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# Trichohyalin-like 1 protein, a member of fused S100 proteins, is expressed in normal and pathologic human skin

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ABSTRACT

Trichohyalin-like 1 (TCHHL1) protein is a novel member of the fused-type S100 protein gene family. The deduced amino acid sequence of TCHHL1 contains an EF-hand domain in the N-terminus, one transmembrane domain and a nuclear localization signal. We generated specific antibodies against the C-terminus of the TCHHL1 protein and examined the expression of TCHHL1 proteins in normal and pathological human skin. An immunohistochemical study showed that TCHHL1 proteins were expressed in the basal layer of the normal epidermis. In addition, signals of TCHHL1 proteins were observed around the nuclei of cultured growing keratinocytes. Accordingly, TCHHL1 mRNA has been detected in normal skin and cultured growing keratinocytes. Furthermore, TCHHL1 proteins were strongly expressed in the peripheral areas of tumor nests in basal cell carcinomas and squamous cell carcinomas. A dramatic increase in the number of Ki67 positive cells was observed in TCHHL1-expressing areas. The expression of TCHHL1 proteins also increased in non-cancerous hyperproliferative epidermal tissues such as those of psoriasis vulgaris and lichen planus. These findings highlight the possibility that TCHHL1 proteins are expressed in growing keratinocytes of the epidermis and might be associated with the proliferation of keratinocytes.



## 1. Introduction

The skin is the first line of defense against chemical, physical and microbial insults [1]. The epidermis is continuously regenerated though a process called epidermal differentiation because of perpetual attacks. The terminal differentiation of a keratinocyte in the epidermis involves the cessation of proliferation and the subsequent migration of the keratinocyte from the basal layer to the suprabasal layers with progressive cornification [2]. This is a complex process that requires the regulated and sequential expression of a variety of genes. Several genes involved in epidermal differentiation are found within a 2 Mb region at chromosome band 1q21.3 called the epidermal differentiation complex (EDC) [3,4]. The EDC contains three clustered families of genes encoding the following: (a) precursor proteins of the cornified cell envelope (involucrin, lorocrin, and small praline-rich proteins) characterized by short tandem peptide repeats in the central region [5–7]; (b) calcium-binding S100 proteins containing EF hand domains [7]; and (c) a family of proteins (profilaggrin, trichohyalin, hornerin, repetin, cornulin and filaggrin-2) described as "fused S100 proteins" containing the EF-hand domain in the N-terminus followed by multiple tandem peptide repeats [8–14]. During the final stages of differentiation, these specific proteins are cross-linked together by transglutaminase to form a cornified envelope.

The role of filaggrin among fused S100 proteins has been well-characterized. Filaggrin is produced by post-translational proteolysis of the precursor protein, profilaggrin in the granular layer of the epidermis, and promotes the aggregation of keratin filaments resulting in the formation of disulfide bonds [8,15,16]. Trichohyalin is cross-linked to either itself or other cornified envelope proteins to form the main constituent of inner root sheaths and the medullae of the hair shafts of hair follicles, contributing to the mechanical strength of these entities [17,18]. Abnormalities in

*Abbreviations:* EDC, the epidermal differentiation complex; TCHHL1, trichohyalin-like 1; RT-PCR, reverse transcription polymerase chain reaction; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; NLS, a nuclear localization signal; TMD, a transmembrane domain; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GST, anti-glutathione S-transferase; DAPI, 6-diamidine-2′-phenylindole dihydrochloride.

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the cornification process cause various human diseases [19,20], including absent granular layer-type ichthyosis vulgaris [21], lamellar ichthyosis [22,23] and Vohwinkel syndrome with ichthyosis [24].

Trichohyalin-like 1 (TCHHL1) protein is a novel member of the fused-type S100 protein gene family [25]. The human *TCHHL1* gene was found to be located at the chromosomal locus between trichohyalin and S100A11 on 1q21.3. In this study, we generated specific antibodies against the C-terminus of the TCHHL1 protein and examined the expression of TCHHL1 proteins in normal and pathological human skin.

## 2. Materials and methods

### 2.1. Clinical materials

Normal human skin tissue samples and tissue samples from patients with skin diseases were obtained from Toyama University Hospital. The skin tissue samples from subjects with diseases included psoriasis vulgaris (PV), lichen planus (LP), actinic keratosis (AK), Bowen's disease (BD), basal cell carcinoma (BCC) or squamous cell carcinoma (SCC). All patients gave their written informed consent and the study protocol complied with all of the Principles of the Declaration of Helsinki. This study was approved by the Medical Ethics Committees of the University of Toyama, Toyama, Japan.

### 2.2. Cell culture

Normal human epidermal keratinocyes (Kurabo Industries Ltd, Osaka, Japan) were cultured in Humedia-KG2 (Kurabo Industries Ltd, Osaka, Japan) in a humidified atmosphere with 5% $CO_2$.

### 2.3. Detection of mRNA

For the reverse transcription polymerase chain reaction (RT-PCR) analysis, total RNA was prepared from cultured human keratinocytes using a method previously described [16]. RNA from adult, fetal and vulval skin was purchased from Agilent Technologies (Santa Clara, CA). All of the RNA samples were pretreated with DNase I (Roche Diagnostics, Basel, Schweiz) and confirmed to give no positive signals without reverse transcription. Reverse transcription was performed with random hexamers and Superscript III (Invitrogen, Carlsbad CA).

In addition, human tissue cDNAs included in the Human MTC Panel 1 and Human MTC Panel 2 (BD Biosciences, Palo Alto, CA) were used for the RT-PCR analysis. The primers used for PCR were as follows: TCHHL1; sense 5′-ATGCCTCAGCTCCTGAGAAATGTC-3′, antisense5′-TTGCTTTGTGGTGCTTGCCTCCCTTTGTA-3′: Keratin 5; sense 5′-TGCTGCAAGTCACTGCCTTC-3′, antisense5′-TTGAACACATTCTGGAGGTAG-3′: Keratin 16; sense5′-GCTGAACAAAGAAGTGGCCTC-3′, antisense5′-TGAAGCTGGATGAGCTCTGCT-3′: GAPDH; sense5′-GAAGGTGAAGGTCGGAGTCAACG-3′, antisense5′-AGTCCTTCCACGATACCAAAGTTG-3′. The amplified DNA fragments were analyzed using 2% agarose gel electrophoresis.

### 2.4. Preparation of specific antibodies against TCHHL1 proteins

To prepare antibodies against TCHHL1 proteins, an oligopeptide (HPQRERLVLQREASTTKQ; Fig. 1**A broken line**) corresponding to part of the C terminal region of the human TCHHL1 protein was synthesized, conjugated with keyhole limpet hemocyanine, and injected with an adjuvant (TyterMax Gold, CytRx) into rabbits. The resulting antibodies were affinity-purified using a Hitrap NHS-activated column (GE healthcare UK Ltd, Buckinghamshire, England) conjugated with the peptides.

### 2.5. Preparation of recombinant TCHHL1 proteins

To prepare recombinant proteins, cDNA fragments covering all of the coding regions were amplified using RT-PCR and subcloned into the pDEST15 gateway vector (glutathione S-transferase gene fusion vector; Invitrogen, Carlsbad, CA). The primers used for PCR were: sense 5′-CACCATGCCTCAGCTCCTGAGAAATGTC-3′, antisense 5′-TCATTGCTTTGTGGTGCTTGCCTCCCTTTGTAG-3′. After inducing protein expression in BL21 cells (Invitrogen, Carlsbad, CA) with 1 mM isopropyl thiogalactopyranoside, the proteins were purified using glutathione–Sepharose 4B (GE healthcare UK Ltd, Buckinghamshire, England).

### 2.6. Western blot analysis

Protein extracts were prepared by homogenizing normal human skin tissues in 0.1% Tris–HCl (pH 7.5), 5 mM EDTA, a protease inhibitor mixture diluted according to manufacturer's instructions (Shigma-Aldrich. CO, St. Louis, MO), 0.2 mM phenylmethylsulfonyl fluoride (Shigma-Aldrich. CO, St. Louis, MO), and 2 mM pepstatin A (Peptide Institute, Osaka, Japan). A trichloroacetic acid solution was added to the homogenates to make the final concentration 10% and the same samples were kept on ice for 15 min. After centrifugation was completed, the precipitates were sonicated in a sample buffer (125 mM Tris–HCl, pH6.8, 2.3% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 10 μg bromophenol blue). Ten or 20 μg equivalents of protein were applied onto 5–20% gradient SDS–polyacrylamide gels (PAGEL, Atto, Tokyo, Japan), electrophoresed and transferred onto PolyScreen Transfer Membranes (NEN life science products, Boston, MA). The membranes were treated with antibodies against TCHHL1 proteins, and positive signals were visualized using ECL-plus Western Blotting Detection Reagents (GE healthcare UK Ltd, Buckinghamshire, England). The following antibodies were also used as primary antibodies: anti-glutathione-S-transferase antibody (Santa Cruz INC, Santa Cruz, CA) and anti-human cytokeratin-14 monoclonal antibody (Biomeda, Foster City, CA). Preimmune antisera and the antibodies absorbed with the immunogen peptides were used as negative controls.

### 2.7. Immunohistochemistry

Human skin tissues were directly dipped into OCT Compound (Ted Pella, Redding, CA) and rapidly frozen in liquid nitrogen. Sections measuring 5 μm in thickness were blocked with Protein Block Serum-Free (DAKO, Carpinteria, CA) for 30 min, then incubated with the primary antibodies. The signals were detected with Envision+ (DAKO, Carpinteria, CA) followed by staining using the Liquid DAB + Substrate Chromogen System (DAKO, Carpinteria, CA). For the immunofluorescent observation, Alexa Fluor 488 goat anti-rabbit IgG (H + L) or Alexa Fluor 555 goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR) was used as second antibodies. The following antibodies were used as primary antibodies: anti-humanTCHHL1 antibody, anti-human cytokeratin-14 monoclonal antibody (Biomeda, Foster City, CA) and anti-human Ki67 antibody (DAKO, Carpinteria, CA). Both preimmune sera and the anti-human TCHHL1 antibodies pre-absorbed with the immunogen peptides gave consistently negative results. The tissue sections were observed using a fluorescence microscopy (Olympus) or a confocal laser microscope, LSM510 (Carl Zeiss). The TCHHL1-positive cells and Ki67-positive cells were counted under a fluorescence microscope under high power field (HPF: X400) and the results were ex-

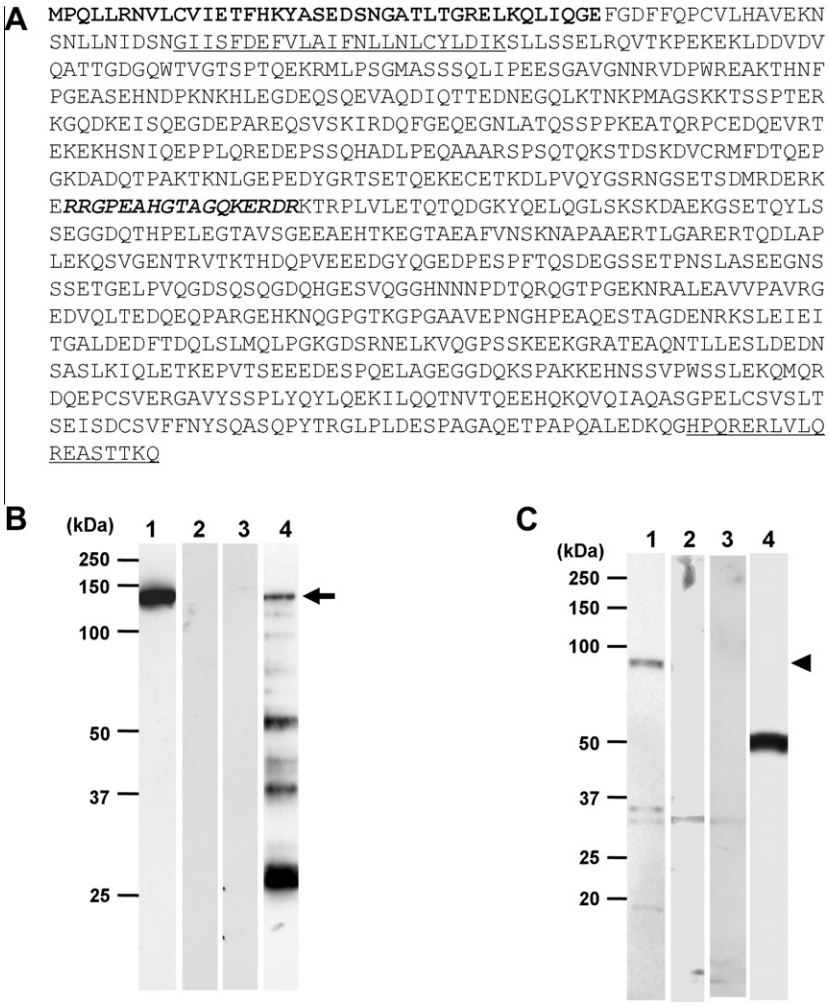


**Fig. 1.** The deduced amino acid sequence of TCHHL1 and the expression in human skin. (**A**) The deduced amino acid sequence of the human TCHHL1 protein. The EF-hand domain in the N-terminus is marked in bold. The putative transmembrane domain is underlined, the nuclear localization signal sequence is italicized and the peptides used for immunization is showed in broken line. (**B**) Specific recognition of the recombinant TCHHL1 proteins by the antibodies. Purified recombinant TCHHL1 proteins were applied onto a 10% SDS gel and blotted with an anti-TCHHL1 antibody (**lane 1**), preimmune serum from the respective rabbits (**lane 2**), anti-serum pre-absorbed with the peptide of the immunogen (**lane 3**) or an anti-glutathione S-transferase (GST) antibody (**lane 4**). The arrow indicates the expected size of the GST-TCHHL1 fusion protein. (**C**) A Western blot analysis of normal human skin. Twenty μg of the protein preparation was blotted with an anti-human TCHHL1 antibody (**lane 1**), preimmune serum from the respective rabbits (**lane 2**), antiserum pre-absorbed with the peptide of the immunogen (**lane 3**), or an anti-human keratin14 antibody (**lane 4**). The arrowhead indicates the expected size of the intact TCHHL1 protein.

pressed as the mean number of cells in five random areas per section from five different cases of each disease.

### 2.8. Statistical analysis

The values are expressed as the means ± standard deviation (SD). The statistical significance of differences between the normal skin and diseased skin was evaluated by Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. The deduced amino acid sequence of human TCHHL1 proteins

The deduced amino acid sequence of TCHHL1 protein contains an EF-hand domain in the N-terminus (Fig. 1**A bold**) followed by a large domain. The RADAR program [26] analysis detected 10 tandem peptide repeats in the large domain. The repetitive segments measured of 35–65 amino acids in length. Although this structural feature might be similar to that found in the fused S100 protein family members, the repetitive segments showed low homology (8–29%) to each other. On the other hand, this large domain also includes one trans-membrane domain (TMD) at amino acids 66–88 (Fig. 1**A, underlined**) and a nuclear localization signal (NLS) at amino acids 394–410 (Fig. 1**A, italicized**). None of the fused S100 protein family members, except profilaggrin, have TMDs or NSLs. Only profilaggrin has an NLS between the EF-hand domain and a large repetitive domain.

### 3.2. Detection of TCHHL1 proteins in human skin

Antibodies against the C-terminus of the TCHHL1 protein were generated in order to examine the expression of TCHHL1 proteins. The antibodies specifically recognized the recombinant glutathione S-transferase (GST) fused TCHHL1 proteins (Fig. 1**B, lane 1**). Anti-GST antibodies also detected the same proteins (**arrow in** Fig. 1**B, lane 4**). The ladder-like bands detected by the anti-GST antibodies are most likely partially degraded proteins. No corresponding bands were detected with preimmune serum or the antibodies pre-absorbed by the peptides used for immunization (Fig. 1**B, lanes 2 and 3**). Using these antibodies, a band corresponding to the expected size of the TCHHL1 protein was detected by a

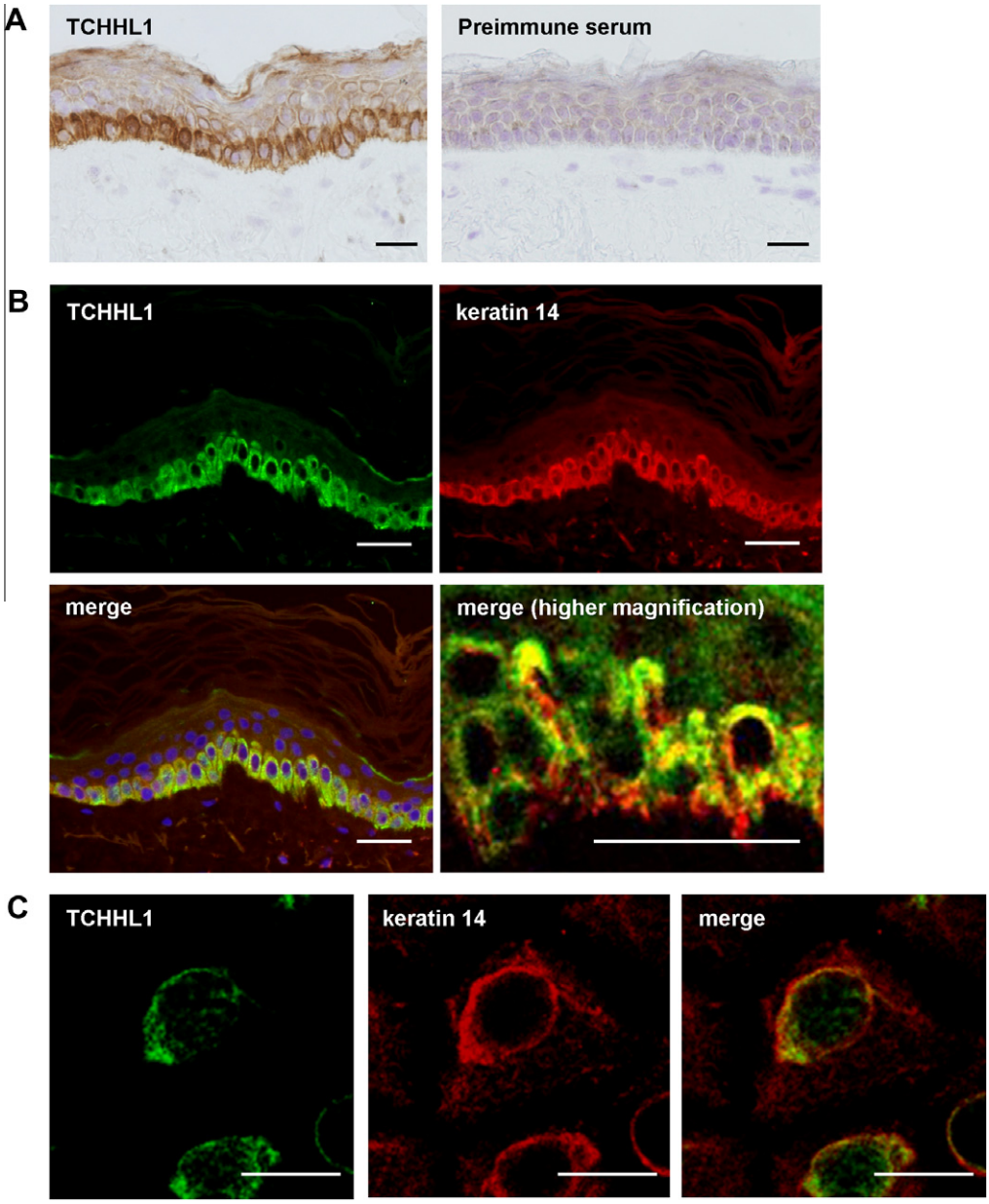


**Fig. 2.** Immunostaining for TCHHL1 proteins in normal human skin tissues and cultured normal human keratinocytes. (**A**) Tissue sections of normal human skin were stained with an anti-TCHHL1 antibody or preimmuneserum. The scale bar, 50 μm, is the same for all panels. (**B**) Normal human skin tissues were doubly immunostained for TCHHL1 and keratin. The tissue sections were also stained with 4′, 6-diamidine-2′-phenylindole dihydrochloride (DAPI) to visualize the nuclei. The scale bar, 50 μm, is the same for all panels. (**C**) Double immunostaining with TCHHL1 and keratin 14 in the cultured growing keratinocytes. The scale bar, 10 μm, is the same for all panels.

Western blot analysis in normal human adult skin **(arrowhead in** Fig. 1**C)**. However, TCHHL1 antibodies did not detect any ladder-like bands, which are usually observed in profilaggrin, filaggrin2 and hornerin and suggest that single repeat units are produced by post-translational proteolysis during the cornification process. No corresponding bands were detected using the preimmune serum or the antibodies pre-absorbed by the peptides used for immunization (Fig. 1**C**, **lanes 2 and 3**). Because keratin 14 proteins at a size of approximately 50 kD were detected, the TCHHL1 antibodies did not cross-react with the keratin 14 proteins (Fig. 1**C, lane 4**).

### *3.3. Localization of TCHHL1 proteins in the epidermis and cultured normal human keratinocytes*

An immunohistochemical study showed that the TCHHL1 protein is strongly expressed in the basal layer of normal skin; however, it is not expressed in the spinous, granular or horny layers of the normal epidermis (Fig. 2**A left panel)**. No signals were detected with preimmune serum (Fig. 2**A right panel)** or the antibodies pre-absorbed by the peptides used for immunization **(data not shown)**. To confirm the localization of TCHHL1 in the epidermis, we doubly stained the tissue sections with fluorescent labels for TCHHL1 and keratin14. TCHHL1 **(green**, Fig. 2**B)** and keratin 14 **(red,** Fig. 2**B)** were observed to form a homogenous distribution in the cytoplasm of basal keratinocyes. When the two panels were merged, most of the signals were yellow **(merge,** Fig. 2**B)**. However, in an image obtained at a higher magnification, the signals of TCHHL1 were unevenly observed and partially co-localized with those of cytokeratin 14 in the cytoplasm. Furthermore, an immunofluorescent study was also performed using human keratinocytes in culture to clarify the expression and localization of TCHHL1 in human keratinocytes. Signals for TCHHL1 proteins were

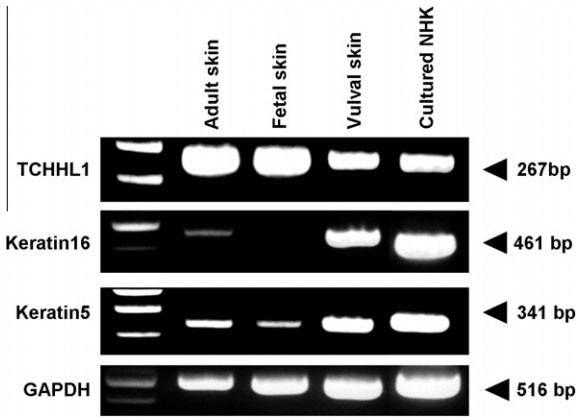


**Fig. 3.** The expression of TCHHL1 mRNA in human skins and cultured keratinocytes. The RT-PCR analysis of TCHHL1 transcripts. The PCR cycles were adjusted to obtain an appropriate band thicknesses, i.e., TCHHL1 5′, 35; keratin 16, 28; keratin 5, 35; glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 25.

primarily observed around the nuclei of growing keratinocytes **(green,** Fig. 2**C)**. Furthermore, TCHHL1 proteins were partially co-localized with keratin 14 **(red,** Fig. 2**C)**, especially around nuclei (**merge,** Fig. 2**C)**.

### *3.4. Expression of TCHHL1 mRNA in human skins and cultured keratinocytes*

To confirm the expression of TCHHL1 in the epidermis and cultured normal human keratinocytes, we examined various human skin tissues for TCHHL1 expression using a reverse transcription polymerase chain reaction (RT-PCR) analysis. TCHHL1 mRNA was detected in normal human adult and fetal skin, vulval skin and cultured normal human keratinocytes as evidenced by a 267 bp-band amplified with 5′-terminal primers **(**Fig. 3). In addition, we examined the expression of TCHHL1 mRNA in various human tissues using a PCR analysis with cDNAs included in Human MTC Panel 1 and Human MTC Panel 2. The Human MTC Panels cover most human tissues (except skin), including the heart, brain, placenta, lungs, liver, skeletal muscles, kidneys, pancreas, spleen, thymus, prostate, testes, ovaries, small intestine and colon with mucosal linings and leukocytes; however, no panels were positive for the expression of TCHHL1 **(data not shown)**.

### *3.5. Expression of TCHHL1 proteins in pathologic skin tissues with hyperproliferation*

The findings of immunostaining in normal skin and keratinocyts suggest that TCHHL1 proteins primarily localize in growing keratinocytes. To clarify this hypothesis, an immunofluorescent study was performed using cancerous/non-cancerous skin samples with hyperproliferation of keratinocytes (five cases of each diseases; PV, LP, AK, BD, BCC and SCC). In the SCC and BCC samples, TCHHL1 proteins were expressed in all of the cells in the tumor nests and the expression was stronger in the peripheral areas than in the center areas (SCC: 204.67 ± 51.29, BCC: 354.2 ± 85.45/HPF; Fig. 4**A)**. Dramatic increases were observed in the number of Ki67-positive cells in the TCHHL1-expressing areas of the tumor nests in the SCC and BCC skin samples (74.8 ± 43.94 and 73.87 ± 27.79/HPF, respectively). In AK and BD skin samples, which included precancerous states of hyperproliferative epidermal cells, the expression of TCHHL1 proteins were widely observed in all layers of the epidermis (AK: 54.83 ± 15.56, BD: 221.53 ± 66.19/HPF; Fig. 4**A)**. In these diseases, signals of Ki67 were observed not only in basal layers, but also in spinus layers of the epidermis, in which TCHHL1 proteins were expressed (AK: 17.8 ± 9.6, BD: 44.53 ± 16.3/HPF). To assess whether up-regulation of TCHHL1 is a cancer-specific phenomenon, non-cancerous hyperproliferative epidermal tissues of PV and LP were used in an immunofluorescent study. The TCHHL1 expression was up regulated and TCHHL1 signals were observed primarily in the basal and suprabasal layers of these skin samples (PV: 128.33 ± 26.70, LP: 58.13 ± 19.94/HPF; Fig. 4**B**). The number of Ki67-positive cells in the suprabasal and basal layers also increased (PV: 46.33 ± 22.22, LP: 17.0 ± 6.97/HPF). The numbers of both TCHHL1-positive cells and Ki67-positive cells in all of the samples from patients with skin diseases were significantly higher compared to those in normal skin ($p < 0.001$). The numbers of TCHHL1-positive and Ki67-positive cells in normal skin were 34.95 ± 8.48 and 4.75 ± 2.75, respectively (Fig. 4**C**).

## 4. Discussion

In this study, we examined the expression of TCHHL1 proteins in normal and pathologic human skin and cultured human keratinocytes. Most fused S100 protein family members including profilaggrin have been shown to localize in the granular layer of the epidermis. Trichohyalin was originally found to be expressed in the inner root sheaths of hair follicles [27,28] and was later shown to be expressed in a variety of pathological human epidermal tissues [29,30]. Surprisingly, the signal of TCHHL1 proteins is clearly observed in the basal layer of the normal epidermis. In addition, TCHHL1 proteins are observed around the nuclei of growing keratinocytes in culture, although the signals of fused S100 protein family members are commonly observed in differentiated keratinocytes. This distribution suggests that TCHHL1 proteins might be associated with nuclear envelopes, which separate a cell's genetic material from the surrounding cytoplasm [31]. The existence of an NLS and a TMD in the TCHHL1 protein sequence is considered to support this hypothesis. Considering the expression patterns, TCHHL1 may have different functions from those of other fused S100 protein family members.

The observations of the present study are different from those of previous one suggesting that the TCHHL1 protein is expressed only in the inner root sheaths of hair follicles [25]. We therefore investigated the expression of TCHHL1 mRNA in skin tissues obtained from both fetuses at 18–20 weeks gestation and cultured keratinocytes. The TCHHL1 mRNAs were strongly expressed in fetal skin, in which hair follicles had not yet been formed [32]. Furthermore, in spite of the fact that cultured keratinocytes include very few cells composed of hair follicles, TCHHL1 mRNA expression was observed in these cells. Accordingly, TCHHL1 proteins were also detected in growing keratinocytes by an immunofluorescent study. These findings support the results that TCHHL1 protein expressed not only in hair follicles but also in epidermis. The differences may be related to differences in the experimental conditions, including the specificity of antibodies.

The expression of TCHHL1 proteins was markedly increased in cancerous/non-cancerous skin samples with hyperproliferation of keratinocytes compared to normal skin samples. More cells expressing Ki-67, a marker of dividing cells, were observed in areas with TCHHL1 expression than in areas without TCHHL1 expression. These findings suggest that TCHHL1 proteins might be associated with the proliferation of keratinocytes. It is known that S100A11, which is another member of the S100 protein family, is expressed in the cytoplasm of basal cells in the human epidermis. S100A11 translocates to the nucleus during the process of cornification and it functions as a key mediator of either $Ca^{2+}$ or transforming growth factor beta-induced growth inhibition of human

keratinocytes [33,34]. Therefore, we speculated that TCHHL1 proteins might also play important roles in the proliferation of keratinocytes, similar to S100A11. Although further study is necessary to obtain to a better understanding of the functions of TCHHL1, our findings of the pattern of TCHHL1 expression in normal skin and the up-regulation of TCHHL1 expression in hyperproliferative keratinocytes will certainly contribute to extending knowledge of TCHHL1.

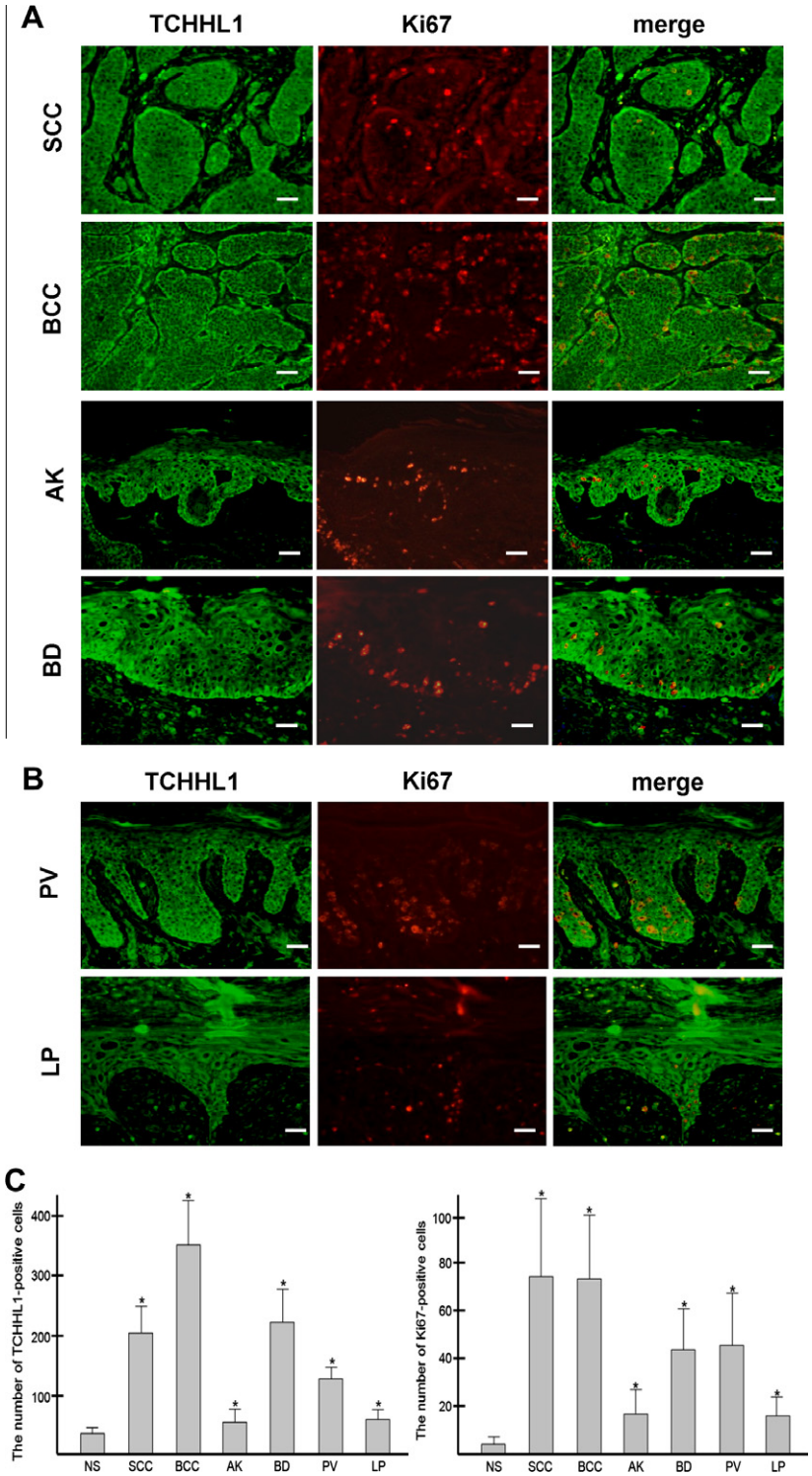


**Fig. 4.** Immunostaining for TCHHL1 proteins in pathologic skin tissues with hyperproliferation. (**A**) Squamous cell carcinoma samples (SCC), basal cell carcinoma (BCC) samples, actinic keratosis (AK) samples and Bowen's disease (BD) samples were doubly immunostained for TCHHL1 and Ki 67. The scale bar, 50 μm, is the same for all panels. (**B**) Psoriasis vulgaris (PV) samples and lichen planus (LP) samples were doubly immunostained for TCHHL1 and Ki 67. The scale bar, 50 μm, is the same for all panels. (**C**) The numbers of TCHHL1-positive cells and Ki67-positive cells in samples from patients with diseases were compared to samples of normal skin. Each value represents the mean ± SD (five sections from patients with each disease). $^{*}p < 0.001$ by Student's *t*-test, versus normal skin.

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