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rhEGF Treatment Improves EGFR Inhibitors-Induced Skin Barrier and Immune Defects

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Abstract

Background: The mechanisms of epidermal growth factor (EGF) effects EGF receptor inhibitor (EGFRI)-related skin toxicities are as yet unknown. We investigated which mechanisms are involved in EGF's positive effects.

Methods: Two types of EGFRIs, cetuximab and gefitinib, were used to treat the cells or 3d-cultured human skin tissue with recombinant human EGF (rhEGF). The expression levels of skin barrier related proteins, inflammatory cytokines, and antimicrobial peptides (AMPs) were measured by using RT qPCR, ELISA, immunohistochemical (IHC) stain, or immunofluorescence..

Results: Using western blot assay, cetuximab decreased EGFR and phosphorylated EGFR (pEGFR) expression. In contrast, rhEGF increased EGFR and pEGFR expression. Also, rhEGF induces EGFR signaling by pAKT and pPI3K expression in gefitinib and rhEGF co-treated cells. Expressions of proliferation and differentiation proteins, both ki-67 and filaggrin, were decreased in EGFRIs -treated tissue. However, in rhEGF and EGFRIs co-treated tissue, those expressions were increased. Pro-inflammatory cytokines, including IL-1α, IL-8, and TNF-α expression, were increased by EGFRIs, and down-regulated by rhEGF. In patients' tissue evaluation, compared with control, patients' Ki-67 and EGFR expression were decreased (P=0.015, P=0.001). Patients' IL-17 and TNF-α expression intensity was higher than that of control group (P=0.038, P=0.037). After treatment with EGF ointment, average values of Ki-67, EGFR, and Melan-A were changed to normal values. Oppositely patients' proportions of IL-17 and TNF-α were decreased to low stain level.

Conclusions: Treatment of rhEGF improved EGFRIs-induced skin eruption via normalizing the proliferation and differentiation of keratinocytes, reducing inflammatory cytokines by the affected EGFRIs.

Statement Of Translational Relevance

In previous study, we demonstrated that EGF ointment is effective for managing EGFR inhibitor-related skin toxicities and improves patients' QoL compared with placebo. Nevertheless, the mechanisms of EGF effects are unknown yet so basic study is needed to clarify the mechanisms. In this study, we revealed that treatment of rhEGF in human epidermal keratinocytes, 3d-cultured human skin tissue and patient lesions improved EGFRIs-induced skin eruption via normalizing proliferation and differentiation of keratinocytes, reducing inflammatory cytokines expression and inducing expression of AMPs especially hBD-2 and -3. These findings, therefore, may support the effect of topical rhEGF treatment on the improvement of EGFRI-derived skin side effects.

1. Background

Epidermal growth factor receptor (EGFR) inhibition is a good target for the treatment of diverse metastatic epithelial cancers, including lung, colon, pancreatic, and head and neck cancers [1, 2]. There are two strategies to inhibit EGFR signaling: monoclonal antibodies, and tyrosine kinase inhibitors (TKI).

Despite the treatment approach, use of EGFR inhibitors (EGFRIs) is associated with adverse side effects [3]. Among the cutaneous toxicities observed in cancer patients treated with EGFRIs are papulopustular rash of the upper trunk and face skin of (60–90%), and dry and itchy skin of (12–16%) [4, 5]. Although the side effects induced by EGFRIs are generally classified as moderate, they are usually chronically persistent, and may significantly impact the patient's quality of life (QoL), and thus necessitate dose reduction, or even interruption of treatment.

The most frequently used formulation for EGFRIs-induced skin reactions is the oral or topical application of antibiotics, such as tetracycline family and corticosteroids, alone, or in combination with moisturizers and sunscreen [6–11]. Antibiotics, including doxycycline and minocycline, are commonly used to treat acneiform rash, and help reduce its symptoms. Topical treatment of corticosteroids is also generally used for treatment of ERSEs, especially for skin rash. Other topical agents, such as retinoid and vitamin K1 cream, have potential roles for the management of ERSEs [12, 13]. However, their efficacies have not been fully investigated through prospective studies. A recent phase III trial has shown that prophylactic use of vitamin K1 cream in combination with doxycycline cannot decrease the incidence of grade ≥ 2 skin rash in patients initiating cetuximab therapy, compared to doxycycline and vehicle [14]. Even though these therapeutics are used to treat ERSE, they could not treat the main reason, but only relieve the symptoms.

EGF plays a key role in wound healing, epidermal keratinocytes are a rich source of EGFR ligands, and EGFR signaling has a major effect on the proliferation and differentiation of keratinocytes [15]. Therefore, EGF plays an important role in skin development and homeostasis [16]. Beyond its role in wound healing and epithelial homeostasis, recent studies have revealed that EGF has a protective effect of skin barrier functions in atopic dermatitis and acne vulgaris [16–19]. Hershey et al. reported that EGF had an immunomodulatory role in inflamed skin tissue, showing that EGFR signaling reduces allergen-induced interleukin (IL)-6 production and Th17 responses in the skin [16]. Similarly, our previous studies revealed that EGF treatment regulates TLR-2 induced inflammatory reaction in human epidermal keratinocytes [19] and topical treatment of EGF relieved S. aureus-induced inflammation and AD-like skin lesions in Nc/Nga mice [17].

In previous study, we demonstrated that EGF ointment is effective at managing EGFR inhibitor-related skin toxicities and improves patients' quality of life (QoL), compared with placebo, via placebo-controlled, double-blind, multicenter, pilot phase III trial [20]. Nevertheless, the mechanisms of EGF effects are as yet unknown, so basic study is needed to clarify the mechanisms. In this study, we therefore investigated which mechanisms are involved in EGF's positive effects on EGFRIs-induced skin irruptions using human epidermal keratinocytes and 3D-cultured human skin tissue. Also, we compared *in vitro* and *ex vivo* results with the tissues of patients who treated EGF topically.

2. Methods

2.1. Reagents

For the purposes of this study, recombinant human EGF (rhEGF, Daewoong Pharmaceutical Co., Ltd., Seoul, Korea) was used. Cetuximab (5 mg/mL, Erbitux®) was purchased from Merck (Darmstadt, Germany), and gefitinib (250 mg, Iressa®) was purchased from AstraZeneca Corporation (San Diego, CA, USA). To induce inflammatory reaction, lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MI, USA) was used, and it was treated for 24 h before EGFRIs or rhEGF treatment.

2.2. Cell culture

Primary human epidermal keratinocytes (Thermo Fisher Scientific, Waltham, MA, USA) were cultured in EpiLife Medium (Thermo Fisher Scientific) with human keratinocyte growth supplement (HKGS, Thermo Fisher Scientific). Cells were maintained in a humidified atmosphere of 5% CO2 and 37 °C, and the medium was replaced every two days. Before reagent treatment, the cells were cultured overnight in EpiLife Medium without HKGS to induce starvation. To induce inflammatory reaction, LPS was treated for 24 h. Then, various concentration of rhEGF with EGFRIs (10 ng/mL cetuximab or 1 μM gefitinib) or only EGFRIs were treated to LPS-treated keratinocytes for (24 or 48) h.

2.3. 3d-cultred human skin tissue

Neoderm® is a 3D human skin tissue model in which human primary keratinocytes and fibroblasts are 3-dimensionally cultured to mimic the morphology and physiology of human skin. To confirm whether rhEGF affects epidermis homeostasis, the Neoderm® tissues were cultured with rhEGF and EGFRIs (10 ng/mL cetuximab or 1 μM gefitinib) co-treatment or only EGFRIs treatment for 48 h. Also, to investigate whether rhEGF affects inflammatory reaction, the Neoderm® tissues were cultured with LPS for 24 h. After that, EGFRIs (10 ng/mL cetuximab or 1 μM gefitinib) and/or 10 ng/mL EGF were treated to LPS-treated Neoderm® tissues for 48 h. The reagents were treated to the top of tissues. Then, each tissue was fixed by 4% formaldehyde solution.

2.4. Real-time quantitative PCR

After reagents treatment for 24 h, the cells were harvested by trypsinization, and the total RNA was extracted using the ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA), and 1 μg of the total RNA was converted to cDNA using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Foster City, CA, USA), under the following reaction conditions: 45 °C for 45 min, and 95 °C for 5 min. Probes were obtained from Applied Biosystems as Assays-on-Demand™ Gene Expression Assays (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]: Hs02758991_g1, IL-1α: Hs00174092_m1, IL-8: Hs00174103_m1, tumor necrosis factor-alpha (TNF-α): Hs01113624_m1, IL-17α: Hs00174383_m1, IL-17β: Hs07287652_m1, hBD-1: Hs00608345_m1, hBD-2: Hs00175474_m1, hBD-3: Hs04195435_g1, hBD-4: Hs00414476_m1, LL37: Hs00189038_m1, RNase 7: Hs00922963_s1). Reactions were carried out on the ABI StepOnePlus™ (Applied Biosystems), and relative transcription levels were determined by GAPDH as the reference gene. The data were analysed by using the ABI StepOnePlus™ software (Applied Biosystems).

2.5. Western blot assay

To confirm whether rhEGF activated EGFR on human keratinocytes when rhEGF and cetuximab were co-treated to cells, EGFR and phosphorylated EGFR (pEGFR) expressions in human keratinocytes were detected by western blot assay. Various concentration of rhEGF and 10 ng/mL cetuximab were co-treated to cells for 4 h, and then the cells were washed by phosphate-buffered saline (PBS). Then, cell pellet was harvested, and stored at -80 °C in deep freezer, before use.

To confirm whether rhEGF activated EGFR on human keratinocytes when rhEGF and gefitinib were co-treated to cells, EGFR signaling markers, including Protein kinase B (AKT), phosphorylated AKT (pAKT), Phosphoinositide 3-kinase (PI3K), and phosphorylated PI3K (pPI3K), were detected by western blot assay. Various concentration of rhEGF and 1 μM gefitinib were co-treated to cells for 40 min, the cells were washed by PBS 1 time, then the cells were harvested, and stored at -80 °C in deep freezer, before use.

To detect the expression of EGFR, pEGFR, AKT, pAKT, PI3K, and pPI3K in reagents-treated keratinocytes, the cells were lysed in RIPA lysis and extraction buffer (Thermo Fisher Scientific), to which was added a protease inhibitor cocktail (Thermo Fisher Scientific), and total protein concentration was measured using the BCA assay (Thermo Fisher Scientific). The 30 g of total protein per sample was resolved using SDS-PAGE on a (4–12) % Bis-Tris gel (Nupage; Invitrogen Corp., CA, USA), using MES SDS running buffer (Nupage, Invitrogen). Then, the resolved protein was transferred to PVDF membrane using iBlot gel transfer device (Thermo Fisher Scientific). Western blot analysis was performed according to standard procedures, using each primary antibody. The reaction product was detected by enhanced chemiluminescence (Amersham Imager 600, GE Healthcare Life Sciences, IL, USA). The primary antibodies of EGFR and pEGFR were purchased at Cell Signaling Technology (MA, USA), while the primary antibodies of AKT, pAKT, PI3K, and pPI3K were purchased from Abcam (Cambridge, UK). The antibodies were diluted according to the manual of each primary antibody.

2.6. Receptor binding affinity test

The binding affinity of rhEGF and cetuximab to human EGFR (R&D Systems, Minneapolis, MN, USA) was measured by Biacore T200 (GE Healthcare, Sweden). HBS-EP was used as running buffer, and 30 mM NaOH was used for regeneration of the chip surface. The concentrations were (1.563, 3.125, 6.25, 12.5, 25, 50, 100, and 200) nM for rhEGF, and (0.012, 0.024, 0.049, 0.098, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, 100, and 200) nM for cetuximab. Because of the difference of molecular weight between rhEGF and cetuximab, rhEGF was analysed by 1:1 model, while cetuximab was analysed by bivalent model. The association constant (Ka) and the equilibrium dissociation constant (KD) were obtained to evaluate the binding affinity by using BIA evaluation software version 3.0 (GE Healthcare).

2.7. Tissue pathology

Twelve patients' ERSEs skin biopsies from randomized clinical trial that were EGF treatment for ERSE were obtained. The same numbers of comparison-controlled skin biopsies who did not undergo related clinical trial were obtained from the Bio-Resource Bank and Dong-A University Hospital. Among them, five patients were treated with EGF ointment for ERSE. We also observed skin changes before and after EGF ointment.

2.7.1. Hematoxylin and eosin

All tissues, including 3D-cultured human tissues and biopsy samples of patients, were fixed by 4% formaldehyde solutions. Then the fixed tissues were embedded in paraffin. The 4 μm tissue sections were deparaffinized, and rehydrated in a graded ethanol series. Then, sections were stained with hematoxylin-eosin (H&E). All slides were examined under light-microscopy (Olympus, Tokyo, Japan).

2.7.2. Immunohistochemical (IHC) assay

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections, according to the manufacturers' instructions. The panel of primary antibodies included: Ki-67 antibody (Dako, Zug, Switzerland); EGFR Pharm Dx Kit (Agilent, Glostrup, Denmark); Melan-A antibody (Fuzhou Maixin Biotechnology Co. Ltd., Fuzhou, China); IL-17 (dilution 1:200; Abcam Inc., Cambridge, UK); and TNF-α antibody (MyBioSource, USA). Immunohistochemistry was performed using a Ventana BenchMark automated stainer (Ventana Medical Systems, Tucson, AZ, USA). The immunohistochemistry assessments were evaluated by a pathologist. Each specimen was analyzed three times for each different field at × 200 magnification.

2.7.3. Immunofluorescence (IF) assay

The paraffin-embedded 3D-cultured human tissue sections were deparaffinized with xylene, dehydrated in gradually decreasing concentrations of ethanol, and then subsequently treated with 3% hydrogen peroxidase in TBS for 30 min to block endogenous peroxidase activity. Then, antigen retrieval was performed by using low pH IHC antigen retrieval solution (Invitrogen) at 90 °C for 30 min. The sections were immediately immersed in TBS for 10 min at 4 °C. Thereafter, the sections were blocked with BlockAid™ Blocking Solution (Invitrogen) for 15 min at room temperature (RT). The sections were rinsed in PBS, and incubated with primary antibody for overnight at 4 °C, and secondary antibody was performed for 2 h at RT. The nuclei stain and mounting were performed with ProLong™ Gold Antifade Mountant with DAPI (Invitrogen). Supplementary Table S1 lists the Primary antibodies and goat anti-rabbit or goat anti-mouse secondary antibody with Alexa Fluor 488 (Invitrogen) that were used in this study.

2.8. Statistical analysis

All experiments were carried out in triplicate, and the results are expressed as mean ± standard deviation. P values < 0.05 were considered statistically significant. One-way analysis of variance with Dunnett's post-test was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. rhEGF activated EGFR signaling when EGFRIs and rhEGF co-treated to keratinocytes.

To investigate whether rhEGF could activate EGFR on keratinocytes when rhEGF and EGFRIs were co-treated to cells, we examined phosphorylated EGFR expression in keratinocytes that were treated with rhEGF and cetuximab. As a result, when various concentration of rhEGF and cetuximab were co-treated to keratinocytes, expressions of both EGFR and phosphorylated EGFR (pEGFR) were increased (Fig. 1 (a)). In particular, the highest expression was in pEGFR in 10 ng/mL rhEGF- and cetuximab-treated cells (Fig. 1 (a)). Then, to investigate whether EGFR could be activated by rhEGF when rhEGF was co-treated with gefitinib, expression of EGFR signaling molecules, including AKT, PI3K, phosphorylated AKT (pAKT), and phosphorylated PI3K (pPI3K), in rhEGF and cetuximab cotreated keratinocytes was detected by western blot assay. Figure 1(b) shows that in rhEGF and gefitinib co-treated cells, pAKT and pPI3K were detected, and the expression of these molecules were increased by rhEGFR in a concentration-dependent manner (Fig. 1 (b)).

Next, we compared the binding affinity between rhEGF and cetuximab using BIACORE T200 (GE Heathcare). Following a result, the value of each association constant (K_A) was 6.07×10^5 (rhEGF) and 3.54×10^5 (cetuximab) (Table 1). This result meant that rhEGF bound to EGFR faster than did cetuximab. However, in the results of the equilibrium dissociation constant (K_D), the value of K_D of rhEGF (1.30×10^{-9}) was lower than that of cetuximab (0.49×10^{-9}), and this result meant that cetuximab bound to EGFR stronger than did rhEGF (Table 1).

Table 1
rhEGF and cetuximab were compared in a surface plasmon resonance (SPR) analysis for their human EGFR binding and approximate kinetic parameters are presented.

Ligand	Analyte	Conc.	K_a (1/Ms)	K_d (1/s)	K_D (M)	Rmax	Chi^2
Human EGFR	rhEGF	1.563, 3.125, 6.25, 12.5, 25, 50, 100, 200 nM	6.07×10^5	7.91×10^{-4}	1.30×10^{-9}	11.8	0.497
	Cetuximab	0.012, 0.024, 0.049, 0.098, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, 100, 200 nM	3.54×10^5	1.75×10^{-4}	0.49×10^{-9}	292.8	8.11

EGFR: epidermal growth factor receptor, rhEGF: recombinant human epidermal growth factor

3.2. rhEGF had regulatory effects on proliferation and differentiation of keratinocytes in *ex-vivo* studies.

To elucidate whether the proliferation/differentiation of keratinocytes altered by EGFRIs was regulated by rhEGF, we analyzed the expression of proliferation and differentiation markers including ki67, filaggrin, K5, and K10 in 3D-cultured human skin tissues using immunofluorescence assay. Figure 2 (a) shows that

compared with un-treated control, ki67 expression in tissues treated with 10 ng/mL cetuximab or 1 μM gefitinib was decreased (Fig. 2 (a)). However, in 10 ng/mL rhEGF- and EGFRIs-treated tissues, ki67 expression was similar to ki67 expression in control (Fig. 2 (a)). Similarly, filaggrin expression was decreased in cetuximab- or gefitinib-treated tissues, compared with untreated control (Fig. 2 (b)). However, in rhEGF and cetuximab or gefitinib co-treated tissues, filaggrin expression was increased, compared with that in tissues treated with cetuximab- or gefitinib-only (Fig. 2 (b)).

Figure 2 (c) shows that K5 expression was not affected by cetuximab treatment or rhEGF and cetuximab co-treatment. However, in tissue treated with gefitinib-only, K5 expression was down-regulated (Fig. 2 (c)). Notably, in rhEGF and gefitinib co-treated tissue, K5 expression level was similar to the expression of K5 in untreated control (Fig. 2 (c)). Interestingly, K10 expression was increased by cetuximab or gefitinib treatment (Fig. 2 (d)), and that increment was not observed in rhEGF and cetuximab or gefitinib co-treated tissue (Fig. 2 (d)). Nevertheless, K10 expression level in rhEGF and cetuximab or gefitinib co-treated tissue was a little higher than in untreated control (Fig. 2 (d)).

3.3. rhEGF had little effects on expression of tight junction proteins of keratinocytes including claudin-1, -3, and occludin.

The effect of EGFRIs and rhEGF on the proliferation and differentiation of keratinocytes was observed. Thus, we evaluated the expression of tight junction proteins, including claudin-1, -3, and occluding, using IF assay to investigate whether rhEGF and EGFRIs can affect tight junction proteins. Figure 3 (a) shows that reduction of claudin-1 expression was observed in EGFRIs-treated, and EGFRIs-only or rhEGF co-treated tissue. Interestingly, there was a difference between cetuximab-treated tissue and gefitinib-treated tissue. In cetuximab-treated tissue, claudin-1 decrement was observed in all epidermal layer, but in gefitinib-treated tissue, claudin-1 expression was decreased above the basal layer (Fig. 3 (a)).

Then, we observed claudin-3 expression in reagent-treated or not-treated 3D-cultured tissues. As a result, claudin-3 expression was observed in the stratum granulosum layer of untreated tissues (Fig. 3 (b)). In cetuximab- or gefitinib-treated tissues, claudin-3 expression was not observed (Fig. 3 (b)); and in cetuximab and rhEGF co-treated tissue, claudin-3 expression was also not detected (Fig. 3 (b)). However, in gefitinb- and rhEGF-treated tissue, claudin-3 expression was observed at the stratum granulosum layer (Fig. 3 (b)).

Next, we investigated the change of occludin expression between reagent untreated control and reagent-treated tissues. Figure 3 (c) shows that occludin was detected in all the epidermal layers of untreated or reagent-treated tissues. In only EGFRIs-treated tissues, it was observed that occluding expression was slightly decreased (Fig. 3 (c)). Also, in rhEGF and EGFRIs co-treated tissues, occludin expression was slightly decreased, compared with untreated control, but it did not differ, compared with only EGFRIs-treated tissues.

3.4. rhEGF inhibited expression of pro-inflammatory cytokines, including IL-1α, IL-8, and TNF-α, in *in vitro* and *ex vivo* studies.

We next examined the effects of EGFRIs and rhEGF on inflammatory cytokine productions from primary human epidermal keratinocytes and 3D-cultured human skin tissues that were stimulated by lipopolysaccharides. messenger ribonucleic acid (Mrna) expression of IL-1α, IL-8, and TNF-α was increased by cetuximab or gefitinib treatment in LPS-pretreated keratinocytes (Figs. 4 (a) and (b)). Hence, in rhEGF and EGFRIs co-treated cells, the increment of mRNA expression of cytokines was inhibited in an rhEGF concentration-dependent manner (Figs. 4 (a) and (b)). Similarly, as a result, protein expression of cytokines was increased by EGFRIs treatment in LPS-pretreated keratinocytes (Figs. 4 (c) and (d)). As a result, protein expression of IL-1α, IL-8, and TNF-α in (1 and 10) ng/mL rhEGF and cetuximab or gefitinib co-treated keratinocytes (Figs. 4 (c) and (d)) was decreased. However, in 20 ng/mL rhEGF and EGFRIs co-treated cells, the protein expression was not decreased.

Then, to confirm the change of protein expression by rhEGF and EGFRIs treatment, we assessed cytokines expression in EGFR or rhEGF and EGFR co-treated 3D-cultured tissues that were pre-treated by LPS. Figure 4 (e) shows that IL-1α was detected in LPS-treated tissue, and cetuximab and gefitinib treatment increased IL-1α expression in the basal layer and stratum corneum of LPS-treated tissues. Similar to previous in vitro results, in rhEGF and EGFRIs co-treated tissues, IL-1α expression was reduced, but it was higher than in untreated control.

The results of IL-8 and TNF-α expression were similar to that of IL-1a. Figure 4 (f) shows that IL-8 expression in LPS-treated tissue was increased at the upper basal layer. It was observed that IL-8 expression in cetuximab- or gefitinib-treated tissue was higher than that of LPS-treated tissue. Interestingly, IL-8 expression in cetuximab-treated tissues was higher than that of gefitinib-treated tissues. Also, it was observed that rhEGF and EGFRIs treatment decreased IL-8 expression. Similarly, TNF-α expression was higher in EGFRIs-treated tissues, than that of LPS-treated tissue (Fig. 4 (g)). In cetuximab-treated tissue, TNF-α was detected in the basal layer and stratum corneum, but TNF-α was detected in all epidermal layers of the gefitinib-treated tissue. Figure 4 (g) shows that rhEGF- and EGFRI-co-treatment decreased TNF-α expression.

3.5. rhEGF increased antimicrobial peptides expression in human epidermal keratinocytes, especially hBD-2 and − 3.

The regulatory effect of rhEGF on EGFRIs-induced inflammatory cytokine expression was observed. Then, we evaluated the expression of anti-microbial peptides (AMPs), including human β defensin (hBD)-1, -2, -3, -4, LL37, and RNase 7. First, we analyzed the mRNA expression in EGFRIs-only, or rhEGF and EGFRIs co-treated keratinocytes that were pre-treated with LPS for 24 h using real-time qPCR. As a result, LPS induced the expression of hBD-2 up to 24.6-fold and hBD-3 up to 13.4-fold, 24 h after treatment (Fig. 5 (a)), but LL37 and RNase7 mRNA expressions were only slightly increased in LPS-treated cells (LL37: 1.7-

fold; and RNase 7: 2.0-fold; Fig. 5 (b)). On the other hand, in cetuximab-treated cells, the increment of mRNA expression was decreased (Figs, 5 (a) and (b)). Notably, rhEGF increased those decreased AMPs mRNA expression in a concentration-dependent manner (Figs. 5 (a) and (b)). Similarly, in gefitinib-treated cells, hBD-2 and hBD-3 mRNA expressions were inhibited; and these were increased by rhEGF treatment in a concentration-dependent manner (Fig. 5 (c)). However, mRNA expression of LL37 and RNase 7 was not affected by gefitinib, or gefitinib and rhEGF co-treatment (data not shown). Next, we could confirm increased hBD-2 and hBD-3 expressions on protein level (Figs. 5 (d) and (e)). hBD-2 and hBD-3 protein expressions were inhibited by cetuximab or gefitinib treatment, and those decrements were increased by rhEGF treatment (Figs. 5 (d) and (e)).

3.6. Skin pathologic change evaluation of the patients who had EGFR inhibitor-related skin adverse events (ERSE)

In addition, in clinical trial results, the immunohistochemistry differences between ERSE group and control group were observed. As a result, in ERSE group, Ki-67 expression (21.2%) was lower than control group's Ki-67 expression (40.8%) ($P = 0.015$, Figs. 6 (a) and (b)). Similarly, EGFR presentation range of epidermis was (98.3 vs 84.6) % in control and ERSEs group, respectively ($P = 0.001$, Figs. 6 (c) and (d)). Also, we could observe (14.2 vs 8.1) % ($P = 0.069$) of Melan-A immunohistochemistry (Figs. 6 (e) and (f)). On the other hand, IL-17 ($P = 0.038$) and TNF-α ($P = 0.037$) expression intensity in ERSE group was higher than control group in dermis (Figs. 6 (e) and (f)).

After treatment of EGF ointment of 5 patients, we could observe the rebuilding of epidermis and decrement of infiltrated inflammatory cells in dermis in the 5 patients' tissues (Fig. 7 (a) and (b)). We also observed the average values of Ki-67, EGFR, and Melan-A, and stain levels of IL-17 and TNF-α. As expected, in EGF ointment-treated patients' tissues, the average values of Ki-67 (28%), EGFR (94%), and Melan-A (8.2%) were changed (Figs. 7 (c)–(h)). Moreover, all of patient's proportion of IL-17 and TNF-α were decreased to low stain level (Figs. 7 (i)–(l)).

4. Discussion

In this study, we used two types of EGFR inhibitors, cetuximab and gefitinib, to induce EGFRIs-derived skin side effects on in vitro and ex vivo, respectively. In addition, we treated cetuximab or gefitinib with rhEGF at the same time, to determine whether EGF could improve the side effects of EGFRIs. To this end, it was necessary to confirm whether the EGFR signaling was activated when EGFR inhibitors (cetuximab and gefitinib) were treated simultaneously. So, after simultaneously treating cetuximab and rhEGF, pEGFR expression was simultaneously treated with gefitnib and rhEGF, and then AKT and PI3K phosphorylation related to EGFR signaling was examined. pEGFR expression was examined after simultaneous treating of cetuximab and rhEGF, and AKT/PI3K phosphorylation related to EGFR signaling was identified in gefitinib and rhEGF co-treated cells. As a result, as shown in Fig. 1, the expression of pEGFR was increased in the group simultaneously co-treated with cetuximab and rhEGF, compared with the cetuximab alone. In addition, EGFR expression was also increased by rhEGF in a concentration-dependent manner.

Furthermore, we compared the binding affinity for human EGFR between cetuximab and rhEGF. As a result, rhEGF binds to hEGFR faster than does cetuximab. Our guess is that although cetuximab maintained binding with hEGFR for a longer time than rhEGF, EGFR phosphorylation occurred in cetuximab and rhEGF co-treated cells because of the faster binding rate of rhEGF with hEGFR than that of cetuximab. Gefitinib is one of the EGFR-TKI inhibitors, and the inhibition of EGFR phosphorylation and phosphorylation of downstream effectors AKT in cells treated with over 0.1 M concentration of gefitinib were observed [21]. Similarly, in this study, gefitinib treatment inhibited the phosphorylation of AKT and PI3K. However, in gefitinib and rhEGF co-treated cells, phosphorylated AKT and PI3K were also observed. We estimate that the lowest concentration of rhEGF (1 ng/mL) in this study exceeds the ability of TKI inhibition of 1 uM gefitinib, and further study is needed.

The skin barrier function is mainly provided by keratinocytes, and it is maintained by a tightly controlled balance between the proliferation and differentiation of keratinocytes [22]. EGFR signaling plays an important role in the final differentiation of keratinocytes by inducing the activation of Transglutaminase (TGase) via Phospholipase C-γ (PLCγ) and Protein kinase C (PKC) [23]. Also, EGF induces keratinocyte proliferation via Raf-MEK-ERK signaling pathway. In this study, in cetuximab- or gefitinib-treated tissue, low expressions of both ki-67 and filaggrin were observed. However, in rhEGF and cetuximab or gefitinib co-treated tissue, those expressions were high, and the expressions were similar to those of un-treated control tissue. These results mean that rhEGF treatment could normalize the proliferation and differentiation of keratinocytes via activating EGFR signaling pathway. Keratins are principal structural proteins in epidermis, and the primary function of keratin is structural and mechanical support. In addition, keratins also modulate the growth, adhesion, migration, and invasion of epithelial cells. So, the dysfunction or mutations of keratin proteins are associated with a remarkable variety of skin disorders, including skin blistering, and inflammatory disorders [22]. In normal skin, epidermis expresses K1 and K10 in suprabasal layers, and K5 and K14 in basal layer. Whereas, in inflammatory skin, such as atopic dermatitis and psoriasis, expression of inflammatory related-keratin, including K6, K16, and K17, are increased [24]. Terrinoni et al. reported that abnormal expression of K10 with respect to control skin was observed in the lesional skin of patient who were under epidermal erythrodermic hyperkeratosis [25]. In this study, K5 expression was inhibited by EGFRIs, and that decrement was induced by rhEGF cotreatment (Fig. 2 (c)). Whereas, the aggregation of K10 in EGFRIs-treated tissues was observed, but in rhEGF- and EGFRIs-treated tissues, aggregation of K10 was not observed (Fig. 2 (d)). These results mean that interruption of EGFR signaling by EGFRIs also affects keratin expression, and further studies of other inflammatory keratins, such as K6, K16, and K17, in the lesional skin of patient under EGFRIs-induced skin toxicity are needed.

EGFR is also implicated in regulating cell–cell contacts. Expression of claudin-3 was reduced in mice deficient for Egfr keratinocytes (*EGFR*^{dEP}) epidermis, as was expression of claudin-1 in human lesional skin[26]. As we expected, we also confirmed the reduction of claudin-1, -3, and occludin expression in EGFRIs-treated tissues. Meanwhile, there were no dramatic changes of tight junction abnormal in rhEGF- and EGFRIs-treated tissues (Fig. 3). However, weak normalization of tight junction expression was

observed in rhEGF and EGFRIs co-treated tissues. Therefore, these results mean that rhEGF may indirectly affect the formation of tight junctions during normalization of the proliferation and differentiation of keratinocytes.

Our previous studies reported that topical treatment of rhEGF relieved S. aureus-induced inflammation and AD-like skin lesions in Nc/Nga mice, and rhEGF treatment attenuated P. acnes-induced inflammatory responses, at least in part, through the modulation of TLR2 signaling in human epidermal keratinocytes [17, 19]. In this study, proinflammatory cytokines, including IL-1α, IL-8, and TNF-α expression, were increased by EGFRIs, and those were down-regulated by rhEGF (Fig. 4). EGFR signaling in keratinocytes affects the expression of chemokines induced by TNF-α through the ERK1/2 signaling pathway. Activated ERK1/2 has been shown to decrease chemokine mRNA stability and decrease chemokine mRNA expression, suggesting that EGFR-dependent ERK1/2 activity in keratinocytes also participates in homeostatic mechanisms that regulate dermatitis response [27].

The skin is not only a physical barrier protecting from infection, but is also an environmental niche hosting a plethora of commensal organisms. Combined with the physical epidermal barrier, antimicrobial peptides (AMPs) are major components of the active innate immune defense against invading microbes in the skin. Several reports revealed that the expression of some kinds of AMPs is under the control of EGFR. *Helicobacter pylori* virulence effector CagA or LPS function via EGFR signaling to either suppress or upregulate the expression of hBD3, respectively [28, 29]. Treatment of human epidermal keratinocytes with erlotinib reduced the expression of hBD3, RNase 7, and CAMP. Similarly, in *egfr*-deficient keratinocytes, it was observed that expression of murin β-defensin 14, the mouse homolog of hBD3, was reduced [30]. In this study, we confirmed that mRNA expression of AMPs, including hBD-2, -3, LL37, and RNase7, was inhibited by EGFRI treatment (Figs. 5 (a)–(d)). Moreover, mRNA expression of AMPs was increased in rhEGF and EGFRI co-treated cells (Figs. 5 (a)–(d)). However, only hBD-2 and − 3 protein expressions were affected by EGFRIs and rhEGF (Figs. 5 (e) and (f)). These results mean that rhEGF may be attributed to improve the innate immunity of the epidermis via inducing the expression of not cathelicidins, but defensins, especially hBD-2 and − 3, through EGFR signaling.

Clinical trial results confirmed that EGFRIs decreased the proliferation and differentiation of keratinocytes. Also, EGFRIs induced inflammatory reactions in skin. As expected, treatment of EGF ointment induced the re-epithelization and proliferation of keratinocytes, and reduced infiltrated inflammatory cytokines in dermis. These results are remarkably similar to the in vitro and ex vivo results in this study. Nevertheless, further studies are needed, because in this study, the expression of tight junction and AMPs in lesional skin was not confirmed.

5. Conclusions

EGFRIs: 1) inhibit the proliferation and differentiation of keratinocytes, 2) cause eruption of the keratinocytes differentiation process, 3) cause tight junction protein expression abnormalities that play an important role in physical barriers, 4) induce inflammatory factors and play an important role in the

innate immunity of the epidermis, inducing the inhibition of AMPs expression. EGFRIs cause epidermal physical and immunological barrier abnormalities, resulting in complex skin side effects, such as xerosis and rash. In this study, we revealed that treatment of rhEGF in human epidermal keratinocytes, 3d-cultured human skin tissue, and patient lesions improved EGFRIs-induced skin eruption via normalizing the proliferation and differentiation of keratinocytes, reducing inflammatory cytokines expression, and inducing the expressions of hBD-2 and hBD-3. These findings may therefore support the effect of topical rhEGF treatment on the improvement of EGFRI-derived skin side effects.

List Of Abbreviations

AKT Protein kinase B

AMPs antimicrobial peptides

EGF epidermal growth factor

EGFR epidermal growth factor receptor

EGFRIs EGFR inhibitors

H&E hematoxylin-eosin

IF Immunofluorescenc

IHC immunohistochemical

IL interleukin

LPS lipopolysaccharide

mRNA messenger ribonucleic acid

pAKT phosphorylated AKT

PBS phosphate-buffered saline

pEGFR phosphorylated EGFR

PI3K phosphoinositide 3-kinase

pPI3K phosphorylated PI3K

QoL quality of life

rhEGF recombinant human EGF

RT room temperature

TKI kinase inhibitors

TNF-α tumor necrosis factor-alpha

Declarations

Ethics approval and consent to participate

We performed the human investigations after approval by Dong-A University Hospital institutional review board (DAUHIRB-19-003). Patients' tissues form prior phase III trial registered to ClinicalTrials.gov (NCT02284139).

Consent for publication

Each of the listed authors have agreed and approved this manuscript for submission.

Availability of data and materials

Please request to corresponding author OSY.

Competing interests

Ji Min Kim, So Yun Ahn and Jung Eun Choo are employee of Daewoong Pharmaceutical Company.

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Authors' contributions

SYO, JEC and MSR conceptualized and designed the study, coordinated, and supervised the data collection. JMK, JHJ and SYO analyzed the data, drafted, and revised the manuscript. SYO and M.K.P. conducted pathological review and data analysis. JMK, SYA, and JEC evaluated mechanism of ERSE with human epidermal keratinocytes and 3D-cultured human skin tissue. KHS provided normal skin tissue. YSK, SJH, SL, CHS, and JHK contributed to execute the patient practice and provided ERSE skin tissue. All authors agreed to be responsible for all aspects of the study. All authors have read and approved the final manuscript.

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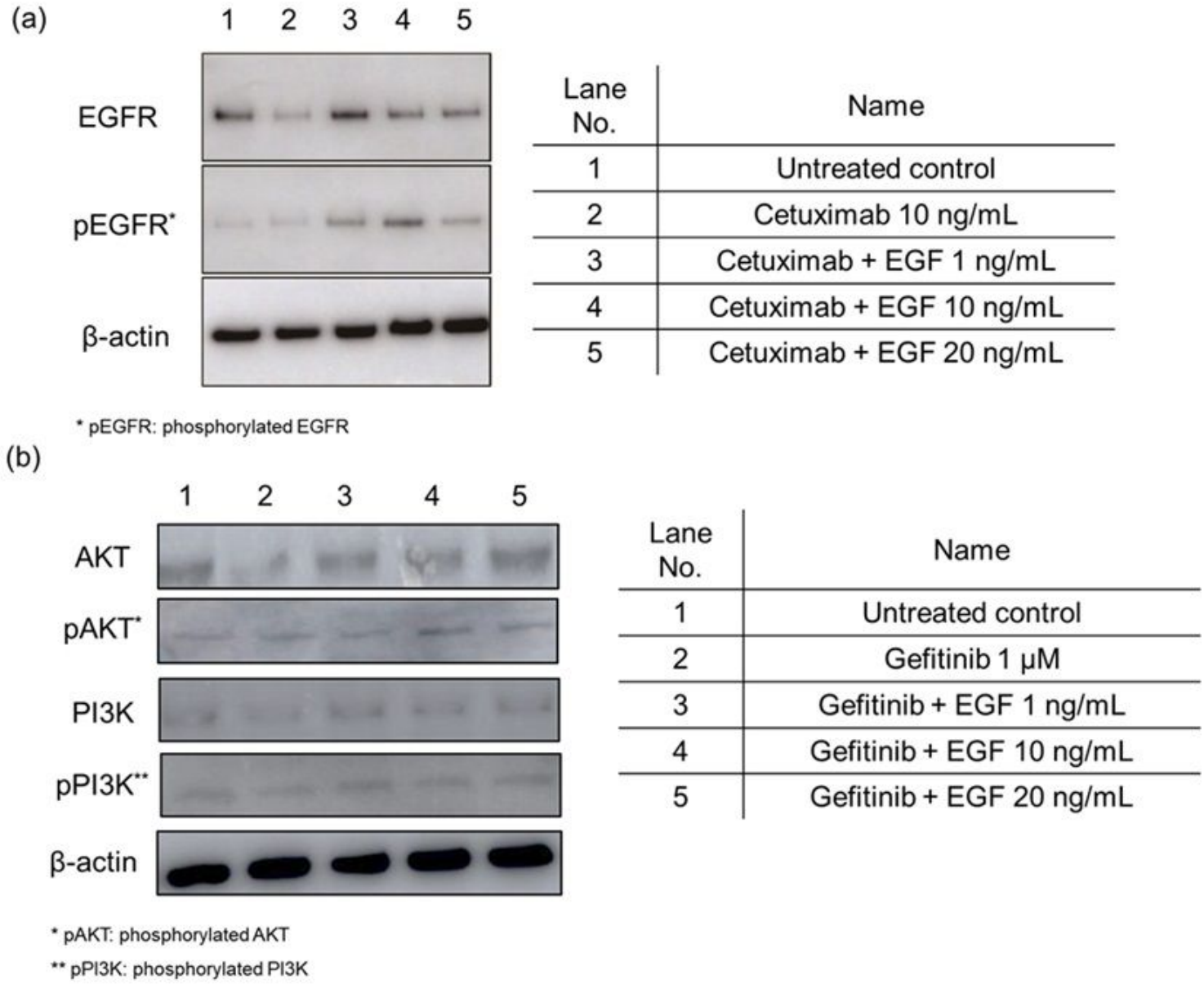
Figures



Lane No.	Name
1	Untreated control
2	Cetuximab 10 ng/mL
3	Cetuximab + EGF 1 ng/mL
4	Cetuximab + EGF 10 ng/mL
5	Cetuximab + EGF 20 ng/mL

Lane No.	Name
1	Untreated control
2	Gefitinib 1 μM
3	Gefitinib + EGF 1 ng/mL
4	Gefitinib + EGF 10 ng/mL
5	Gefitinib + EGF 20 ng/mL

Figure 1

rhEGF increased EGFR and EGFR signaling interrupted by EGFR inhibitors. Expression of EGFR of human epidermal keratinocytes was decreased by cetuximab treatment. However, EGFR and phosphorylated EGFR expression was induced by rhEGF treatment (a). Similarly, in gefitinib treated keratinocytes, it was observed that phosphorylated AKT and PI3K was slightly decreased. On the other hands, EGFR and gefitnib co-treatment induced AKT and PI3K phosphorylation (b).

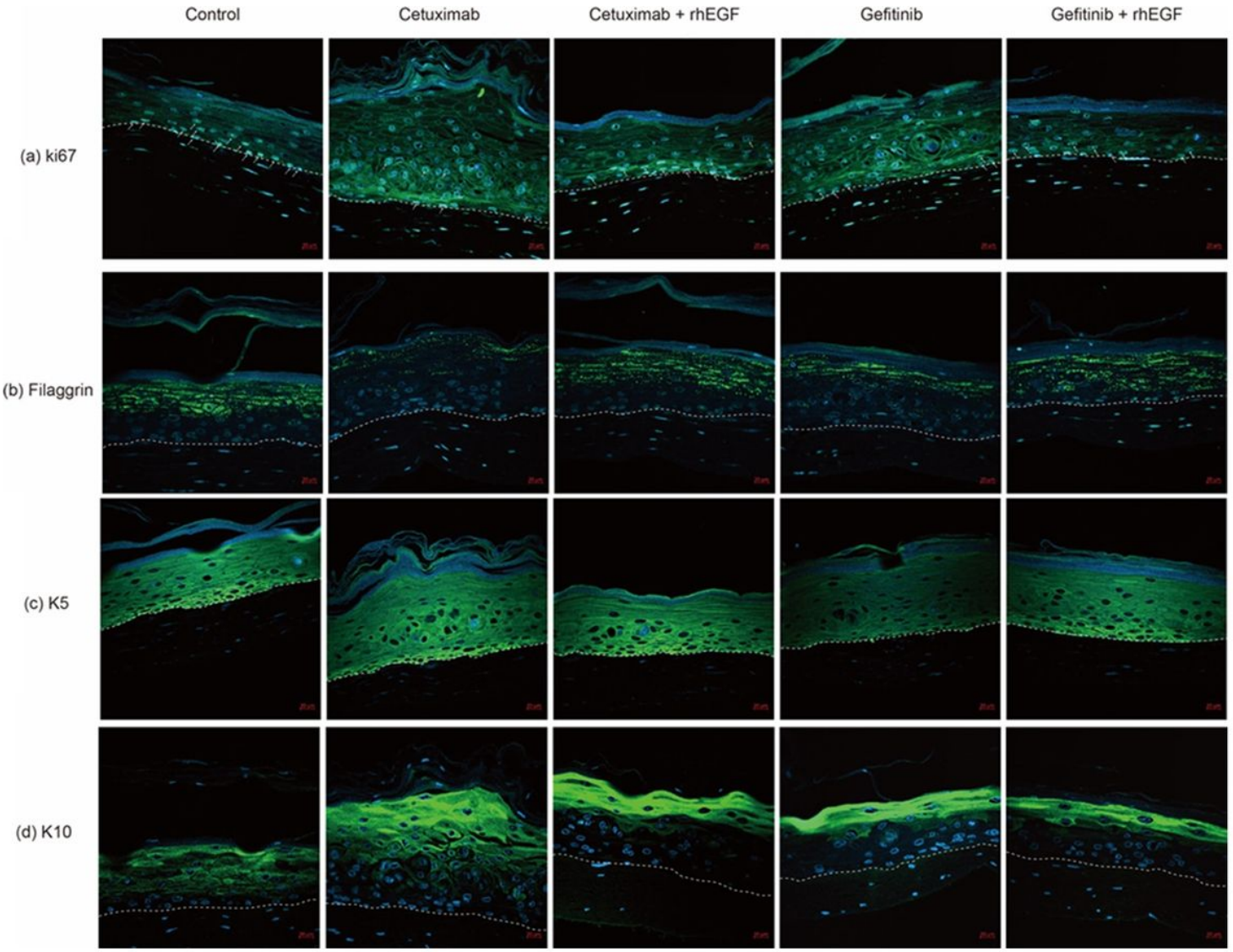


Figure 2

rhEGF normalized proliferation and differentiation of epidermis erupted EGFRIs. Expression of ki67 in the nucleus of keratinocytes in the epidermal basal, para-basal cell layer (a). Expression of filaggrin in upper epidermis (b). K5 expression was seen in the epidermal basal layer (c). K10 expression was observed in upper epidermis especially stratum corneum (d).

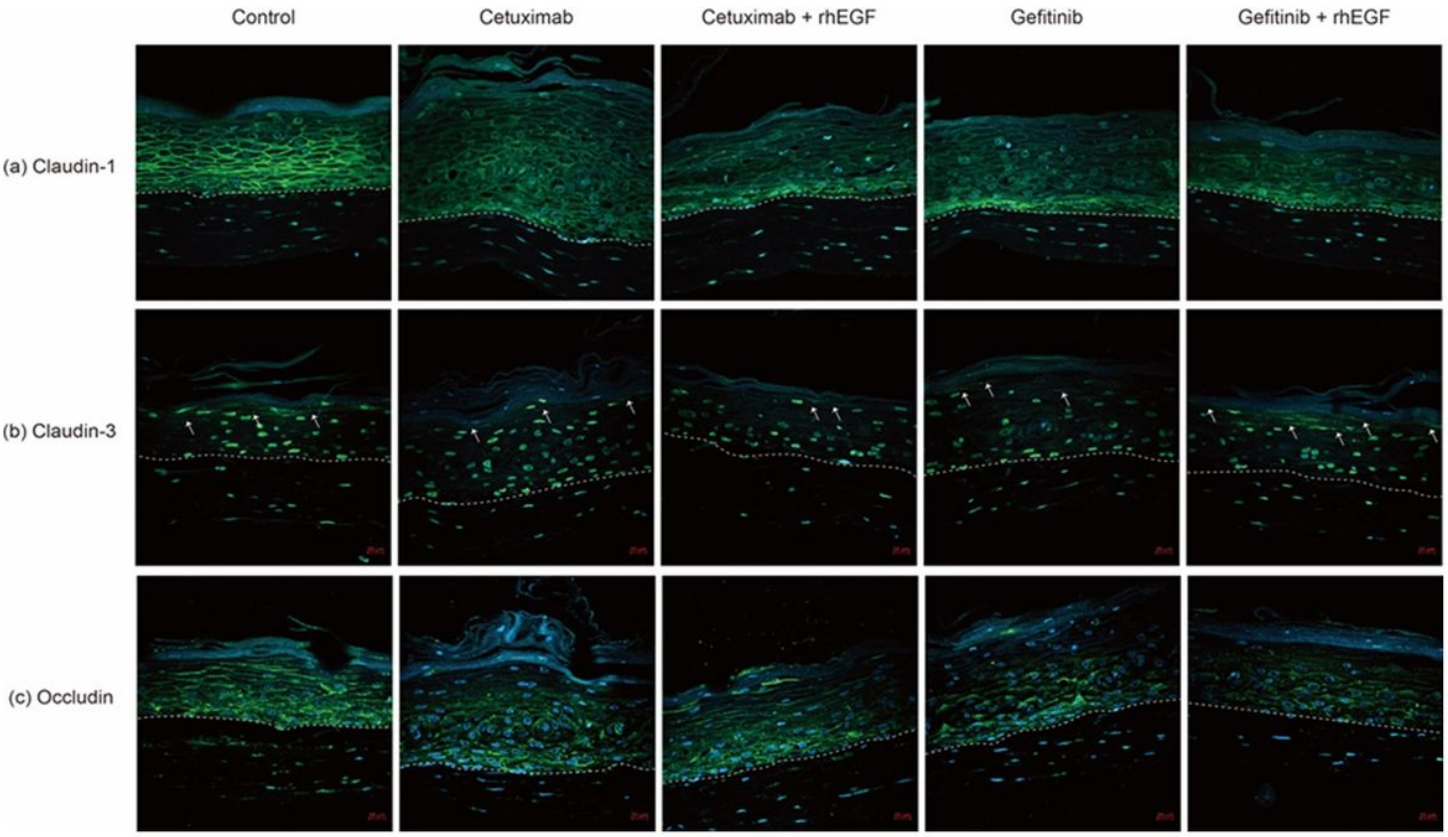


Figure 3

Tight junction expression was erupted by EGFRIs and that was weekly normalized by rhEGF. Claudin-1 expression was seen in epidermal layer especially suprabasal layer (a). Expression of claudin-3 in stratum corneum and stratum granulosum junction (b). Expression of occluding was observed in all epidermal layer (c).

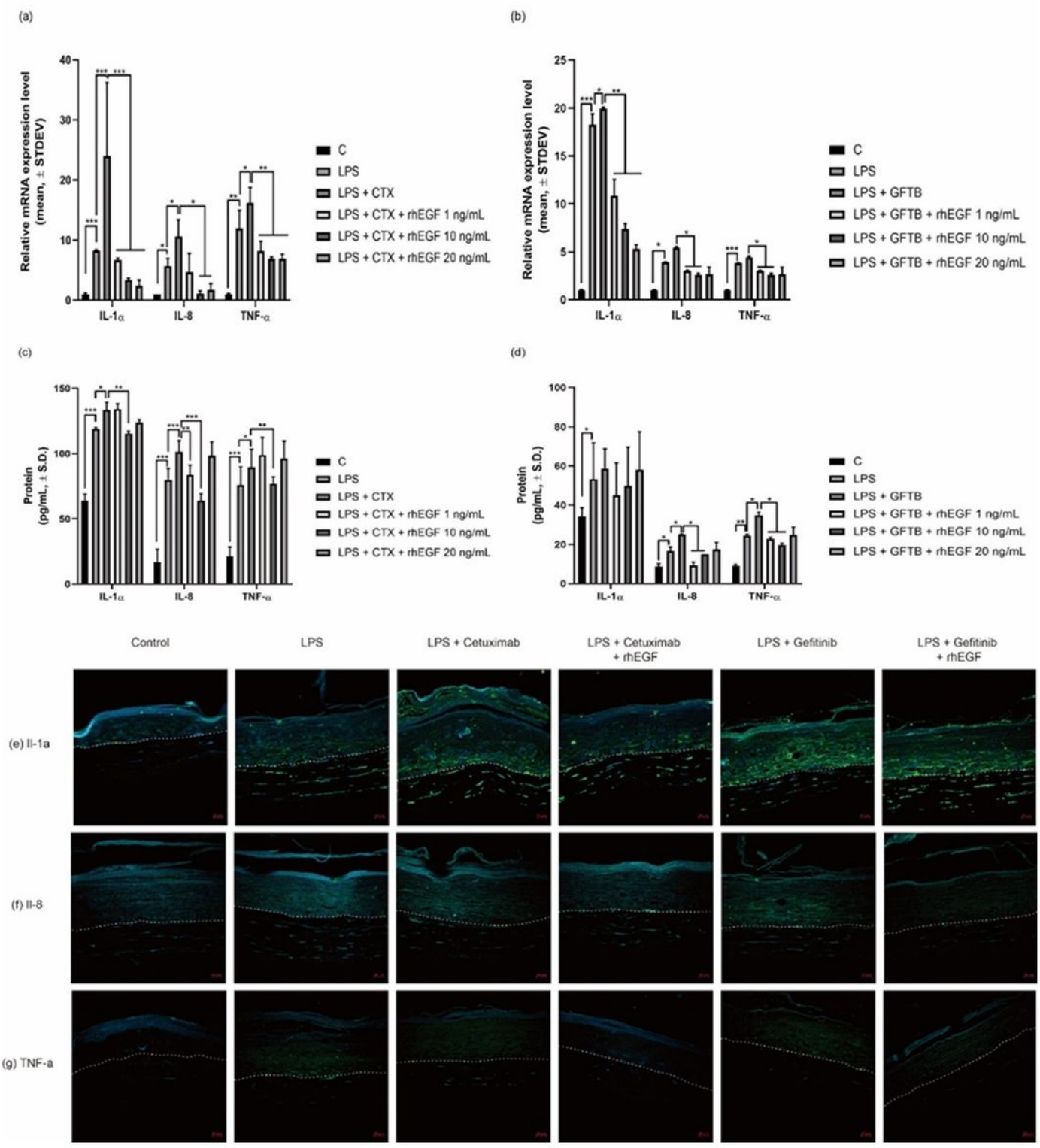


Figure 4

EGFRIs induced inflammatory cytokine expression was inhibited by rhEGF treatment. IL-1α, IL-8 and TNF-α mRNA expression was increased in keratinocytes treated with cetuximab or gefitinib (a, b). However, that increment was decreased by rhEGF and cetuximab (a) or gefitinib (b) co-treatment. Protein expression of IL-1α, IL-8, and TNF-α also induced in cetuximab (c) or gefitinib (d) treated keratinocytes (c, d) and that was inhibited by rhEGF and EGFRIs co-treatment (c, d). IL-1α expression was observed in

epidermal layer (e). Expression was IL-8 was slightly observed in epidermis (f). TNF-α expression was seen in epidermal layer (g). Results are presented as mean ± standard deviation (S.D.), representative of three separated experiments. Asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01, ***P < 0.001)

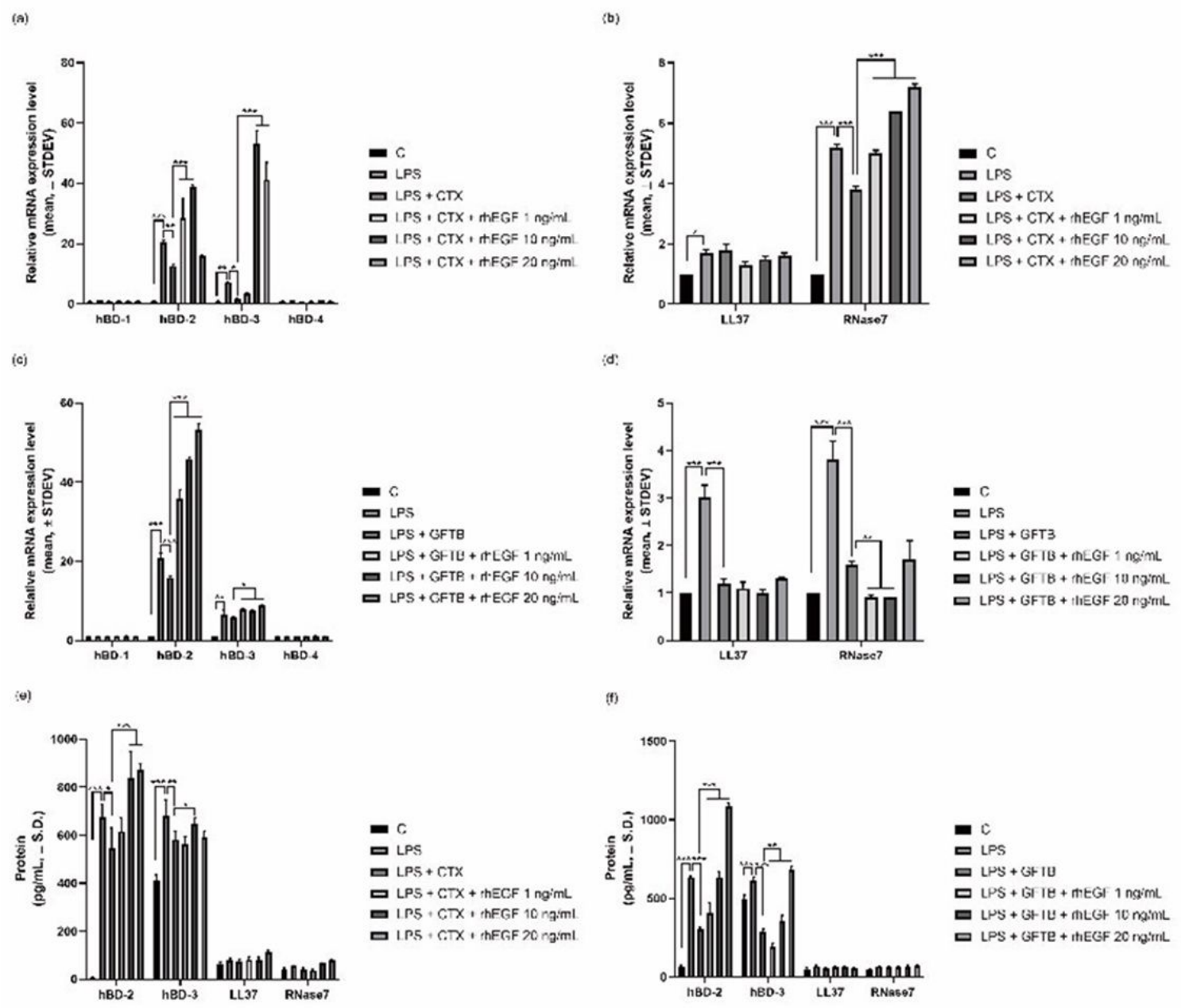


Figure 5

EGFRIs-inhibited AMPs expression was increased by rhEGF treatment. LPS increased mRNA and protein expression of antimicrobial peptides including hBD-2, -3, LL37, and RNase 7 in human epidermal keratinocytes (a, b, c, d, e, f). It was observed that mRNA and protein expression of hBD-2 and -3 was decreased in LPS and EGFRIs treated cells. Also, rhEGF increased hBD-2 and -3 mRNA and protein expression respectably (a, c, e, f). However, in rhEGF and cetuximab treated cells, rhEGF only increased RNase 7 but not LL37 mRNA expression (b). Unlikely results of mRNA expression, rhEGF did not affect

protein expression of not only LL37 but also RNase 7 (e). Similarly, in rhEGF and gefitinib treated cells, rhEGF affected only RNase 7 mRNA expression (d) but not affected protein expression of both LL37 and RNase 7 (f). Results are presented as mean ± standard deviation (S.D.), representative of three separated experiments. Asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01, ***P < 0.001)

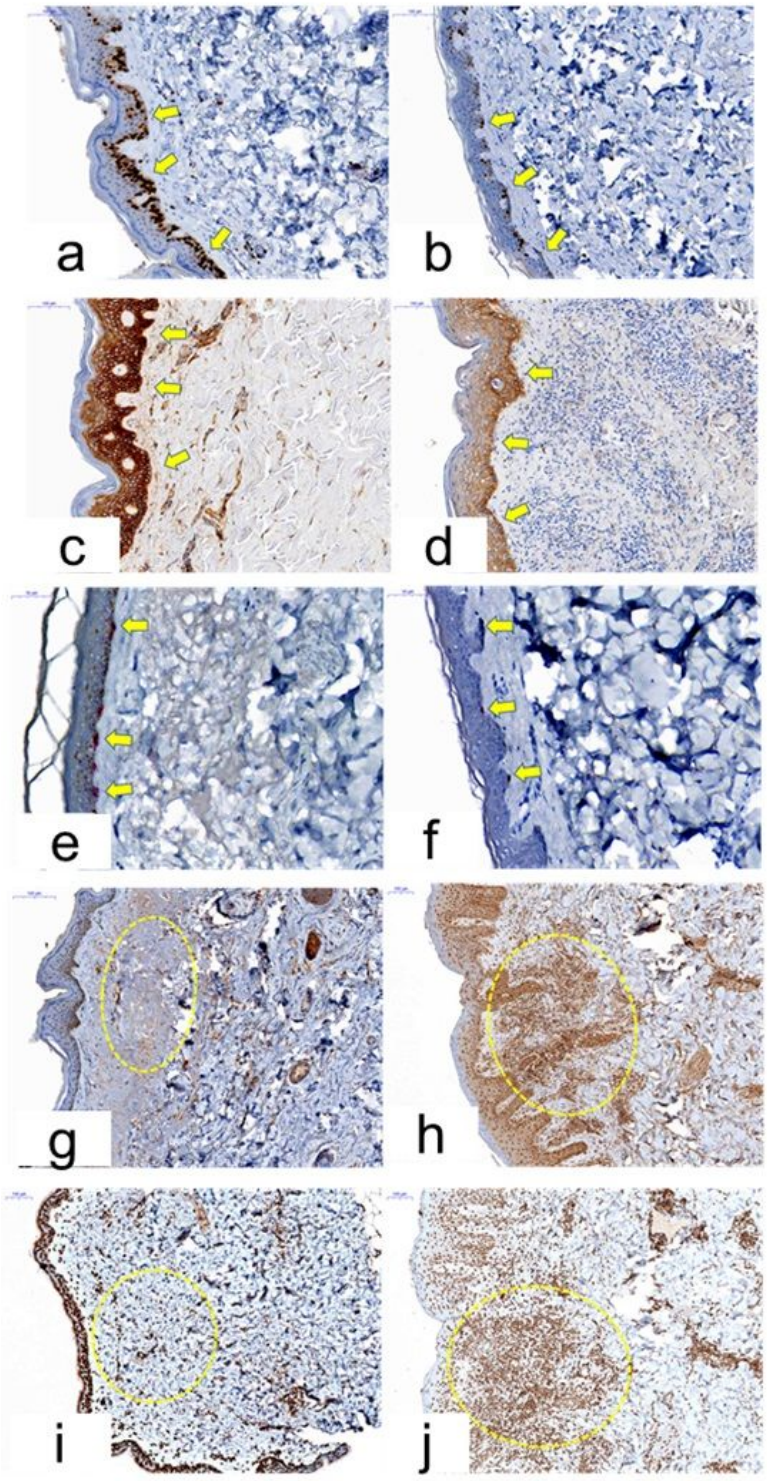


Figure 6

Pathologic comparison between normal control and patient who had epidermal growth factor receptor inhibitor related side effect (ERSE). (x 200) Expression of Ki-67 in the nucleus of keratinocytes in the epidermal basal, para-basal cell layer (a: control, b: ERSE). Expression of EGFR in the membrane of keratinocytes in the epidermal basal cell layer (c: control, d: ERSE). Cytoplasmic Melan-A expression was seen in the basal melanocytes of the epidermis (e: control, f: ERSE). Cytoplasmic IL-17 expression in dermis (g: control, h: ERSE) and Nuclear with or without cytoplasmic TNF-α expression in dermis and epidermis (i: control, j: ERSE)

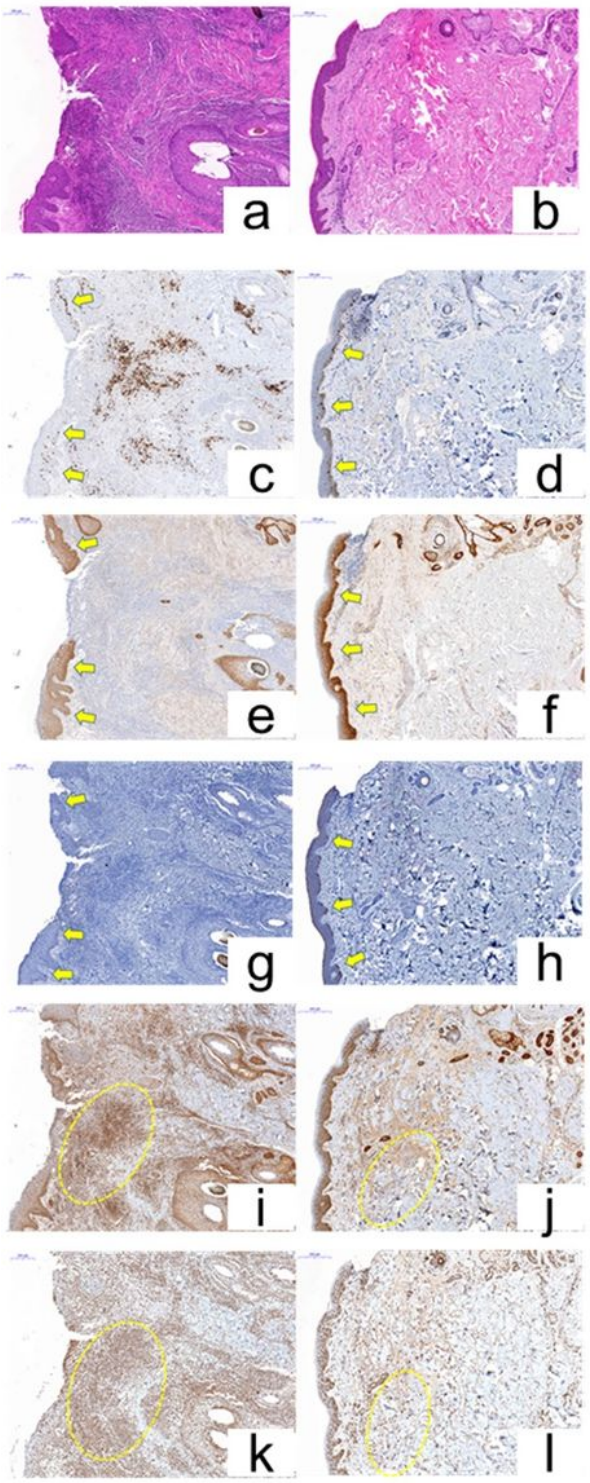


Figure 7

ERSE skin pathologic change pre- and post EGF ointment treatment. (x 200) H&E stain (a: pre-, b: post EGF treatment). Expression of Ki-67 in the nucleus of keratinocytes in the epidermal basal, para-basal cell layer (c: pre-, d: post EGF treatment). Expression of EGFR in the membrane of keratinocytes in the epidermal basal cell layer (e: pre-, f: post EGF treatment). Cytoplasmic Melan-A expression was seen in the basal melanocytes of the epidermis (g: pre-, h: post EGF treatment). Cytoplasmic IL-17 expression in dermis (i: pre-, j: post EGF treatment) and Nuclear with or without cytoplasmic TNF-α expression in dermis and epidermis (k: pre-, l: post EGF treatment)

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