Research Article

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Keratin 17 is induced in prurigo nodularis lesions

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Abstract: Prurigo nodularis (PN) is a highly pruritic chronic inflammatory dermatosis with unknown pathogenesis. It is characterized by the existence of many hyperkeratotic, erosive papules and nodules, and the development of lesions may be associated with hyperproliferation and aberrant differentiation of keratinocytes. Keratin 17 (K17) is overexpressed selectively in human proliferative skin diseases, promoting keratinocyte proliferation not found in normal epidermis. In this study, we investigated the mRNA levels and protein levels of K17 in lesional and perilesional skin using quantitative real-time polymerase chain reaction and western blot. We demonstrate that K17 is induced in lesional and perilesional skin in PN. The mRNA expression level of K17 was upregulated in PN lesions ($P < 0.01$), with multifold changes in the PN lesion (normalized to glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene) showing a median positive correlation with PRUNOSI ($P < 0.05$). The protein level of K17 was also markedly increased in PN lesions ($P < 0.01$). In conclusion, K17 is highly induced in PN lesions, which may contribute to the proliferation of keratinocytes and the pathogenesis of PN.

Keywords: prurigo nodularis, skin lesion, keratinocyte, keratin

1 Introduction

Prurigo nodularis (PN) is a chronic disorder of the skin characterized by multiple, pruritic, and hyperkeratotic nodules that tend to distribute symmetrically with a predilection on the extensor surfaces of the limbs [1,2]. Recently, PN was defined as a subtype of chronic prurigo and may possibly be triggered by neuronal sensitization to itch and the development of an itch–scratch cycle [3]. Various disorders have been considered to be linked to PN (e.g., atopic predisposition, systemic diseases, infections, neurological disorders, xerosis cutis, and psychiatric comorbidities) [1,4–10]. However, its exact pathophysiology remains unclear. Most patients with PN suffer from a high itch intensity with limited existing management, resulting in a reduced quality of life [1,11].

In the epidermis, keratinocytes, resident immune cells, and innervating somatosensory nerve fibers interact in direct and indirect ways and are involved in itch [12]. There is an aberrant expression of histamine-independent pruritogenic mediators in keratinocytes, underlining the importance of keratinocytes in the pathogenesis of PN [13]. The behavior of keratinocytes in normal and diseased skin is determined mostly by its state of activation or differentiation [14]. Histopathological studies of prurigo nodularis have shown thick epidermis, irregular epidermal hyperplasia, focal parakeratosis, and hypergranulosis in the epidermis [11,15], suggesting that hyperproliferation and aberrant differentiation of keratinocytes may be involved in the formation of PN.

The keratins are the typical intermediate filament proteins of epithelia. More than half of keratin genes are expressed in mature mammalian skin tissues and are expressed in highly specific patterns related to the epithelial type, stage of cellular differentiation, and striking modulation upon wounding, infection, or disease [16–18]. In the

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homeostatic state, only basal keratinocytes expressing keratin 5 and keratin 14 (K5/K14) can regenerate and differentiate through the spinous and granular layers of the epidermis to become corneocytes; complete terminal differential keratinocytes express keratin 1 and keratin 10 (K1/K10). In response to various stressors observed in wounding, KC activation is triggered, expressing keratin 6 and keratin 16 (K6/K16) as well as keratin 17 (K17), which leads to different phenotypes, including keratinocyte hyperproliferation, migration, and cytoskeleton alteration [14,17,19,20].

K17 was originally discovered in epidermis cells as a specific cytoskeleton in basal cell skin carcinoma [19]. It has limited expression in certain healthy epithelia such as sweat glands and the outer root sheath. However, it is strongly induced in challenged keratinocytes [21]. K17 stimulates protein synthesis and epithelial cell growth by binding to the adaptor protein 14-3-3-sigma [22]. K17 can induce epidermal hyperplasia by regulating the immune response in the skin and promoting the Th1-/Th17-dominated inflammatory response, which contributes to the development of basal skin tumor [23]. In psoriasis, K17 is overexpressed in psoriatic lesional epidermis and can act as an autogenic target for autoreactive T cells in the lesional epidermis, causing epithelial proliferation [24,25]. Hence, K17 is considered to be the hallmark of the psoriasis.

In this work, to discover the pattern of keratin gene expression in PN skin lesions and to understand dysregulated keratinocytes in PN pathogenesis, we detected the K17 expression pattern in PN lesional and perilesional skin by using quantitative real-time polymerase chain reaction (qRT-PCR), Western blot and immunohistochemistry studies.

2 Materials and methods

2.1 Patient population

This retrospective study was carried out on patients with PN presenting to the Department of Dermatology at Peking University Shenzhen Hospital in China. All patients were examined and diagnosed by different dermatologists according to clinical features and histopathological results. Following a 2-week washout period without active treatment (systemic immunosuppressants, corticosteroids, topical steroids, and immunomodulators), the participating dermatologist completed the questionnaire to assess patient clinical data, including demographic data, total serum immunoglobulin E (IgE) level, verbal rating scale (VRS), visual analog scale (VAS), dermatology life quality index (DLQI), and the extent of scratch lesions via PRUNOSI [26] (PRUNOSI evaluated the type [papules, nodules, lichenification excoriations, and crusts] and the percentage of lesions in the affected body area versus the whole body). Lesional skin and perilesional skin biopsy specimens from the extensor of the upper/lower extremities of patients with PN were obtained. PN lesional skin biopsies of 3–4 mm in diameter were taken from the arm, leg, or thigh, and perilesional skin biopsies were taken near the PN lesional skin. The skin specimen was divided into two parts (Figure S1): one part was used for hematoxylin and eosin staining to confirm the diagnosis, and the other part was used for mRNA analysis or protein analysis. We obtained PN specimens from 38 individuals and normal skin specimens from nine individuals (four men and five women). The normal healthy skin volunteers had no history of skin disease, including skin pruritus or atopic dermatitis (AD). Biopsies were taken from similar anatomical locations when the patient underwent surgery for some other reason. All of the experimental protocols for this research were approved by the local Ethical Committee of the Peking University Shenzhen Hospital (Figure S2). Written informed consent was obtained from all participants. The study was conducted in accordance with the ethical standards of the institutional research committee (approval no. PKUSZHEC(R)201915).

3 Methods

3.1 RNA extraction and qRT-PCR

Specimens of PN lesional skin, perilesional skin, and healthy skin were obtained. Total RNA was extracted with TRIzol (Cat. No. T9424; Sigma-Aldrich, St. Louis, MO, USA). RNA quality was detected with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA). The RNA product was reverse-transcribed into first-strand cDNA according to the protocol performed using a transcription kit (Cat. No. R212-02, HiScript 1st Strand cDNA Synthesis Kit; Vazyme Biotech, Nanjing, China). The expression of K17 and other keratin genes was quantified using the iTaq™ Universal SYBR®Green Supermix (Cat. No. 172-5124; Bio-Rad, Hercules, CA, USA) according to the qRT-PCR protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control to normalize the gene expression data. The qRT-PCR (40 cycles of denaturation at 98°C for 15 s and annealing at 58°C for 15 s) was performed based on the settings of the Bio-Rad system (CFX96TM Real-Time System; Bio-Rad). All reactions were performed in triplicate. The results of the difference in expression were calculated using the comparative cycle threshold (Ct) method by standardizing against GAPDH

expression. Quantification of the target gene expression was performed using the $2^{-\triangle\triangle Ct}$ method, ΔCt value = target gene Ct value – GAPDH gene CT value, ΔΔCt value = experimental group ΔCt mean value – control group ΔCt mean value. Sequence-specific primers were designed by Primer3 online as follows: keratin 5, sense: 5′-GGACAACAA CCGCAACC-3′, antisense: 5′-TGCTTGGTGTTGCGGA GGT-3′; keratin 6, sense: 5′-AGGCTGAATGGCGAAGG-3′, antisense: 5′-AGGAGGTGGTGGTGTACTTGATGGT-3′; keratin 16, sense: 5′-GGTGGTGATGGGCTTCTG-3′, antisense: 5′-CGATGGTCTT GAAGTAGGGA-3′; keratin 17, sense: 5′-GGTGGGTGGTGA GATCAATGT-3′, antisense: 5′-CGCGGTTCAGTTCCTC TGTC-3′; and GAPDH, sense: 5′-GGAGTCAACGGATTTGGT CGTA-3′, antisense: 5′-GCAACAATATCCACTTTACCAGAGT TAA-3′.

3.2 Western blot and immunohistochemistry analysis

Total protein of the skin tissues was extracted according to the methods of Wang et al. (2016) using 6.5 M urea buffer with the addition of protease and phosphatase inhibitors (Cat. No. 04693132001; Roche Applied Science, Germany). Protein concentration was measured using the Pierce™ BCA protein assay kit (Cat. No. 23225; Thermo Fisher, Waltham, MA, USA). Protein (10–20 μg) was separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked in 5% bovine serum albumin for 45 min, followed by incubation in specific antibodies against K17 (Cat. No. 12509; Cell Signaling Technology, Beverly, MA, USA) and GAPDH (Cat. No. 2118; Cell Signaling Technology) for 2 h at room temperature or 4°C overnight. Consequently, incubation with anti-mouse IgG antibody conjugated with horseradish peroxidase (1:2,000; Cat. No. A9044; Sigma-Aldrich; secondary antibody) was performed at room temperature for 2 h. Protein bands were detected using the SuperSignal West Pico (Plus) Chemiluminescent Substrate (Cat. No. 34580; Thermo Fisher, MA, USA). The protein level of K17 was normalized against GAPDH. Immunohistochemical staining was conducted as previously described [13] with K17 rabbit polyclonal antibody (Cat. No. 103765; GeneTeX, Irvine, CA, USA).

3.3 Statistical analysis

The clinical data were expressed as mean ± standard deviation or median, while expression data were expressed as mean ± standard error of the mean (SEM). qRT-PCR expression values were normalized to the mRNA expression of GAPDH. The relative protein expression levels were quantified using Image J software (National Institutes of Health) and normalized to the controls (GAPDH).

Statistical analysis was performed with GraphPad Prism5 (GraphPad Software Inc., San Diego, CA, USA). Unpaired Student's t test was used as an appropriate approach to compare the data between the lesional skin and healthy control groups, and paired Student's t test was performed to determine the significant differences between lesional and perilesional skin. $P < 0.05$ was accepted as statistically significant. Spearman correlation was used to analyze the association among clinical variables and genes. All tests were two-tailed and considered statistically significant when $P < 0.05$. Statistical software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for analysis.

4 Results

4.1 Demographics

We recruited 38 patients with PN in this study, including 24 men (63.2%) and 14 women (36.8%), with a mean age at first visit of 46.5 ± 14.6 years (range, 14–76 years) and average PN duration of 4.5 years (range, 0.2–40 years). Most of the patients suffered from high-intensity pruritus and accompanying sensations (e.g., pruritus, stinging, burning, or tingling). VAS (median = 5) and VRS (median = 2) scores indicated that most of the patients suffered from moderate or higher pruritus intensity. PRUNOSI scores ranged from 3 to 15, and the total serum IgE level of patients ranged from 8 to 5,500 IU/mL; 23 (60.5%) patients showed increased total IgE level (10–15 years old >200 IU/mL, adult >100 IU/mL; Table 1).

4.2 Location of lesions and histological characteristics

In this study, 32% of patients with PN had an atopic background with no signs of active dermatosis, lesions of PN manifested in circumscribed areas, and symmetrical distribution of the extensor surfaces of extremities and trunk. On the back, the untouched skin area that was free of lesions resembled a butterfly shape and is thus called the "butterfly sign" [2,27]. In addition, lesions affected patients' face, scalp, buttock, or palms (Figure 1a–e). The biopsies from the representative

Table 1: Demographic and clinical information of patients with prurigo nodularis

Patient (*n*)	Gender (male:female)	Age (year) (mean ± SD) (range)	Duration (year) (mean ± SD) (range)	Atopic history	PRUNOSI (median) (range)	VRS (median) (range)	VAS (median) (range)	IgE (IU/ml) (mean ± SD) (range)	DLQI (median) (range)
38	24:14	46.6 ± 14.6 (14–76)	4.5 ± 7.8 (0.2–40)	12	9 3–15	2 0–3	5 0–10	539.4 ± 967.3 8.7–5,500	8 1–26

DLQI: dermatology life quality index; IgE: total IgE; *n*: number; VAS: visual analog scale (scores from 0 to 10); and VRS: verbal rating scale (score range 0–3).

lesions showed typical features, thick orthohyperkeratosis, irregular acanthosis hyperplasia, irregular elongation of the rete ridges, and focal parakeratosis in the epidermis (Figure 2a); in the dermis, mild perivascular lymphocytic infiltrate, an increased number of capillaries, and vertically oriented collagen bundles were observed (Figure 2b).

4.3 K17 expression is induced in PN lesional skin

We first investigated the K17 transcription in the lesional and the perilesional skin area of patients with PN (eight men and six women). K17 mRNA levels were quantified by qRT-PCR analysis. In PN lesional skin, K17 transcriptions were significantly higher than those in perilesional skin ($P < 0.01$; Figure 3a). The average mRNA levels in the lesional skin were about 2.8-fold higher than those in perilesional skin. K17 mRNA expression was also reduced in the healthy group ($n = 9$) as compared with the PN group (change between the lesional skin and healthy skin was more than 40-fold, $P < 0.0001$; Figure 3b).

To confirm the difference in K17 expression levels between the lesional and perilesional skin, we also checked the K17 protein levels in other patients with PN (five men and five women) and normal controls ($n = 9$) using Western blot analysis. Compared with those in the healthy controls and the perilesional skin, K17 protein levels were sharply increased in the PN lesional skin (Figure 4a), exhibiting a 3.3-fold upregulation compared with the perilesional skin by quantification of relative protein levels (Figure 4c). Among the samples, K17 protein was hardly detected in 40% of the perilesional skin samples; similarly, K17 protein levels in most of the normal skin were absent (Figure 4b). We then performed an immunohistochemical analysis of PN lesions and found that K17 was expressed in the lesional epidermis (Figure 4d). The K17 expression level in lesions was higher than in the perilesional skin.

4.4 Higher levels of K6/K16 and K5 mRNA expression are detected in PN lesions

K17 together with K6/K16 is considered to be the marker for activated keratinocytes [28]. The mRNA expressions of K6/K16 were increased in the lesional skin (Figure S3a and b). In healthy epidermis, the expressions of specific keratin genes K5 and K14 were used as markers for the basal layer, whereas higher K5 mRNA expression levels were detected in the PN lesional skin as well (Figure S3c).

4.5 Correlations between clinical markers and K17 mRNA expression in PN lesions

We evaluated the correlations between the clinical markers and K17 relative to the mRNA expression level in PN lesions. We found that the PRUNOSI score (the extent of scratch lesions) was positively correlated with the VAS, VRS, and DLQI in a larger group of 38 patients (Figure S4). A median positive correlation was found between the K17 fold change of relative mRNA expression and PRUNOSI ($r = 0.547$, $P < 0.05$; $n = 14$; Figure 3c).

5 Discussion

Previous histopathological studies on PN skin lesion revealed changes in most types of skin cells, including mast cells, Merkel cells, epidermal keratinocytes, dendritic cells, and endothelial cells [15,29]. Keratinocytes are the predominant cell type in the epidermis, forming an integrated physical barrier. Epidermal keratinocytes may play an important role in the pathogenesis of PN. To date, no research has focused on the proliferation of abnormal keratinocytes in PN skin lesions, and discovery of keratin

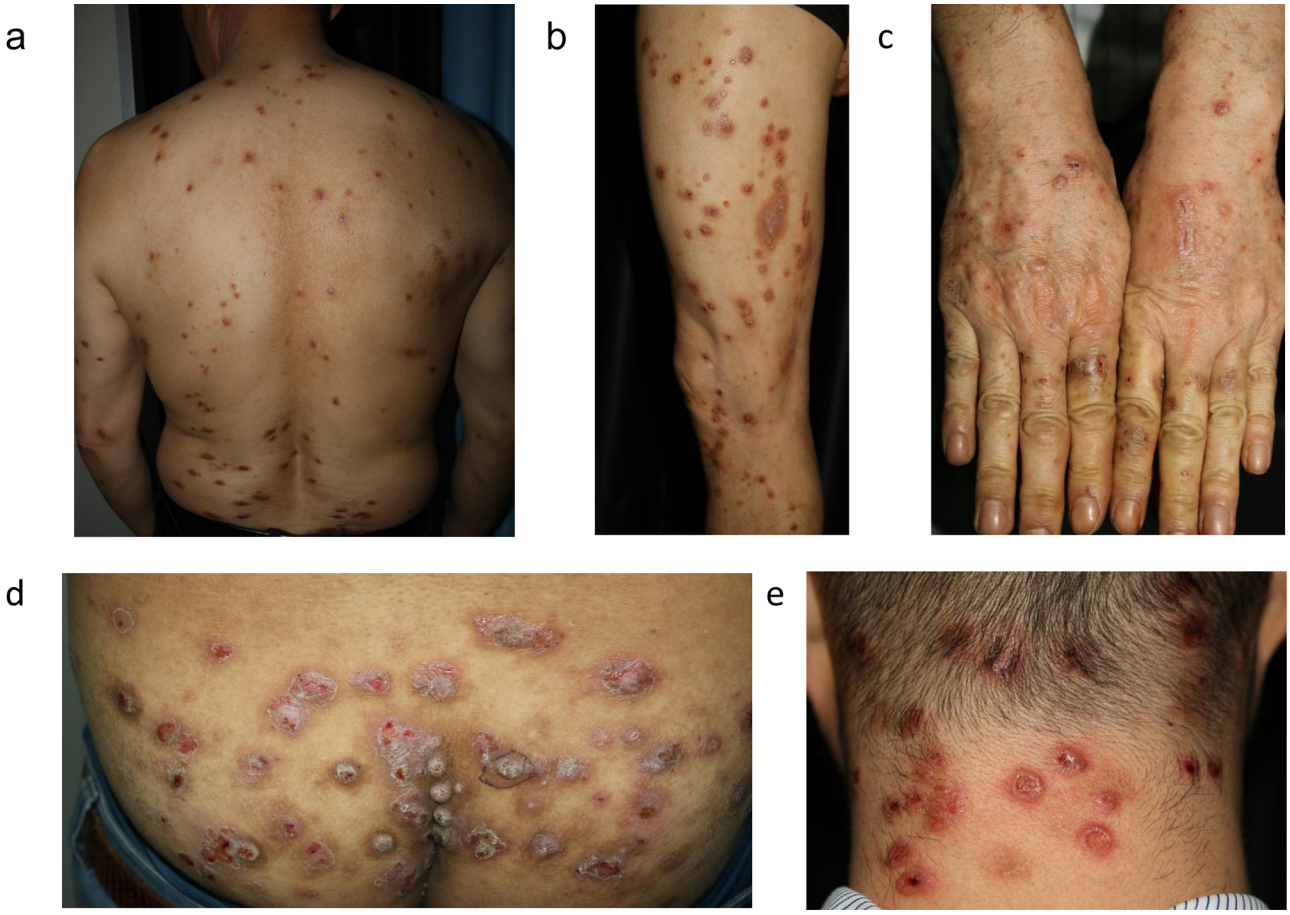

Figure 1: Clinicopathological features of prurigo nodularis. (a–e) Multiple excoriated papules or nodules involving typical distribution of trunk with the "butterfly sign" (no lesion on the center of the back), extensors of the extremities, palms, buttocks, and neck. These images were taken from five different patients with PN: (a) P36, (b) P23, (c) P26, (d) P18, and (e) P25.

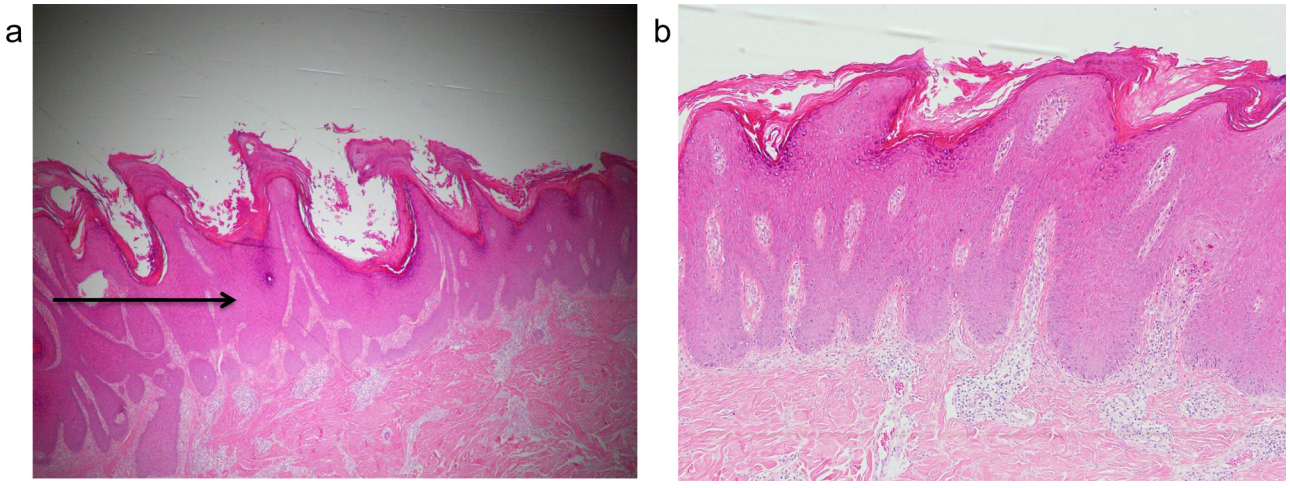

Figure 2: Histological observations of lesional skin biopsy from prurigo nodularis. Paraffin-embedded sections of lesional skin from PN stained with hematoxylin and eosin. Lesional skin biopsy showed orthohyperkeratosis, hypergranulosis, and epidermal hyperplasia (arrow); the epidermis mostly consisted of keratinocytes. (a) Original magnification ×40; (b) original magnification ×100.

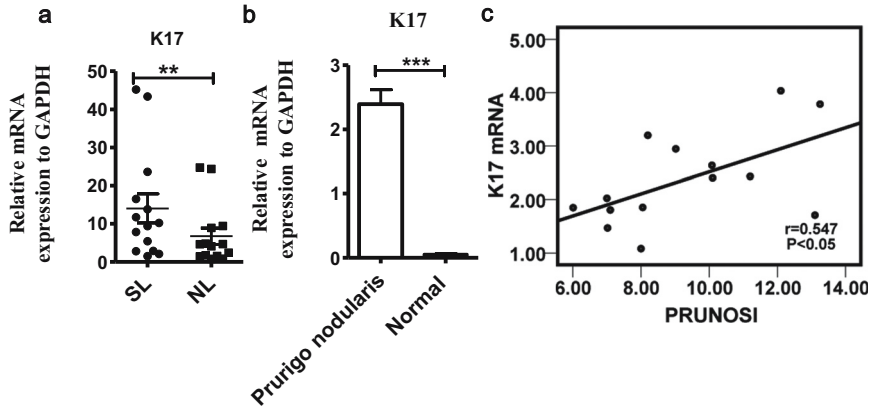

Figure 3: Keratin 17 (K17) mRNA expression levels in lesional and perilesional skin of patients with PN compared with those of healthy volunteers. (a) Real-time PCR analysis of K17 mRNA expression levels (n = 14). The levels are compared between the lesional skin and perilesional skin. (b) K17 mRNA expression levels are compared between the PN lesional skin and normal healthy skin. (c) Spearman analysis of PRUNOSI (x-axis) and K17 gene expression (y-axis); in the scatter plot, r = 0.547, *P < 0.05, regression line $Y = 1.551x + 5.616$, and regression coefficients R^2 = 0.32. SL, lesional skin; NL, perilesional skin. Values show the expression/GAPDH level and are presented as mean ± SEM. Asterisks above the error bars denote comparisons between the matched lesional and perilesional skin. *P < 0.05, **P < 0.01, and ***P < 0.001, by paired Student's t test.

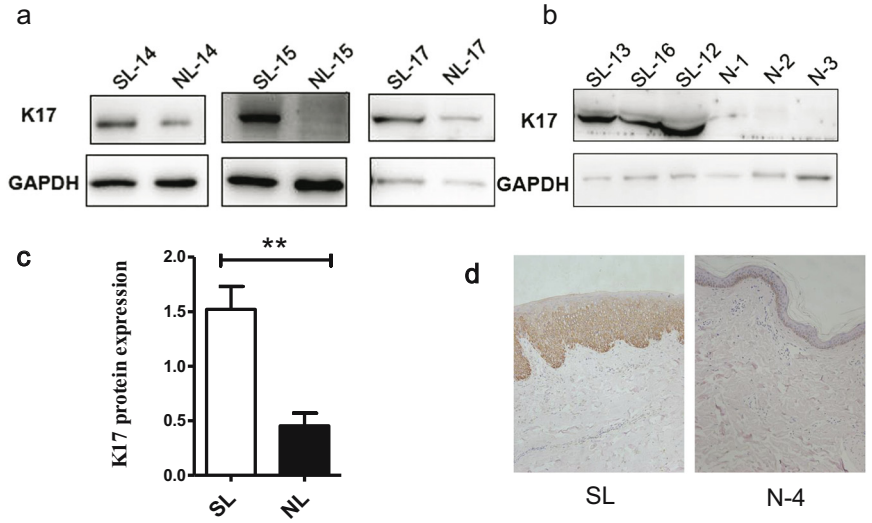

Figure 4: K17 expression is elevated in PN lesional skin. (a) Protein levels of K17 were detected in lesional skin and perilesional skin of patients with PN (n = 10) by Western blot and (b) protein levels of K17 from patients with PN and healthy controls (n = 9); (c) Image J analysis of K17 protein levels in lesional and perilesional skin. Band intensity was quantified by Image J. Values presented are the average of the K17 protein levels normalized to GAPDH from ten patients. (d) K17 expression in the PN lesional skin sample. Representative images of K17 expression are shown at 200 × magnification. SL, lesional skin; NL, perilesional skin; and N, normal control. **P < 0.01, by paired Student's t test.

expression patterns may benefit the understanding of epidermal hyperplasia and PN pathogenesis.

K17 is a member of the type I acidic epithelial keratin family and can modulate keratinocyte growth during skin development by positively regulating protein synthesis [22,30]. K17 promotes the growth of basaloid skin tumors, oral squamous cell carcinoma, and so on [23,31]. High K17 expression is a powerful negative prognostic biomarker associated with poor outcome in patients with endocervical neoplasia [32]. In this study, we investigated the expression of K17 in PN skin lesions and its potential role in epidermal hyperplasia in PN.

In this work, comparison of K17 mRNA expression in PN skin lesions, perilesional skin, and healthy skin

showed that the mRNA expression of K17 in the lesional skin was upregulated compared with the perilesional skin (Figure 3a) and healthy controls (Figure 3b). In addition, the comparison of K17 protein level in PN lesions confirmed the results of K17 mRNA expression, as the level of K17 protein was markedly increased in PN lesional skin. Compared with the healthy controls, K17 protein levels were elevated not only in the lesional skin but also in parts of the perilesional skin samples (6/10). These findings remind us that we initially selected newly developing nodules for this study, and these nodules tend to cover the healthy-appearing perilesional skin region and grow to larger nodules. These results may provide evidence for the clinical observation that the lesion frequently started as a smaller papule and rapidly processed to a larger nodule.

Overexpressed K17 in the lesion challenges the homeostasis of the skin epidermis in response to pathologic conditions. K17 together with K6/K16 is considered to be the marker for activated keratinocytes [21], and specific keratins (K6/K16) are inducible to expression in the suprabasal interfollicular epidermis [5,33], which is distinct from the keratins of healthy epidermis.

In this study, we analyzed the K6/K16 mRNA expression levels of PN skin, and the results revealed that the K6/K16 was significantly upregulated in lesional skin than in perilesional skin ($P < 0.01$ and $P < 0.05$), showing 12-fold ($n = 9$) and 5-fold ($n = 6$) increases on average, respectively (Figure S1a and b). Furthermore, positive trends of the relative mRNA expression of K6 correlated with K17 ($r = 0.617$, $P = 0.077$; $n = 9$; Figure S1d). K17 was mainly expressed in the epidermal keratinocytes of PN lesional skin (Figure 4d). Taken together, as the activated keratinocyte markers [21], the increased expression of K17 and K6/K16 implied that keratinocytes in PN lesions may be in the activated state. In basaloid skin tumors, co-polymerization of K17 with K5 was induced, where K17 as an immunomodulator accelerated epithelial proliferation and tumor formation in situations with an activated Hedgehog signaling pathway [23]. We also checked the K5 mRNA levels in both the lesional and perilesional skin and observed 19-fold higher K5 mRNA expression levels in the lesional skin ($P = 0.089$; $n = 9$; Figure S1c). This finding reflected the preferential activation and proliferation of the basal keratinocytes. We proposed that the activated basal keratinocytes in lesions expressed the K6/K16; consequently, some cytokines induced K17 expression [21]. These activated keratinocytes may lead to the hyperkeratotic epidermis of the PN.

From the correlation analysis, the K17 relative mRNA expression levels (fold change) in the 14 samples were positively correlated with the PRUNOSI score (Figure 3c), suggesting that K17 may play a role in the severity of PN skin lesions. During follow-up detection in a larger group of 38 patients, we found that the PRUNOSI score was positively correlated with the VAS, VRS, and DLQI (Figure S4), which indicates that severe itching leads to scratch-induced skin lesions [13], and damaged lesional skin negatively affects a patient's quality of life. Therefore, we hypothesize that K17 could be critical to PN pathogenesis and a potential therapy target.

In this research, 60.5% of patients showed increased total serum IgE levels (Table 1). Spearman analysis showed that no correlations were detected between the total serum IgE levels and disease severity (PRUNOSI, VAS, and VRS). Specific IgE reactivities against a variety of bacterial antigens were observed in a subgroup comprising a third of AD patients [28], as an elevated serum IgE level is one of the clinical criteria of AD [20,34–36], and significantly high total serum IgE is also a hallmark of visceral infections by parasites [37]. Fifty percent of the patients with PN showed an atopic predisposition [4], and infection (hepatitis C and HIV) has been considered to be linked to PN. In this research, 31.6% of patients with PN had an atopic history; half of these patients with PN showed insect-bite dermatitis, and the IgE levels of the AD patients were greater than 100 IU/mL. Because increased levels of total IgE may be partly associated with PN pathogenesis, we will investigate the details of this in the future.

Therapies for PN generally focus on interrupting the itch–scratch cycle. In addition to addressing the cause of chronic pruritus [38], PN lesion healing should be considered as the goal of PN therapy. As far as we are concerned, there has been no standard criterion of treatment for itch until now. Treatment typically relies on the use of topical or intralesional steroids, although more severe or recalcitrant cases require novel therapeutic agents for treatment according to the underlying factors of PN [39]. Calcipotriol ointment, a synthetic form of vitamin D, acts to inhibit skin keratinocyte cell proliferation and enhance cell differentiation in the skin of patients with psoriasis, and downregulation of K17 is vital for its effect [40]. In a small randomized controlled trial comparing calcipotriol ointment with betamethasone valerate 0.1% ointment, calcipotriol ointment showed greater efficacy and more rapid clearance of the PN lesions [41].

In summary, we preliminarily identified that K17 was upregulated in PN lesions. Along with an aberrant expression of K6/K16 and K5, we speculated that

keratinocytes may be in the activated cycles, and K17 might contribute to the proliferation of keratinocytes in PN epidermis and lesion progression. However, all of these results were obtained from a small sample size; in the future, we will use immunochemistry staining studies of keratins in the lesional epidermis to understand the dysregulated patterns of keratinocytes. The K17 regulation mechanism and its exact biological role in PN remains to be elucidated in future studies.

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Conflict of interest: There are no conflicts of interest.

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