

Enhanced Expression of Kallikrein-related Peptidase 7 in Lichen Planus, Lichen Sclerosis, Porokeratosis and Psoriasis Vulgaris

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ABSTRACT

AIM: Kallikrein-related peptidase KLK7 is implicated in desquamation and homeostasis of epidermis. In this study, we aim to determine KLK7 protein expression in four different skin diseases

with abnormal keratinization including lichen planus, lichen sclerosis, porokeratosis and psoriasis vulgaris. We also investigate whether skin diseases –related inflammatory cytokines and transcription factor ZNF750 regulates KLK7 gene expression in normal human epidermal keratinocytes (NHEK), and whether ZNF750 protein levels in psoriasis are correlated with KLK7 protein levels.

MATERIALS AND METHODS: Human skin biopsies were obtained from normal subjects and patients with lichen planus, porokeratosis, lichen sclerosis and psoriasis vulgaris. Immunohistochemistry staining, NHEK cell culture, Ca₂Cl-induced differentiation, small siRNA interfering technology, real-time PCR and western-blot assays were performed in this study.

RESULTS: KLK7 protein levels are significantly enhanced in skin lesions of lichen planus, porokeratosis, lichen sclerosis and psoriasis vulgaris as compared to normal skin epidermis. Addition of a single cytokine of TNF α , IFN γ , IL1 α , IL1 β , IL17A don't affect KLK7 protein expression, but a combination of these cytokines reduces both KLK7 mRNA and protein levels in differentiated NHEK. KLK7 is significantly reduced in ZNF750 silenced differentiated NHEK cells. ZNF750 protein expression levels in psoriasis vulgaris lesions are not correlated with enhanced KLK7 expression levels.

CONCLUSION: Enhanced protein expression of KLK7 is a common characteristics for both hyperkeratosis and parakeratosis disorders. The enhanced KLK7 expression is not associated with increased inflammatory cytokines and ZNF750.

Key words: KLK7; ZNF750; Lichen planus; Lichen sclerosis; Porokeratosis; Psoriasis vulgaris

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List of Abbreviations

KLK: Kallikrein-related peptidase; LEKTI: Lympho-epithelial

Kazal-type related inhibitor; NS: Netherton syndrome; NHEK: normal human epidermal keratinocytes; UD: undifferentiated NHEK; D: Differentiated NHEK; Loricrin: LOR.

INTRODUCTION

The human tissue Kallikrein-related peptidase (KLK) gene family consists 15 secreted serine proteases (KLK1-15). KLK7 along with KLK3 and KLK9 are chymotrypsin-like enzymes, whereas other KLKs are trypsin-like enzymes^[1]. To date, eight different KLKs have been detected in the stratum corneum (SC) of human epidermis by enzyme-linked immunosorbent assay (ELISA)^[2]. These KLKs are often expressed in the highly differentiated cells including SC and stratum granulosum and skin appendages^[3,4]. Among these KLKs, KLK7 has been well studied for its biological functions. KLK7 is secreted into the intercellular space via lamellar granules^[5,6]. In vitro biochemistry studies demonstrated that KLK7 can degrade corneodesmosomal cadherins: desmoglein 1, desmocollin 1, and corneodesmosin^[7,8]. Therefore, KLK7 along with other epidermal expressed KLKs are postulated to have functions in desquamation and skin homeostasis.

The importance of KLK7 in skin homeostasis was unveiled by recent studies on Netherton syndrome (NS), a disease is characterized by ichthyosis, hair abnormalities and atopic manifestations. NS patients carry loss-of function mutations in *SPINK5*, a gene encoding the protein of Lympho-epithelial Kazal-type related inhibitor (LEKTI)^[9,10]. LEKTI is a serine protease inhibitor which is expressed in the human epidermis^[11]. KLK7 is a target of LEKTI^[12,13]. Loss-of-function mutations in *SPINK5* in Netherton syndrome led to reduced serine protease inhibition and subsequently enhanced KLK7 serine protease activities^[14]. Recently, Kasparek et al found that ablation of both KLK5 and KLK7 together could fully rescue the phenotype of NS^[15]. This study further validated that KLK7 is one of the major KLKs for skin barrier homeostasis.

Aside from Netherton syndrome, aberrant KLK levels were reported in atopic dermatitis, psoriasis and rosacea diseases^[16,17]. In atopic dermatitis, KLK7 has been reported to be the major abnormally expressed KLK, it is increased in AD lesional skin, however, the secretion of KLK7 from lamellar granules is reduced^[18,19]. This impairment may lead to abnormal proteolysis of corneodesmosomes and compact hyperkeratosis. Although psoriatic lesions display mainly as hypokeratosis, multiple KLKs were increased measured by ELISA assays^[17]. In rosacea disease, enhanced KLK5 was believed to play a role in process of cathelicidin, which may be responsible for significant increased LL37 in the skin lesions of rosacea^[20-22].

Abnormal keratinization is a common phenomenon for various skin diseases. In this study, we investigated KLK7 protein expression in four different kinds of skin diseases of lichen planus, lichen sclerosis, porokeratosis and psoriasis vulgaris. we further investigated the influence of inflammatory cytokines to KLK7 gene expression in normal human epidermal keratinocytes (NHEK), and whether transcription factor ZNF750 regulates KLK7.

MATERIALS AND METHODS

Patients and biopsies

Human skin biopsies were obtained with informed consent from normal human subjects and human subjects with a keratinization disorder at the First Affiliated Hospital of Jinan University at Guangzhou, China. This study was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University and conducted according to the Declaration of Helsinki (Approval

number# 2015-056). Subjects included 19 healthy individuals with no history of skin diseases (10 males and 9 females, mean age 35.9 ± 17.5), 20 lichen planus patients (10 males and 10 females, mean age 41.3 ± 12.7), 8 porokeratosis (7 males and 1 females, mean age 45.8 ± 28.7), 4 lichen sclerosis et atrophicus (2 males and 2 females, mean age 43.7 ± 19.3), 20 patients with psoriasis vulgaris (12 males and 8 females, 47.68 ± 18.35). None of the subjects had received topical corticosteroids or calcinerin inhibitors for at least two weeks before biopsies.

Immunohistochemically staining

Skin biopsies were routinely fixed in 10% formalin and then embedded in paraffin. Serial paraffin sections (4 μ m) were used for staining. Rabbit polyclonal anti-KLK7 (ab 96710) (used as 1:100) and anti-ZNF750 (ab 121685)(used as 1:30) antibodies were purchased from Abcam (Cambridge, MA). After sequential steps of deparaffinization, antigen retrieval, and inactivation of endogenous peroxidase with 0.3 % H₂O₂, skin sections were blocked with 5% BSA, then incubated with primary antibody at 4 C overnight. The following day, skin sections were washed by 1xPBS, incubated with secondary antibody conjugated HRP for 30 minutes, and then developed with 3, 3'-Diaminobenzidine at room temperature, counterstained with hematoxylin solution, dehydrated, clarified and mounted. Semi-quantification of KLK7 positive cells was performed using the Image-Pro PLUS program (Media Cybernetics, Inc., Shanghai, China).

NHEK cell culture and treatment

Primary human neonatal foreskin keratinocytes from 6 different donors were purchased from Thermo Fisher Scientific and maintained in EpiLife Medium containing 0.06 mM CaCl₂ and S7 supplemental reagent under standard tissue culture conditions. Human recombinant TNF α , IFN γ , IL1 α , IL1 β , and IL17A were purchased from R&D Systems. The cells were seeded in 24 well-dishes at 2×10^5 /well to form a confluent monolayer. The following day, the cells were subjected to differentiation by increasing CaCl₂ to 1.3mM in the culture media with or without different human recombinant cytokines including TNF α , IFN γ , IL1 α , IL1 β and IL17A at 25ng/ml, as well as a combination of these 5 cytokines. The cells were harvested for protein extraction before differentiation and differentiated for 5 days.

Total RNA extraction and real-time PCR

Total RNA was extracted using RNeasy mini kit according to the manufacture's guideline (QIAGEN, MD). RNA was then reverse transcribed into cDNA using superScript[®] III reverse transcriptase from Invitrogen (Portland, OR) and analyzed by real time RT-PCR using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). Primers and probes for human *ZNF750* (Hs01563263_g1), *LOR* (Hs01894962_s1), *KLK7* (Hs00192503_m1) and *18s* (Hs99999901_S1) were purchased from Applied Biosystems (Foster City, CA). Quantities of all target genes in test samples were normalized to the corresponding 18S levels.

Western-blot protein expression

Cells were lysed in protein lysis buffer (20mM Tris-HCl pH7.4, 150 mM NaCl, 1mM EDTA, 1% Triton-X100) supplemented with protease inhibitors. The proteins were then used for western-blot assay following the standard procedure. The antibodies against human KLK7 were purchased from Abcam (Cambridge, MA). The mouse monoclonal antibody against human β -actin was purchased from Sigma Aldrich (St. Louis, MO).

siRNA knockdown gene expression

ZNF750 siRNA duplexes and control non-targeting scrambled siRNA duplexes were purchased from Life Technologies. The sequence for ZNF750 siRNA are: sense: CCUCA AUGUUGUGAACGGAtt; antisense: UCCGUUCACAACAUUGAGGct. KCs were plated in 24 well plates at 1×10^5 per well the day before transfection. Cells were transfected with siRNA duplexes at final concentration of 10 nM using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After 24 hours incubation, the cells were replaced with EpiLife supplemented either with 0.06mM or 1.3 mM calcium for up to 120 hours. Then the cells were used for total RNA and protein extraction.

Statistical analysis

The statistical analysis was conducted using Graph Pad prism, version 5.03 (San Diego, CA). Comparisons of expression levels were performed using ANOVA techniques and independent sample *t* tests as appropriate. Differences were considered significant at $p < 0.05$.

RESULTS

KLK7 protein expression in normal epidermis and skin diseases.

KLK7 protein positive staining signals are only shown in the out-most layers of granular layer in the normal human epidermis, usually

only 1 or 2 layers of granular cells and stratum corneum have pronounced KLK7 positive staining signals. The rest of epidermis is negative (Figure 1a).

The lichen planus skin lesions showed two different types of KLK7 positive staining signals. One group ($n=8$ subjects) has slightly increased numbers of strong positive stained cell layers varied from 2 to 4 layers cross the entire lesion area of the subjects; we designated this group as type 1 lichen planus (Figure 1b); Another group ($n = 12$ subjects) have significantly increased cell layers of KLK7 positive signals, the numbers of layers with strong positive signals increased from 5 layers up to 17 layers (Figure 1 c). We designated this group as type 2 lichen planus. Compared to the type 1 lichen planus, the type 2 lichen planus patients seem have much more pronounced hyperkeratosis. The infiltrated lymphocytes are KLK7 negative (Figure 1 b and c).

KLK7 protein positive staining signals are also significantly enhanced in porokeratosis and lichen sclerosis skin lesions, displayed as increased positive stained cell layers as compared to the normal human skins (Figure 1d and e). In porokeratosis lesions, KLK7 shows strong intensity of positive signals in the epidermis around the cornoid lamella (Figure 1d). Beneath of the hyperkeratotic lesions of lichen sclerosis, KLK7 protein expression is also significantly enhanced (Figure 1e). The staining intensity of KLK7 is significantly increased in type 2 lichen planus, porokeratosis and lichen sclerosis skin lesions (Figure 1f).

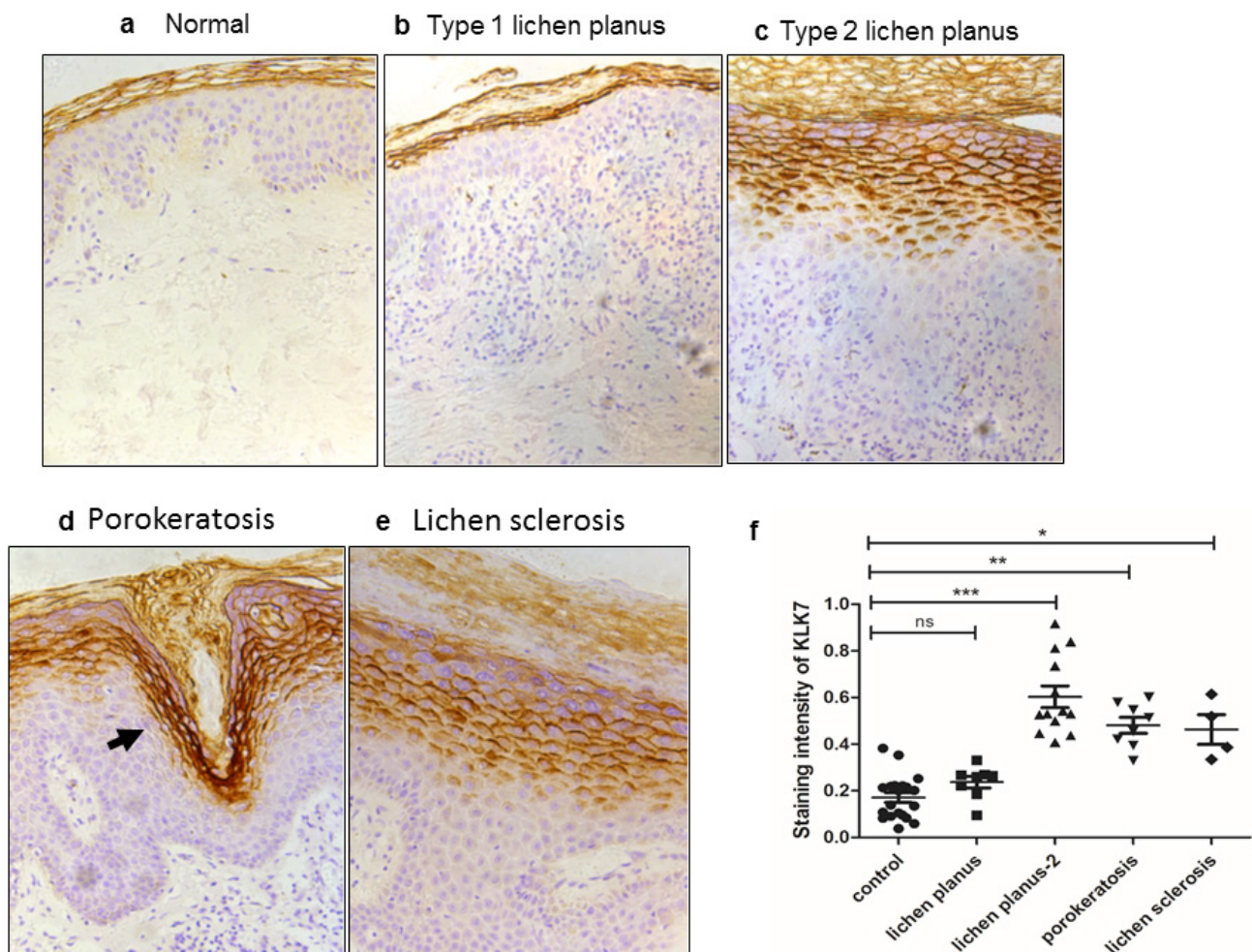


Figure 1. Increased KLK7 protein expression in the skin lesions of lichen planus, porokeratosis and lichen sclerosis. Typical images of immunohistochemistry staining of KLK7 in normal epidermis (a), lichen planus (b) & (c), porokeratosis (d) and lichen sclerosis (e). The Arrow points to the cornoid lamella of porokeratosis. (f) Semi-quantitative analyses results of immunohistochemistry staining of KLK7 positive signals. Pictures' magnification x 200. ns indicates not significant; * indicates $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

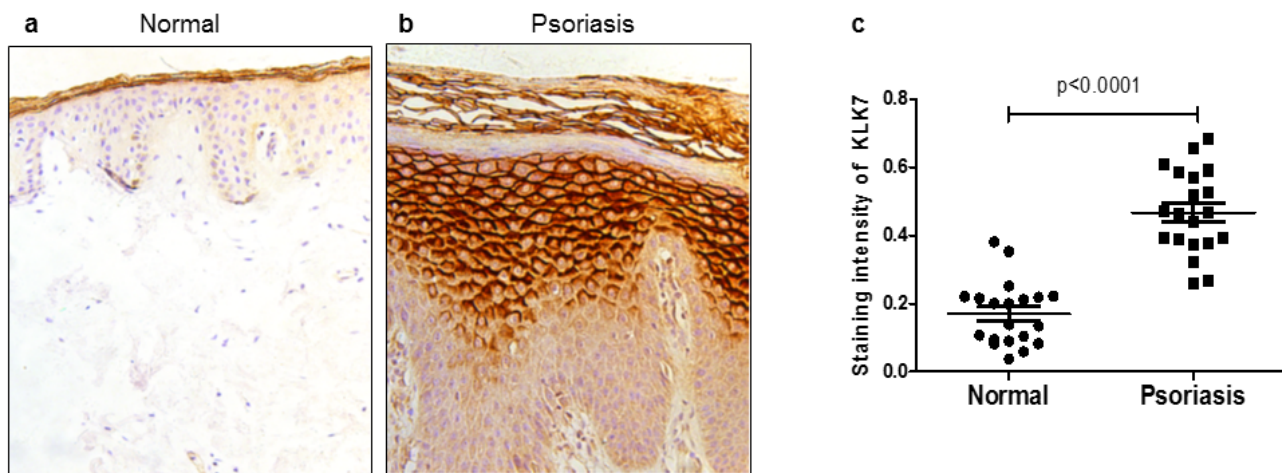


Figure 2 Increased KLK7 protein expression in skin lesions of psoriasis vulgaris. Typical images of immunohistochemistry staining of KLK7 in normal epidermis (a) and psoriasis lesions (b). (c) Semi-quantitative analyses results of immunohistochemistry staining of KLK7 positive signals. Pictures' magnification x 200.

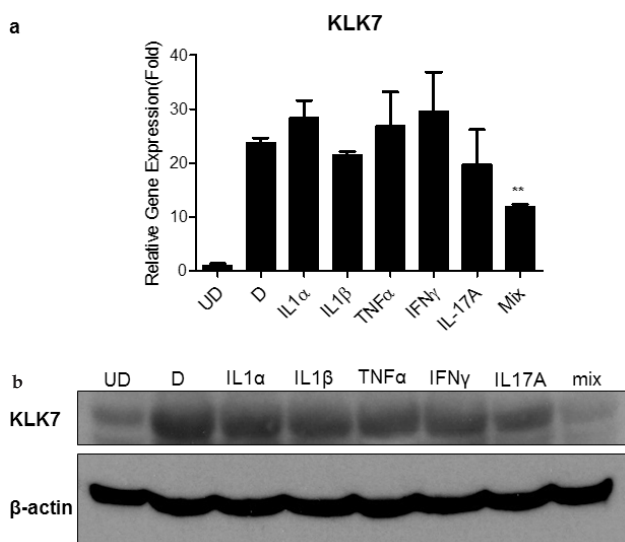


Figure 3 The diseases-associated cytokines do not up-regulate KLK7 expression in differentiated NHEK. (a) KLK7 mRNA expression levels were evaluated by real-time PCR. (b) KLK7 protein expression levels were evaluated by western blot. Mix: a combination 25 ng/mL of the 5 cytokines were added to the differentiating NHEK. UD: undifferentiated NHEK; D: differentiated NHEK.

KLK7 protein is also significantly enhanced in overall psoriasis vulgaris skin lesions including the epidermal cells underneath the parakeratosis regions (Figure 2). These data together with the data from other skin diseases tested in this study suggest that KLK7 enhancement is a common characteristic for both hyperkeratosis and parakeratosis.

Inflammatory cytokines' effects on the gene expression of KLK7
Lichen planus, lichen sclerosis and psoriasis are associated with increased lymphocytes infiltration and increased inflammatory cytokines in skin lesions. Increased IFN γ , TNF α and IL1 α were reported in these skin lesions^[23-25]. Recently, IL17A was indicated in psoriasis and lichen planus^[26,27]. We therefore further investigated whether these inflammatory cytokines could affect KLK7 gene expression in normal human epidermal keratinocytes (NHEK). NHEK cells were differentiated by addition of 1.3 Mm CaCl₂ in culture medium. The cells were then differentiated in the presense and absence of IFN γ (25 ng/mL), TNF α (25 ng/mL), IL1 α (25 ng/mL),

IL1 β (25 ng/mL) and IL17A(25 ng/mL) for 5 days. KLK7 mRNA levels and protein levels were evaluated by real-time PCR and western blot assays, respectively. As shown in Figure 3a and b, KLK7 are significantly increased in differentiated NHEK cells as compared to undifferentiated cells. However, KLK7 mRNA and proteins are not significantly altered by addition of a single inflammatory cytokine, but reduced significantly under treatment of the 5 combined cytokines.

KLK7 is regulated by epidermal transcription factor ZNF750, but enhanced KLK7 in psoriasis skin lesions are not correlated with ZNF750 protein levels.

ZNF750 promotes epidermal keratinocyte differentiation^[28]. Furthermore, ZNF750 genetic variants in its gene promoter were reported to be associated with increased risk of psoriasis^[29-31]. We then investigated whether ZNF750 regulates KLK7 gene expression, and tested whether ZNF750 protein expression levels in psoriasis skin lesions are correlated with increased KLK7 expression. As shown in Figure 4a and b, silencing ZNF750 in NHEK led to reduced KLK7 mRNA and protein expression as compared to cells transfected with scrambled siRNA in differentiated NHEK, suggesting that ZNF750 indeed transcriptionally regulates KLK7 gene expression during epidermal keratinocyte differentiation. Loricrin as a skin terminal differentiation marker, its gene expression was also reduced in ZNF750 silenced cells (Figure 4 a and b). We examined ZNF750 protein expression in skin lesion biopsies from 20 patients with psoriasis vulgaris as compared to normal controls. In normal epidermis, ZNF750 protein starts to express in spinous layer, gradually enhanced to the most intensity in the most upper layer of granulosum (Figure 4c). Semiquantification analyses of ZNF750 protein intensity didn't show significant difference between normal skin tissues and psoriasis vulgaris lesions (Figure 4g).

DISCUSSION

In this study, we carefully investigated protein levels and distribution of KLK7 in 4 different types of skin diseases with either hyperkeratosis or parakeratosis conditions. Lichen planus and lichen sclerosis often have hyperkeratosis, while psoriasis vulgaris and parakeratosis often have parakeratosis. Our result demonstrated that both hyperkeratosis and parakeratosis skin lesions have enhanced KLK7 proteins expression, suggesting that enhanced KLK7 gene expression is a common feature for skin lesions with a disturbed desquamation process.

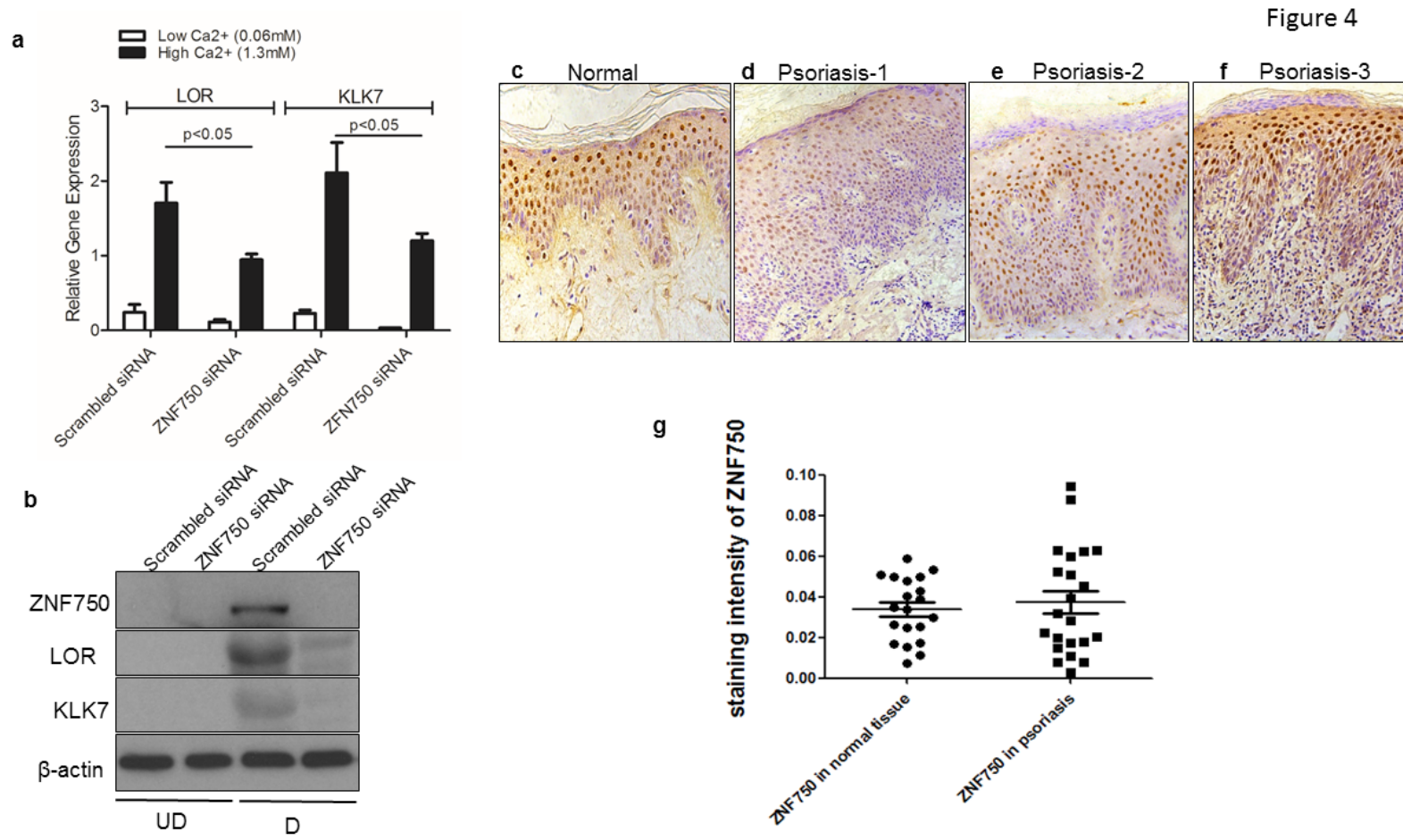


Figure 4 ZNF750 regulates KLK7 gene expression and its expression in psoriasis lesions is not correlated with KLK7 levels. (a) KLK7 mRNA is reduced in ZNF750-silenced differentiated NHEK cells evaluated by real-time PCR. (b) KLK7 proteins is reduced in ZNF750-silenced differentiated NHEK cells evaluated by western blot. (c)(d)(e)(f) Typical image of immunohistochemistry staining of ZNF750 in normal epidermis and psoriasis lesions. (g) Semi-quantitative analyses results. Pictures' magnification x 200.

Previous studies have found that excessive KLK5 and KLK7 in epidermis could initiate inflammatory responses through PAR2 activation and TSLP production^[32,33], which may explain the type 2 inflammation characterized in atopic dermatitis and Netherton syndrome, as TSLP is an epithelia-derived Th2 promoting cytokine^[34]. However, psoriasis, lichen planus and lichen sclerosis are all related to Th1- and Th17- associated inflammation, whether and how KLK7 are involved in Th1- and Th17- inflammation will need further investigation.

Although a few studies have reported that KLKs are increased in psoriasis lesions^[2,17], the mechanism underlying this phenomenon has not been investigated. In this study, we explored the hypothesis whether psoriasis-associated inflammatory cytokines would up-regulate KLK7 in NHEK cells *in vitro*. The panel of cytokines we tested, including IFN γ , TNF α , IL1 α , IL1 β and IL17A are not only associated with psoriasis, but also associated with lichen planus and lichen sclerosis. Our results demonstrated none of these cytokines could significantly enhance the gene expression KLK7 in differentiated NHEK as compared to cells without cytokines treatment (Figure 3). These results suggest that increased KLK7 in skin lesions is not caused by increased inflammatory cytokines. Our results demonstrated that KLK7 enhancement is common in Th1 – associated chronic skin diseases such as psoriasis vulgaris, lichen planus and lichen sclerosis. In combination with the findings from Th2 – associated skin diseases of atopic dermatitis and Netherton syndrome, it seems that KLK7 expression enhancement is a common characteristic for all chronic inflammatory skin diseases.

The fact that KLK7 is significantly increased in the terminally differentiating keratinocytes of granular layer in normal human epidermis, implicates that KLK7 gene expression is under the regulation of epidermal terminal differentiation program in normal epidermis. ZNF750 is one of the transcription factors that promotes human epidermal terminal differentiation^[35]. Previous studies report that genetic variants in ZNF750 were associated with increased risk of psoriasis disease^[29-31]. A few of promoter variants are functionally confirmed to reduce promoter activity of ZNF750^[29,30], presumably lead to reduced gene expression. Indeed, we found that ZNF750 regulated KLK7 gene expression in NHEK using the siRNA silencing method (Fig 4a & 4b). However, results of immunohistochemistry staining of ZNF750 protein expression in psoriasis lesions were not correlated well with KLK7 protein levels. These data suggest that KLK7 in skin diseases with abnormal keratinization is not through the transcription factor of ZNF750.

CONCLUSION

In conclusion, our study demonstrated that increased KLK7 protein expression in skin lesions are the common feature for skin diseases with abnormal keratinization. The Type 1 and type 17 inflammatory cytokines do not up-regulate KLK7 gene expression. ZNF750 does regulate KLK7 gene expression, however, the increased KLK7 protein levels in psoriasis is not correlated with protein levels of ZNF750, demonstrating that dysregulation of KLK7 in skin diseases is diverged from ZNF750 involved differentiation regulatory program. Our current study warrants a new avenue of research to investigate whether KLK7 is involved in general skin inflammatory diseases and whether correction of KLK7 would have therapeutic meanings to skin diseases with abnormal keratinization.

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