreased the expression of AMPs.

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Key Words: Antimicrobial peptide, Atopic dermatitis, Skin barrier, TEWL

INTRODUCTION

Atopic dermatitis (AD) is a highly pruritic,

recurring inflammatory skin disease. It can be exacerbated by bacterial, viral, and fungal infections¹. In fact, many AD patients suffered from recurrent infections of skin lesions. Especially, *Staphylococcus aureus*(*S. aureus*) is found in more than 90% of AD skin lesions, but in only 5% of normal subjects². The high frequency of skin infection in AD may be due to impairment of immune system, such as antimicrobial lipids, peptides, toll-like receptors and chemokines. Among them, Ong and co-workers have shown that the levels of AMPs were dramatically suppressed in AD¹. AMPs are small peptides produced by keratinocytes in the host

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skin³. A number of endogenous AMPs have been shown to play an integral part in innate immunity³. They exhibit potent killing of a broad range of micro-organisms, including *gram-negative and gram-positive* bacteria, *fungi*, and *viruses*⁴. The lowered AMPs may explain the susceptibility of patients in AD to skin infections.

Recently there were some reports why the expressions of AMPs were decreased in AD. Leung et al. reported that increased IL-10 expression accounts for reduced AMPs production in AD^{3,4}. Ong et al reported that excess of T helper 2 (TH2) cytokines may underlie the deficient AMP expression¹. Elias et al suggested the hypothesis that the elevated stratum corneum pH in AD increased activity of serine protease (SP), which have a role in degradation of AMPs^{5,6}. The skin has been known to have an acidic surface that is thought to play a key role in preventing infection⁴. However, the atopy skin displays a neutral pH because of disrupted permeability barrier. pH influences barrier function directly through its effects on membrane bilayer organization and secondarily through its regulation of extracellular lipid processing and SP signaling⁷. Increased SP activity due to neutral pH could reduce expressions of AMPs⁸.

Our hypothesis is that permeability barrier disruption could lead to antimicrobial barrier dysfunction. The assessment of epidermal permeability barrier function, we were performed measurements of transepidermal water loss (TEWL), which have been presented to provide information about status of permeability barrier under either normal, experimentally perturbed, or diseased conditions³.

In this study, we have examined the level of expression of AMPs according to changes TEWL levels in AD patients. Through these results, we evaluated the relationship between permeability barrier and antimicrobial peptide barrier in AD.

Materials and methods

Subjects

Punch skin biopsies were taken from involved and adjacent uninvolved skin of 7 patients with AD after TEWL measuring. AD was diagnosed according to the standard criteria (Hanifin and Rjaka, 1982). Normal skin was obtained from healthy donor. Informed consent was obtained from all subjects after they were fully informed about the details and the

potential risk of the study, and the study was approved by the Ethical Committee of Chung-Ang University Hospital Institutes Review Board.

TEWL assessment

All subjects condition were first stabilized for 15 to 20 minutes in a climate and humidity-controlled room. Ambient temperatures ranged between 21° and 24° , with a mean relative humidity of 45%. TEWL was measured with a Tewameter TM 210 (Courage & Khazaka, Cologne, Germany) and estimated over representative involved skin sites as well as adjacent clinically normal appearing uninvolved skin with AD. TEWL levels were also measured over normal skin of healthy donor.

Preparation of primer

We synthesized the PCR primer from the basis of Gene Bank data. Primers were chemically synthesized by using DNA synthesizer (Pharmacia, Bjö gatan, Uppsala, Sweden). Their sequences were as follows:

hBD-2 (128bp):

5'-ATC TCC TCT TCT CGT TCC TC-3' (sense),

5'-ACC TTCTAG GGC AAA AGA CT-3 (anti-sense)

LL-37 (208bp):

5'-CTG ATG CCT CTT CCA GGT GT-3' (sense),

5'-GAG GGA GCC CTT TCT GAA TC-3' (anti-sense)

GAPDH (593bp):

5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense),

5'-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3' (anti-sense)

Reverse transcription-polymerase chain reaction (RT-PCR)

The tissues were cut (hash) by scissor. Total RNA was isolated from skin using TRIZol reagent (Invitrogen, Carlsbad, CA, USA) after adding 1 ml of TRIZol reagent. And the tissue homogenized by homogenizer. After 5 minutes at room temperature, 0.2 ml of chloroform per 1 ml of TRIZol reagent was added. Tubes were shaken vigorously by hands for 15 seconds and incubated at 15° to 30° for 3 minutes. The mixtures were centrifuged with 12,000 rpm at 4° for 15 minutes, the upper aqueous phase were transferred to a fresh tube, and the same amount of 2-propanol was added. After mixtures were incubated at 4° for 15 minutes, it was centrifuged with 12,000 rpm at 4° for 15

minutes. The supernatant was removed, then washed 500 μ l of 70% ethanol with 12,000 rpm at 4° for 5 minutes, the RNA pellet was briefly dried. The purified RNA was dissolved in DEPC-DW 30 μ l. Three μ g of total cellular RNA was reverse transcribed at 42° for 30 minutes in a 20 μ l volume containing 1 μ l reverse transcriptase (TaKaRa, Shiga, Japan), 10 \times buffer 2 μ l, 10mM dNTP 2 μ l (dNTP mix), oligo dT primer 1 μ l, RNase inhibitor 0.5 μ l, 25 mM MgCl₂ 4 μ l.

2 μ l of each cDNA sample from the RT-PCR was amplified by PCR in 25 μ l containing 10 \times buffer 2.5 μ l, 25 mM MgCl₂ 2.5 μ l and 10 pmol 0.75 μ l primer

Thermal cycle profiles were as follows; 94° for 5 minutes, 35 cycles of 94° for 1 minutes, 59° for 1 minutes, 72° for 1 minutes, final extension step of 72° for 10 minutes. RT-PCR analysis of LL-37 and hBD-2 mRNA using specific primers were performed

Electrophoresis

The products were run on 1.5% agarose gel contain 1 μ g ethidium bromide per millimeter. 20 μ l of reaction mixture was mixed with loading buffer, separated by electrophoresis for 15 minutes at 100 voltages and visualized by UV transillumination.

Quantitative analysis

PCR products of hBD-2, LL-37 were normalized with GAPDH on DIG chemiluminescent film by using densitometer (volume of hBD-2/volume of GAPDH \times 100, volume of LL-37/volume of GAPDH \times 100).

Western blotting

The tissue were cut(hash) by scissor. Skin were lysed in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μ g/ml phenylmethanesulfonyl fluoride (PMSF), 1 μ g/ml aprotinin, 1% Triton \times 100, the tissue was homogenized by homogenizer. After centrifuged with 12,000 rpm at 4° for 30 minutes. The supernatant was transferred into new tube, 30 μ g of soluble protein were loaded in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with sample buffer containing 1 M Tris, glycerol 50%, samples were heated at 95° for 5 minutes prior to gel loading. For hBD-2 detection, separated protein on gel electrophoresis was transferred to nitrocellulose membrane (Osmonics, Minnesota, MN, USA) at

0.16 A for 1 hour. The membrane were washed 3 times with Tris-buffered saline tween 20 (TBST), and blocked with 5% skim milk for 1 hour at room temperature. Following this, the membrane were incubated overnight at 4° with goat anti human hBD-2 polyclonal antibody (1:1500 in 5% bovine serum albumin, SantaCruz, Delaware, CA, USA) and goat anti human LL-37 polyclonal antibody (1:1500 in 5% bovine serum albumin, SantaCruz, Delaware, CA, USA) and then washed 3 times with TBST. The secondary mouse anti-goat peroxidase conjugated antibody (1:2000 in blocking solution, SantaCruz, Delaware, CA, USA) was incubated for 1 hour at room temperature. After washing the membrane with TBST, the membrane was developed with ECL solution (SantaCruz, Delaware, CA, USA) for 3 minutes then exposed to X-ray film (Roche, Indianapolis, IN, USA).

Immunohistochemistry (IHC)

IHC were carried out using sections of frozen tissues of uninvolved and involved sites in AD. In brief, 4 μ m thick sections were deparaffinized in xylene three times for 5 minutes each, and epitopes were retrieved by autoclaving (121°) for 10 minutes in citrate-buffered saline (pH 6.0). After 20 minutes of cooling at room temperature, the activity of endogenous peroxidase was quenched by treatment with 3% H₂O₂ for 5 minutes. The sections were blocked with normal goat serum for 1 hour, and incubated with mouse anti-human LL-37 and hBD-2 polyclonal antibody, respectively. After five washes with PBS, the sections were incubated with peroxidase-conjugated anti-mouse secondary antibody, FITC-anti-mouse secondary antibody and color was developed with diaminobenzidine.

Statistical analysis

The amounts of hBD-2 and LL-37 for skin between involved skin and noninvolved skin were statistically compared by t-test.

RESULTS

1) TEWL measurement between involved and uninvolved skin

For comparisons of TEWL levels between involved and uninvolved sites in AD, measurements were performed 5 times for each site, repeatedly. The

mean TEWL level of healthy donor was 7 g/hm². The mean TEWL levels increased in both uninvolved and involved sites than that of healthy donors, especially higher increased in involved sites (Table 1, $p < 0.05$).

2) Expression of LL-37 and hBD-2 mRNA in involved and uninvolved sites of AD using RT-PCR method

Compared to the expression of AMPs of healthy donor, the expressions of hBD-2 and LL-37 were decreased. LL-37 and hBD-2 mRNA expressions more decreased in involved sites than in uninvolved sites (Fig. 1, $p < 0.05$).

3) Expression of LL-37 and hBD-2 protein in involved and uninvolved sites of AD using Western blotting

Compared to the expression of AMPs of healthy donor, the expression of hBD-2 and LL-37 were decreased. The production of hBD-2 and LL-37 in involved sites were markedly decreased compared to that in uninvolved sites (Fig. 2, $p < 0.05$).

4) IHC for LL-37 and hBD-2 in involved and uninvolved sites of AD

The intensity of stainings of both AMPs in involved skin were significantly weaker than those in uninvolved skin (Fig. 3). The IHC revealed the

Table 1. Comparison of mean TEWL levels between involved and uninvolved sites in atopic dermatitis. average levels of TEWL in age and anatomical site matched normal control was 7 g/hm²

No.	TEWL (g/hm ²)		
	Involved	Uninvolved	> EWL
1	36	22	14
2	24	8	16
3	55	30	25
4	60	47	13
5	60	20	40
6	34	10	24
7	46	30	16

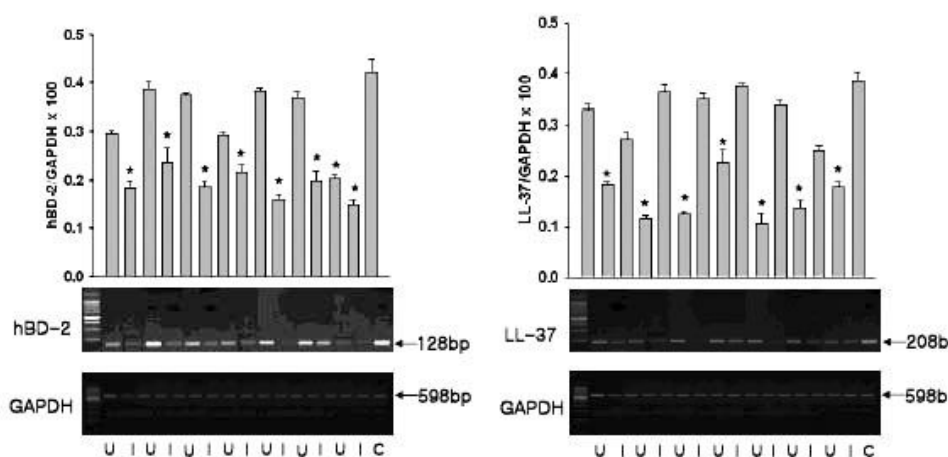


Fig. 1. RT-PCR for hBD-2, LL-37 mRNA in specimens from uninvolved(U), involved(I) sites and normal control(C) with atopic dermatitis. The expression of hBD-2, LL-37 mRNA were down-regulated in both uninvolved and involved sites, more decreased in involved sites (* $p < 0.05$)

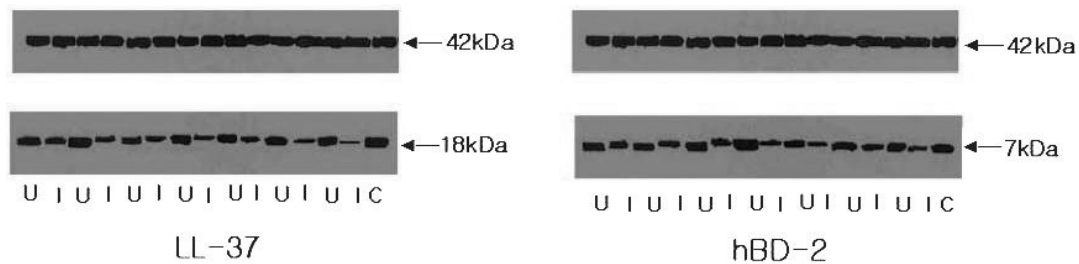


Fig. 2. Expression of hBD-2, LL-37 proteins in specimens from uninvolved (U), involved (I) sites and normal control (C) with atopic dermatitis by Western blot. The production of hBD-2 in involved skin was markedly decreased compared to that in uninvolved skin. The upper lanes are actins.

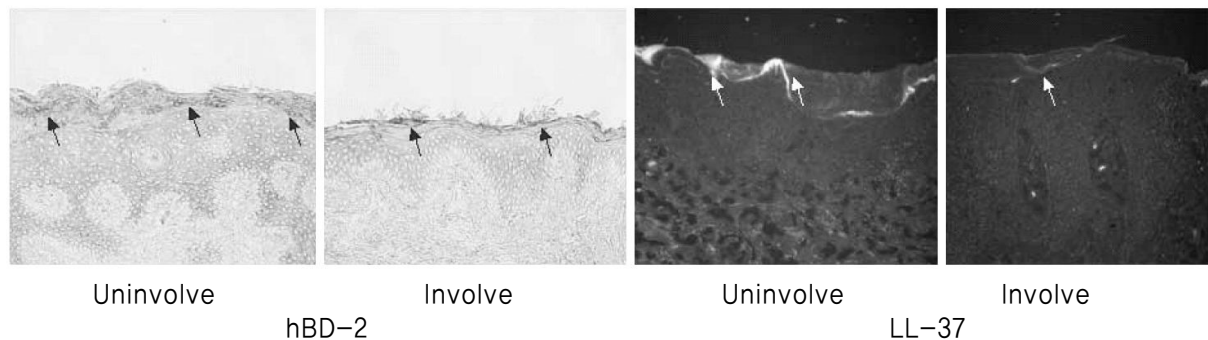


Fig. 3. Immunohistochemical staining For hBD-2 and LL-37 in frozen sections from patients with atopic dermatitis. The IHC confirmed the presence of more abundant hBD-2 and LL-37 in the uninvolved sites than involved sites.

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5) Regression curves

For evaluation of the expression of AMPs in according to TEWL levels changes in AD, the subtraction values of AMPs expressions and TEWL levels, which were measured from involved and noninvolved sites of each patient, were calculated, respectively. When these results compared to each other using regression curve, subtraction values of AMPs expressions were more decreased according to increase of TEWL levels. It showed inverse proportional relationship (Fig. 4, 5).

DISCUSSION

AD is multifactorial, polygenetic skin disorder and is commonly viewed as immunologic in pathogenesis¹. Examination of skin biopsy samples from patients with AD has shown that *S. aureus* grows in colonies in the upper layers of the epidermis

between keratinocytes^{1,9}. This suggests that an exponential increase in *S. aureus* could result from failure of the innate immune defense system. Naturally occurring AMPs are a critical component of this innate immune system^{1,10,11}. Among numerous AMPs, LL-37 and hBD-2 are the major classes of peptides in human skin³. hBD-2 and LL-37 in human are normally produced by keratinocytes in response to inflammatory stimuli such as bacteria, viruses, and fungi^{5,9,12}. In fact, *S. aureus* can be isolated from skin lesions of most patients with AD⁵. The mechanisms leading to increased *S. aureus* colonization in atopic dermatitis are unknown until now^{13,14}. A number of processes could contribute to increase in *S. aureus* colonization. These include disruption of skin barrier function from scratching, exposure of inflamed underlying skin from scratching, loss of certain innate antibacterial activities from changes in lipid composition or hBD levels^{5,6}.

Both patients with AD and psoriasis have altered skin barrier function, but patients who are psoriatic are more resistant to skin infections than healthy individuals⁶. About 30 percent of patients with

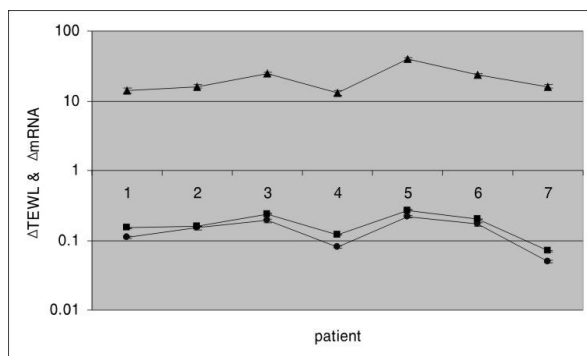


Fig. 4. Δ RNA expression amounts of hBD-2 (circle) LL-37(square) and Δ EWL(triangle)

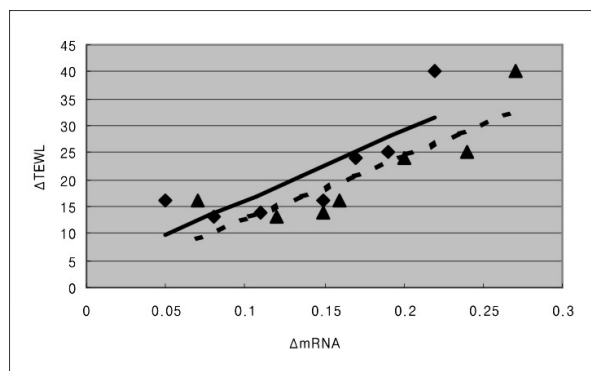


Fig. 5. Regression curves Δ RNA expression amounts of hBD-2(diamond, solid line), LL-37 (triangle, dotted line) and Δ EWL (Δ RNA : mRNA amount of uninvolved site mRNA amount of involved site).

atopic dermatitis have bacterial or viral infections of the skin, as compared with only 7 percent of patients with psoriasis^{7,15}. That's why both LL-37 and hBD-2 have been known to be decreased significantly in acute and chronic lesions of AD compared to psoriasis⁴. In psoriatic skin, keratinocytes were increased in number and differentiated acceleratively, thus, the epidermis became thickening. So, quantitatively, abundant antimicrobial peptides are expressed in the psoriatic epidermis^{7,8}. However, in AD, why the expressions of AMPs decreased are still unknown. a deficiency in the expressions of AMPs may account for the susceptibility of AD patients to skin infection, such as *S. aureus*^{3,5}. The inability to increased AMPs may be caused by suppression by TH2 cytokines that are elevated in AD. In vitro, keratinocytes in culture could be shown to lose the ability to increase hBD-2

expression when exposed to IL-4 or IL-13^{11,12}. Enhanced TH2 cell activity is a hallmark of acute AD^{3,4,16}. Increased production of IL-4, IL-5 and IL-13 by TH2 cells, in turn, inhibit TH1 cytokine production, including generation of interferon-gamma and IL-18, which are two beneficial antimicrobial mediators^{17,18,19}. IL-4, IL-13 have also direct inhibitory effects for AMPs expression^{16,17}.

In the addition, Elias et al suggested that the changes of stratum corneum pH due to disruption of permeability barrier function increased the activity of SP which could degrade AMPs^{3,10}. There is a reduction in ceramides in the epidermis of patients with AD, with an abnormal expression of sphingomyelin deacylase. This enzyme hydrolyzes sphingomyelin to yield sphingosylphosphorylcholine rather than ceramide¹¹. Insufficiency of ceramides in the stratum corneum is an etiologic factor in atopic dry skin. Dry skin is a feature of AD with increased TEWL, which is a reflection of impaired barrier function of stratum corneum^{9,10}. Several studies have shown a significant increase in basal TEWL, even in clinically uninvolved skin in AD¹¹. The low levels of ceramide makes skin surface pH altered toward to alkalinity. As a result of alkalinized or neutral skin surface pH, recovery of permeability barrier may be delayed and activity of SP are increased, thus amounts of AMPs may decrease by SP^{13,16,17}. According to Elias et al, the substitution of a ceramide-dominant barrier repair formulation for standard, nonphysiologic lipid-based emollients and moisturizers is beneficial not only for the uninvolved 'dry' skin in AD but also for the inflammatory skin in recalcitrant AD patients³. It implicated that emollients are helpful in not only skin hydration but also ceasing to cytokine cascade leading to inflammation through normalization of permeability barrier function.

As our results were shown, TEWL levels were increased both involved and uninvolved sites compared to normal control, which these were more significantly decreased in involved sites. These finding showed that the permeability barrier were disrupted in both involved and uninvolved sites in AD, obviously more significant in involved sites. In RT-PCR analyses of AMP mRNA expressions, the expression levels of hBD-2 and LL-37 mRNA were down-regulated in both uninvolved and involved sites compared to healthy donor, more decreased in involved sites. These tendency were also confirmed

by Western blot and IHC. These results suggested that AMPs expressions were downregulated in AD, even in uninvolved sites and more decreased in involve sites. For comparing relationship between the expression of AMPs and permeability barrier function, we calculated the subtraction values of TEWL levels and AMPs expressions between involved and uninvolved sites, respectively. We supposed that these subtraction values could reflect a contribution for decreasing AMPs expressions according to disrupted degrees of permeability barrier. And we also expected that the subtraction values of expressed AMPs would be in inverse proportion with severity as measured TEWL levels. It implicated that permeability barrier function have a role in modulation the expression of AMPs²⁰. In other words, more significant permeability barrier disrupted, less the AMPs expressed in AD.

In conclusion, we were able to conclude there was in proportion between TEWL levels and the permeability barrier disruption. Thus, our results demonstrated that AMPs barrier may co-localize to permeability barrier. The co-localization of the permeability and antimicrobial peptide barrier had demonstrated by the translocation of hBD-2 from the endoplasmic reticulum to epidermal lamellar body(LB) following IL-1 α stimulation². These findings showed in agreement with our results. Although our results have a statistical limitation because patient group were only 7, however, we believe that it is first results about relationship between skin barrier function and AMPs. It should be necessary to carry out further studies that after restoration of skin barrier function through using a ceramide dominant emollients on atopy skin, how the expression of AMPs changes.

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