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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-