major mediator of skin innervation density,[10] with higher local NGF concentrations found in the lesional skin of patients with AD, psoriasis, contact dermatitis and xerosis.[5–9] In adult rat primary sensory neurones, NGF has been shown to upregulate neuropeptides, especially substance P (SP) and calcitonin-gene-related peptide,[11] both of which are involved in hypersensitivity to itch and in neurogenic inflammation.[4]

Neurotropin (NTP) is an analgesic drug currently used in Japan and China for the treatment of chronic refractory pain. The analgesic effects of NTP are believed to be due to the activation of the descending pain inhibitory system, but without affecting prostaglandins.[12,13] NTP also has antiallergic effects through its suppression of eosinophilic infiltration and its regulation of cholinergic receptors on the nasal mucosae.[14,15] The antipruritic effects of NTP on pruritus in eczema, dermatitis, urticaria and haemodialysis have also been validated by Japanese clinical studies, although the mechanisms underlying its antipruritic effects remain enigmatic.

In the present study, we examined the pharmacological effect of NTP on release of SP from cultured dorsal root ganglion (DRG) neurones and on NGF-induced neurite outgrowth.

## Methods

### Dorsal root ganglion neurone culture

The wells of 96-well tissue-culture plates were coated with poly-D-lysine and laminin (both Sigma, St. Louis, MO, USA). DRG neurones ($5 \times 10^3$ cells; Cambrex, Walkersville, MD, USA) were plated into each well for measurements of SP release, neurite outgrowth and methylthiazole tetrazolium (MTT) cytotoxicity. The DRG neurones were maintained in serum-free medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 ng/mL NGF, 87.5 ng/mL 5-fluoro-2′-deoxyuridine, 37.5 ng/mL uridine, 50 U/mL penicillin and 50 μg/mL streptomycin (all Sigma) and 5% N-2 supplement (Invitrogen) at 37 °C in 5% $CO_2$.

### Measurement of substance P content in the culture medium

DRG neurones cultured in 96-well tissue-culture plates for 5 days were incubated for 10 min at 37 °C in 5% $CO_2$ with 10–300 milli neurotropin units (mNU)/mL NTP in the presence or absence of 100 nmol/L capsaicin$_2$. Phosphate-buffered saline (PBS, pH 7.4) was used for the NTP-untreated controls. The culture medium was collected from each well and SP concentration was measured using a commercial substance P assay (R & D Systems, Minneapolis, MN, USA), based on the competitive binding technique, according to the manufacturer's instructions.

### Neurite outgrowth assay

DRG neurones were cultured in serum-free medium with 10–300 mNU/mL NTP or PBS (control) in the presence of NGF. After 24 or 48 h, the cells were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 10 min. The fixed cells in each well were examined under phase-contrast microscopy (DMIL; Leica, Wetzlar, Germany). For quantification of neurite outgrowth, nine random photographs were taken per well and the length of the longest process of DRG neurones was measured with BZ-H2A software (Keyence, Osaka, Japan). At least 100 cells from 2 replicate wells per group were analysed in each experiment. The data from five independent experiments (at least 500 cells per group) were used for statistical analysis.

### Methylthiazole tetrazolium cytotoxicity assay

The cytotoxicity of NTP toward cultured DRG neurones was assessed using an MTT assay (MTT Cell Growth Assay; Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. DRG neurones were cultured in serum-free medium with NTP (10–300 mNU/mL) or PBS (control). At designated time points, the cells were incubated with 100 μL MTT solution in medium for 4 h at 37 °C, 5% $CO_2$. The absorbance of MTT formazan produced in wells containing live cells was measured at 560 nm in a plate reader (1420 ARVO; PerkinElmer, Waltham, MA, USA).

### Statistical analysis

All values are presented as the mean ± SD of three or five independent experiments. Comparisons were made by one-way ANOVA with Tukey's multiple comparison test. $P < 0.05$ was considered significant.

## Results

### Effects of neurotropin on capsaicin-induced substance P release from cultured dorsal root ganglion neurones

At concentrations up to 100 mNU/mL NTP dose-dependently inhibited the capsaicin-induced release of

SP from cultured DRG neurones. SP release was not elicited by NTP alone (Fig. 1).

### Effects of neurotropin on nerve growth factor-induced neurite outgrowth in cultured dorsal root ganglion neurones

NTP dose-dependently inhibited NGF-induced neurite outgrowth in DRG neurones cultured for 24 h (Fig. 2a). This inhibitory effect was also seen in neurones cultured for 48 h with 300 mNU/mL NTP (Fig. 2b). In contrast, NTP alone had no effect on neurite outgrowth in the absence of NGF (Fig. 2c).

### Cytotoxicity of neurotropin toward cultured dorsal root ganglion neurones

Although we found that NTP significantly inhibited both capsaicin-induced SP release and NGF-induced neurite outgrowth in cultured DRG neurones, these inhibitory effects may have been due to the neuronal cytotoxicity of NTP. We therefore assessed NTP in MTT cytotoxicity assays on cultured DRG neurones. We found no significant differences in the degree of neuronal cytotoxicity between control and NTP-treated cells cultured for 24 or 48 h (Fig. 3).

## Discussion

NTP is an analgesic drug currently used in Japan and China for the treatment of chronic refractory pain. The antipruritic effects of NTP on pruritus of eczema, dermatitis, urticaria and haemodialysis have also been validated by Japanese clinical studies, although the mechanisms underlying this effect are incompletely understood.

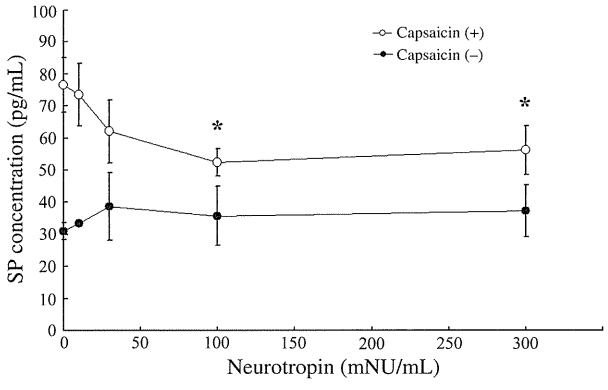


**Figure 1** Inhibition of capsaicin-induced substance (SP) release from cultured dorsal root ganglion (DRG) neurones by neurotropin (NTP). SP concentration was measured in culture media collected from cells incubated with NTP (10–300 mNU/mL) in the presence or absence of capsaicin (100 nmol/) for 10 min at 37 °C. Values are expressed as the mean ± SD of five independent experiments. *$P < 0.01$ (vs. capsaicin alone).

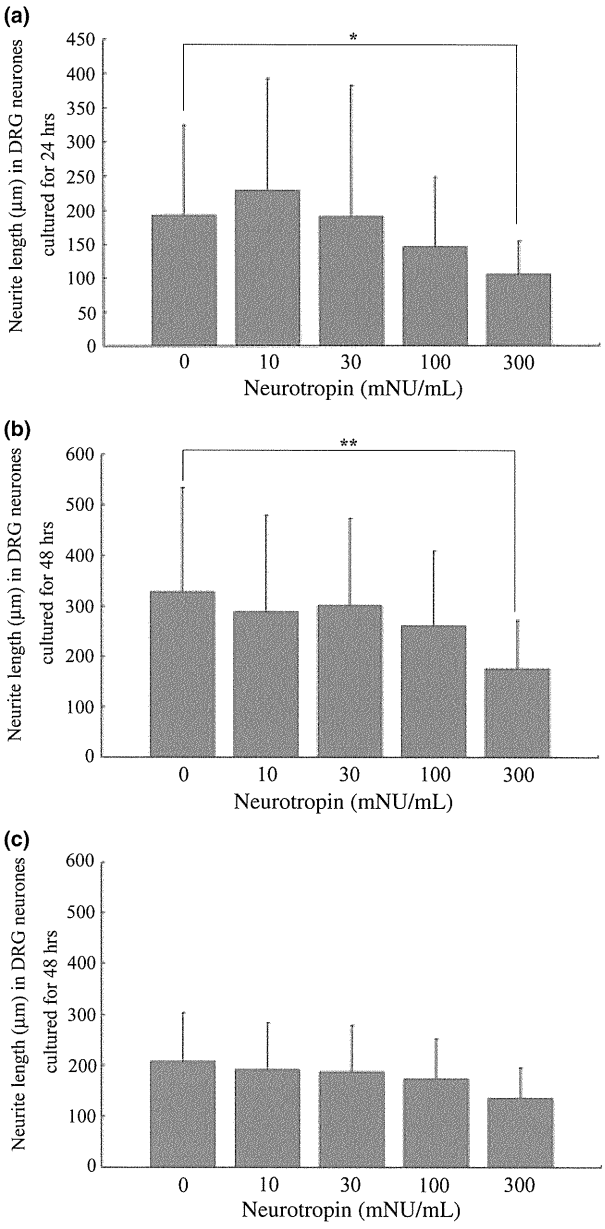


**Figure 2** Inhibition of nerve growth factor (NGF)-induced neurite outgrowth in cultured dorsal root ganglion (DRG) neurones by neurotropin (NTP). The length of the longest process was measured in DRG neurones incubated with NTP (10–300 mNU/mL) in the presence of NGF (10 ng/mL) for (a) 24 (b) or 48 h, or (c) in the absence of NGF for 48 h at 37 °C. Values are expressed as the mean ± SD of five independent experiments. *$P < 0.05$; **$P < 0.01$.

 

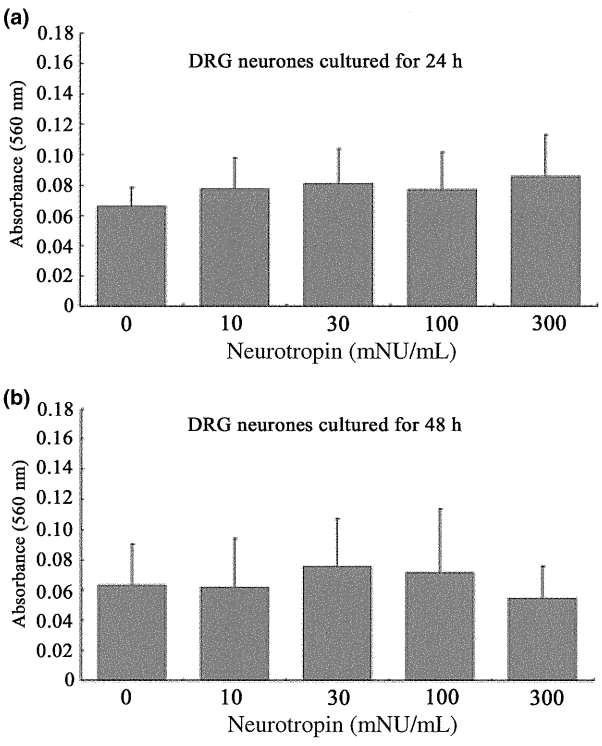


**Figure 3** Measurement of neurotropin (NTP) cytotoxicity on cultured DRG neurones. Methylthiazole tetrazolium (MTT) cytotoxicity assays were performed using dorsal root ganglion (DRG) neurones cultured with NTP (10–300 mNU/mL) in the presence of nerve growth factor (10 ng/mL) for (a) 24 h or (b) 48 h. Values are expressed as the mean ± SD of three independent experiments.

Using cultured DRG neurones, we found that NTP had was able to inhibit (i) capsaicin-induced SP release and (ii) NGF-induced neurite outgrowth. SP is a neuropeptide involved in neurogenic inflammation and pruritus, and has cutaneous effects on keratinocytes, including hair follicles, mast cells, fibroblasts and endothelial cells.[2–4] Increased epidermal SP-immunoreactive nerve fibres have been seen in human skin diseases such as AD and psoriasis. SP also stimulates release of tumour necrosis factor-α, histamine, prostaglandin $D_2$ and leucotrien $B_4$ from skin mast cells.[3] Moreover, acute immobilization stress induces skin mast-cell degranulation via SP release from sensory nerves.[4] Olopatadine hydrochloride, a selective histamine H1 receptor antagonist, significantly decreases serum SP levels in patients with AD due to modulatory effects of tachykinin release on cutaneous sensory nerves, resulting in the suppression of pruritus.[16] Thus, our findings suggest that the antipruritic effects of NTP are due to its inhibition of SP release from sensory nerve fibres in the skin. This may also explain the ability of NTP to decrease the plasma concentrations of SP in patients on haemodialyis who have pruritus and to decrease flare reactions to SP.[17–19]

NGF has been found to induce the sprouting of epidermal nerve fibres in atopic and dry skin.[3–9] Increased NGF expression has also been seen in the epidermis of patients with psoriasis and allergic contact dermatitis, concomitant with the increase of epidermal nerve densities.[5,6] In some cases, the pruritus of these dermatoses cannot be controlled by treatment with antihistamines.[1–3] Recent studies of atopic NC/Nga mice found that anti-NGF antibodies and NGF receptor inhibitors significantly inhibited both the sprouting of epidermal nerve fibres and the scratching behaviour of the mice.[20,21] These findings suggest that epidermal nerve growth is partly related to intractable pruritus, uncontrolled by antihistamines. Moreover, ultraviolet phototherapy has been found to reduce the number of cutaneous nerve fibres, especially those in the epidermis of patients with AD or psoriasis,[22] and the intense itch experienced by patients with these dermatoses can also be controlled by phototherapy. Taken together, the results of our neurite outgrowth assays suggest that the antipruritic effects of NTP are due to its inhibition of NGF-induced neurite outgrowth. Additionally, SP and neurokinin A directly induce NGF expression in epidermal keratinocytes.[23] Therefore, NTP may also inhibit SP-inducible NGF expression in keratinocytes by modulating the effects of SP release on cutaneous sensory nerves.

Although NTP inhibited both capsaicin-induced SP release and NGF-induced neurite outgrowth in cultured DRG neurones, the inhibitory mechanisms remain unclear. Aptamers are oligonucleic acid or peptide molecules that bind a specific target molecule. These molecules are usually selected from large random sequence pools, but natural aptamers may also be present in riboswitches.[24] Recently, aptamers have been used both for basic research and for clinical purposes as macromolecular drugs.[25] NTP contains small molecules such as nucleic acids, amino acids and sugars (Nippon Zouki Pharmaceutical Company, unpublished observations). Therefore, this drug may contain aptamers that bind certain molecules involved in neuromodulation. If purified, the components of the NTP biological extract that have inhibitory effects on SP release and/or neurite outgrowth may act as effective antipruritic drugs.

In conclusion, our *in vitro* results using DRG neurones found that NTP inhibits both capsaicin-induced SP release and NGF-induced neurite outgrowth without neuronal cytotoxicity. These findings may increase understanding of the antipruritic effects of NTP in skin diseases and/or haemodialysis.

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# Keratinocyte-derived anosmin-1, an extracellular glycoprotein encoded by the X-linked Kallmann syndrome gene, is involved in modulation of epidermal nerve density in atopic dermatitis

Suhandy Tengara [a], Mitsutoshi Tominaga [a], Atsuko Kamo [a], Kenichi Taneda [b], Osamu Negi [a,b], Hideoki Ogawa [a], Kenji Takamori [a,b,*]

[a] Institute for Environmental and Gender Specific Medicine, Juntendo University Graduate School of Medicine, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan
[b] Department of Dermatology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan

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ABSTRACT

*Background:* Epidermal nerve density is increased in atopic dermatitis (AD), suggesting that the hyperinnervation is partly responsible for abnormal itch perception. It is probably controlled by axonal guidance molecules produced by keratinocytes. An extracellular matrix glycoprotein anosmin-1 encoded by *KAL1* has chemoattractive or chemorepulsive effects on different neuronal types.
*Objective:* This study was performed to investigate the roles of anosmin-1 in skin innervation.
*Methods:* Rat dorsal root ganglion (DRG) neurones were cultured in conditioned medium from control or *KAL1*-overexpressing cells for neurite outgrowth assay. *KAL1* expression in cultured epidermal keratinocytes or human skin was examined by quantitative RT-PCR (qRT-PCR). Anosmin-1 distribution in normal and atopic skin was examined immunohistochemically. The effects of calcium concentrations and cytokines on *KAL1* expression in cultured normal human epidermal keratinocytes (NHEK) were analysed by qRT-PCR.
*Results:* Neurite outgrowth in cultured DRG neurones was inhibited by conditioned medium from *KAL1*-overexpressing cells, while it was rescued by addition of recombinant fibroblast growth factor receptor 1 for capturing anosmin-1. *KAL1* transcripts were expressed in cultured keratinocytes or in normal skin. Anosmin-1 was strongly expressed in the basal cell layer of normal skin, but decreased in atopic skin, concomitant with increases of epidermal nerve fibres. *KAL1* expression was downregulated during keratinocyte differentiation. The expression was also upregulated by interleukin-4 (IL-4), IL-13 or transforming growth factor (TGF)-β1. TGF-β1 acted synergistically with IL-13 to enhance *KAL1* expression, while interferon-γ inhibited its expression.
*Conclusion:* Anosmin-1 produced by epidermal keratinocytes in response to calcium concentrations or cytokines may modulate epidermal nerve density in AD.


## 1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease with pruritus as one of the most important symptoms. Histamine is the best-known pruritogen and has been regarded as a target of antipruritic therapy. However, there remain some patients whose pruritus is not satisfactorily improved by antihistamines [1–3].

Histological observations have indicated that cutaneous unmyelinated nerve fibres are present at higher densities in the epidermis of AD patients with intractable pruritus, suggesting that the hyperinnervation is partly responsible for abnormal itch perception [2–4]. Hyperinnervation in AD is probably caused by nerve growth factor (NGF) released from keratinocytes. Moreover, recent studies have demonstrated that amphiregulin, gelatinase

*Abbreviations:* AD, atopic dermatitis; DRG, dorsal root ganglion; FGFR1, fibroblast growth factor receptor 1; GnRH, gonadotropin-releasing hormone; IFN-γ, interferon-γ; IL, interleukin; K10, keratin 10; K14, keratin 14; *KAL1*, Kallmann syndrome 1 sequence; NHEK, normal human epidermal keratinocytes; NGF, nerve growth factor; PBS, phosphate buffered saline; PGP9.5, protein gene product 9.5; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; Sema3A, semaphorin 3A; STAT6, signal transducer and activator of transcription 6; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α.

* Corresponding author at: Department of Dermatology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan. Tel.: +81 47 353 3171; fax: +81 47 353 3178.
*E-mail address:* ktakamor@juntendo.ac.jp (K. Takamori).

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and tumour necrosis factor, which are nerve elongation factors, and semaphorin 3A (Sema3A), which is a nerve repulsion factor, are involved in modulation of nerve density in atopic skin [5–8]. Thus, various nerve elongation and repulsion factors are related to the pathogenesis of AD.

The *KAL1* gene (Kallmann syndrome 1 sequence), encoding the extracellular matrix glycoprotein anosmin-1, is responsible for the X chromosome-linked recessive form of Kallmann syndrome [9,10]. This molecule has been shown to play a number of roles in different events during neural development. It promotes the migration of gonadotropin-releasing hormone (GnRH)-producing neurones, guides the navigation of axons from mitral cells, as well as participating in the formation of their collaterals and stimulates outgrowth and branching of Purkinje axons *in vitro* [11–14]. Interestingly, anosmin-1 also has an inhibitory effect on neurite outgrowth in cerebellar granular neurones co-cultured with anosmin-1-overexpressing CHO cells [11]. These findings indicate that anosmin-1 plays various roles in a neurone type-specific manner. One study also indicated that *KAL1* transcripts are overexpressed in dermatomyositis, suggesting a fibrogenic effect of chronic inflammation [15]. However, the expression patterns and roles of anosmin-1 in inflammatory skin diseases such AD are unknown.

In the present study, we examined the expression patterns in human skin and the roles of anosmin-1 in skin innervation of patients with AD using skin biopsies, cultured epidermal keratinocytes and rat DRG neurones. Here, we report a possible role of anosmin-1 in modulation of epidermal nerve density in AD.

## 2. Materials and methods

### 2.1. Antibodies and reagents

Primary antibodies used in this study were as follows: mouse anti-anosmin-1 antibody (1:100 dilution, Abnova, Taipei, Taiwan), mouse anti-neuronal class III β-tubulin (Tuj1, 1:500 dilution; Convance, Berkeley, CA, USA), mouse anti-type IV collagen (1:20 dilution; Progen Biotechnik GmbH, Heidelberg, Germany), guinea pig anti-keratin-14 (K14, 1:200 dilution; Progen Biotechnik GmbH, Heidelberg, Germany), rabbit anti-protein gene product 9.5 (PGP9.5, 1:4000 dilution; BIOMOL International L.P., PA, USA). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were purchased from Molecular Probes (Eugene, OR, USA). For Western blotting, horseradish peroxidase-conjugated rabbit anti-mouse IgG was purchased from DAKO (Glostrup, Denmark).

All recombinant human cytokines and recombinant fibroblast growth factor receptor 1 (rhFGFR1) used in this study were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human anosmin-1 fragment (residues 548–658) was purchased from Abnova. Dulbecco's Modified Eagle's Medium (DMEM), bovine serum albumin, poly-D-lysine, recombinant human nerve growth factor (rhNGF), 5-fluoro-2′-deoxyuridine, uridine and Ponceau solution were purchased from Sigma (St. Louis, MO, USA). Minimal Essential Medium Alpha (MEM-α), DMEM/F12, N-2 supplement and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA, USA). Trypsin/ethylenediaminetetraacetic acid (EDTA) solution, Keratinocyte Basal Medium (KBM-2) and KGM-2 SingleQuots were purchased from Clonetics (Walkersville, MD, USA). Trypsin solution was purchased from Life Technologies (Karlsruhe, Germany). Normal donkey serum was purchased from Chemicon (Temecula, CA, USA). VECTASHIELD mounting medium was purchased from Vector Laboratories (Burlingame, CA, USA). An enhanced chemiluminescence detection kit was purchased from Amersham Biosciences (Piscataway, NJ, USA).

### 2.2. Skin biopsies

Three-millimetre punch biopsies were taken with informed consent from the lesional skin of 10 patients (8 men and 2 women, average age 31.3 years) with AD diagnosed according to Hanifin and Rajka criteria [16], and 8 healthy male volunteers (average age 29.8 years) with no history of skin disease. The degree of pruritus was evaluated by visual analogue scale score, and its mean in the patients with AD was 82.7%. None of the patients took oral corticosteroid or cyclosporine, and topical corticosteroids were not applied for more than 1 week before the collection of skin samples.

All of the protocols used to obtain skin biopsies were approved by the Medical Ethical Committee of Juntendo University Urayasu Hospital for the Protection of Human Subjects. The clinical investigation was conducted according to the Principles of the Declaration of Helsinki.

### 2.3. Animals

Male ICR mice (8–12 weeks; SLC Japan, Shizuoka, Japan) were maintained in Juntendo University Graduate School of Medicine's experimental animal facility. They were kept under a 12-h light:12-h dark cycle at 22–24 °C. Food and tap water were provided ad libitum. Mice care and handling conformed to the NIH guidelines for animal research. All animal procedures were approved by the Institutional Animal Care and Use Committee at Juntendo University Graduate School of Medicine.

### 2.4. Cell cultures

HaCaT cells were cultured in DMEM containing 10% heat-inactivated foetal bovine serum supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin.

Normal human epidermal keratinocytes (NHEK) were purchased from Cambrex (Walkersville, MD, USA). They were cultured in KBM-2 supplemented with KGM-2 SingleQuots (0.4% bovine pituitary extract, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% hydrocortisone and 0.1% gentamicin). Cells at passages 1–3 were used for *in vitro* experiments. Trypsin/EDTA solution was used for detaching the cells during passages. NHEK were grown until 80–90% confluency. For keratinocyte differentiation, the cells were cultured in the presence of 1.4 mM $CaCl_2$ for 5 or 14 days.

CHO cells were cultured in MEM-α containing heat-inactivated foetal bovine serum at 10% supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. Cells at passages 1–3 were used for *in vitro* experiments. Trypsin solution was used for detaching the cells during passages. All cells were cultured at 37 °C, 5% $CO_2$.

### 2.5. Immunohistochemistry

Skin samples were embedded in Optimal Cutting Temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and cryosections 8 μm thick were cut using a CM1850 cryostat (Leica, Wetzlar, Germany). The sections were mounted on silane-coated glass slides and then were fixed with ice-cold acetone for 10 min at −20 °C. They were washed three times with phosphate buffered saline (PBS, pH 7.4) and blocked in PBS with 5% normal donkey serum and 2% bovine serum albumin. The sections were incubated with primary antibodies overnight at 4 °C. After washing with PBS, secondary antibodies were added to the sections for 1 h at room temperature. The sections were mounted in VECTASHIELD mounting medium. As controls, the primary antibodies were either omitted or replaced with normal IgG. Immunoreactivity was visualized with a confocal laser-scanning microscope (DMIRE2; Leica). Immunostanning for cutananeous nerve fibres was performed as described previously [5].

### 2.6. Total RNA preparation

Total RNA from cultured cells was isolated by using an RNeasy Mini Kit (Qiagen KK, Tokyo, Japan). Total RNA from skins of human or mouse was isolated using an RNeasy Fibrous Mini Kit (Qiagen KK) according to the manufacturer's guidelines. Universal Human Reference RNA (UHR RNA; Stratagene, La Jolla, CA, USA) and mouse skin RNA were used as positive and negative controls for quantitative reverse transcription-polymerase chain reaction (qRT-PCR), respectively.

### 2.7. QRT-PCR

The protocols for qRT-PCR analysis were described previously [5]. The primers used in this study are listed in Table 1. They were designed to meet specific criteria and were synthesised using Perfect Real Time support system (TaKaRa Bio Inc., Kyoto, Japan). The specificity of PCR was confirmed by dissociation curve analysis and gel electrophoresis. QRT-PCR was performed in triplicate, and the measured mRNA levels are expressed relative to the internal reference ribosomal protein S18 (*RPS18*) mRNA level and further adjusted to the level in the control group.

### 2.8. Transfection of plasmid into CHO cells

*KAL1* cDNA (accession no. NM_000216.1) in the mammalian expression vector pCMV6-XL4 was purchased from OriGene (Rockville, MD, USA). For transfection of plasmid into CHO cells, the cells were grown in culture plates in DMEM/F12 for 24 h and incubated with pCMV6-XL4-KAL1 or mock vector in the presence of the Lipofectamine™ 2000 reagent according to the manufacturer's instructions. After 24 h, conditioned media were collected from cultures of transfected cells for neurite outgrowth assay. The levels of anosmin-1 in the conditioned media were confirmed by Western blot analysis.

### 2.9. Western blot analysis

Conditioned media were collected from CHO cells transfected with pCMV6-XL4-KAL1 or with vector alone, and then concentrated with Amicon Ultra-4 (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Equal amounts of total protein (50 μg per lane) were applied to 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred onto Immobilon-P Transfer Membranes (Millipore) by Electroblotting Trans Blot (Bio-Rad Laboratories, Hercules, CA, USA). After blocking, the membranes were incubated with anti-anosmin-1 antibody (1:1000 dilution; Abnova, Taipei, Taiwan) overnight at 4 °C. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was used as the secondary antibody. The membrane was developed with an enhanced chemiluminescence detection kit. Autoradiographs were prepared by exposing the membranes to X-ray film (Kodak Japan, Tokyo, Japan). After Western blotting, the membranes were stained with Ponceau solution.

**Table 1**
Sequences of primer pairs used for quantitative RT-PCR analyses.

| Gene | Product (bp) | Primer | Sequence (5′–3′) |
|---|---|---|---|
| *KAL1* | 108 | Forward | CAACCGGATCAGAGGCATCA |
| | | Reverse | TTGGCCGTATTGGTTGGACA |
| *K14* | 136 | Forward | CAAGACCATTGAGGACCTGAGGA |
| | | Reverse | GCGCAGGTTCAACTCTGTCTCATA |
| *K10* | 111 | Forward | AGGCTGGCAGCTGATGACTTC |
| | | Reverse | CAGGGTCAGCTCATCCAGCA |
| *Human RPS18* | 123 | Forward | ACTCAACACGGGAAACCTCA |
| | | Reverse | AACCAGACAAATCGCTCCAC |
| *Mouse RPS18* | 128 | Forward | AGGATGTGAAGGATGGGAAG |
| | | Reverse | ACGAAGGCCCCAAAAGTG |

KAL1, Kallmann syndrome 1 sequence; K14, keratin 14; K10, keratin 10; RPS18, ribosomal protein S18.

### 2.10. Neurite outgrowth assay

Bio Falcon™ CultureSlides (BD Falcon, Bedford, MA, USA) were coated with poly-D-lysine, and then neonatal rat DRG neurones ($4 \times 10^3$; Cambrex) were cultured in conditioned medium from cells transfected with pCMV6-XL4-KAL1 or vector alone. Neuronal survival factors (0.1 ng/ml rhNGF and 0.5% N-2 supplement) and cell growth inhibitors (87.5 ng/ml 5-fluoro-2′-deoxyuridine and 37.5 ng/ml uridine) were added to the conditioned media. In some experiments, rhFGFR1 (50 ng/ml) was added to the culture system to capture free anosmin-1 for 24 and 48 h. Immunocytochemistry was performed using anti-neuronal class III β-tubulin antibody (Tuj1) to visualize neurites as described previously [17]. Immunoreactivity was observed by fluorescence microscopy (BIOREVO BZ-9000; Keyence, Osaka, Japan). Neurite lengths in whole culture area (0.69 $cm^2$) were measured using BZ Analyzer II software (Keyence) and then mean total neurite length per neurone was determined.

### 2.11. Treatment of cultured cells with cytokines

NHEK were cultured in triplicate in 6-well plates in 2 ml KBM-2 supplemented with KGM-2 SingleQuots for 24 h to allow adherence. The cells were washed and incubated with the indicated concentrations of cytokines. After incubation for 24 h, total RNA was isolated from the cultured cells as described above.

### 2.12. Statistical analysis

Statistical analyses were performed by two-tailed Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparison test.

## 3. Results

### 3.1. Anosmin-1 inhibited neurite outgrowth in cultured DRG neurones

The effects of anosmin-1 on neurite outgrowth of sensory neurones were examined in rat DRG neurones cultured with conditioned media from *KAL1*-overexpressing cells. After 24 h in culture, no differences were observed between neurones cultured in conditioned media from control cells and *KAL1*-overexpressing cells with or without rhFGFR1 (data not shown). After 48 h, the neurones cultured in conditioned medium from control cells displayed long and ramified neurites (Fig. 1a), whereas the neurite length of the neurones cultured in conditioned medium from *KAL1*-overexpressing cells was reduced relative to the controls (Fig. 1b and d). The neurones cultured in the conditioned medium with rhFGFR1 showed longer neurites compared to that in controls (Fig. 1c and d). We also confirmed by Western blot analysis whether anosmin-1 was secreted into the conditioned media. Anosmin-1 was detected in at approximately 100 kDa (full-length) and 40 kDa (C-terminal part) in conditioned media from *KAL1*-overexpressing cells, but was consistently absent in the conditioned media from control cells (Fig. 1e).

### 3.2. Expression and distribution of anosmin-1 in skin from healthy controls and patients with AD

In RT-PCR analyses, *KAL1* transcripts were expressed in cultured keratinocytes and normal skin (Fig. 2a). To further determine the

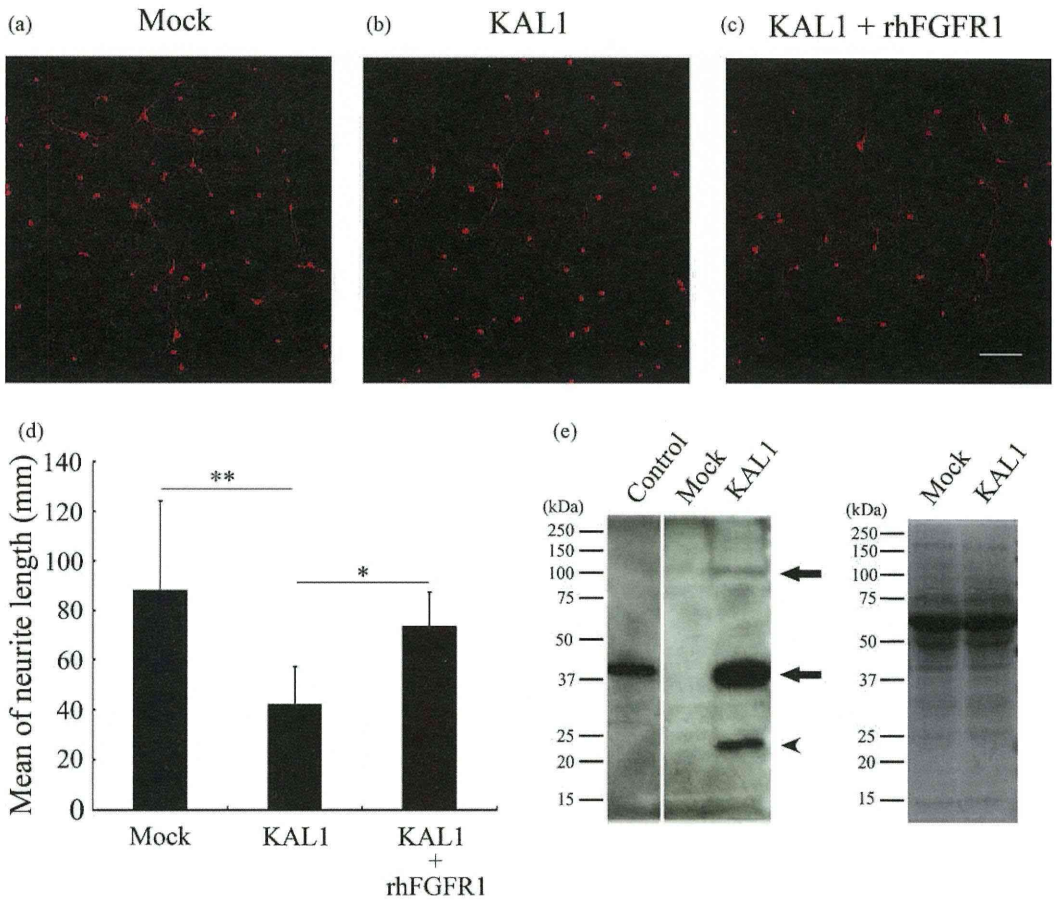


**Fig. 1.** Effects of anosmin-1 on neurite outgrowth of cultured DRG neurones. (a–c) DRG neurones were cultured for 48 h in conditioned medium from mock vector-transfected cells (a) or pCMV6-XL4-KAL1-transfected cells (b) and pCMV6-XL4-KAL1-transfected cells with 50 ng/ml rhFGFR1 (c). The cultured neurones were immunostained with Tuj1 antibody (red). Scale bar: 200 μm. (d) Mean total neurite length per neurone at 48 h in culture was determined over the whole culture area (0.69 $cm^2$). The neurite length of the neurones cultured in conditioned medium from *KAL1*-overexpressing cells was significantly reduced compared with the controls, while its inhibitory effect was blocked by addition of rhFGFR1. Values are means ± SD from four independent experiments ($^*P < 0.05$; $^{**}P < 0.01$). (e) Western blot analysis of anosmin-1 secreted into the conditioned media from CHO cells transfected with vector alone or with pCMV6-XL4-KAL1. Immunoreactive bands of anosmin-1 were detected at approximately 100 and 40 kDa (arrows in left panel). A degradation product was also detected (arrowhead). Recombinant anosmin-1 fragment was used as a positive control (0.15 μg per lane). After Western blotting, the membranes were stained with Ponceau solution (right panel).

distribution of anomin-1 in human skin, skin specimens from healthy controls and patients with AD were subjected to immunolabelling with anti-anosmin-1 antibody. In the normal skin, anosmin-1 was strongly expressed in the basal layer (Fig. 2b). Anosmin-1 was also expressed in some dermal cells, such as vascular endothelial cells and fibroblasts, as described previously [15]. Weak signals were detected in the suprabasal cell layer and stratum corneum with normal IgG (data not shown).

In comparison with healthy controls, the expression of anosmin-1 in the basal layer of atopic skin was reduced, and the distribution was diffuse in the epidermis (Fig. 3a and b). On qRT-PCR analysis, *KAL1* expression levels tended to be lower in patients with AD compared with healthy controls although the differences were not statistically significant (Fig. 3c). We also found the increasing of epidermal nerve fibres in patients with AD compare with healthy controls (Fig. 3d–f).

### 3.3. Expression of KAL1 is downregulated by keratinocyte differentiation

*KAL1* expression during keratinocyte differentiation was examined in NHEK treated with 1.4 mM $CaCl_2$ for 5 or 14 days. In qRT-PCR analysis, *KAL1* expression was significantly downregulated in the differentiated cells (Fig. 4a). The differentiation status was confirmed by examining expression of the proliferation-specific marker keratin 14 (Fig. 4b) and the differentiation marker keratin 10 (Fig. 4c).

### 3.4. Regulation of KAL1 expression by cytokines in cultured NHEK

To further examine the effects of cytokines on *KAL1* expression, qRT-PCR analyses were performed on NHEK cultured in low-calcium serum-free medium with several cytokines for 24 h. The analyses showed that *KAL1* expression was significantly upregulated in the cultured NHEK stimulated by IL-13, IL-4, or TGF-β1 but not with the others, in comparison with controls (Fig. 5a). These cytokines also dose-dependently induced *KAL1* expression (Fig. 5b, c, and d).

The cross-regulation in the effects of cytokines on *KAL1* expression was examined in NHEK treated with IL-13 and in combination with the other cytokines mentioned above for 24 h. QRT-PCR analyses showed that IL-13 and TGF-β1 acted synergistically to enhance *KAL1* expression in the cultured NHEK, while IFN-γ inhibited gene expression induced by IL-13 (Fig. 5e). These effects were also dose-dependent (Fig. 5f and g).

## 4. Discussion

*KAL1* encodes anosmin-1 and underlies the X-linked form of Kallmann syndrome, a neurological disorder that impairs development of the olfactory and GnRH systems [9,10]. Anosmin-1 has

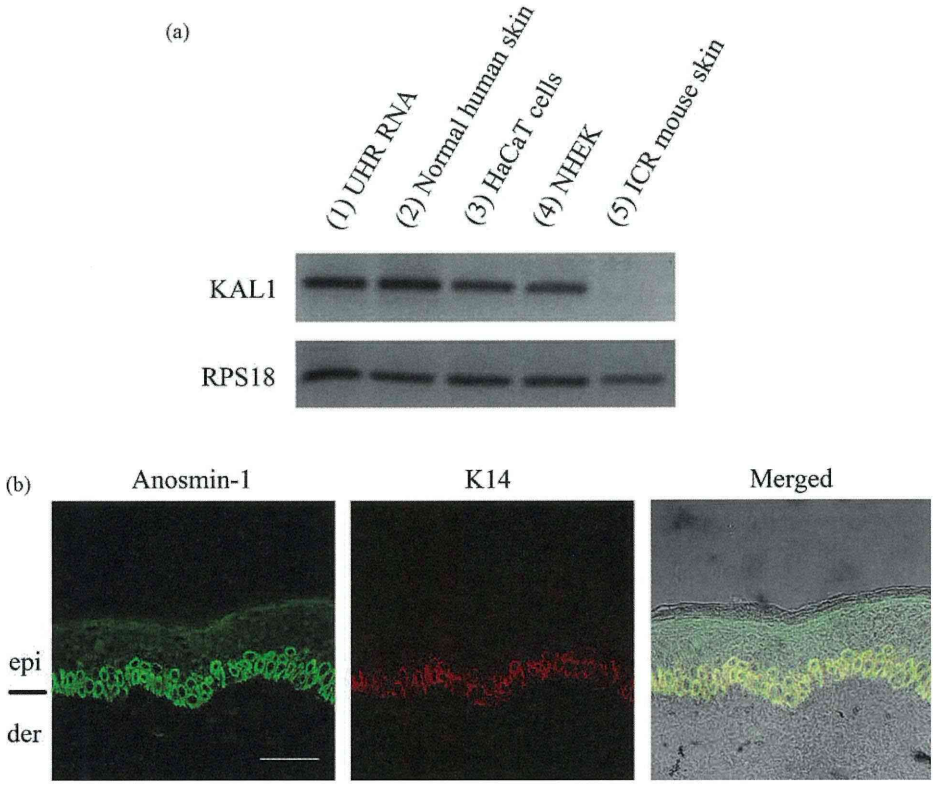


**Fig. 2.** Expression analyses of *KAL1* in cultured keratinocytes and normal human skin. (a) Total RNA was isolated from cultured HaCaT cells and NHEK, and RT-PCR was performed with specific primers for *KAL1*. Lane 1, Universal Human Reference (UHR) RNA (positive control); lane 2, normal human skin; lane 3, HaCaT cells; lane 4, NHEK; lane 5, ICR mouse skin (negative control). *RPS18* was used as an internal control for mRNA intensity and quality. (b) Double-labelling for anosmin-1 (green) and keratin-14 (K14, red) was performed on cryosections of normal human skin. Strong anosmin-1 immunoreactivity was detected in K14-positive cells and in some dermal cells. These images of anosmin-1 and K14 were superposed. Yellow areas in the merged image are double-labelled. Scale bar: 50 μm. epi, epidermis; der, dermis.

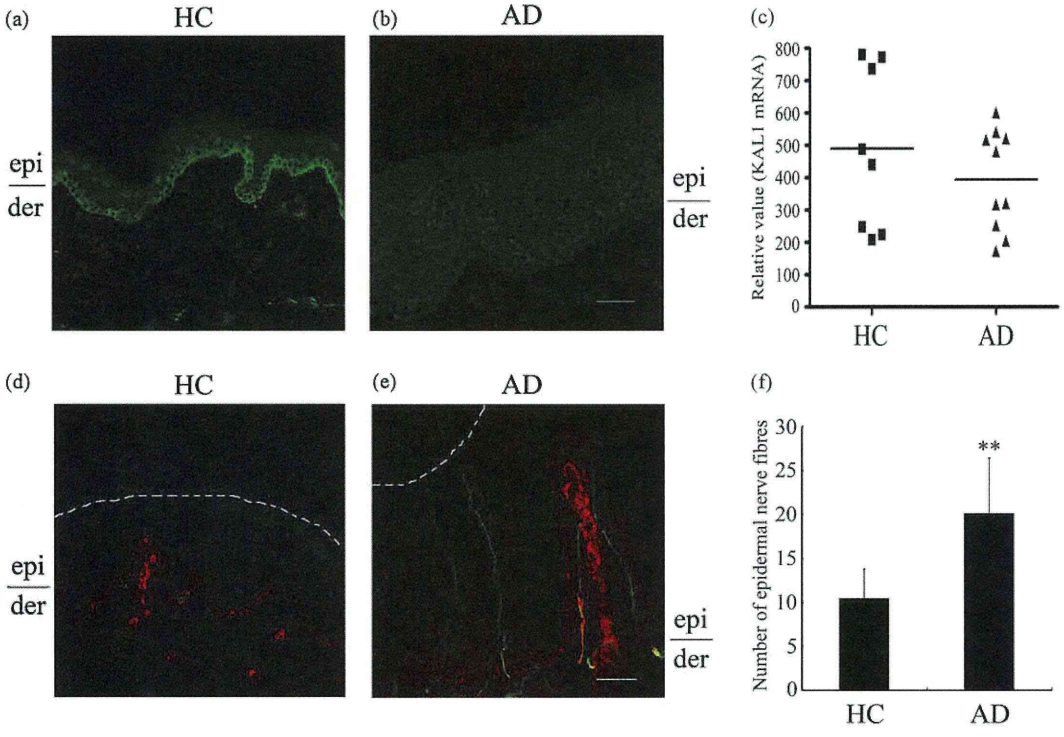


**Fig. 3.** Decreased expression of anosmin-1 in the lesional skin of patients with AD. Immunolabelling with anti-anosmin-1 antibody (green) in the skin of healthy control (a) and AD patient (b). Anosmin-1 was strongly expressed in the basal cell layer of normal skin, while the expression was decreased in the basal cell layer of atopic skin. (c) QRT-PCR analysis for *KAL1* mRNA expression was performed in the skin of healthy controls (HC) ($n = 8$) and patients with AD ($n = 10$). The levels of *KAL1* mRNA expression were calculated by the comparative $C_t$-method compared to *RPS18*. (d and e) Double immunostanning with anti-PGP9.5 (green) and type IV collagen (red) in skins from healthy controls (d) and AD patients (e). PGP9.5 positive fibres were occasionally present in the normal epidermis. In contrast, the epidermal nerve fibres were observed at higher densities in AD patients. White broken lines in panels (d) and (e) indicate the skin surface. Scale bars = 50 μm. (f) The number of epidermal nerve fibres was significantly increased in AD patients compared with that in healthy controls. Values are mean ± SD from three experiments (**$P < 0.01$).

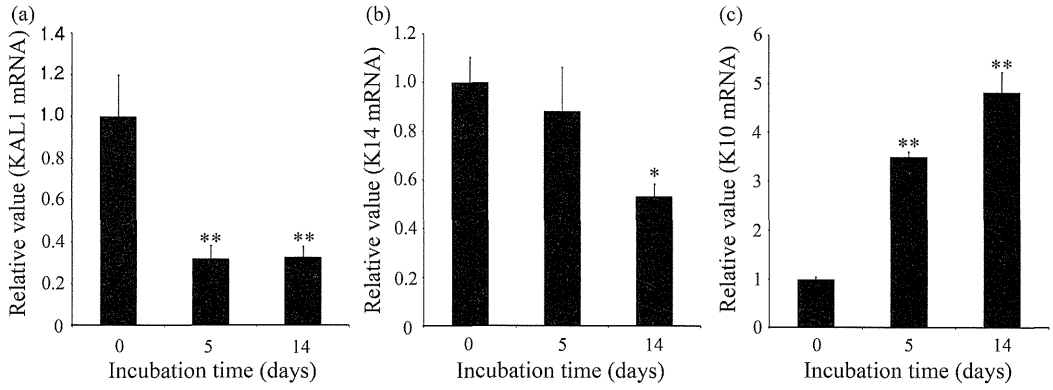


**Fig. 4.** *KAL1* expression is regulated by calcium concentration. NHEK treated with 1.4 mM $CaCl_2$ for 5 or 14 days, and qRT-PCR was performed using *KAL1* (a), *K14* (b) and *K10* (c) primers. *KAL1* expression was significantly downregulated in the differentiated cells. *K14* and *K10* genes were used as markers of proliferation and differentiation, respectively. The levels of mRNA expression were calculated by the comparative Ct-method in comparison to *RPS18*. Results are shown relative to the levels of gene expression in cells cultured with low-calcium serum-free medium (0 day). Values are means ± SD from three independent experiments (*$P < 0.05$; **$P < 0.01$).

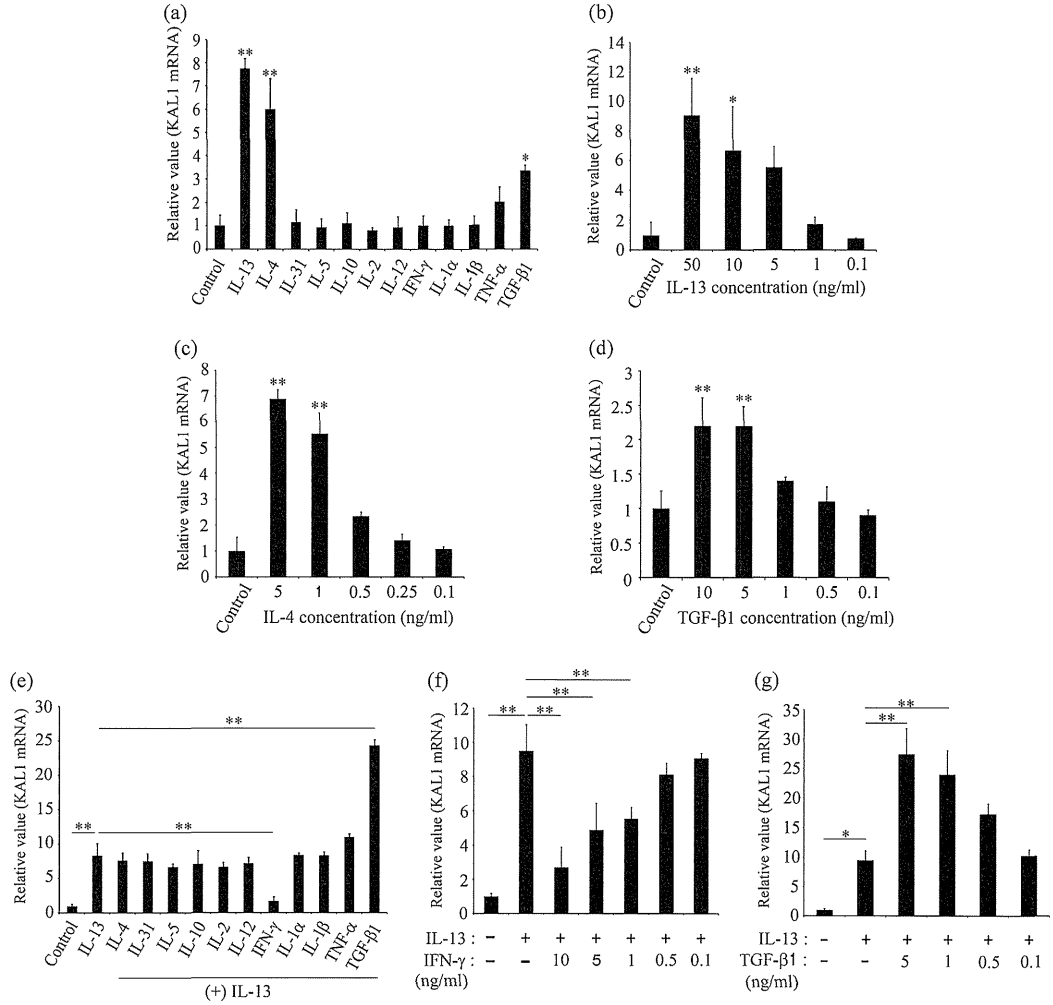


**Fig. 5.** Regulation of *KAL1* expression by cytokines in cultured NHEK. (a) Total RNA was isolated from NHEK stimulated with IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-31, IFN-γ, TNF-α and TGF-β1 at 50 ng/ml, or IL-1β and IL-1α at 50 pg/ml for 24 h, and then the expression level of *KAL1* was determined by qRT-PCR analysis. (b–d) *KAL1* expression was significantly upregulated in the cultured NHEK stimulated by IL-13, IL-4, or TGF-β1. IL-13 (b), IL-4 (c) and TGF-β1 (d) dose-dependently increased the expression levels of *KAL1* in cultured NHEK. (e) QRT-PCR analyses were performed on NHEK treated with IL-13 (50 ng/ml) and in combination with the other cytokines and concentrations mentioned above for 24 h. IFN-γ inhibited IL-13-induced *KAL1* expression, while TGF-β1 enhanced its expression in the presence of IL-13. (f and g) IFN-γ (f) and TGF-β1 (g) showed dose-dependent effects on IL-13-induced *KAL1* expression. The levels of mRNA expression were calculated by the comparative Ct-method in comparison to *RPS18*. Results are shown relative to the levels of gene expression in non-treated cells (control). Values are means ± SD from three independent experiments (*$P < 0.05$; **$P < 0.01$).

been shown to play roles in cell adhesion, cell migration, axonal guidance and branching activities [11–14,18].

The present study demonstrated the inhibitory effect of anosmin-1 on neurite outgrowth in cultured DRG neurones. Such inhibitory effects have been demonstrated in cerebellar neurones co-cultured with anosmin-1-producing cells. Thus, anosmin-1 may act as a nerve repulsion factor in human skin. Meanwhile, the neurite outgrowth was promoted in the cerebellar neurones cultured on anosmin-1-coated plates [11]. This reciprocal result suggests an allosteric mode of action of anosmin-1 on neurite outgrowth.

A recent study has indicated that anosmin-1 binding to fibroblast growth factor receptor 1 (FGFR1) inhibits FGF2–FGFR1–heparan sulfate complex formation [19]. In our DRG neuronal culture system, the neurite outgrowth was also rescued by addition of rhFGFR1. Activation of FGFR1 on adult sensory neurones is involved in promotion of neurite outgrowth [20]. However, we did not use FGF2 in the DRG neuronal culture system. Therefore, although we could not exclude FGF2 produced by CHO cells, this might imply the existence of other receptors or targets for anosmin-1 related to neurite outgrowth.

Unlike our observations, previous studies also indicated that anosmin-1 had no effect on axonal outgrowth of rodent DRG neurones [11,12]. This discrepancy may be explained by different responses to anosmin-1 in cultured neurones derived from different developmental stages.

Sensory nerve fibres are acceptors of itch (or pain) sensations in the skin. A number of studies have demonstrated that epidermal nerve fibres are present at higher densities in the skin of patients with AD than in control individuals [2]. Similar findings have been observed in animal models, such as NC/Nga mice [5]. Our recent studies also demonstrated that the epidermal nerve density is regulated by nerve elongation factors (e.g., NGF, amphiregulin, gelatinase) and repulsion factors (e.g., Sema3A) [5–7]. These findings are indicative of increases in sensory receptors responsive to exogenous trigger factors and to various endogenous pruritogens from immune cells and keratinocytes, suggesting that hyperinnervation is partly responsible for intense itch sensations [2]. This was also supported by recent studies indicating that anti-NGF and recombinant Sema3A replacement approaches suppressed pruritus in NC/Nga mice [21,22].

In all patients with AD examined in this study, immunoreactivity of anosmin-1 reduced in the basal cell layer compared with healthy controls. Our immunohistological data showed increases of epidermal nerve fibres in the all patients. Therefore, these imply a correlation between anosmin-1 levels in the basal cell layer and epidermal nerve number. Unlikely anosmin-1 levels, the levels of *KAL1* mRNA were slightly decreased in the skin of AD patients. This might suggest the existence of post-transcriptional regulation in anosmin-1 expression. Another possibility is a complex regulation of *KAL1* expression by several Th1 and Th2 cytokines *in vivo*. This idea is supported by our data using cultured human keratinocytes.

Epidermal innervation is probably regulated by nerve elongation factor levels in the atopic skin, and not only by nerve repulsion factor levels [7]. In our preliminary data, no close relationship between number of epidermal nerve fibres and Sema3A levels was observed in psoriasis patients with pruritus (Taneda et al., unpublished observations). Although patients with Kallmann syndrome do not express anosmin-1 due to the lack of the *KAL1* gene [9,10], there have been no reports of itchy skin in Kallmann syndrome [23]. Taken together, epidermal innervation could be regulated by combination of several axonal guidance molecules in many skin diseases with pruritus. Therefore, altered balance of expression of these molecules will be needed to investigate in the skin diseases with pruritus.

It has been shown that Sema3A is mainly distributed in upper layers of epidermis [7]. Meanwhile, we showed that anosmin-1 was mainly distributed in the basal cell layer. Additionally, anosmin-1 may function in neurite outgrowth by different mechanisms of Sema3A [19]. Therefore, the different axon guidance regulators may be involved in the topography formation of sensory nerves in the human skin [24].

The mammalian epidermis shows a characteristic calcium gradient, and normal keratinocyte differentiation can be reproduced *in vitro* by culturing cells in media containing high-calcium concentrations [25,26]. In our experiments, *KAL1* expression was downregulated in NHEK cultured in a high-calcium environment in comparison with a low-calcium environment. This was also consistent with our immunohistochemical observation that basal cells mainly express anosmin-1. Thus, *KAL1* expression may be regulated by calcium concentration in normal skin during keratinocyte differentiation. Therefore, further studies are required to elucidate the function of *KAL1* in regulation of keratinocyte proliferation and/or differentiation.

Moreover, we found that *KAL1* expression was upregulated by IL-13 or IL-4 in cultured NHEK. These biological effects may be mediated by a shared receptor composed of the IL-4Rα and the IL-13Rα1 receptor chains [27]. Intracellular signal transduction in response to IL-4/IL-13 occurs through activation of signal transducer and activator of transcription 6 (STAT6) [28]. Therefore, although the *KAL1* promoter is not completely understood, these findings raise the possibility that *KAL1* expression is controlled by the STAT6 signalling pathway. Increased TGF-β1 expression is found in chronic lesions compared with acute lesions in AD, suggesting a role in the development of tissue remodelling [29]. Our data showed that TGF-β1 slightly induced *KAL1* expression in cultured NHEK as compared with IL-4/IL-13 stimulation. This growth factor also acts as an inducer of *KAL1* expression in human muscle cells and fibroblasts [15]. However, in combination with IL-13, it acted synergistically to enhance *KAL1* expression in the present study. The synergistic effect of IL-13 and TGF-β1 has been validated by a recent study on *CCL5* expression using cultured keratinocytes [30]. Therefore, these findings suggest that TGF-β1 acts as an enhancer rather than an inducer of *KAL1* expression in the presence of Th2 cytokines. This may be explained by a previous report that the TGF-β signalling pathway can modify the cellular responses to several cytokines and stress signals [31].

IFN-γ inhibited *KAL1* expression induced by IL-13 in cultured NHEK, suggesting that this cytokine functions as a negative regulator of Th2 cytokine-induced *KAL1* expression. This was strongly supported by previous reports that IFN-γ inhibits the development of Th2 cytokine responses [32,33]. AD is an inflammatory skin disorder characterised by local expression of Th2 cytokines, whereas the lesional skin in chronic AD has a mixed Th1 and Th2 pattern [34,35]. Indeed, the expression levels of *KAL1* tended to decrease in the atopic skin. Thus, our observations provide the first evidence regarding cross-regulation of *KAL1* expression by Th1/Th2 cytokine balance with modulation of TGF-β1 in epidermal keratinocytes.

In conclusion, the results of the present study showed that anosmin-1 has an inhibitory effect on neurite outgrowth in cultured DRG neurones. We also demonstrated the distribution and expression patterns of anosmin-1 in human skin. The downregulation of anosmin-1 in the basal cell layer may also cause penetration of nerve fibres into the epidermis of patients with AD. Moreover, the regulation system of *KAL1* by cytokines may be involved in the pathogenesis of AD.

## Acknowledgements

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CONCISE COMMUNICATION

# Impact of disease severity on work productivity and activity impairment in Japanese patients with atopic dermatitis

**Chizuko YANO, Hidehisa SAEKI, Takaoki ISHIJI, Yozo ISHIUJI, Junko SATO, Yukari TOFUKU, Hidemi NAKAGAWA**

*Department of Dermatology, The Jikei University School of Medicine, Tokyo, Japan*

## ABSTRACT

**Atopic dermatitis (AD) is a common inflammatory skin disease that is characterized by chronic and persisting pruritic and eczematous lesions. There has been no study of work productivity and activity in AD patients in relation to disease severity. The purpose of this study was to examine the impact of disease severity on work productivity and activity impairment (WPAI) in adult AD patients using the Japanese version of the questionnaire. Data were collected from 112 AD patients who visited the Jikei University Hospital. Outcomes as measured by the questionnaire included employment status, total work productivity impairment (TWPI) and total activity impairment (TAI). We investigated the correlation between TWPI or TAI scores and severity scoring of AD (SCORAD) for disease severity and dermatology life quality index (DLQI) for quality of life impairment. Both TWPI and TAI scores were significantly correlated with the SCORAD and DLQI scores ($P < 0.001$), indicating disease severity is significantly associated with WPAI in Japanese adult AD patients. Further studies are necessary to evaluate the effects of treatments on WPAI for severe AD patients.**

**Key words:** atopic dermatitis, dermatology life quality index, Japanese, severity scoring of atopic dermatitis, work productivity and activity impairment.

## INTRODUCTION

Atopic dermatitis (AD) is a common allergic inflammatory skin disease that is characterized by chronic and persisting pruritic and eczematous lesions and is among the allergic diseases such as asthma and allergic rhinitis/conjunctivitis.[1] The work productivity and activity impairment-specific health problem (WPAI-SHP) questionnaire has been used to evaluate various diseases and its validity and reliability has been established.[2] The WPAI-AD is a six-item tool used to assess the impact of AD on work productivity and activity impairment. Based on this questionnaire, total work productivity impairment (TWPI), calculated from the combined effects of absenteeism (i.e. percentage of work hours missed) and "presenteeism" (i.e. percentage of impairment while working) and total activity impairment (TAI) other than work due to AD during the previous week can be evaluated. The impact of allergic diseases such as allergic rhinitis on work productivity and activity using WPAI allergy-specific (WPAI-AS) has been assessed.[3,4] In addition, the impact of certain skin diseases such as chronic spontaneous urticaria, chronic hand dermatitis and psoriasis using WPAI has been evaluated in Western countries.[5–7] Murota *et al.*[8,9] also determined the impact of antihistamines on the impaired productivity of patients with pruritic skin diseases including eczema/dermatitis, urticaria, AD, pruritus cutanea, prurigo and

**Table 1.** Patient characteristics

| | |
|---|---|
| Age, year, mean ± SD | 35.6 ± 10.8 |
| Male, *n* (%) | 56 (50.0) |
| Duration, year, mean ± SD | 25.2 ± 12.1 |
| BMI, mean ± SD | 21.4 ± 3.5 |
| Family histories, *n* (%) | |
| Atopic dermatitis | 39 (34.8) |
| Bronchial asthma | 27 (24.1) |
| Allergic rhinitis/conjunctivitis | 37 (33.0) |
| Past histories, *n* (%) | |
| Bronchial asthma | 42 (37.5) |
| Allergic rhinitis/conjunctivitis | 70 (62.5) |
| SCORAD, mean ± SD | 35.5 ± 21.9 |
| DLQI, mean ± SD | 7.8 ± 5.1 |
| WPAI, mean ± SD | |
| Absenteeism, % | 0.5 ± 2.3 |
| Presenteeism, % | 32.6 ± 23.5 |
| TWPI, % | 32.8 ± 23.7 |
| TAI, % | 42.9 ± 25.2 |

BMI, body mass index; DLQI, dermatological life quality index; SCORAD, scores and severity scoring of atopic dermatitis; SD, standard deviation; TAI, total activity impairment; TWPI, total work productivity impairment.

Correspondence: Hidehisa Saeki, M.D., Department of Dermatology, The Jikei University School of Medicine, 3-25-8 Nishishimbashi, Minato-ku, Tokyo, 105-8461, Japan. Email: saeki-der@jikei.ac.jp
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psoriasis using the WPAI-AS in Japan. However, there has been no study of work productivity and activity in adult AD patients using the WPAI-AD in relation to disease severity. Herein, we sought to examine the impact of disease severity on the WPAI in Japanese adult AD patients using the Japanese version of the questionnaire (WPAI-AD-Japan).

## METHODS

### Patients

Data were collected from 112 adult AD patients (56 male and 56 female) aged 35.6 ± 10.8 years who visited the Jikei University Hospital in the period between July 2011 and November 2012. All patients gave written informed consent to participate in the study which was approved by the ethics committee at the Jikei University.

### Measures

The WPAI-AD-Japan was adapted from the WPAI-SHP version 2.0 (available at http://www.reillyassociates.net/WPAI_SHP.html). The questionnaire first identifies current employment status and asks the following five questions about the past 7 days: (i) the number of hours the patient was absent from work due to AD; (ii) the number of hours absent from work for other reasons; (iii) the number of hours the patient actually worked; (iv) the extent to which AD affected the patient's productivity while at work; and (v) the extent to which AD affected the patient's ability to engage in regular daily activities other than work.[7]

To assess the relationship between WPAI outcomes and severity of AD, two different clinical severity indexes were used: severity scoring of AD (SCORAD) for disease severity, and dermatology life quality index (DLQI) for quality of life

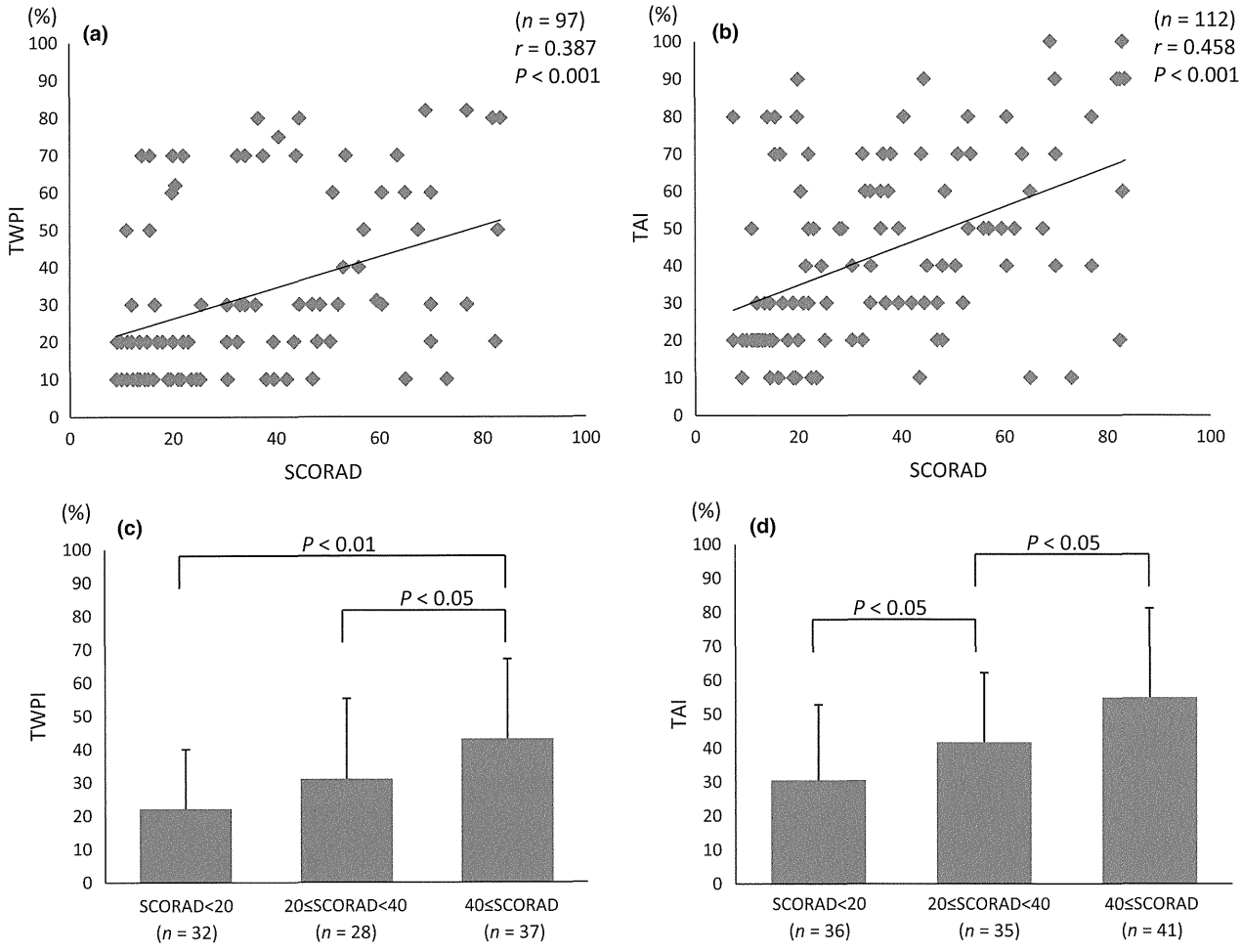


**Figure 1.** Associations between work productivity and activity impairment (WPAI) outcomes (total work productivity impairment [TWPI] and total activity impairment [TAI]) and scores and severity scoring of atopic dermatitis (SCORAD). (a) TWPI and SCORAD. (b) TAI and SCORAD. (c) TWPI and categorical SCORAD. (d) TAI and categorical SCORAD.

(QOL) impairment. The SCORAD is a severity index combining extent, intensity and subjective symptoms and it is scored from 0 to 103, with a larger number indicating greater severity.[10] The DLQI is an instrument that assesses the effect of a skin disease on six different aspects of a patient's QOL. It is scored from 0 to 30, with higher scores indicating a greater impairment on the patient's quality of life.[11] In this study, both continuous SCORAD/DLQI scores and categorical SCORAD (<20, 20–40, and ≥40) as well as DLQI (<5, 5–10, and ≥10) scores were used to assess severity.

### Statistical analyses

Correlation and linear regression analyses were used to assess the association between the continuous WPAI outcomes (TWPI and TAI) and the severity of AD (SCORAD and DLQI). The associations between WPAI outcomes and categorical SCORAD (<20, 20–40, and 40≥) or DLQI (<5, 5–10, and ≥10) scores were also assessed using the ANOVA with SNK test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Patient characteristics

Patient characteristics are shown in Table 1. Patients' mean age was 35.6 ± 10.8 years, duration of AD 25.2 ± 12.1 years, SCORAD 35.5 ± 21.9, DLQI 7.8 ± 5.1, absenteeism 0.5 ± 2.3%, presenteeism 32.6 ± 23.5%, TWPI 32.8 ± 23.7% and TAI 42.9 ± 25.2%.

### Associations between WPAI and SCORAD

Associations between WPAI outcomes (TWPI and TAI) and SCORAD score are shown in Figure 1(a,b). Both TWPI and TAI scores were significantly correlated with the SCORAD score. Associations between WPAI outcomes and categorical

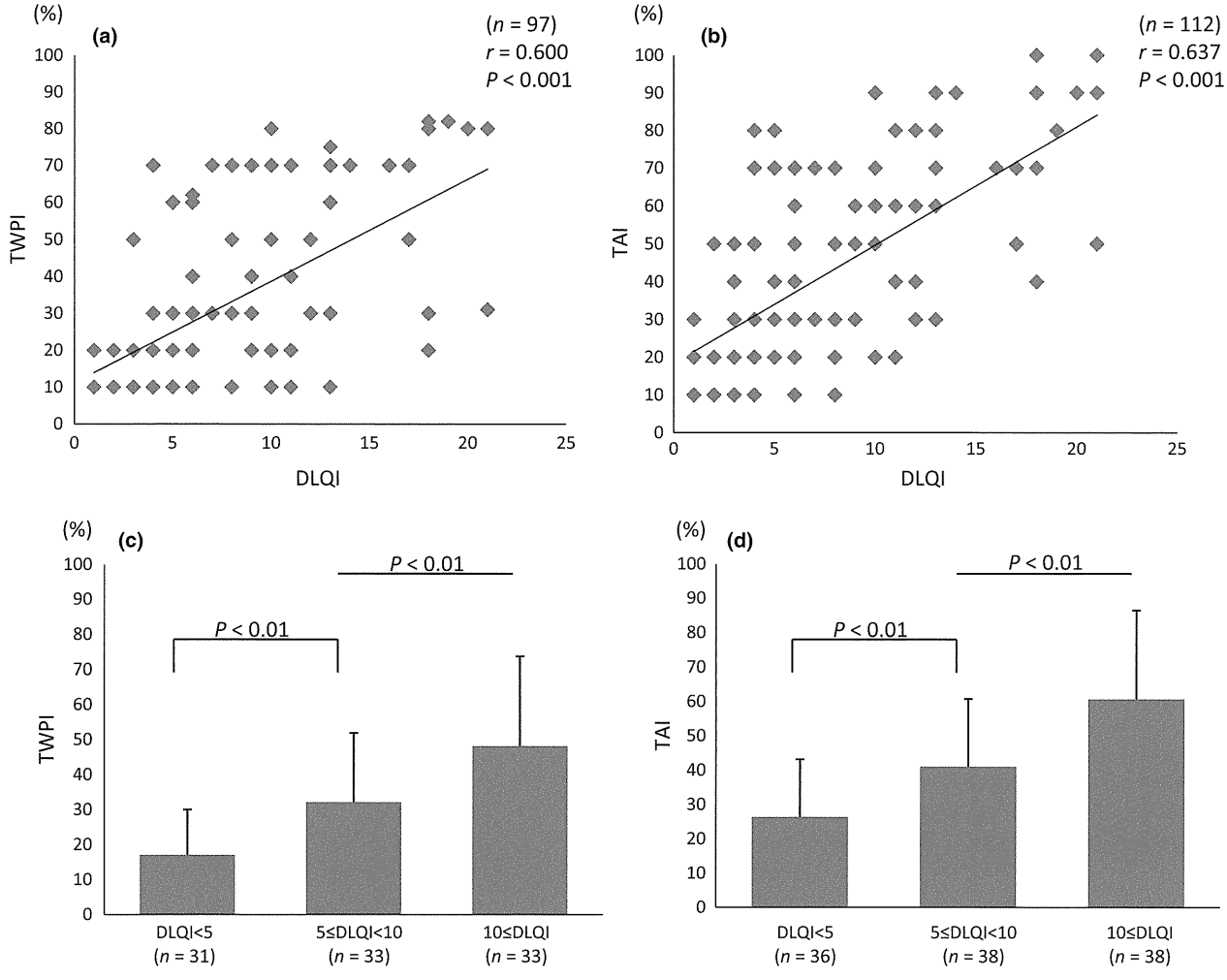


**Figure 2.** Associations between work productivity and activity impairment (WPAI) outcomes (total work productivity impairment [TWPI] and total activity impairment [TAI]) and dermatology life quality index (DLQI). (a) TWPI and DLQI. (b) TAI and DLQI. (c) TWPI and categorical DLQI. (d) TAI and categorical DLQI.

SCORAD score are depicted in Figure 1(c,d). TWPI in the SCORAD ≥ 40 group was higher than those in SCORAD < 20 and 20 ≤ SCORAD < 40. TAI in the 20 ≤ SCORAD < 40 group was higher than that in SCORAD < 20 and TAI in SCORAD ≥ 40 was higher than that in 20 ≤ SCORAD < 40.

### Associations between WPAI and DLQI

Associations between WPAI outcomes and DLQI score are shown in Figure 2(a,b). Both TWPI and TAI scores were significantly correlated with the DLQI score. Associations between WPAI outcomes and categorical DLQI score are depicted in Figure 2(c,d). TWPI in the 5 ≤ DLQI < 10 group was higher than that in DLQI < 5, and TWPI in DLQI ≥ 10 was higher than that in 5 ≤ DLQI < 10. Likewise, TAI in the 5 ≤ DLQI < 10 group was higher than that in DLQI < 5, and TAI in DLQI ≥ 10 was higher than that in 5 ≤ DLQI < 10.

## DISCUSSION

The impact of psoriasis on work productivity and activity has been well assessed in Western countries. Wu *et al.*[7] showed that psoriasis patients had significantly more overall work impairment and more impairment in activity than non-psoriasis patients using the WPAI instrument. Kimball *et al.*[12] evaluated the association between psoriasis severity and WPAI outcomes and showed that all WPAI measures were positively and significantly associated with psoriasis severity. Very recently, we demonstrated that psoriasis severity was also significantly associated with WPAI in Japanese psoriasis patients.[13] However, there has been no study to evaluate the association between WPAI and the severity of other skin diseases including AD. This is the first study to assess the impact of disease severity on WPAI in adult AD patients using WPAI-AD. We demonstrated that both TWPI and TAI were positively and significantly associated with AD severity.

Murota *et al.*[8,9] evaluated the impact of sedative and non-sedative antihistamines on the impaired productivity in patients with pruritic skin diseases using WPAI-AS. Their study included patients with eczema/dermatitis ($n$ = 75), urticaria ($n$ = 50), AD ($n$ = 43), pruritus cutanea ($n$ = 14), prurigo ($n$ = 8), psoriasis ($n$ = 7) and others ($n$ = 9). Due to the relatively small sample size of each disease group, statistically significant differences in impairment at baseline between these groups were not detected. However, their results indicate that TWPI and TAI tended to be larger in the AD, eczema/dermatitis and urticaria disease groups.[9] For example, TWPI in AD ($n$ = 31) tended to be higher than that in psoriasis ($n$ = 3; 40.4 ± 26.8% vs 28.9 ± 21.7%), and TAI in AD ($n$ = 43) was also higher than that in psoriasis ($n$ = 7; 50.2 ± 26.9% vs 44.3 ± 28.8%). Interestingly, our previous and present results showed the same tendency. TWPI in AD ($n$ = 97) tended to be higher than that in psoriasis ($n$ = 134; 32.8 ± 23. 7% vs 27.2 ± 24.6%), and TAI in AD ($n$ = 112) was significantly higher than that in psoriasis ($n$ = 213; 42.9 ± 25.2% vs 35.9 ± 27.8%, $P$ < 0.05 by an unpaired Student's $t$-test), although the age and sex were not matched between two groups. The reason WPAI in AD tended to be higher than that in psoriasis is not clear, but we speculate that one reason might have been the more frequent and more severe itching in AD.

In summary, AD negatively impacts a patient's work productivity and activity and the impact tends to be larger than that of psoriasis and is even larger in patients with severe AD. Further studies are necessary to evaluate the effects of treatments including cyclosporin on WPAI for severe AD patients.

## ACKNOWLEDGMENTS

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Haruka Goto[a,1,*], Maya Hongo[a,1], Hiroshi Ohshima[a], Masumi Kurasawa[a], Satoshi Hirakawa[a], Yasuo Kitajima[b]

[a]*Skin Research Department, POLA Chemical Industries, Inc., Yokohama, Japan*; [b]*Kizawa Memorial Hospital, Gifu, Japan*

*Corresponding author at: Skin Research Department, POLA Chemical Industries, Inc., 560 Kashio-cho, Totsuka-ku, Yokohama 244-0812, Japan.
Tel.: +81 45 826 7134; fax: +81 45 826 7249
*E-mail address:* haruka-goto@pola.co.jp (H. Goto)

[1]These authors contributed equally to this work.

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## Letter to the Editor

### Genetic polymorphisms in the *IL22* gene are associated with psoriasis vulgaris in a Japanese population

*Keywords:*
Genetic polymorphisms; *IL22* gene;
Psoriasis vulgaris; Japanese population

*To the Editor,*

Psoriasis vulgaris (PsV) is an inflammatory skin disease histologically characterized by epidermal hyperplasia, inflammatory cell infiltration and vascular changes in which T-lymphocytes and associated cytokines play a central role [1]. A dysregulated cutaneous immune response occurs in genetically susceptible individuals and the features of inflammation are characterized by tumor necrosis factor (TNF)-α dependence and exaggerated helper T cell 1 (Th1) and 17 (Th17) activation. Interleukin (IL)-22 is an IL-10 family cytokine member produced by Th17 cells and plays a role in the promotion of inflammation and tissue repair at barrier surfaces [2]. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation [3], and circulating IL-22 levels are significantly higher in psoriatic patients than in normal subjects [4,5]. Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease that is basically considered to be a Th-2 type disease. However, a recent study suggests a possible role of Th17 cells in AD [6]. The study has shown that the number of Th17 cells is increased in the peripheral blood and acute lesional skin of AD and that IL-17 and IL-22 synergistically enhance the production of IL-8 from keratinocytes [6]. Since there are few genetic studies of the polymorphisms of *IL22* in populations of Asian and European ancestry, we conducted association studies to assess whether *IL22* gene variants contribute to the susceptibility to PsV or AD in a Japanese population.

We recruited a total of 236 patients with PsV (mean age 53, 11–85 years, male:female ratio = 1.0:2.8), and all subjects were diagnosed by clinical and histopathological findings. A total of 916 patients with AD (mean age 30, 3–77 years, male:female ratio = 1.0:2.2) and 844 controls (mean age 50, 20–75 years, male:female ratio = 1.0:1.3) were recruited as described [7]. Patients with AD were diagnosed according to the criteria of Hanifin and Rajka, and control subjects were never diagnosed with AD or PsV. All individuals were unrelated Japanese and gave written informed consent to participate in the study. The study was approved by the ethical committees at the Institute of Physical and Chemical Research (RIKEN), the University of Tokyo and the Jikei University School of Medicine. Genomic DNA was prepared in accordance with standard protocols.

We resequenced the *IL22* gene regions with genomic DNA from 36 individuals and identified a total of 32 polymorphisms (Table 1). We next examined the linkage disequilibrium (LD) between identified SNPs (Fig. S1). Pairwise LD coefficients $D'$ and $r^2$ were calculated among the 24 SNPs with minor allele frequencies (MAF) of greater than 5% using Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview/). We selected a total of 11 tag SNPs for association studies using tagger in Haploview 4.2, and the 11 tag

**Table 1**
Frequencies of polymorphisms of the *IL22* gene in a Japanese population.

| | SNP[a] | Allele | Location | MAF[b] | NCBI[c] |
|---|---|---|---|---|---|
| 1 | −2479 | T/C | 5′-Flanking region | 0.319 | rs57947370 |
| 2 | −2378 | C/T | 5′-Flanking region | 0.278 | rs11177135 |
| 3 | −2375 | T/C | 5′-Flanking region | 0.014 | rs77156535 |
| 4 | −2161 | G/A | 5′-Flanking region | 0.319 | rs7139027 |
| 5 | −1905 | A/G | 5′-Flanking region | 0.278 | rs2227472 |
| 6 | −1810 | G/A | 5′-Flanking region | 0.319 | rs2227473[d] |
| 7 | −1588 | T/A | 5′-Flanking region | 0.292 | rs2227476[d] |
| 8 | −1536 | C/T | 5′-Flanking region | 0.028 | rs2227477 |
| 9 | −1394 | T/C | 5′-Flanking region | 0.431 | rs2227478[d] |
| 10 | −1114 | C/T | 5′-Flanking region | 0.111 | rs2227480 |
| 11 | −1113 | C/T | 5′-Flanking region | 0.278 | rs2227481 |
| 12 | −1089 | AT/del | 5′-Flanking region | 0.000 | rs35774195 |
| 13 | −1075 | AT repeat | 5′-Flanking region | 0.000 | rs10699698 |
| 14 | −948 | T/A | 5′-Flanking region | 0.292 | rs2227483 |
| 15 | −701 | C/T | 5′-Flanking region | 0.111 | rs2227484[d] |
| 16 | −485 | C/T | 5′-Flanking region | 0.278 | rs2227485[d] |
| 17 | −201 | A/G | 5′-Flanking region | 0.014 | rs141972126 |
| 18 | 393 | T/A | Intron 1 | 0.264 | rs17224704[d] |
| 19 | 708 | A/G | Intron 2 | 0.278 | rs2227491 |
| 20 | 1254 | A/C | Intron 3 | 0.375 | rs2046068[d] |
| 21 | 1366 | G/T | Intron 3 | 0.028 | rs3782552 |
| 22 | 1945 | G/C | Intron 4 | 0.014 | |
| 23 | 2178 | G/C | Intron 4 | 0.361 | rs1179251[d] |
| 24 | 2385 | T/C | Intron 4 | 0.097 | rs1179250[d] |
| 25 | 2449 | C/A | Intron 4 | 0.097 | rs1179249 |
| 26 | 2611 | T/A | Intron 4 | 0.278 | rs1012356 |
| 27 | 3270 | C/A | Intron 4 | 0.278 | rs2227501 |
| 28 | 3531 | A/G | Intron 4 | 0.278 | rs2227503 |
| 29 | 3635 | T/C | Intron 4 | 0.014 | rs976748 |
| 30 | 5301 | A/T | 3′-Flanking region | 0.097 | rs2227508[d] |
| 31 | 5433 | CT/del | 3′-Flanking region | 0.014 | |
| 32 | 5697 | A/T | 3′-Flanking region | 0.444 | rs1182844[d] |

[a] Numbering according to the genomic sequence of IL-22 (NC_000012.11). Position 1 is the A of the initiation codon.
[b] MAF (minor allele frequencies) in the screening population ($N = 36$).
[c] NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).
[d] SNPs were genotyped in this study. Genotyping of the 11 SNPs in IL-22 were performed by the TaqMan™ allele-specific amplification (TaqMan-ASA) method (Applied Biosystems) and multiplex-PCR based Invader assay (Third Wave Technologies).

**Table 2**
Summary of association results for the *IL22* gene.

| dbSNP allele 1/2 position | Subject | Genotype | | | Total | Frequency | | | MAF | 1 vs. 2 alleles | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 11 | 12 | 22 | | 11 | 12 | 22 | | *P* value | OR | 95% CI |
| rs2227473 | PsV | 130 | 94 | 10 | 234 | 0.556 | 0.402 | 0.043 | 0.244 | 0.0095 | 1.38 | 1.08–1.76 |
| G/A | AD | 604 | 274 | 32 | 910 | 0.664 | 0.301 | 0.035 | 0.186 | 0.79 | 1.02 | 0.86–1.21 |
| −1810 | Control | 550 | 262 | 28 | 840 | 0.655 | 0.312 | 0.033 | 0.189 | | | |
| rs2227476 | PsV | 136 | 88 | 9 | 233 | 0.584 | 0.378 | 0.039 | 0.227 | 0.0051 | 1.43 | 1.11–1.84 |
| T/A | AD | 633 | 256 | 26 | 915 | 0.692 | 0.280 | 0.028 | 0.168 | 0.84 | 1.02 | 0.85–1.21 |
| −1588 | Control | 578 | 242 | 23 | 843 | 0.686 | 0.287 | 0.027 | 0.171 | | | |
| rs2227478 | PsV | 98 | 101 | 37 | 236 | 0.415 | 0.428 | 0.157 | 0.371 | 0.00014 | 1.51 | 1.22–1.88 |
| T/C | AD | 470 | 363 | 82 | 915 | 0.514 | 0.397 | 0.090 | 0.288 | 0.60 | 1.04 | 0.90–1.20 |
| −1394 | Control | 429 | 353 | 59 | 841 | 0.510 | 0.420 | 0.070 | 0.280 | | | |
| rs2227484 | PsV | 187 | 41 | 7 | 235 | 0.796 | 0.174 | 0.030 | 0.117 | 0.30 | 1.18 | 0.86–1.64 |
| C/T | AD | 735 | 166 | 12 | 913 | 0.805 | 0.182 | 0.013 | 0.104 | 0.74 | 1.04 | 0.83–1.29 |
| −701 | Control | 674 | 154 | 7 | 835 | 0.807 | 0.184 | 0.008 | 0.101 | | | |
| rs2227485 | PsV | 92 | 105 | 38 | 235 | 0.391 | 0.447 | 0.162 | 0.385 | 0.024 | 1.27 | 1.03–1.57 |
| C/T | AD | 289 | 447 | 179 | 915 | 0.316 | 0.489 | 0.196 | 0.440 | 0.84 | 1.01 | 0.89–1.16 |
| −485 | Control | 258 | 418 | 163 | 839 | 0.308 | 0.498 | 0.194 | 0.443 | | | |
| rs17224704 | PsV | 146 | 82 | 7 | 235 | 0.621 | 0.349 | 0.030 | 0.204 | 0.023 | 1.35 | 1.04–1.75 |
| T/A | AD | 653 | 242 | 18 | 913 | 0.715 | 0.265 | 0.020 | 0.152 | 0.54 | 1.06 | 0.88–1.27 |
| 393 | Control | 595 | 225 | 22 | 842 | 0.707 | 0.267 | 0.026 | 0.160 | | | |
| rs2046068 | PsV | 104 | 102 | 26 | 232 | 0.448 | 0.440 | 0.112 | 0.332 | 0.0018 | 1.42 | 1.14–1.78 |
| A/C | AD | 501 | 342 | 71 | 914 | 0.548 | 0.374 | 0.078 | 0.265 | 0.69 | 1.03 | 0.89–1.20 |
| 1254 | Control | 458 | 329 | 53 | 840 | 0.545 | 0.392 | 0.063 | 0.259 | | | |
| rs1179251 | PsV | 124 | 85 | 26 | 235 | 0.528 | 0.362 | 0.111 | 0.291 | 0.54 | 1.07 | 0.86–1.34 |
| G/C | AD | 454 | 372 | 90 | 916 | 0.496 | 0.406 | 0.098 | 0.301 | 0.75 | 1.02 | 0.89–1.18 |
| 2178 | Control | 408 | 351 | 82 | 841 | 0.485 | 0.417 | 0.098 | 0.306 | | | |
| rs1179250 | PsV | 134 | 84 | 17 | 235 | 0.570 | 0.357 | 0.072 | 0.251 | 0.23 | 1.15 | 0.91–1.46 |
| T/C | AD | 487 | 354 | 74 | 915 | 0.532 | 0.387 | 0.081 | 0.274 | 0.77 | 1.02 | 0.88–1.19 |
| 2385 | Control | 439 | 338 | 66 | 843 | 0.521 | 0.401 | 0.078 | 0.279 | | | |
| rs2227508 | PsV | 187 | 41 | 7 | 235 | 0.796 | 0.174 | 0.030 | 0.117 | 0.11 | 1.31 | 0.94–1.81 |
| T/A | AD | 737 | 162 | 12 | 911 | 0.809 | 0.178 | 0.013 | 0.102 | 0.32 | 1.12 | 0.90–1.40 |
| 5301 | Control | 694 | 141 | 7 | 842 | 0.824 | 0.167 | 0.008 | 0.092 | | | |
| rs1182844 | PsV | 83 | 108 | 43 | 234 | 0.355 | 0.462 | 0.184 | 0.415 | 0.54 | 1.07 | 0.87–1.31 |
| A/T | AD | 327 | 428 | 158 | 913 | 0.358 | 0.469 | 0.173 | 0.407 | 0.61 | 1.04 | 0.91–1.19 |
| 5697 | Control | 310 | 391 | 140 | 841 | 0.369 | 0.465 | 0.166 | 0.399 | | | |

SNPs captured 24 of the 24 alleles with $r^2 > 0.92$. We genotyped the 11 SNPs in *IL22* gene by the TaqMan® SNP Genotyping Assays (Life Technologies).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2013.04.002.

The results for genotype frequencies of the 11 tag SNPs in the case and control group are shown in Table 2. All 11 SNPs were in Hardy–Weinberg equilibrium, and we then compared differences in the allele frequencies by using a contingency $\chi^2$ test. Odds ratios (ORs) with 95 percent confidence intervals (95% CI) were calculated. We applied Bonferroni corrections, the multiplication of *P* values by 11, the number of tag SNPs. In the association study, corrected *P* values of less than 0.05 were judged to be significant.

We identified significant associations between *IL22* gene variants and PsV under the allelic model (rs2227478; corrected $P = 0.0015$; OR = 1.51, rs2046068; corrected $P = 0.020$; OR = 1.42) (Table 2). Weger et al. evaluated a total of 10 common polymorphisms of the *IL22* gene in an Austrian population and reported no association between the variants and chronic plaque psoriasis [8]. Recent genome-wide association studies (GWASs) of PsV revealed a total of 36 psoriasis-associated regions in individuals of European ancestry, and the regions encode several proteins engaged in the TNF, IL-23 and IL-17 signaling pathways [9]. However, the *IL22* locus did not contain the susceptible regions identified by European GWASs. Since heterogeneous association signals are often seen among different ethnic populations, further genetic studies using Asian populations seem to be needed for further focusing attention of polymorphisms of the *IL22* gene in this disease. Although a validation study in an independent population is needed, our findings imply that *IL22* variants play a role in the pathogenesis of PsV in the Japanese population.

A number of features shared by AD and PsV including a Th17 cell pathway and common gene loci, were reported [10], but we did not find a significant association in this study between *IL22* SNPs and susceptibility to AD ($P = 0.32–0.84$) (Table 2). Since a recent study has shown a role of Th17 cells in exacerbation of AD [6], genetic variants of *IL22* might influence the exacerbation of the disease rather than susceptibility to it.

In summary, our data suggested important genetic influences of the polymorphisms in *IL22* on the susceptibility to PsV but not to AD in the Japanese population. Higher concentrations of IL-22 are observed in the peripheral blood and tissues of patients with PsV [2,4,5], and expression of IL-22 and IL-22-regulated genes in keratinocytes is reduced by antipsoriatic therapies [4]. Further evaluation of the clinical significance of the susceptible *IL22* gene variants would help better understand the etiology of PsV.

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Hidehisa Saeki[a,1,*], Tomomitsu Hirota[b,1], Hidemi Nakagawa[a], Yuichiro Tsunemi[c], Toyoaki Kato[d], Sayaka Shibata[d], Makoto Sugaya[d], Shinichi Sato[d], Satoru Doi[e], Akihiko Miyatake[f], Kouji Ebe[g], Emiko Noguchi[h], Tamotsu Ebihara[i], Masayuki Amagai[i], Hitokazu Esaki[j], Satoshi Takeuchi[j], Masutaka Furue[j], Yusuke Nakamura[k], Mayumi Tamari[b]

[a]*Department of Dermatology, The Jikei University School of Medicine, Japan*; [b]*Laboratory for Respiratory Diseases, Center for Genomic Medicine, RIKEN, Japan*; [c]*Department of Dermatology, Tokyo Women's Medical University, Japan*; [d]*Department of Dermatology, Faculty of Medicine, University of Tokyo, Japan*; [e]*Osaka Prefectual Medical Center for Respiratory and Allergic Diseases, Japan*; [f]*Miyatake Asthma Clinic, Japan*; [g]*Takao Hospital, Japan*; [h]*Graduate School of Comprehensive Human Sciences, University of Tsukuba, Japan*; [i]*Department of Dermatology, Keio University School of Medicine, Japan*; [j]*Department of Dermatology, Graduate School of Medical Sciences, Kyushu University, Japan*; [k]*Institute of Medical Science, The University of Tokyo, Japan*

*Corresponding author at: Department of Dermatology, The Jikei University School of Meicine, 3-25-8, Nishishimbashi, Minato-ku, Tokyo 105-8461, Japan.
Tel.: +81 3 3433 1111; fax: +81 3 5401 0125
*E-mail address:* saeki-der@jikei.ac.jp (H. Saeki)

[1]These authors contributed equally to this article.

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## Letter to the Editor

### *HLA-B*58:01* strongly associates with allopurinol-induced adverse drug reactions in a Japanese sample population

*To the Editor,*

Allopurinol, an inhibitor of xanthine oxidase, is widely used for the treatment of hyperuricemia associated with chronic gout, acute uric acid nephropathy, recurrent uric acid stone formation, certain enzyme/blood disorders, and cancer chemotherapy. It has been shown that severe cutaneous adverse drug reactions (ADRs) caused by allopurinol were strongly associated with HLA-B*58:01 in a Han Chinese sample population [1]. Odds ratio (OR) for the association of HLA-B*58:01 with allopurinol-induced severe cutaneous ADR in this population was 580.3 and 95% CI was 34.4–9780.9. Although the relationship between HLA-B*58:01 and allopurinol-induced Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) has subsequently been studied in European and Japanese patients, the association was much weaker than that reported in Han Chinese patients [2,3]. The association study in Japanese patients was examined in only a limited number of allopurinol-induced ADR cases. We therefore conducted a case-controlled study to determine HLA types associated with allopurinol-induced ADR in a Japanese sample population.

All patients were recruited from Shimane University Hospital between 2010 and 2012. These included 7 patients with allopurinol-induced ADR (3 patients with SJS and 4 patients with erythema exudativum multiforme (EEM)) and 25 patients who had been receiving allopurinol for more than 3 months without drug eruption. Diagnoses of SJS were made according to the diagnostic criteria established by Roujeau [4]. Allopurinol-induced ADR was diagnosed using medical histories, indicating that symptoms occurred within 3 months of starting allopurinol administration, and the symptoms resolved upon the withdrawal of allopurinol. If the patients were given other drugs, in addition to allopurinol, 3 months prior to the appearance of symptoms, a drug-induced lymphocyte stimulation test and a patch test were performed with allopurinol/oxypurinol. Allopurinol-induced ADRs were diagnosed by the single medication of allopurinol in 4 of the 7 patients (No. 1, 3, 4, 7), by the positive allopurinol-induced lymphocyte stimulation test in 2 of the 7 patients (No. 2, 6), and by the positive patch test with allopurinol in the patient No. 5. The indication for which drug had been prescribed was the level of hyperuricemia detected in all the patients. All patients were interviewed by investigators regarding the histories of their biological parents and grandparents, and were confirmed as being ethnically Japanese. This study was approved by the ethics committee of Shimane University Faculty of Medicine (approval no. 221).

Low-resolution HLA typing with DNA extracted from peripheral blood was performed using the reverse sequence-specific oligonucleotide with polymerase chain reaction (PCR-rSSO) method [5]. High-resolution HLA-B genotyping was determined using the polymerase chain reaction-sequence based typing (PCR-SBT) method [5]. Statistical analysis of the differences in each allele frequency among patients with ADR and control subjects was performed by Fisher's exact test. The strength of association was estimated by calculating the OR. The OR was determined using Haldane's modification, which adds 0.5 to all cells to accommodate

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Kazumitsu Sugiura[a], Takuya Takeichi[a], Kana Tanahashi[a], Yasutomo Ito[b], Tomoki Kosho[c], Ken Saida[d], Hisashi Uhara[e], Ryuhei Okuyama[e], Masashi Akiyama[a,*]

[a]*Department of Dermatology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*;
[b]*Department of Division for Medical Research Engineering, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*;
[c]*Department of Medical Genetics, Shinshu University, Graduate School of Medicine, Japan*;
[d]*Department of Pediatrics, Shinshu University, Graduate School of Medicine, Japan*;
[e]*Department of Dermatology, Shinshu University, Graduate School of Medicine, Japan*

*Corresponding author. Tel.: +81 52 744 2314; fax: +81 52 744 2318
*E-mail address:* makiyama@med.nagoya-u.ac.jp (M. Akiyama)

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## Letter to the Editor

### Impact of disease severity on sleep quality in Japanese patients with atopic dermatitis

*Keywords:*
Atopic dermatitis
Dermatology life quality index (DLQI)
Severity scoring of atopic dermatitis (SCORAD)
Pittsburgh Sleep Quality Index (PSQI)

*To the Editor,*

Atopic dermatitis (AD) is a common allergic skin disease that is characterized by chronic, pruritic and eczematous lesions. The relationship between AD and sleep disturbances has been studied and the current theory is that sleep problems are related to nocturnal itching and scratching behavior [1]. The Pittsburgh Sleep Quality Index (PSQI) is a self-related questionnaire assessing sleep quality and disturbances over a 1-month time period [2]. Doi et al. developed the Japanese version of PSQI (PSQI-J) and disclosed the utility of the PSQI-J as a reliable and valid measure for sleep quality in clinical practice and research. They showed that the PSQI-J mean scores were significantly higher in psychiatrically disordered subjects than control subjects [3]. There has, however, been no study evaluating sleep quality in Japanese adult AD patients using PSQI-J in relation to disease severity. The aim of this study is to examine the impact of disease severity on sleep quality in this group of patients using PSQI-J.

The PSQI has 19 questions that generate 7 component scores: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. The sum of the 7 component scores generates a global score, ranging from 0 to 21. Higher scores indicate worse sleep quality, and a global PSQI score greater than 5 is consistent with poor sleep quality [2]. To assess the relationship between PSQI outcomes and severity of AD, two different clinical severity indexes were used: severity scoring of AD (SCORAD) for disease severity, and dermatology life quality index (DLQI) for quality of life (QOL) impairment. SCORAD is a severity index combining extent, intensity and subjective symptoms and is scored from 0 to 103, with a larger number indicating greater severity [4]. DLQI is an instrument that assesses the effect of a skin disease on six different aspects of a patient's QOL. It is scored from 0 to 30, with higher scores indicating a greater impairment of the patient's QOL [5]. In this study, both continuous SCORAD/DLQI scores and categorical SCORAD (<20, 20–40, and ≥40) as well as DLQI (<5, 5–10, and ≥10) were used to assess severity.

We conducted a self-administered questionnaire survey of 112 adult AD patients (56 males and 56 females) who visited the Jikei University Hospital. All patients gave written informed consent of their participation in the study which was approved by the ethical committees at the Jikei University. PSQI-J was adapted from "http://www.sleepmed.jp/q/meq/psqi_form.php" with permission of the copyright holder. Correlation analysis was used to assess the association between the continuous PSQI outcomes and the severity of AD (SCORAD and DLQI). The associations between PSQI and categorical SCORAD (<20, 20–40, and ≥40) or DLQI (<5, 5–10, and ≥10) were also assessed using the ANOVA with SNK test. A $P$-value of <0.05 was considered statistically significant.

Patients' mean age was 35.6 ± 10.8 years, SCORAD was 35.5 ± 21.9, DLQI was 7.8 ± 5.1, and PSQI was 7.3 ± 2.8. Associations between PSQI and SCORAD/DLQI scores are shown in Fig. 1(a) and (b). The PSQI score was significantly correlated with both SCORAD and DLQI scores. Association between the PSQI score and categorical SCORAD/DLQI scores are depicted in Fig. 1(c) and (d). PSQI in the SCORAD ≥ 40 group was significantly higher than those in SCORAD < 20 and 20 ≤ SCORAD < 40. PSQI in 5 ≤ DLQI < 10 was significantly higher than that in DLQI < 5 and PSQI in DLQI ≥ 10 was significantly higher than that in 5 ≤ DLQI < 10. Associations between PSQI component scores and SCORAD/DLQI are shown in Table 1. The SCORAD score was significantly associated with subjective sleep quality and sleep latency. The DLQI score was significantly associated with subjective sleep quality, sleep latency, habitual sleep efficiency, sleep disturbance and daytime dysfunction. Additional analyses disclosed that there is no significant association between PSQI and gender ($P = 0.47$, unpaired $t$-test), and a weaker association between PSQI and age ($r = 0.214$, $P = 0.02$, correlation analysis) than that between PSQI and SCORAD ($r = 0.332$, $P = 0.0003$) or between PSQI and DLQI ($r = 0.430$, $P = 2.2 \times 10^{-6}$).