의학석사 학위논문

Transient receptor potential vanilloid (TRPV)

억제와 염증성 과색소침착의 치료

Transient receptor potential vanilloid (TRPV) inhibition
is related to suppression of
inflammation-associated hypermelanosis

울 산 대 학 교 대 학 원

의 학 과

문 익 준

Transient receptor potential vanilloid (TRPV) inhibition is related to suppression of inflammation-associated hypermelanosis

지 도 교 수　　최　지　호

이 논문을 의학석사 학위 논문으로 제출함

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울 산 대 학 교 대 학 원

의　　학　　과

문　익　준

문익준의 의학석사학위 논문을 인준함

심사위원 장 성 은 인

심사위원 이 미 우 인

심사위원 최 지 호 인

울 산 대 학 교 대 학 원

2017년 11월

Abstract

Background and Objectives: Pigmented contact dermatitis (PCD) is a skin disorder caused by inflammation-related hyperpigmentation. Little is known of the pathogenesis of PCD, making its treatment quite challenging. Patients with PCD often report pruritus and burning sensation on affected areas. These clinical features suggest the possible role of transient receptor potential vanilloid (TRPV) channels in the pathogenesis of PCD as these calcium-modulating channels participate in diverse processes such as neurogenic inflammation, perception of pain and itch, and even cutaneous melanogenesis. Therefore, we sought to evaluate the expression level of TRPVs in the skin of patients with PCD aiming at identifying crucial receptors related to inflammation-associated melanogenesis.

Methods: Immunohistochemical analysis was performed to evaluate cutaneous expression of TRPVs in PCD patients' lesional skin in comparison to the corresponding peri-lesional skin. The mRNA levels of various melanogenesis-related genes were measured using Next-generation sequencing (NGS). Capsaicin, a potent TRPV1 activator, and AMG9810, a selective TRPV1 inhibitor, were used as TRPV regulators in *in vitro* assays using normal human melanocytes. The expression of melanogenesis-related proteins was assessed using Western blot assay.

Results: TRPVs were increased in the epidermal and dermal cells in the lesions of PCD. Furthermore, Pmel, TYRP1 and S100A8 were increased in the lesions of PCD compared to peri-lesion. qRT-PCR analysis showed that tyrosinase, along with TRPV1 and TRPV3, was up-regulated in patients with PCD. We also demonstrated that TRPV1 regulates melanogenesis in human melanocytes as well as B16 melanoma cells and co-culture. TRPV1 activation promoted melanin synthesis in human melanocytes *in vitro*, while this effect could be reversed by TRPV1 blockade using AMG9810. A notable downmodulation of PKC-βII signaling was observed, subsequently decreasing the activity of tyrosinase. This resulted in the anti-melanogenic effect of TRPV1 inhibitors.

Conclusion: Our findings suggest that elevated expression of TRPVs in the skin links the clinically reported symptoms of PCD to its causative inflammation-related hyperpigmentation. TRPVs, particularly TRPV1, may play a crucial role in the development, aggravation and chronicity of PCD through modulation of ca^{2+}/PKC/tyrosinase activity.

Keywords: Transient receptor potential vanilloid 1, Pigmented contact dermatitis, Melanogenesis, PKC

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INTRODUCTION

Pigmented contact dermatitis (PCD), also sometimes referred as a variant of Riehl's melanosis, mostly affects middle to old-aged Asian women with darker skin phototypes, characterized by diffuse reticulated brown to gray discoloration of the face and neck with a predilection for the forehead, temples and zygomatic areas. However, it is recently not rare to see young women in their 20 to 30's or even men exhibit features similar to those of PCD. It is also striking that the prevalence of PCD has been abruptly increasing in Korea, drawing much attention to dermatologists. It is possible that the marked increase of PCD is associated with a more and more frequent use of irritating, itch-provoking cosmetic products, especially henna hair dye and laser procedures which have been popular since mid-2000's in Korea.

The pathogenesis and exact etiology of PCD, a distinct inflammation-associated melanogenesis, still remain largely unknown. Previously, Japanese authors suggested that allergic sensitization caused by repeated exposure to sub-threshold concentrations of particular chemicals including perfume or dye commonly found in cosmetic products has been appointed to be a major clinical event in the development of PCD.(1-3)

Clinically, Korean patients with PCD are somewhat different, as observed in recent observation studies. In fact, first, they tend to present with brown to gray pigmentation over ill- defined erythematous patches specially, on the most dry area such as the side of neck or bone-protruding (possibly less buffered to heat or irritation) facial areas. In the original observation of Riehl's melanosis, erythema was not observed.(4) In this situation, we suggest that our case series patients are better termed as PCD rather than Riehl's melanosis. Being secondly differently from the original observation of usually asymptomatic Riehl's melanosis, the majority of Korean PCD patients complain of quite severe itching, burning and heat sensation with simultaneous aggravation of PCD lesions. Laser treatments have demonstrated variable degree of success while neither systemic nor topical therapy was reported to be satisfactorily effective. Rather, they reported aggravation of skin lesions by over-heat accumulating laser procedures such as strong dose of 1064 nm Nd:YAG laser toning or fractional lasers. When we performed patch tests in these patients, PCD patients

had more irritation results or combined irritation contact dermatitis (ICD) and allergic contact dermatitis (ACD) profiles than true ACD. When considering that impaired barrier, dry skin and ICD collectively tend to bring about ACD, these preceding factors also could be the main causative features of PCD.

Based on these clinical observations, we have tried to investigate what common factors cause PCD. On the background to look for ideal agents for the treatment of hyperpigmentary skin disorders, we previously studied anti-melanogenic action of diverse chemicals and natural extracts by high-throughput screening (HTS) screening. Among them, we found ion channel modulation regulated melanogenesis profoundly and one of the most strong and consistent action was observed with AMG 9810, a specific transient receptor potential vanilloid (TRPV) antagonist. (Fig 1) We consequently focused on TRPV channels as alteration of TRPVs is the key process involved in compromise of skin barrier, itching, burning, heat sensation, inflammation and pigmentation clinically. Pathomechanistically, TRPVs are known to be associated with calcium signal, histamine, neuropeptides and inflammatory cascades. We thus hypothesize that TRPVs are key which may link with the peculiar heating sensation, inflammation and the post-inflammatory hyperpigmentation(PIH) of PCD. Therefore, we sought to evaluate the expression of immunohistochemical and mRNA level of TRPV1 and 3 in the lesional skin of patients with PCD comparing with peri-lesional skin of the corresponding patients. Further, we assessed whether and how TRPV activators or inhibitors affect melanin synthesis.

MATERIALS AND METHODS

Materials and reagents

The reagents were purchased from the following sources: 2E-N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-Propenamide (AMG9810; Sigma-Aldrich, St. Louis, MO), capsaicin (Sigma-Aldrich), DWP05195 (Daewoong pharmaceutical company, Seoul, Korea). The antibodies used in this study were purchased from the following sources: TRPV1 (Genetex, Los Angeles, CA), TRPV3 (Santa Cruz Biotechnology , Santa Cruz, CA), Tyrosinase (Santa Cruz), MITF (Thermo Fisher Scientific, Grand Island, NY), Tyrosinase-related protein 1 (TRP-1; Abcam, Cambridge, UK), Dopachrome tautomerase (DCT ; Abcam), β-actin (Sigma-Aldrich), PKC-βII (Santa Cruz) and Integrin-αV (Santa Cruz).

Patient demographics

The vast majority were middle-aged women (mean age of 54.3 years) with longstanding PCD, with a mean disease duration of 23.9 months. Five (33%) patients reported pruritus associated with PCD while only one patient reported heating sensation related to the condition. Hyperpigmentation was localized on either the face or the neck in 5 (33%) and 2 (13%) patients respectively. Hyperpigmentation was noted on both face and neck in 8 (53%) patients.

Hypertension, psoriasis, Crohn's disease and dyslipidemia were identified as underlying conditions although none demonstrated evident association with PCD.

Skin specimens were obtained in PCD patients undergoing skin biopsy for pathologic confirmation of the condition. After local injection of 0.1 mL 2% lidocaine, skin biopsy was performed using 4-mm biopsy punch. The specimen was embedded in 10% neutral buffered formalin for histologic evaluation. An additional skin specimen was obtained using a 2-mm biopsy punch at the margin of the original biopsy wound, embedded in RNA stabilization solution (RNAlater™, Merck, Darmstadt, Germany) for molecular analysis.

Next-generation sequencing in PCD patients

We performed punch biopsies of lesional skin and adjacent peri-lesional one obtained from 3 PCD patients. Total RNA was isolated from tissue using Trizol based method. One μg of total RNA was processed for preparing mRNA sequencing library using TruSeq stranded mRNA sample preparation kit (Illumina, San Diego, CA) according to manufacturer's instruction. The first step involves purifying the poly-A containing mRNA molecules using poly-T oligo attached magnetic beads. Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. Strand specificity is achieved by replacing dTTP with dUTP in the Second Strand Marking Mix (SMM), followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments then have the addition of a single 'A' base and subsequent ligation of the adapter. The products are then purified and enriched with PCR to create the final cDNA library. Finally, quality and band size of libraries were assessed using Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA). Libraries were quantified by qRT-PCR using CFX96 Real Time System (Biorad, Hercules, CA). After normalization, sequencing of the prepared library was conducted on the Nextseq system (Illumina) with 75 bp paired-end reads. The result was aligned using the reference human genome (hg19).

Cell culture

Normal human epidermal melanocytes (NHMs) were obtained from Invitrogen and maintained in Medium 254 (Invitrogen, Carlsbad, CA) containing human melanocyte growth supplement (HMGS; Invitrogen). Normal human keratinocytes (NHKs; Invitrogen) were cultured in keratinocyte growth media (EpiLife; Invitrogen) supplemented with Human Keratinocyte Growth Supplement (HKGS; Invitrogen).

The co-culture model of NHMs and NHKs were to proceed as the following method: NHMs were into a 6-well plate at a density of 6×10^4 cells per well. Next day, NHKs were added to each well at a density of 3×10^5 cells for the co-culture. Co-cultures of NHMs and NHKs were cultured in keratinocyte growth media, the seeding ratio of NHKs to NHMs being 5:1.

After 24 h, TRPV1 regulators were treated. Melanin contents were measured after 5 days.

Immunohistochemistry and semiquantitative image analysis

Immunohistochemical staining with TRPV1 and TRPV3 were performed on 2-mm punch skin biopsy specimens (lesional and peri-lesional skin pair) of patients with PCD. Samples were fixed in 10% neutral-buffered formalin overnight and then embedded in paraffin. These specimens were cut into 4-μm sections and serial sections were prepared. For antigen retrieval, sections were autoclaved in Antigen unmasking solution (Vector laboratories, Burlingame, CA) and then immunostained using Vector Elite ABC Kit (Vector) according to the manufacturer's instruction. The AEC substrate was used for detection.

Both epidermal and dermal intensity and the number of immunostained dermal cells were rated on a scale of 0 to 4 (0=absent, 1=weak/low, 2=moderate, 3=strong, and 4=very strong staining).

Expression analysis by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Then, 1 μg of RNA was reverse transcribed using a RevertAid First Strand cDNA Syntesis Kit (Thermo Scientific). qRT-PCR was performed using the LightCycler® 480II machine coupled with SYBR Green chemistry (Roche Applied Science, Indianapolis, IN). In terms of qRT-PCR settings, initial denaturation was performed at 95°C for 5 min, followed by amplification at 95°C for 10 sec, 60°C for 10 sec, 72°C for 10 sec for 45 cycles. Primers specific for RPLP0 were used for loading control amplifications. Specific primer sets used for each gene were as follows;

TRPV1-F: 5′-GGCTGTCTTCATCATCCTGCTGCT-3′,

TRPV1-R: 5′-GTTCTTGCTCTCCTGTGCGATCTTGT-3′,

TRPV2-F: 5′-CTTCCTTTTCGGCTTCGCTGTAG-3′,

TRPV2-R 5′-GCACTGACTCTGTGGCATTGG-3′,

TRPV3-F: 5′-TCCTCACCTTTGTTCTCCTCCT-3′,

TRPV3-R: 5′-CGCAAACACAGTCGGAAATCAT-3′,

TRPV4-F: 5′-CTACGCTTCAGCCCTGGTCTC-3′,

TRPV4-R: 5′-GCAGTTGGTCTGGTCCTCATTG-3′,

Tyrosinase-F: 5’-GGCCTCAATTTCCCTTCACA-3′

Tyrosinase-R: 5’-CAGAGCACTGGCAGGTCCTAT-3′

RPLP0-F: 5′- GGCGACCTGGAAGTCCAACT-3′,

RPLP0-R: 5′-CCATCAGCACCACAGCCTTC-3′.

Cell viability assay

Cell viability was determined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay. The cells were treated with various concentrations of TRPV1 regulators for 3 days, and then added with MTT solution (2.5 mg/ml in PBS). After incubation for 2 hours (hr), the absorbance was determined at 570 nm using microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of melanin content

The cells were incubated in 6-well plate with TRPV1 regulators for 5 days. Cells were dissolved in 550 μL of 1 N NaOH at 100°C for 30 min. The optical densities (OD) of the supernatants were measured at 405 nm using a microplate reader.

Western blot analysis

Cells were lysed in protein extraction solution (PRO-PREPTM; iNtRON, Seongnam, Korea) and halt protease inhibitor (Thermo Scientific, Rockford, IL). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich). Briefly, 20 μg of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes, which were then blocked with 5% skim milk in Tris-buffered saline containing 0.5% Tween-20 for 1 hr at room temperate. Blots were incubated with the primary antibodies for overnight at 4℃. Antibody recognition was detected with the following secondary antibody linked to horseradish peroxidase. Bound antibodies were detected using an enhanced chemiluminescence substrate (Thermo Scientific).

Cell Fractionation

After cell washing with PBS, 1 ml of hypoosmotic solution [20mM Tris/HCl (pH 7.5), 2 mM β-mercaptoethanol, 2 mM EDTA, 1 M NaOH dissolved 5 mM EDTA] containing a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A and 20 μg/ml PMSF) was applied to the culture plate. The cell homogenate was centrifuged at 14,000 rpm for 30 min at 4 °C. The resulting supernatant represented the cytosolic fraction. The membrane fraction was collected by solubilizing the pellet in RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl 1% (V/V) Nonidet P40, 10 mM NaF/2 mM Na3V04] containing the protease inhibitor cocktail. RIPA lysates were centrifuged at 15,000 g for 30 min at 4℃, denatured with SDS sample buffer, then boiled and analyzed by SDS-PAGE.

Intracellular and cell-free tyrosinase activity assay

The cells were lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100, then disrupted by freezing and thawing. After quantifying the protein levels of the lysate and adjusting the protein concentrations with lysis buffer, 90 μL of each lysate containing the same amount of protein was placed in each well of a 96-well plate, and 10 μL of 10 mM L-DOPA was then added to each well. Following incubation for 30 min at 37°C, absorbance at 475 nm was measured using a microplate reader. A cell-free assay system was used to examine the direct effects of AMG9810 on tyrosinase activity. Thus, A 70 μL of phosphate buffer containing AMG9810 was mixed with 20 μL of 10 μg/mL mushroom tyrosinase, and 10 μL of 10 mM L-DOPA was added. Following incubation for 30 min at 37°C, absorbance was measured at 475 nm.

Statistical analysis

All values are expressed as means and standard deviations (SD). Differences between results were assessed for significance using the Tukey's t-test. In this study, $p<0.05$ and $p<0.01$ represent * and ** respectively and were considered statistically significant.

RESULTS

1. TRPV expression in PCD patients

1.1 Elevated TRPV expression and melanogenic factors in PCD lesions compared to peri-lesional skin.

Epidermal keratinocytes as well as some dermal fibroblasts and inflammatory cells were positively stained with both TRPV1 and TRPV3. Compared to the peri-lesional skin, PCD lesions demonstrated more prominent positive epidermal and dermal stainings with TRPV1 and 3. Increased epidermal and dermal TRPV1 immunostaining was observed in 9 (60%) and 7 (47%) of a total of 15 patients, respectively. Epidermal and dermal TRPV3 stainings were more intense in lesional skin than in peri-lesional skin in 6 (40%) and 7 (47%) patients, respectively. On statistical analysis, epidermal TRPV1 expression was higher in lesion compared to peri-lesion. No significant difference was noted in epidermal TRPV3, dermal TRPV1 and dermal TRPV3 compared to peri-lesional counterpart. (Fig. 2)

1.2 RNA Sequencing and qRT-PCR of melanogenic molecules and ion channel-related genes in PCD lesions compared to peri-lesional skin.

We performed RNA Sequencing of lesions compared to peri-lesional skin of a PCD patient for determining the expression of ion channel-related genes. This showed that TRPV1, 2, 3,4 and VDAC1 were up-regulated (Table 2A). mRNA sequencing results of other patients' lesions compared to peri-lesion showed significant differences of melanogenesis-related genes including Pmel, TYRP1 and S100A8 (Table 2B).

To validate the results of RNA Sequencing, we performed qRT-PCR analysis using 3 patients of lesion and peri-lesion skin samples. mRNA expression of TRPV1 was significantly up-regulated in 2 patients and TRPV3 was also up-regulated in 3 patients. The expression of tyrosinase, a critical melanogenenic enzyme was significantly up-regulated in 2 patients (Fig. 3A).

We examined these genes of mRNA detection using qRT-PCR in primary human skin cells, and then the mRNA expression of TRPV1 was stronger than other TRPV genes in

melanocyte than other skin cells (Fig. 3B).

2. TRPV modulation affects melanogenesis

2.1 Capsaicin resulted in reduction of NHMs co-cultured with NHKs viability proportionally to its concentration.

Capsaicin, a potent TRPV1 activator, on the cell viability NHMs co-cultured with NHKs. Cells were treated with varying concentrations of capsaicin for 72 hr. Detrimental effect of capsaicin on the cell viability was noted at concentrations higher than 20 μM. The effect of capsaicin was found to be proportional to its concentration (Fig 4A).

2.2 Treatment with capsaicin increased melanin synthesis in NHMs co-cultured with NHKs

NHMs co-cultured with NHKs treated with capsaicin to activate TRPV1 showed increased melanin synthesis for 5 day treatment. The melanin content was increased by treatment with capsaicin at concentrations equal to or higher than 20 μM (Fig. 4B)

2.3 TRPV blockade did not affect NHMs viability

Different concentrations of TRPV antagonists were used to treat NHM in order to determine their effects on cell viability. On the fifth day after incubation with AMG9810, a TRPV1 antagonist, at varying concentrations from 0.1 μM to 1 μM the viability of neither NHMs was not affected. (Fig. 5A)

2.4 TRPV blockade down-regulated melanin synthesis in NHMs.

NHMs were incubated for 5 days in various concentration of AMG9810 to verify the effect of TRPV1 blockade on melanin production. The melanin content was significantly reduced by treatment with AMG9810 concentrations of 0.25 μM and higher. The overall trend of decrease in melanin content exhibited a dose-dependent relationship (Fig. 5).

2.4 The effect of TRVP1 activator was reversed by TRPV blockade.

Further experiments were carried out by adding AMG9810 after having treated the cells with

capsaicin. Compared to the control, treatment with both 10 μM and 30 μM capsaicin increased the melanin content in NHMs co-cultured with NHKs. By adding AMG9810, this increase in melanin content was reversed, with the highest AMG9810 concentration (1 μM) showing the strongest effect. In this experiment, the inhibitory effect of AMG9810 on melanogenesis following TRPV stimulation with capsaicin displayed a dose-dependent relationship. (Fig. 5C)

2.5 Another TRPV1 blockade down-regulated melanin synthesis.

To examine the effect of another TRPV1 blockage, NHMs co-cultured with NHKs treated with TRPV1 blockade, DWP 05195 down-regulated melanin synthesis for 5 days treatment. The melanin content was decreased by DWP 05195 concentrations of 100 ng/ml and higher. (Fig. 5D)

3. AMG9810 inhibited melanogenesis via PKC-βII

3.1 AMG9810 inhibited melanogenesis-associated protein

We investigated whether AMG9810 decreases the expression levels of MITF, tyrosinase TRP-1 and DCT. NHMs were treated with 1 μM AMG9810 for 1~ 5 days and analyzed by using western blot. As shown in Fig. 6A, treatment with 1 μM AMG9810 decreased the level of tyrosinase, TRP1 and TRP2 protein. However, MITF expression did not significantly decrease (Fig 6A).

3.2 AMG9810 inhibited melanogenesis via PKC-βII, the regulator of tyrosinase activity.

As AMG9810 reduced melanin synthesis and the expression of melanogenesis-associated protein, we further investigated the signaling pathways associated with regulation of melanogenesis. Especially, PKC-βII is known to increase melanogenesis and the activity of PKC-βII is determined by the membrane localization. NHMs were treated with 1 μM AMG9810 for 15 min and cell lysates were fractioned into cytoplasm and membrane. Following stimulation with AMG9810, membrane PKC-βII reduced, in other words, cytoplasmic PKC-βII did not translocate to the membrane (Fig. 6B) . Because PKC-βII is a

regulator of tyrosinase activity, we investigated whether AMG9810 decreases the Tyrosinase activity

The effects of AMG9810 on cellular tyrosinase activity were examined by evaluating L-DOPA oxidation activity. The results showed that cellular tyrosinase activity of AMG9810-treated cells was reduced time-dependently (Fig. 6C). Next, to investigate whether AMG9810 directly inhibits tyrosinase, we measured the effect of AMG9810 on mushroom tyrosinase in a cell-free system. AMG9810 did not show any inhibitory effect on mushroom tyrosinase (Fig. 6D).

DISCUSSION

Transient receptor potential (TRP) channels are distributed widely across human tissue including the skin.(5-7) They are classified into six subfamilies according to their chemical structures and consequently differ in their distribution and function. As mediator of calcium influx into the cytoplasm of a variety of cells, TRPs are linked to diverse essential physiological processes and thus many conditions of abnormal calcium homeostasis including Hailey-Hailey disease, Darier's disease, and Olmsted syndrome.(8, 9) Of particular importance is the TRP vanilloid (TRPV), which is the most comprehensively characterized TRP subfamily in mammals. The presences of TRP vanilloid (TRPV) 1, TRPV2, TRPV3 and TRPV4 in human skin have been demonstrated, and the specific roles of these cations channels in skin physiology are being readily reported.(10-13) Among these, TRPV 1 and TRPV3 are reported to be involved in heat and itching sense in skin.(14, 15) In the skin, TRPVs are expressed in many cell types, including keratinocytes, sensory neurons, melanocytes, and immune/inflammatory cells. TRPVs are have been focused by recent investigations in the background that dermatologists and researchers in related industries have been making substantial efforts to reduce burning sensation and itching, which result from dermatologic procedures producing skin resurfacing, contact dermatitis to irritants/allergens or irritated/sensitive skin conditions. Thus, inhibitors of TRPVs have been developed to ameliorate these sensation and conditions and are used or are developed to be used as ingredients of cosmetics or topicals.

As previously mentioned in the introduction, PCD is a cosmetically debilitating disorder causing detrimental effects to the patients. Unfortunately, the pathogenesis and appropriate treatment are still under debate. The development of pigmented contact dermatitis (PCD) are also associated with dermatologic procedures such as heat producing or mechanically irritating laser, contact dermatitis in easily irritated and pigmented skin type. Use of hair dye with heating (especially henna), cosmetic products, peels, perfumes and jewelry is conjectured as precipitating or casual events on PCD.(1-3) Patients with PCD frequently report heating sensation and itching with somewhat apparent erythema on the lesions.

Histaminergic itch may be involved in hyperpigmentation in PCD as histamine acts through receptors on effector cells and H1 and H2 receptors exist on human melanocytes. Itching occurs also via TRPVs which sense mechanical or nociceptive stimuli such as heat, clothing or contact irritation, sweat, some environmental factors such as dryness or humidity, scratching, scrubbing and so on. TRPVs and ions are also related to inflammation-triggered itch. Histologically, PCD is primarily caused by cutaneous inflammation confined to the basal layer of epidermis (lichenoid dermatitis), increased pigmentation and vessel dilatation (6). Accordingly, the basal melanocyte damage or cytotoxic response may trigger an inflammatory response that eventually entails abnormal vessel triggered/inflammation-associated hypermelanosis. In fact, more than half of PCD patients tend to be clinically accompanied by flushing and/or rosacea and sensitive skin syndrome, in which increased expression of cutaneous TRPVs have been newly characterized.(16, 17) As a consequence of either excessive or deficient TRPVs activity, cutaneous pathologic conditions such as chronic pain and itch, dermatitis, vitiligo, alopecia, wound healing, skin carcinogenesis, and skin barrier compromise may occur.(12, 18, 19) Human skin melanocytes modulate skin color in response to stress and skin barrier perturbation results in hyperpigmentation. A representative of TRPV 1 activator, capsaicin is notorious for causing burning/itch sensation by provoking action potential of sensory nerves and releasing substance P(SP) which mediates neurogenic inflammation, vasodilatation and hyperpigmentation.(20) As such, topical calcineurin inhibitors elicit burning sensation probably via activating TRPVs. These specific clinical observations had shed light on the possibility of TRPVs being involved in both the clinical signs and symptoms such as heating sense and itching of PCD. Therefore, we sought to evaluate the expression of level of TRPV 1 and TRPV 3 in the skin of patients with PCD aiming at identifying crucial receptors and channels related to development of PCD. In results, TRPVs were increased in the epidermal and dermal cells in the lesions of PCD which means there are more mechanical or nociceptive stimuli in the lesions and the cellular environments surrounding melanocytes. In our present study, in addition to change of TRPVs, other genes were increased in the lesions of PCD compared to control or peri-lesion suggesting that PCD may result from complex sum of pathogenesis involving

numerous alterations of cellular milieu. Further, we identified TRPV 1 and TRPV 3 in human melanocytes, a finding which was consistent with previous reports and the latest research suggesting a putative role of TRP channels in cutaneous pigmentation. (18, 21-23) Although other members of the TRP family are known to be involved in melanocyte and melanoma function, our finding was the first one that TRPV1 regulates melanogenesis in human melanocytes as well as B16 melanoma cells and co-culture. As mechanism, intracellular calcium concentration has been presumed to be the key through which TRPVs affect melanin production. We herein also demonstrated downmodulation of PKC-βII signaling which in turn, decreases activity of tyrosinase enzyme in the anti-melanogenic effect of TRPV1 inhibitors. Overproduction of melanin can cause skin hyperpigmentary disorders, such as solar lentigo, melasma and post-inflammatory hyperpigmentation(PIH). Tyrosinase, which is the rate-limiting enzyme in melanin biosynthesis, controls melanogenesis by catalyzing the hydroxylation of L-tyrosine to L-dihydroxydroxyphenylalnine (L-DOPA) as well as the oxidation of DOPA to DOPA-quinone.(22) The central transcription factor involved in the regulation of tyrosinase expression as well as the growth and survival of melanocytes is microphthalmia-associated transcription factor (MITF).(24-26) Several signaling pathways are reportedly involved in melanogenesis including the cAMP signal pathway, PI3K/Akt signal pathway, MEK/Erk signal pathway, and Wnt/β-catenin pathway. In our present study, MITF and these signals were not affected by TRPV1 inhibition. We previously screened anti-melanogenic functions of ion-channel related agents in B16F10 cells media and found several candidates (Fig 1).

Other studies using mouse and zebrafish mutants have identified novel encoding ion transport proteins that regulate melanogenesis. (27, 28) For example, Ca2+ can act as a modulator in the synthesis and storage of pigment in melanosomes, by regulating tyrosinase activity and other melanosomal enzyme activities with change of membrane voltage and/or luminal pH. It is suggested that increase of intracellular Ca2+ contributes to an increase of melanin content.(29, 30) Ion transport could change membrane voltage which might initiate intracellular signaling that modulates melanogenesis. Ca2+ may activate inositol trisphosphate (IP3)/diacylglycerol (DAG) pathway and subsequently localizing protein

kinase C (PKC) enzymes to plasma membrane. PKC-βII, a subtype of classic PKC, is known to regulate melanin synthesis by activating tyrosinase and modulating its location in melanosome. We demonstrated that TRPV1 inhibitor treatment localized PKC-βII from plasma membrane to cytosol, thereby reducing its activity.

Taken together, TRPVs, particularly TRPV1 may participate in the development and aggravation and chronicity of PCD through Ca2+/PKC/tyrosinase activity. In the meantime, activation of TRPVs could explain persistent inflammation and associated burning/itch sensation. These diverse functions of TRPVs, coupled with the fact that TRP channels are pharmacologically accessible and feasible, make this family of proteins appealing therapeutic targets for skin disorders. Among them, TRPV1 could be a major therapeutic target of PCD, a refractory inflammatory hyperpigmentary skin disorders.

국문요약

배경 및 목적: 색소성 접촉피부염은 대표적인 피부의 염증반응에 의한 과색소증으로 아직까지 원인이 불명하며 국소 도포제, 경구 약물 및 레이저치료 등의 단일 또는 복합 치료에도 불구하고 임상적 호전을 기대하기가 어려운 피부 질환이다. 염증에 의한 피부 색소침착과 연관된 다양한 신경전달물질, 채널, 수용체들이 현재까지 밝혀져 왔으나 염증성 색소침착의 기전은 아직 명확하지 않다. 피부와 신경 조직 등에 분포하는 transient receptor potential (TRP) 채널은 세포 내 칼슘 이온 농도 조절을 통해 세포 내 신호전달을 관장하는데 TRP vanilloid(TRPV)는 포유류의 다양한 세포들에서 발현이 확인되어 그 기능에 대한 연구가 진행 중이다. 피부과 영역에서 TRPV는 주사(rosacea) 및 민감성피부(sensitive skin)에서 발현이 증가되어있음이 최근에 확인되었다. 색소성 접촉피부염 환자들 중 상당 비율이 안면 홍조, 열감, 피부 민감도 증가를 호소하여 저자들은 색소성 접촉피부염의 병태생리에 TRPV가 관여할 것으로 예상하고 본 연구에서는 환자들의 피부 조직검체의 TRPV 발현 정도를 확인하고 세포실험을 통한 TRPV이 멜라닌 합성에 미치는 영향을 관찰할 것이다.

재료 및 방법: 면역조직화학염색을 시행하여 색소성 접촉 피부염 환자들의 병변부에서 TRPV의 발현 정도를 분석하였다. 환자들의 과색소 병변 조직을 병변부 주변 피부조직과 비교하였다. Next-generation sequencing을 통해 환자의 병변부와 병변부 주변 피부간 멜라닌 합성 관련 유전자들의 mRNA 증폭여부를 확인하였다. 정상 사람 표피 멜라닌 세포를 TRPV1 작용물질인 capsaicin과 TRPV1 억제제인 AMG9810으로 처리하였고 세포 생존능과 멜라닌 함유 정도를 분석하였다. 멜라닌 합성과 관련된 단백질 발현 정도에 대해서는 웨스턴 블롯으로 분석하였다.

결과: TRPV1는 색소성 접촉피부염 환자들의 과색소 병변 조직에서 정상 또는 저색소 조직에 비해 발현이 증가한 것으로 확인되었으나 TRPV3의 경우 발현 양상의 유의한 차이를 확인할 수 없었다. 아울러 색소성 접촉피부염 환자들의 병변부 피부 조직에서 TRPV1,3를 비롯한 Pmel, TYRP1, S100A8과 같은 멜라닌생성관련

유전자들의 messenger RNA(mRNA)가 환자의 병변부 주변 피부 조직보다 증폭되어있음을 확인하였다. 추가적으로 시행한 qRT-PCR에서는 TRPV1과 TRPV3, 그리고 tyrosinase의 발현이 증가됨이 나타났다. Cultured human melanocyte를 이용한 세포실험에서는 TRPV1의 활성이 멜라닌 세포의 멜라닌 합성을 촉진하는 것으로 확인되었으며 이러한 작용은 AMG9810을 처리함으로써 역전이 가능했다. 또한 AMG9810는 멜라닌 세포의 PKC-βII 활성 저해를 통한 tyrosinase 활성을 억제하였다.

결론: TRPV1은 색소성 접촉피부염 환자의 병변부 피부에서 발현이 증가해있으며 TRPV1의 활성은 멜라닌 세포의 멜라닌 합성을 촉진한다. TRPV1의 선택적 억제제인 AMG9810을 이용한 멜라닌 합성 억제 작용은 PKC-βII 경로를 통한 tyrosinase의 활성억제를 통해 일어나며, TRPV1이 Ca2+/PKC/tyrosinase의 활성도를 통한 멜라닌 합성 과정을 조절하는 것을 확인하였다. 본 사실을 통해 향후 TRPV의 조절을 이용한 색소성 접촉피부염과 그 외 피부염증반응에 의한 과색소증의 치료가 유망할 것으로 기대한다.

중요단어: Transient receptor potential vanilloid 1, Pigmented contact dermatitis, Melanogenesis, PKC

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FIGURES AND FIGURE LEGENDS

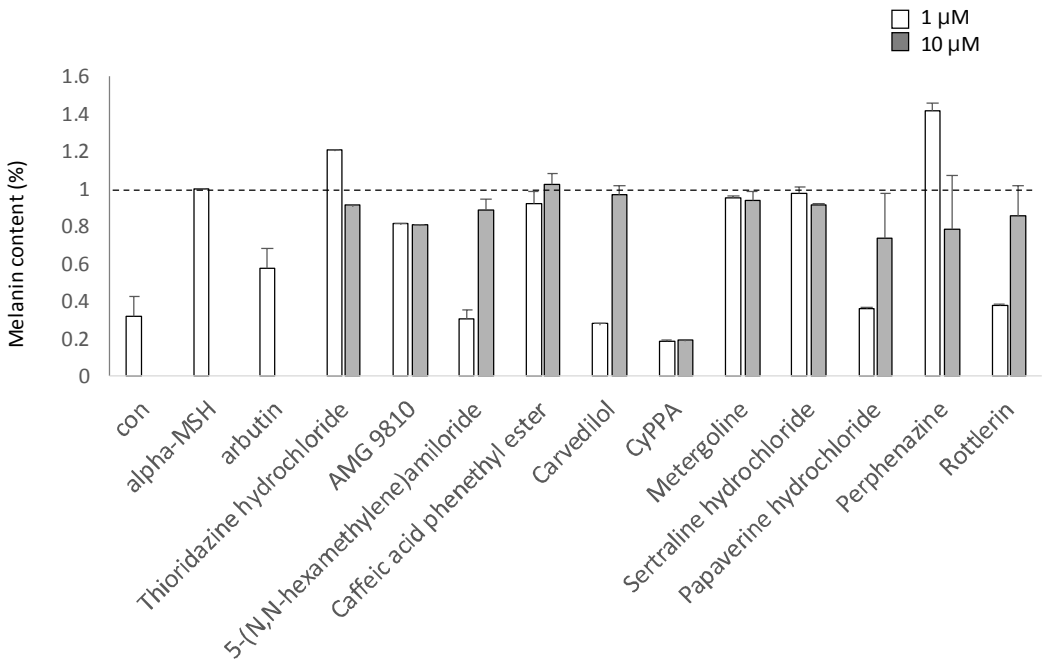

Fig.1 Inhibitors of melanogenesis in a high-throughput screening (HTS) of ion channel-modulating chemical agents in B16F10 cells.

We screened anti-melanogenesis in B16F10 melanoma cells with ion channel-modulating chemical agents from the HTS library, including LOPAC (Sigma-Aldrich). In results, the other 11 chemicals including, thioridazine hydrochloride, AMG 9810, 5-(N,N-hexamethylene) amiloride, caffeic acid phenethyl ester, carvedilol, CyPPA, metergoline, sertraline hydrochloride, papaverine hydrochloride, perphenazine and rottlerin reduced the amount of secreted melanin in the media without causing cytotoxicity in B16F10 cells screening. Alpha-MSH (500 nM) stimulated B16F10 cells were treated with library chemicals at 1 and 10 μM and arbutin (100 μg/ml) as a positive control for 3 days.

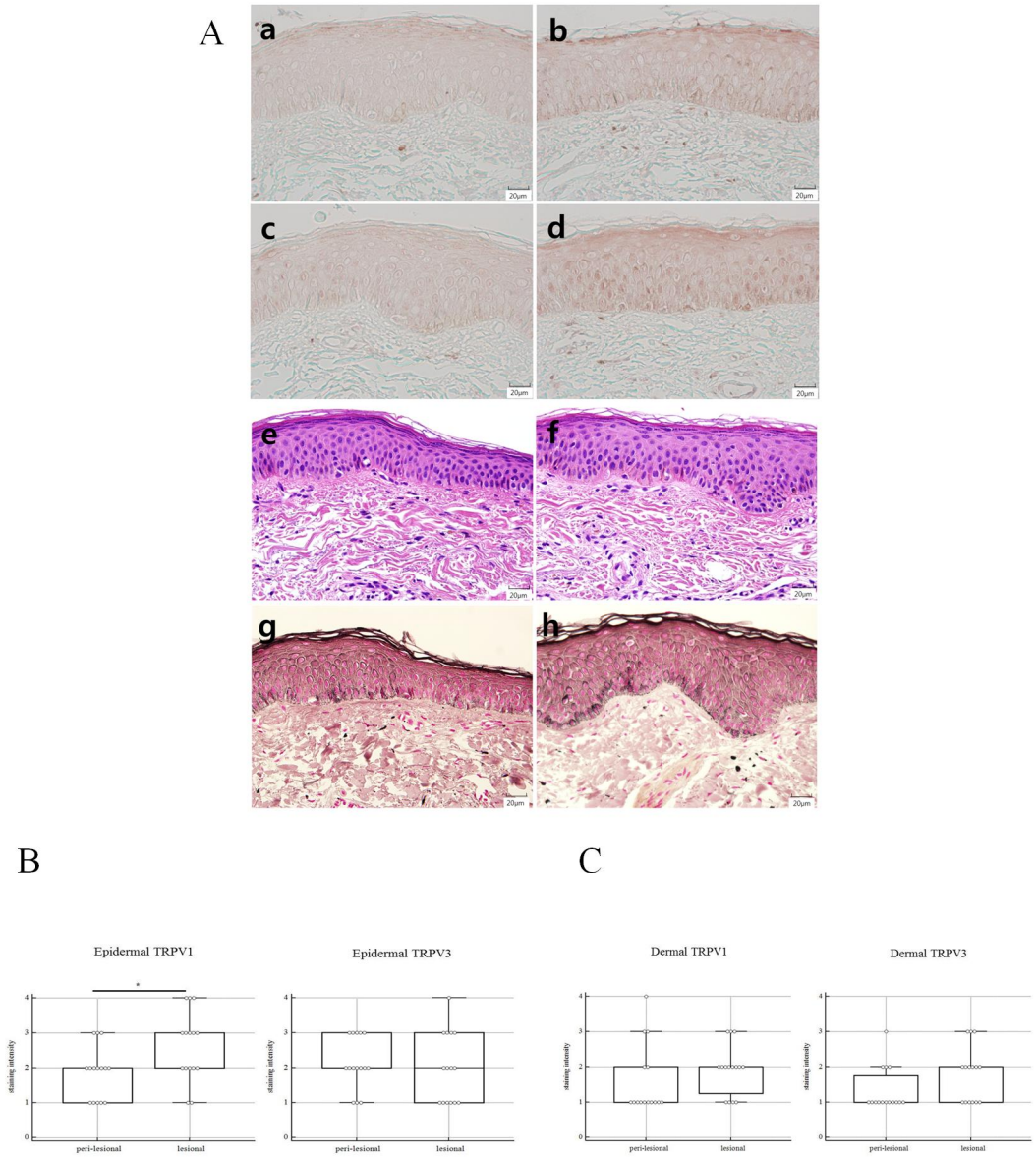

Fig. 2

A: Expression of transient receptor potential vanilloid subfamily 1 (TRPV1) and 3 (TRPV3) in hypopigmented lesion (a,c) and hyperpigmented lesion (b,d) of patient with PCD, respectively. Both TRPV1 and TRPV3 immunostaining was observed in the epidermis of all samples. Stronger immunoreactivity was noted on the hyperpigmented lesion compared to the hypopigmented lesion. (e,f) Hematoxylin and eosin staining of hypopigmented lesion and hyperpigmented lesion, respectively. Increased basal pigmentation on the

hyperpigmented lesion was noted without apparent increase in number of epidermal melanocytes. (g,h) Fontana-Masson staining of hypopigmented lesion and hyperpigmented lesion, respectively. These more clearly demonstrate the increase in the basal pigmentation in peri-lesional specimen. (All figures: x400, Bar: 20 μm)

B: Box-and-whisker plots of the semiquantitative examinations of epidermal TRPV1 and TRPV3 staining intensity comparing peri-lesional to lesional skin specimens. While epidermal TRPV3 expression did not differ from peri-lesional to lesional skin, notably higher epidermal TRPV1 staining intensity was observed in lesional skin. (*: $P < 0.05$)

C: Box-and-whisker plots of the semiquantitative examinations of dermal TRPV1 and TRPV3 staining intensity comparing peri-lesional to lesional skin specimens.

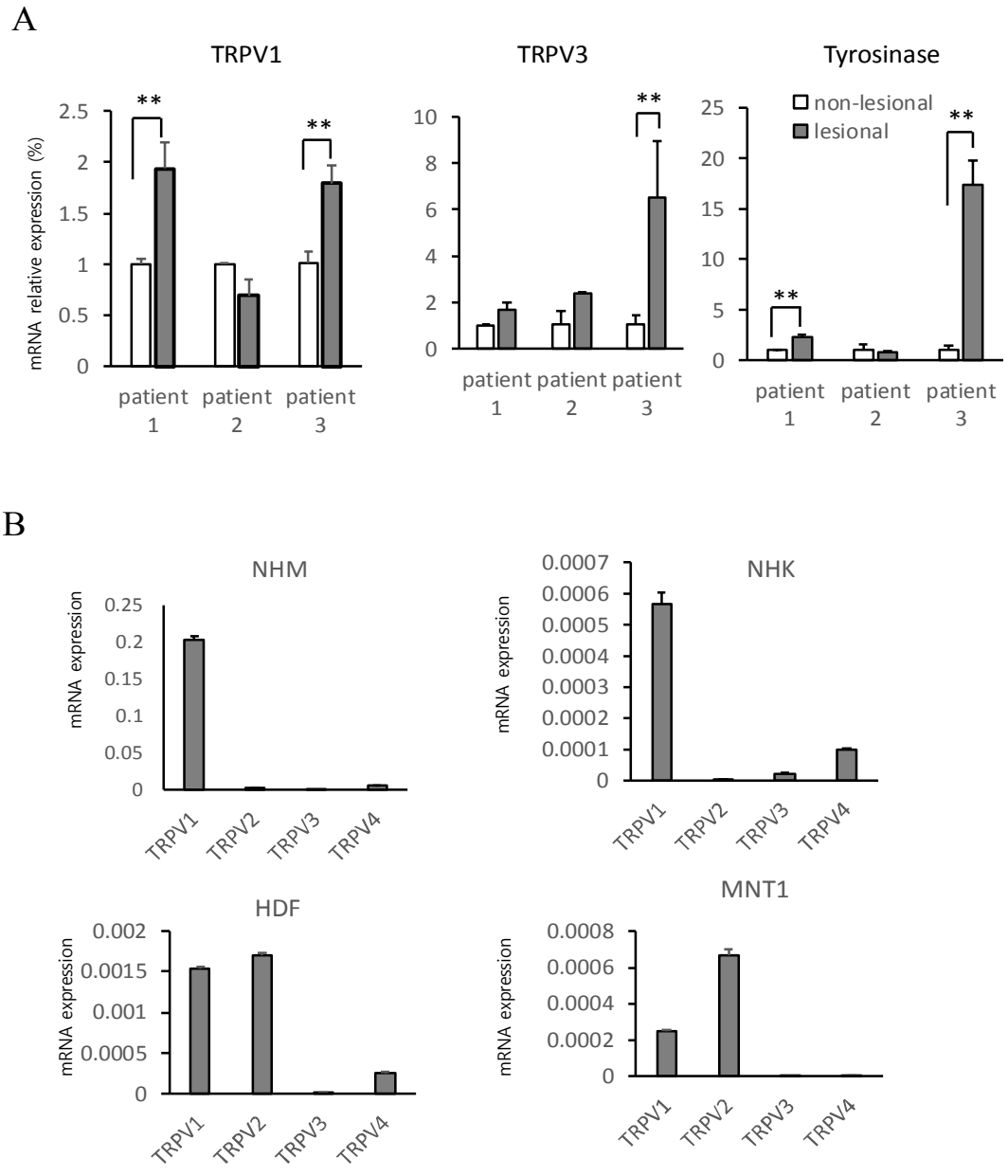

Fig. 3

A: mRNA expression of TRPV1, TRPV3 and Tyrosinase comparing 3 patients' peri-lesional to lesional skin specimens. mRNA expression of TRPV1 was significantly up-regulated in 2 patients and TRPV3 was also up-regulated in 3 patients. The expression of Tyrosinase, a critical melanogenenic enzyme was significantly up-regulated in 2 patients. qRT-PCR which was normalized by that of RPLPO gene (large ribosomal protein). (**: $P < 0.01$)

B: mRNA expression of TRPV1, TRPV2, TRPV3, and TRPV4 in NHK, NHM, HDF and MNT1.

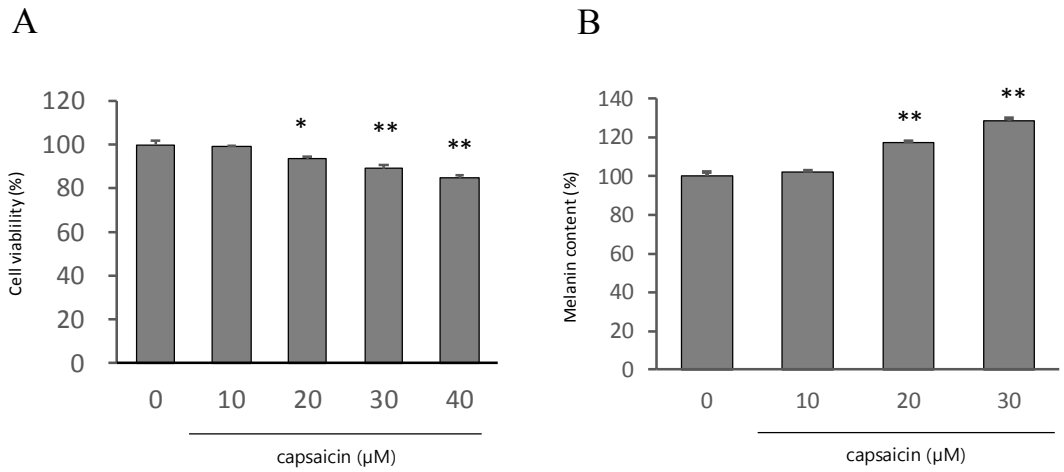

Fig. 4

A: NHMs co-cultured with NHKs were treated with 10-40 μM of capsaicin, for 3 days. Cell viability was examined using MTT testing to show the detrimental effect of capsaicin 40 μM. B: NHMs co-cultured with NHKs were treated with various concentrations (10-30 μM) of capsaicin, a potent TRPV1 activator, for 5 days. The melanin content was increased by treatment with capsaicin at concentrations equal to or higher than 20 μM. (*: $P < 0.05$, **: $P < 0.01$)

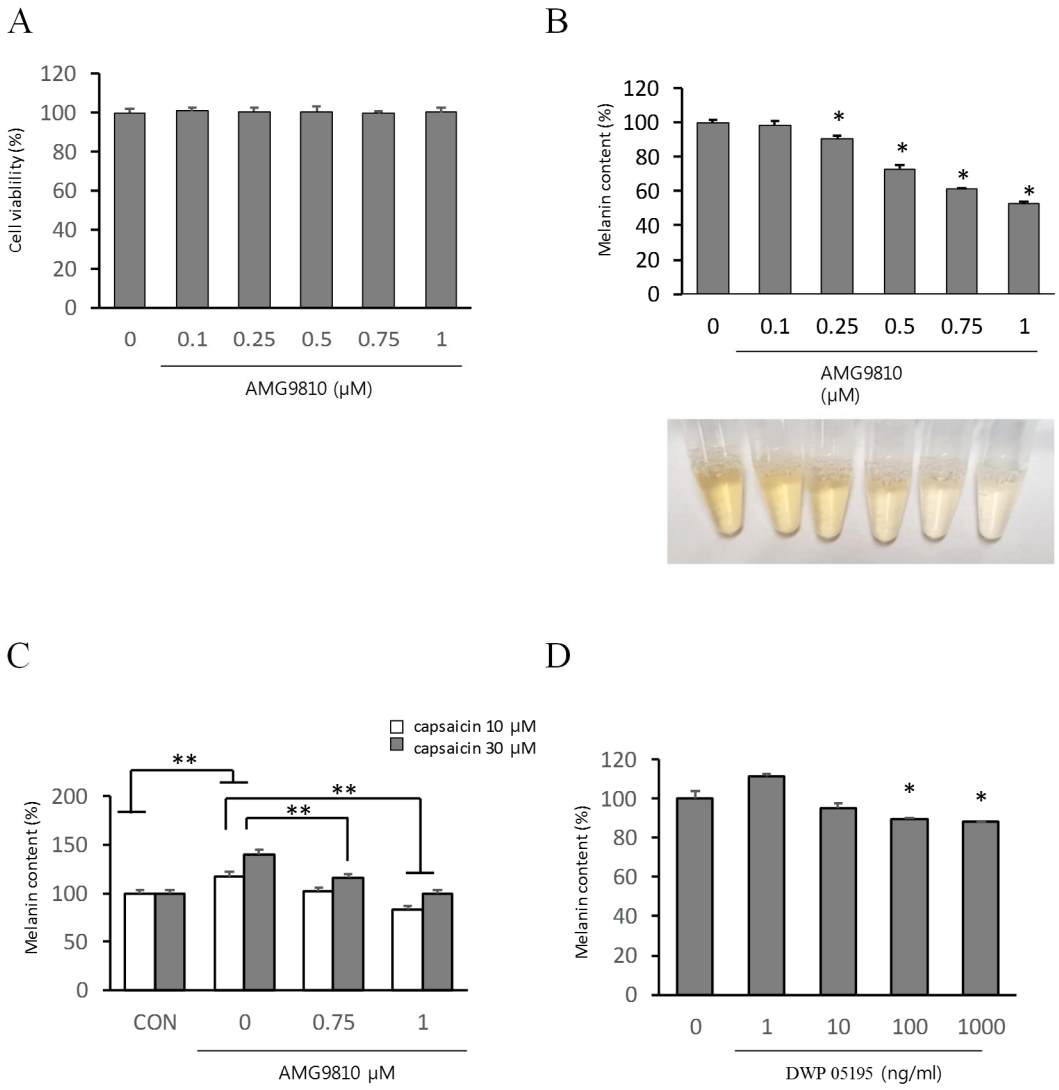

Fig. 5

A: NHMs were treated with various concentrations (0.1-1μM) of TRPV1 antagonist, AMG9810, for 5 days. Cell viability was examined using MTT assay and not affected by AMG9810.

B: NHMs were treated with various concentrations (0.1-1 μM) of AMG9810 for 5 days. The melanin content was significantly decreased by treatment with AMG9810 at concentrations equal to or higher than 0.25 μM. The lysates of NHMs were photographed.

C: NHMs co-cultured with NHKs were treated with various concentrations (10-30 μM) of capsaicin were pretreated with two different concentration of capsaicin, 10 μM and 30 μM.

Then they were treated with of AMG9810 (0.75 μM and 1 μM) for 5 days. The Melanin content was promoted by treatment with capsaicin, an effect which was reversed by addition of AMG9810.

D: NHMs co-cultured with NHKs treated with TRPV1 blockade, DWP 05195 down-regulated melanin synthesis for 5 days treatment. The melanin content was decreased by DWP 05195 concentrations of 100 ng/ml and higher. (*: $P < 0.05$, **: $P < 0.01$)

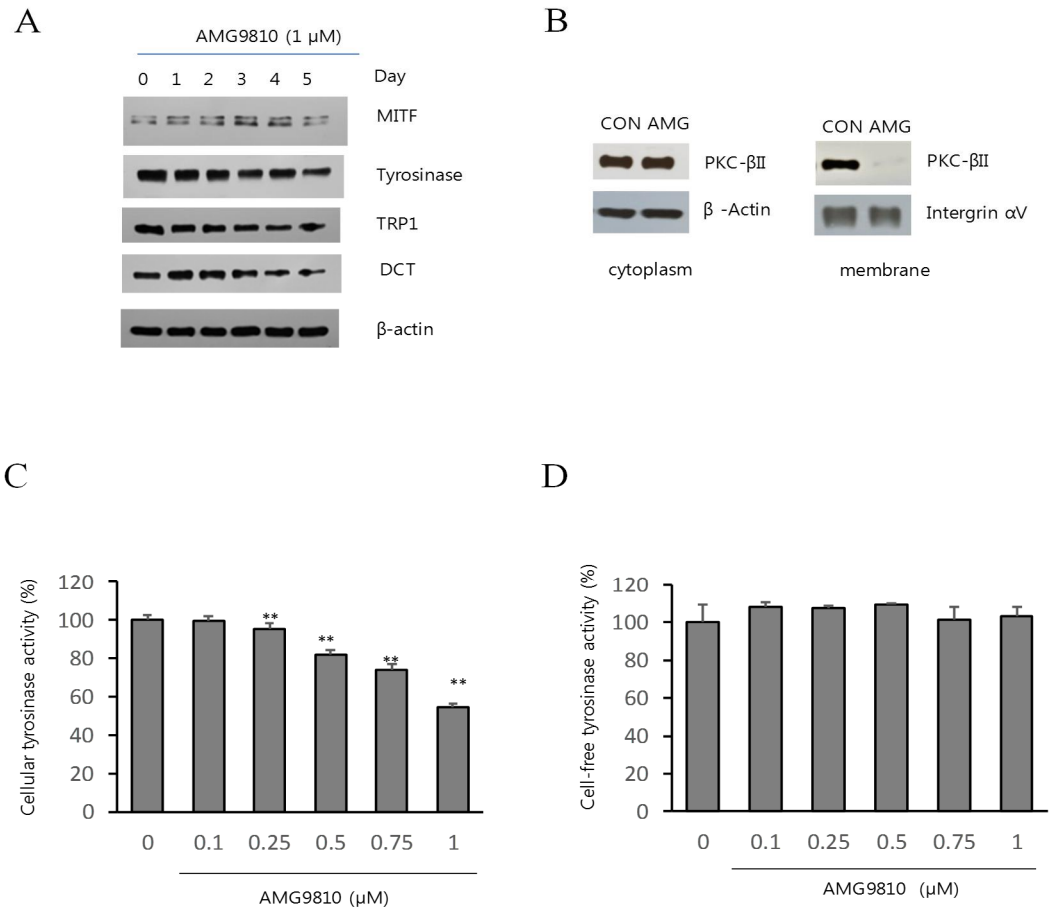

Fig. 6

A: NHMs were treated 1 μM AMG9810 at the indicated times. Cell lysates were assessed using western blot analysis using antibodies against MITF, tyrosinase, TRP-1 and DCT. The β-actin antibody was used as a control for equal protein loading. AMG9810 down-regulated tyrosinase, TRP-1 and DCT.

B: NHMs were stimulated with 1 μM of AMG9810 for 15 min and cell lysates were then assessed using western blot analysis using antibodies against PKC-βII.

C: Cells were treated with AMG9810 (0.1-1 μM) for 5 days, and then cellular tyrosinase activity was assessed.

D: To analyze the direct effect on tyrosinase, its activity in a cell free system was measured. (*: $P < 0.05$, **: $P < 0.01$)

TABLE LEGENDS

Table 1. Demographic and clinical characteristics of the fifteen subjects with PCD investigated in this study.

Onset (mean ±SD, months)	23.9 ± 16.5
Symptom	
Pruritus	5 (33%)
Heating sensation	1 (7%)
Distribution	
Face only	5 (33%)
Neck only	2 (13%)
Face and neck	8 (53%)

Table 2. The expression of ion channel-related genes of PCD patient

Gene Symbol	up/ down	Log2(FC)	descriptions of the gene
TRPV1	up	0.122223	transient receptor potential cation channel subfamily V member 1 [Source:HGNC Symbol;Acc:HGNC:12716]
TRPV2	up	1.05085	transient receptor potential cation channel subfamily V member 2 [Source:HGNC Symbol;Acc:HGNC:18082]
TRPV3	up	0.237425	transient receptor potential cation channel subfamily V member 3 [Source:HGNC Symbol;Acc:HGNC:18084]
TRPV4	up	0.233277	transient receptor potential cation channel subfamily V member 4 [Source:HGNC Symbol;Acc:HGNC:18083]
TRPV5	.	0	transient receptor potential cation channel subfamily V member 5 [Source:HGNC Symbol;Acc:HGNC:3145]
TRPV6	down	-1.39282	transient receptor potential cation channel subfamily V member 6 [Source:HGNC Symbol;Acc:HGNC:14006]
VDAC1	up	0.144531	voltage dependent anion channel 1 [Source:HGNC Symbol;Acc:HGNC:12669]

Table 3. Two fold change differently expressed genes of at least 2 out of 3 PCD patients samples

A: Up-regulated genes

Gene Symbol	#1		#2		#3		average Log2 (FC)	descriptions of the gene
	Log2(FC)	P-value	Log2(FC)	P-value	Log2(FC)	P-value		
ADAMDEC1	1.444	0.036		0.598	3.291	0.005	2.368	ADAM like decysin 1 [Source:HGNC Symbol;Acc:HGNC:16299]
ARG1	1.914	0.046		0.239	1.873	0.035	1.893	arginase 1 [Source:HGNC Symbol;Acc:HGNC:663]
ATP12A	1.671	0.04	1.48	0.049		0.749	1.575	ATPase H+/K+ transporting non-gastric alpha2 subunit [Source:HGNC Symbol;Acc:HGNC:13816]
BST2	1.449	0.04		0.592	2.126	0.009	1.787	bone marrow stromal cell antigen 2 [Source:HGNC Symbol;Acc:HGNC:1119]
CCL18	2.298	0.007		0.799	1.646	0.021	1.972	C-C motif chemokine ligand 18 [Source:HGNC Symbol;Acc:HGNC:10616]
CCL19	1.81	0.01		0.769	2.606	0	2.208	C-C motif chemokine ligand 19 [Source:HGNC Symbol;Acc:HGNC:10617]
CNFN	2.269	0.003		0.539	1.596	0.017	1.933	cornifelin [Source:HGNC Symbol;Acc:HGNC:30183]
COMP		0.437	1.464	0.046	3.208	0.005	2.336	cartilage oligomeric matrix protein [Source:HGNC Symbol;Acc:HGNC:2227]
CXCL10	2.648	0.019		0.251	2.004	0.01	2.326	C-X-C motif chemokine ligand 10 [Source:HGNC Symbol;Acc:HGNC:10637]
CXCL9		0.127	3.314	0.021	3.274	0	3.294	C-X-C motif chemokine ligand 9 [Source:HGNC Symbol;Acc:HGNC:7098]
DEFB4A	Inf	0		1	6.044	0.004	6.044	defensin beta 4A [Source:HGNC Symbol;Acc:HGNC:2767]
ELOVL3	-6.385	0.003	1.749	0.034	5.126	0	0.164	ELOVL fatty acid elongase 3 [Source:HGNC Symbol;Acc:HGNC:18047]
EPGN	5.111	0.008		0.163	3.148	0.048	4.129	epithelial mitogen [Source:HGNC Symbol;Acc:HGNC:17470]
FABP5	2.528	0		0.515	2.477	0.001	2.502	fatty acid binding protein 5 [Source:HGNC Symbol;Acc:HGNC:3560]
FGFBP1	2.487	0.001		0.474	1.396	0.038	1.942	fibroblast growth factor binding protein 1 [Source:HGNC Symbol;Acc:HGNC:19695]
FGFBP1	2.487	0.001		0.474	1.396	0.038	1.942	fibroblast growth factor binding protein 1 [Source:HGNC Symbol;Acc:HGNC:19695]
GABRP	2.571	0.05		0.198	1.604	0.04	2.087	gamma-aminobutyric acid type A receptor pi subunit [Source:HGNC Symbol;Acc:HGNC:4089]
GDA	Inf	0		0.109	4.723	0.002	4.723	guanine deaminase [Source:HGNC Symbol;Acc:HGNC:4212]
GGCT	2.287	0.004		0.782	1.488	0.041	1.888	gamma-glutamylcyclotransferase [Source:HGNC Symbol;Acc:HGNC:21705]
GSTA1	-10000	0	2.056	0.05	5.754	0.004	3.905	glutathione S-transferase alpha 1 [Source:HGNC Symbol;Acc:HGNC:4626]
GZMA	1.477	0.049		0.249	3.166	0.024	2.321	granzyme A [Source:HGNC Symbol;Acc:HGNC:4708]
GZMB	2.735	0.004		0.317	4.069	0.014	3.402	granzyme B [Source:HGNC Symbol;Acc:HGNC:4709]
HEPHL1	4.18	0.009	2.798	0.043	1.349	0.045	2.776	hephaestin like 1 [Source:HGNC Symbol;Acc:HGNC:30477]
HRASLS2	5.089	0.009		1	Inf	0.037	5.089	HRAS like suppressor 2 [Source:HGNC Symbol;Acc:HGNC:17824]
IDO1	1.691	0.04		0.422	3.807	0.008	2.749	indoleamine 2,3-dioxygenase 1 [Source:HGNC Symbol;Acc:HGNC:6059]
IL36A	Inf	0.002		1	Inf	0.034	inf	interleukin 36, alpha [Source:HGNC Symbol;Acc:HGNC:15562]
IL36G	2.793	0		0.482	2.47	0.007	2.632	interleukin 36, gamma [Source:HGNC Symbol;Acc:HGNC:15741]
IL36RN	1.872	0.007		0.585	1.586	0.02	1.729	interleukin 36 receptor antagonist [Source:HGNC Symbol;Acc:HGNC:15561]
IRF8	1.444	0.039		0.738	1.844	0.017	1.644	interferon regulatory factor 8 [Source:HGNC Symbol;Acc:HGNC:5358]
IVL	1.742	0.021		0.507	1.697	0.012	1.719	involucrin [Source:HGNC Symbol;Acc:HGNC:6187]
JCHAIN		0.601	1.822	0.008	3.815	0.007	2.819	joining chain of multimeric IgA and IgM [Source:HGNC Symbol;Acc:HGNC:5713]
KLK10	1.669	0.05		0.168	1.932	0.011	1.8	kallikrein related peptidase 10 [Source:HGNC Symbol;Acc:HGNC:6358]
								kallikrein related peptidase 6 [Source:HGNC

(Table 3A continued)

KLK6		0.917	1.653	0.024	3.145	0	2.399	Symbol;Acc:HGNC:6367]
KRT16	2.972	0.012		0.052	3.05	0.017	3.011	keratin 16 [Source:HGNC Symbol;Acc:HGNC:6423]
KRT25		1	3.937	0	7.872	0.004	5.904	keratin 25 [Source:HGNC Symbol;Acc:HGNC:30839]
KRT27		1	4.173	0	6.007	0.001	5.09	keratin 27 [Source:HGNC Symbol;Acc:HGNC:30841]
KRT31		0.654	3.329	0.005	2.971	0	3.15	keratin 31 [Source:HGNC Symbol;Acc:HGNC:6448]
KRT32		1	3.642	0.018	4.141	0.008	3.891	keratin 32 [Source:HGNC Symbol;Acc:HGNC:6449]
KRT35		1	Inf	0	Inf	0	inf	keratin 35 [Source:HGNC Symbol;Acc:HGNC:6453]
KRT6C	4.116	0		0.256	4.485	0	4.301	keratin 6C [Source:HGNC Symbol;Acc:HGNC:20406]
KRT71		1	4.79	0	7.295	0.004	6.042	keratin 71 [Source:HGNC Symbol;Acc:HGNC:28927]
KRTAP11-1		1	4.397	0.008	2.359	0.004	3.378	keratin associated protein 11-1 [Source:HGNC Symbol;Acc:HGNC:18922]
LCE3A	Inf	0	Inf	0.004	Inf	0	inf	late cornified envelope 3A [Source:HGNC Symbol;Acc:HGNC:29461]
LCE3D	4.751	0	2.626	0.049	3.696	0	3.691	late cornified envelope 3D [Source:HGNC Symbol;Acc:HGNC:16615]
LCE3E	5.747	0.005		0.424	3.178	0	4.463	late cornified envelope 3E [Source:HGNC Symbol;Acc:HGNC:29463]
LYZ	1.801	0.028		0.935	2.462	0.003	2.132	lysozyme [Source:HGNC Symbol;Acc:HGNC:6740]
NLRP10	1.566	0.029		0.195	1.441	0.033	1.504	NLR family pyrin domain containing 10 [Source:HGNC Symbol;Acc:HGNC:21464]
OASL	1.953	0.019		0.449	3.19	0.002	2.571	2'-5'-oligoadenylate synthetase like [Source:HGNC Symbol;Acc:HGNC:8090]
PI3	3.608	0		0.174	3.088	0	3.348	peptidase inhibitor 3 [Source:HGNC Symbol;Acc:HGNC:8947]
PLIN1		0.065	1.907	0.005	3.903	0.012	2.905	perilipin 1 [Source:HGNC Symbol;Acc:HGNC:9076]
PMEL	2.082	0.004		0.956	2.243	0.003	2.163	premelanosome protein [Source:HGNC Symbol;Acc:HGNC:10880]
PRR9		1	2.869	0	6.605	0.003	4.737	proline rich 9 [Source:HGNC Symbol;Acc:HGNC:32057]
S100A8	5.212	0	1.682	0.047	2.097	0.03	2.997	S100 calcium binding protein A8 [Source:HGNC Symbol;Acc:HGNC:10498]
S100A9	5.179	0.002	1.653	0.026	2.885	0.035	3.239	S100 calcium binding protein A9 [Source:HGNC Symbol;Acc:HGNC:10499]
SPINK7	3.475	0.013		0.742	5.811	0.049	4.643	serine peptidase inhibitor, Kazal type 7 (putative) [Source:HGNC Symbol;Acc:HGNC:24643]
SPRR1A	3.171	0.023		0.069	3.563	0.024	3.367	small proline rich protein 1A [Source:HGNC Symbol;Acc:HGNC:11259]
SPRR1B	3.623	0	1.582	0.026	2.23	0.007	2.478	small proline rich protein 1B [Source:HGNC Symbol;Acc:HGNC:11260]
SPRR2B	9.827	0.004		0.124	3.66	0.001	6.744	small proline rich protein 2B [Source:HGNC Symbol;Acc:HGNC:11262]
SPRR2D	4.555	0	2.276	0.04	2.064	0.003	2.965	small proline rich protein 2D [Source:HGNC Symbol;Acc:HGNC:11264]
SPRR2F	Inf	0	Inf	0.047		0.195	inf	small proline rich protein 2F [Source:HGNC Symbol;Acc:HGNC:11266]
SPRR2G	5.869	0.002		0.161	2.418	0.003	4.144	small proline rich protein 2G [Source:HGNC Symbol;Acc:HGNC:11267]
TCHH		1	3.131	0	3.008	0	3.069	trichohyalin [Source:HGNC Symbol;Acc:HGNC:11791]
TMEM45B	1.74	0.027		0.979	1.497	0.03	1.619	transmembrane protein 45B [Source:HGNC Symbol;Acc:HGNC:25194]
TYRP1	2.179	0.003		0.843	3.251	0	2.715	tyrosinase related protein 1 [Source:HGNC Symbol;Acc:HGNC:12450]
WARS	2.008	0.022		0.709	2.874	0	2.441	tryptophanyl-tRNA synthetase [Source:HGNC Symbol;Acc:HGNC:12729]
WFDC12	3.243	0		0.311	1.348	0.046	2.295	WAP four-disulfide core domain 12 [Source:HGNC Symbol;Acc:HGNC:16115]

B: Down-regulated genes

Gene Symbol	#1		#2		#3		average Log2 (FC)	descriptions of the gene
	Log2(FC)	P-value	Log2(FC)	P-value	Log2(FC)	P-value		
AL590560.1		1	-Inf	0.002	-Inf	0.002	-Inf	HCG1995379; Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:M0R2Y0]
GABRB3	-2.688	0.044		0.524	-2.429	0.049	-2.559	gamma-aminobutyric acid type A receptor beta3 subunit [Source:HGNC Symbol;Acc:HGNC:4083]
NRN1		0.319	-1.543	0.026	-2.411	0.005	-1.977	neuritin 1 [Source:HGNC Symbol;Acc:HGNC:17972]
PAIP2B	-1.651	0.028		0.845	-1.573	0.036	-1.612	poly(A) binding protein interacting protein 2B [Source:HGNC Symbol;Acc:HGNC:29200]
PIK3C2G	-3.193	0		0.358	-1.599	0.018	-2.396	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma [Source:HGNC Symbol;Acc:HGNC:8973]
POSTN	-1.587	0.017		0.548	-2.183	0.005	-1.885	periostin [Source:HGNC Symbol;Acc:HGNC:16953]
PTGFR	-1.397	0.034	-1.332	0.045		0.053	-1.365	prostaglandin F receptor [Source:HGNC Symbol;Acc:HGNC:9600]
SCARA5		0.882	-1.484	0.019	-1.443	0.03	-1.464	scavenger receptor class A member 5 [Source:HGNC Symbol;Acc:HGNC:28701]
SCGB2A2	-Inf	0.021	-2.168	0.005		0.083	-Inf	secretoglobin family 2A member 2 [Source:HGNC Symbol;Acc:HGNC:7050]
UGT3A2	-4.155	0.008		0.557	-2.678	0.02	-3.416	UDP glycosyltransferase family 3 member A2 [Source:HGNC Symbol;Acc:HGNC:27266]
WIF1	-Inf	0		0.136	-7.171	0.01	-Inf	WNT inhibitory factor 1 [Source:HGNC Symbol;Acc:HGNC:18081]
WISP2		0.453	-1.479	0.039	-1.676	0.018	-1.578	WNT1 inducible signaling pathway protein 2 [Source:HGNC Symbol;Acc:HGNC:12770]