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Acral Peeling in Nagashima Type Palmo-plantar Keratosis Patients Reveals the Role of Serine Protease Inhibitor B 7 in Keratinocyte Adhesion

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Abstract

Acral peeling skin syndrome (APSS) is a heterogenous group of genodermatoses, manifested by peeling of palmo-plantar skin, occasionally associated with erythema and epidermal thickening. A subset of APSS is caused by mutations in protease inhibitor encoding genes, resulting in unopposed protease activity and desmosomal degradation and/or mis-localization, leading to enhanced epidermal desquamation. We investigated two Arab-Muslim siblings with mild keratoderma and prominent APSS since infancy. Genetic analysis disclosed a homozygous mutation in *SERPINB7*, c.796C>T, which is the founder mutation in Nagashima type palmo-plantar keratosis (NPPK). Although not previously formally reported, APSS was found in other patients with NPPK. We hypothesized that loss of SERPINB7 function might contribute to the peeling phenotype through impairment of keratinocyte adhesion, similar to other protease inhibitor mutations that cause APSS. Mis-localization of desmosomal components was observed in a patient plantar biopsy compared to a biopsy from an age and gender matched healthy control. Silencing of SERPINB7 in normal human epidermal keratinocytes led to increased cell sheet fragmentation upon mechanical stress. Immunostaining showed reduced expression of Desmoglein

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1 and Desmocollin 1. This study shows that in addition to stratum corneum perturbation, loss of SERPINB7 disrupts desmosomal components, which could lead to desquamation, manifested by skin peeling.

Keywords

SERPINB7; Keratoderma; Cell Adhesion; Protease Inhibitor; Acral Peeling Skin Syndrome

Introduction-

The epidermis plays a critical role in the establishment of an efficient barrier between the human body and the environment. This barrier confers mechanical integrity, blocks foreign body intrusion, and prevents water loss[1,2]. For this purpose, the epidermis goes through a highly coordinated process of differentiation, which guarantees the transition of proliferating cells in the basal layer to a sealed, anucleated cell layer in the stratum corneum (SC)[3].

Epidermal differentiation also includes a dynamic evolution of intercellular junctions, including desmosomes, adherens junctions, tight junctions (TJ) and gap junctions. Desmosomes link intermediate filaments (keratins) to the plasma membranes at sites of cell-cell adhesion[4-6]. Desmosomal cadherins, consisting of desmogleins (DSGs) and desmocollins (DSCs) interact in trans outside the cell, while their cytoplasmic tails associate with armadillo proteins [Plakoglobin (PG), Plakophilins] and the keratin anchoring plakin, Desmoplakin (DSP)[5]. Desmosomal composition varies in the stratified layers of the epidermis, and while DSG3 and DSC3, are expressed mainly in the lower layers, DSG1 and DSC1 expression increases as cells stratify[5]. The adherens junctions, which anchor actin to plasma membranes, are expressed across the epidermis, up to the level of the second layer of the stratum granulosum (SG2)[5]. In the interface between the living epidermis and the SC, cell adhesion is achieved through the assembly of DSG1, DSC1 and corneodesmosin (CDSN) to form corneodesmosomes[6,7].

Epidermal proteases are involved in early and late stages of epidermal differentiation, and regulate important processes, such as cornified envelope assembly, profilaggrin processing and desquamation[8]. Epidermal protease activity is regulated by protease inhibitors (PIs), and the importance of the interplay between proteases and PIs has been demonstrated during the past decades. Several genetic skin diseases caused by mutations in PI encoding genes have been identified[8-15].

The clinical phenotypes of those diseases are varied but can be categorized as either generalized or localized cutaneous diseases. The prototype of the former is Netherton Syndrome, caused by recessive mutations in the *SPINK5* gene, encoding the serine PI lymphoepithelial Kazal-type-related inhibitor (LEKTI), which presents with ichthyosis, hair shaft defects, atopic diathesis and multiple allergies[14,15]. These features are also present in patients with SAM (severe dermatitis, multiple allergies and metabolic wasting) syndrome and generalized peeling skin syndrome caused by mutations in *DSG1*[16,17] and *CDSN*[18,19], respectively. At the molecular level, it has been shown that loss of LEKTI function results in unopposed activity of the extracellular proteases Kallikrein 5, 7 and 14, leading to the

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degradation of DSG1 and CDSN, which provides a molecular explanation for the phenotypic mimicry[3,16-20].

Localized diseases caused by mutations in PI genes commonly present with peeling of palms and soles[8]. Mutations in the *CSTA* gene, encoding Cystatin A, underlie exfoliative ichthyosis, presented by dry, scaly skin, palmo-plantar keratoderma (PPK) and acral peeling skin syndrome (APSS)[9]. APSS was also reported in patients with bi-allelic mutations in *SERPINB8*, encoding serine protease B8[11]. Interestingly, the clinical peeling effect of both the above diseases was explained *in vitro* through impairment of desmosome function, secondary to unopposed protease activity[9,11].

We investigated two siblings, who suffered from erythema, peeling and mild thickening of their palms and soles since early infancy. Genetic investigation revealed a previously reported homozygous mutation in *SERPINB7*, c.796C>T, predicted to cause premature protein termination (p.266x). This mutation is known to be the founder mutation in Japanese and Chinese patients with Nagashima type palmo-plantar keratosis (NPPK), the most frequent keratoderma among Asians[21,22]. Previous *in vitro* studies have shown that the truncated SERPINB7 mutant had negligible expression due to mRNA decay and enhanced proteasomal degradation[21,23]. A careful clinical search revealed focal and peripheral peeling in other patients with NPPK, suggesting it is an under-reported phenomenon. The pathogenesis of NPPK is still obscure, and the role of SERPINB7 in epidermal homeostasis is unclear. Based on SERPINB7's function as a PI, loss of SERPINB7 expression due to the mutation and the patients' shared phenotype with other APSS caused by mutations in PI encoded genes, we hypothesized that loss of SERPINB7 might also impair desmosomal expression or function, contributing to the NPPK phenotype and pathogenesis.

Materials and Methods-

Patients-

Patients were recruited from the Department of Dermatology of Emek Medical Center (EMC), Afula, Israel in 2019. Patients provided informed consent prior to their inclusion in the study, according to a protocol approved by the EMC Helsinki committee (IRB Protocol #0086-15). Patients signed informed consents for publication of clinical photos. De-identified control plantar skin biopsies were provided by the department of pathology in EMC (IRB Protocol#0086-15). Clinical photos of other patients with NPPK were provided by the Department of Dermatology, Keio University, Tokyo, Japan.

DNA isolation and Exome sequencing analysis-

Germline DNA was extracted from participants' leukocytes using Qiagen DNA isolation Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Exome sequencing was performed on a patient I DNA sample. DNA sequencing enrichment was carried out with Agilent Sureselect V5 (Agilent, Santa Clara, USA) and sequenced on Illumina HiSeq4000, at Macrogen laboratory (USA). The bioinformatics analysis was processed by Variantyx Intellegence platform version 1.16.0.0. The identification of the disease-causing variant was carried out by filtering the variants with MAF >1% using an

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aggregated data from ExAc, genomAD, ESP6500, 1K genome, and selecting variants with damaging scores based on multiple prediction tools including Polyphen2, SIFT, GERP, MT, FATHMM. Validation of the variant and its co-segregation within the family was performed through Sanger sequencing, using the primers (Forward-GAAAACTATGTATCTCTGTAGC, Reverse- AGAGAGATCTGCTTTGGATT).

Immunostaining of paraffin embedded skin sections and staining visualization-

Formalin fixed tissues were processed by the EMC pathology core. Plantar biopsies of patient I and an age and gender matched control skin were obtained and processed on the same day, and by the same technician. Paraffin-embedded sections were baked and deparaffinized in sequential xylene and ethanol washes, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. Antigen retrieval was performed by heating samples to 95°C in 0.01 M citrate buffer. Sections were blocked in 0.5% Bovine Serum Albumin (BSA) in PBS and incubated with primary antibodies diluted in 0.5% BSA for 16h at 4°C followed by PBS washes. Alexa Fluor conjugated secondary antibodies (goat anti-mouse IgG, and goat-anti-rabbit and anti-chicken linked to fluorophores of 488 and 568 nm) diluted 1:300 in 0.5% BSA in PBS were added and incubated at 37°C for 60 min followed by PBS washes. For Filaggrin IHC, I-View DAB detection kit of Ventana (Roche, San Jose) was used according to the manufacturer's instructions.

After mounting in ProLong Gold Antifade Mountant (Thermo Fisher), sections were visualized with a digital camera (Axio-Cam MRm; Carl Zeiss), an Apotome slide module (Carl Zeiss), and Axio-Vision software (Carl Zeiss).

The images were obtained with identical conditions and analytically processed uniformly by ImageJ software (NIH). For IF localization quantifications, five images of each section were analyzed. To evaluate localization, intensity was quantified in supra-basal layers using a rectangle around cell borders (plasma membrane) and in the cytoplasm (cytoplasm), then the ratio of plasma membrane to cytoplasm was calculated.

IF Primary antibodies - monoclonal mouse anti-human DSG1 (diluted 1:1, P124, Progen Biotechnik, #651111, Heidelburg, Germany), mouse anti-human DSG1 (c-terminal) (4B2, Dusek, R.L. et al. The differentiation-dependent desmosomal cadherin desmoglein 1 is a novel caspase-3 target that regulates apoptosis in keratinocytes. J. Biol. Chem. 281, 3614–3624 (2006)). monoclonal mouse anti-human DSC1 (diluted 1:100, U100, Progen Biotechnik, #61092), monoclonal mouse anti-human DSP, diluted 1:50, 115F (diluted 1:100, 91121236-1VL, Sigma Aldrich), rabbit anti-human Claudin 1 diluted 1:100, (51-9000, Thermo Fisher), rabbit anti-human CDSN Ab (Proteintech, Catalog# 13184-1-AP), rabbit anti-human Loricrin (A gift from J.Segre, NIH) and mouse anti-human Filaggrin (NCL-Filaggrin, Novacastra TM, Leica biosystems).

Cell Cultures-

Primary normal human epidermal keratinocytes (NHEKs) were isolated from human foreskin as previously described[24] and grown in KGM Gold basal media (Lonza Walkersville, MD, USA) supplemented with 0.07 mM $CaCl_2$, human keratinocyte growth

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supplements, and gentamicin/amphotericin B solution (Lonza). NHEKs were grown in 6/12 well plates to reach 50% confluency, when they were transfected with 5nM pre-designed silence-select siRNA oligonucleotides against SERPINB7 (Ambion, Life Technologies, #16591)), or negative control non targeted siRNA (siNT) (Sigma Aldrich, Saint-Louis MO, USA) using Lipofectamine 2000 (Thermo Fischer, Waltham, MA, USA), according to the manufacturer's instructions. Keratinocytes were then grown to confluency in KGM media (0.07mM $CaCl_2$) and switched to KGM media supplemented with 1.2 mM $CaCl_2$ for 72h to induce differentiation.

Dispase based dissociation assay-

NHEKs were transfected as described above with siNT or siSERPINB7. Cells were grown to confluence in triplicates on 6-well plates for at least 48h. After reaching 100% confluence, M154 media supplemented with 1.2 mM $CaCl_2$ was added for 72h. NHEK cultures were washed twice with PBS, incubated in 2 ml of dispase II (2.4 units/ml, Roche Diagnostics) at 37 °C for 30 minutes and detached from the plate as epidermal sheets. Cell sheets were first rotated, in parallel, on an orbital shaker for 20 minutes (150 rpm) followed by trituration of the monolayers by gently pipetting monolayers 8 x each with 1000 μl wide bore pipette tips. Fragments were visualized using a dissecting microscope and images obtained using a digital camera followed by processing using Adobe Photoshop CS3. Fragments were counted using the cell counter function in Image J (NIH).

Western blotting-

For analysis of protein expression levels, cells were washed twice with PBS and lysed in urea sample buffer (10 M deionized urea, 1% sodium dodecyl sulfate, 10% glycerol, 60 mM Tris, pH 6.8, and 5% β-mercaptoethanol). Total protein concentrations were equalized and samples were run on 7.5–12.5% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes to be probed with primary and secondary antibodies (Goat anti mouse/rabbit/chicken peroxidase, 1:5000, Rockland KPL, Gaithersburg, MD) against proteins of interest. Chemiluminescent imaging was performed using a G-Box imaging system (Syngene) and densitometry was performed using ImageJ (NIH). Densitometry quantification was normalized using β–Actin or Tubulin as loading controls.

Primary antibodies for western blot: Rabbit anti-human SERPINB7 (diluted 1:250, #HPA024200, Sigma Aldrich), Rabbit anti-human DSP (diluted 1:1000, NW6), rabbit anti-human DSG1 (diluted 1:1000, ab124798, abcam, USA), mouse anti-human DSC1 (diluted 1:200, A4, Santa Cruz Biotechnology, SC- 398590), mouse anti-human β actin (diluted 1:2000, #4970, Cell Signaling, Danver, MA, USA), monoclonal mouse anti-human α Tubulin (diluted 1:4000, #T5168, Sigma-Aldrich), rabbit anti-human Claudin 1 (diluted 1:1000, 51-9000, Thermo Fisher), rabbit anti-human Occludin (diluted 1:500, ab31721, Abcam), mouse anti-human Filaggrin (diluted 1:200, Santa Cruz, SC-66192), Chicken anti-PG antibody (diluted 1:5000, 1407, Aves laboratories, Tigard, OR), Rabbit anti E cadherin antibody #795 (diluted 1:1000, a gift from Randy Marsh).

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qPCR of SERPINB7, DSG1 and DSC1-

Total RNA was isolated from cells using the TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Samples were then equalized for total RNA concentration, and cDNA was synthesized using the qscript (QuantaBio, USA). Primers for SERPINB7 (5'-3'- AACCAGCGGCCAACTTCCT, 3'-5'- AGAGGTCATTCTCAGGCAGC), DSG1 (5'-3'- AGCCTGTCGTGAAGGTGAAG, 3'-5'- TGTTCGGTTCATCTGCGTCA) and DSC1 (5'-3'-TCCCCATGCAGACATCCAAC , 3'-5'- TCCTCTAATGGATTCTTCGCCA) were supplied by Sigma-Aldrich, Israel. qPCR was performed using SYBR Green PCR master mix (Applied Biosystems, Thermo Fisher) along with the appropriate primers using a StepOne-Plus instrument (Applied Biosystems, Thermo Fisher). Calculations for relative mRNA levels were performed using the ΔΔCt method using GAPDH mRNA as a control. Relative levels were presented as fold-change values compared with control samples.

Statistics-

Statistics were analyzed using Prism 8 (GraphPad Software, San Diego, CA). Biological replicates were quantified separately and compared as repeated measures. All quantifications are presented as mean ±SD. For data comparing two conditions, statistical differences were analyzed with a two-tailed Student's t test. $P < 0.05$ was considered significant. For analysis presented as fold change (bar graphs), the mean of data from control samples was assigned a value of 1, and all other samples were calculated relative to the control.

Results-

Clinical report-

Acral peeling in patients with NPPK—Two brothers (patients I and II) were referred to our dermatology department due to peeling and erythema of their palms and soles. They were 18 and 20 years old, respectively, born to first degree cousins of Arab-Muslim ethnicity (Figure S1A). Since infancy, they suffered from erythema and diffuse peeling of their palmo-plantar skin. Upon adulthood, they reported gradual thickening of their palmo-plantar skin, accompanied by central and peripheral peeling (Figure 1A, B). Recurrent fungal cultures were negative and skin biopsy disclosed non-epidermolytic hyperkeratosis. Accordingly, a clinical diagnosis of APSS was suspected. Preliminary molecular investigation included the direct sequencing of *CSTA*, which has been associated with APSS among Arab-Muslims in Israel. *CSTA* was normal in both patients. Subsequently, whole exome sequencing was performed for patient I. Following filtering for homozygous rare variants with minimal allele frequency of less than 1%, we identified a homozygous mutation in *SERPINB7* c.796C>T, causing a premature stop codon in amino acid 266 (Figure S1B). The variant was validated through Sanger sequencing. Family segregation disclosed that patient II was homozygous for the same variant and the patients' parents were heterozygous (Figure S1B). This variant is considered the founder mutation causing NPPK in Japanese and Chinese populations. Variants in other known genes associated with APSS or PPK were not detected. Interestingly, although peeling has not previously been considered a feature of NPPK, we investigated the clinical files of other genetically diagnosed NPPK patients of Japanese origin, in which focal peeling was reported in addition to the classical manifestations (Figure 1C, D), suggesting that this clinical phenotype might be under-diagnosed[18].

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Plasma membrane expression of several junctional proteins is perturbed in a patient with NPPK—Other peeling skin syndromes caused by mutations in genes encoding PIs result in secondary impairment of desmosome expression and/or localization. Accordingly, we stained for desmosomal components in skin sections obtained from the lesional area of patient I's plantar skin compared to the skin of an age- and gender-matched plantar biopsy from a healthy control. Paraffin embedded tissues were stained with antibodies against the desmosomal cadherins, DSG1 and DSC1, the desmosomal plakin, DSP, CDSN, PG, and E cadherin.

We examined the localization of DSG1 in suprabasal epidermal layers, staining the tissue with an antibody recognizing the N-terminus of the molecule and found an altered plasma membrane and cytoplasmic localization of DSG1 in the patient's section compared to specific membrane localization in the control biopsy (Figure 2A, A'). Staining with an antibody recognizing the C-terminus showed similar results, with a higher cytoplasmic signal than that observed in the control tissue (Figure S2B, B'). Similarly, the localization of DSC1, CDSN and PG in the patient's tissues were perturbed compared to the control (Figure 2B, B', Figure S2). The localization pattern of DSP was unchanged between the patient and control tissue (Figure 2C, C'). IF staining of the adherens junction protein E cadherin revealed an impaired localization in the patient biopsy compared to control (Figure S2D, D'). The mis-localization was prominent in the basal layers, while in supra-basal layers membranous staining was evident, albeit still reduced in the patient's biopsy compared to control.

Loss of SERPINB7 results in reduced intercellular adhesion—Based on the staining results showing disruption of desmosome protein localization in Patient I, and prior evidence that the truncated SERPINB7 mutant has negligible expression[19], we aimed to determine if loss of SERPINB7 would result in a keratinocyte adhesion defect *in vitro*. Real time PCR and immunoblotting demonstrated the decrease of SERPINB7 mRNA and protein expression upon siRNA-mediated silencing compared to siNT transfected control cells (Figure 3A, A', A"). Epidermal sheets were lifted from the substrate using dispase and subjected to mechanical manipulation. SERPINB7-silenced keratinocytes resulted in significantly more fragments following mechanical stress compared to siNT- transfected keratinocytes, suggesting that loss of SERPINB7 impairs keratinocyte adhesion (Figure 3B, C).

Reduced expression of DSG1 and DSC1 in SERPINB7-silenced NHEKs—Next, we tested the impact of SERPINB7 loss on expression of junctional proteins in differentiated keratinocytes. Immunoblot showed significantly decreased expression of DSG1 and DSC1 in SERPINB7-silenced NHEKs compared to siNT-transfected NHEKs (Figure 4A, B). In contrast, the expression of DSP, E cadherin and PG were not impacted (Figure 4A, B).

DSG1 and DSC1 mRNA expression was not significantly impacted by SERPINB7 loss (Figure 4C), consistent with the proposed mechanism of desmosomal protein degradation through unopposed protease activity, secondary to SERPINB7 silencing.

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In addition, we analyzed the expression levels of the TJ proteins (Claudin 1 and Occludin) and the differentiation marker Filaggrin and found no differences between siSERPINB7-transfected NHEKs and controls (Figure S3A, A'). These results were consistent with the immunostaining of Claudin 1, Loricrin, and Filaggrin (Figure S3B-D) in the patient tissue sections.

Discussion-

In this study, we report two related cases of NPPK with APSS and demonstrate the involvement of SERPINB7 in desmosome protein expression and keratinocyte adhesion.

As mentioned earlier, PIs are involved in the regulation of desquamation, which was underscored by the identification of APSS caused by mutations in PI encoding genes[3,8,9,11,12,13,21]. Ultrastructural analysis of biopsies obtained from patients with exfoliative ichthyosis caused by mutations in the *CSTA* gene showed widening of intercellular spaces in basal and supra-basal layers and aggregates of tonofilaments[9]. Further, silencing of Cystatin A in keratinocytes showed reduced intercellular adhesion and breakage of keratin filaments upon stress, suggesting the role of Cystatin A in the regulation of supra-basal epidermal adhesion. *In vitro* silencing of *SERPINB8*, another APSS causing gene, revealed increased fragmentation compared to control upon mechanical stress, surprisingly associated with upregulation of DSG1 and DSP[11]. Further IF analysis showed that under stretch, SERPINB8-silenced keratinocytes showed an aberrant shift of DSP from the plasma membrane to the cytoplasm, as a possible explanation for impaired adhesion. A similar DSP upregulation and mis-localization was shown in skin biopsies of a patient with PLACK syndrome, caused by mutations in the PI encoding gene *CAST*, which includes a phenotype of non-acral skin peeling[12]. In our study, a skin biopsy from a patient with APSS and a homozygous *SERPINB7* truncating mutation showed a decreased plasma membrane localization of DSG1, DSC1 and PG compared to a control, while DSP localization was normal. In addition, we found that silencing of SERPINB7 *in vitro* resulted in increased monolayer fragmentation using the dispase-based cell dissociation assay. Further, we evaluated the expression of desmosomal proteins and found that DSG1 and DSC1 expression were significantly reduced in siSERPINB7- transfected NHEKs, while DSP, E cadherin and PG expression was preserved. Similarly, reduced DSG1 protein expression was recently reported by Mohamad et al, who identified a new autosomal recessive keratoderma associated with peripheral peeling, caused by recessive mutations in the PI encoding gene *SERPINA12* (VAPSIN)[13].

Using electron microscopy, Chassain et al[25] identified visible intercellular spaces in the stratum corneum in an NPPK patient biopsy, either by simple expansion of the intercellular space or by cleavage between corneocytes, suggesting a superficial peeling, which is consistent with the clinical appearance in our study (Figure 1). Based on these observations, several mechanisms for the peeling pathogenesis could be hypothesized. One explanation could involve the target protease of SERPINB7, which is unknown and might potentially include the serine proteases Kallikreins 5, 7 and 14[3]. Supporting that idea, localization of CDSN was altered in the patient's biopsy, and its SC intensity was reduced compared to the control (Figure S2A, A'). Nevertheless, the fact that SERPINB7 is an intracellular enzyme,

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while Kallikreins are extracellular, argues against that hypothesis. An alternative hypothesis might highlight the potential compensation of adhrens junctions (eg. E cadherin) for the reduced expression of DSG1 and DSC1, maintaining intercellular adhesion up to the level of SG2, but not in SG1 and the SC, where adhesion is maintained solely by desmosomes. However, the perturbed plasma membrane expression of E cadherin in the patient's biopsy (Figure S2D), does not support its compensatory role.

The pathogenesis of skin thickening and inflammation in NPPK remains unclear. Some ultrastructural studies failed to discover major defects in the SC and SG[21], while others found accumulation of lipid bodies in both[25]. An important clinical clue - skin whitening upon water immersion, was correlated with higher trans-epidermal water loss (TEWL) in NPPK lesional areas compared to controls[21]. Hence, it has been proposed that loss of SERPINB7 leads to protease hyperactivation in the SC, resulting in an epidermal barrier defect facilitating water permeation, further manifested by hyperkeratosis and inflammation[21].

Since SERPINB7-silenced NHEKs expressed reduced levels of DSG1 compared to control NHEKs, and since DSG1 promotes epidermal differentiation[4], we sought to evaluate whether SERPINB7- associated DSG1 reduction led to disturbed epidermal differentiation. Filaggrin, a late epidermal differentiation marker, was not impacted in SERPINB7-silenced NHEKs compared to the control (Figure S3A). This observation is consistent with intact staining for epidermal differentiation markers- Filaggrin and Loricrin in our patient (Figure S3C, D) and in other patients with NPPK[21].

TJs are important components of the skin barrier, providing an effective functional barrier in the epidermal SG2 layer, which regulates the paracellular movement of molecules[26]. Generally, TJs are composed of four-transmembrane proteins (Claudins), forming TJ strands on the plasma membranes of opposing cells. In addition, scaffolding proteins ZO1-3 and transmembrane proteins (Occludin, JAM-A, Tricellulin and Angulin) are required for the establishment of functional TJs[26]. We found similar expression levels of Claudin 1 and Occludin in siSERPINB7-transfected NHEKs, compared to siNT control cultures, which was consistent with a similar localization pattern of Claudin1 in skin sections of the NPPK patient and the control (Figure S3). Nevertheless, it is still possible that loss of SERPINB7 affects TJ function, without changing the level of protein expression in 2D cultures or in the patient's skin samples assessed by IF staining, which might contribute to the pathogenesis of NPPK.

In conclusion, we have highlighted an under-reported phenomenon of skin peeling in patients with NPPK. Further, we found that loss of SERPINB7 function impairs DSG1 and DSC1, both *in vivo* and *in vitro*, and disrupts intercellular adhesion. Further studies are needed to elucidate other factors contributing to the pathogenesis of NPPK, which might expand our understanding regarding other roles of SERPINB7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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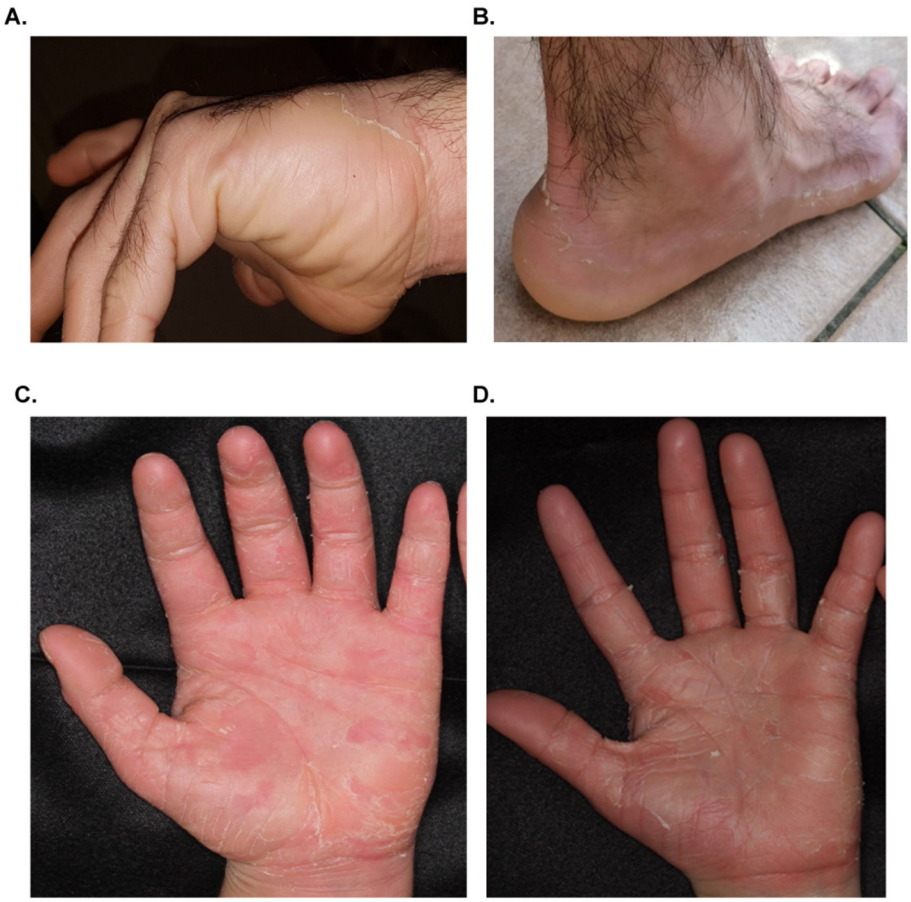

Figure 1. Acral peeling in patients with Nagashima Type Palmo-plantar Keratosis (NPPK).

A, B. Peripheral peeling in palms and soles of Patients I and II, respectively. C, D. Japanese patients with NPPK demonstrate a focal, central peeling of palms (*SERPINB7* mutations-compound heterozygote of c.796C>T (p.R266*) / c.218_219del2ins12 (p.Gln73Leufs*17) and homozygous c.796C>T (p.R266*), respectively).

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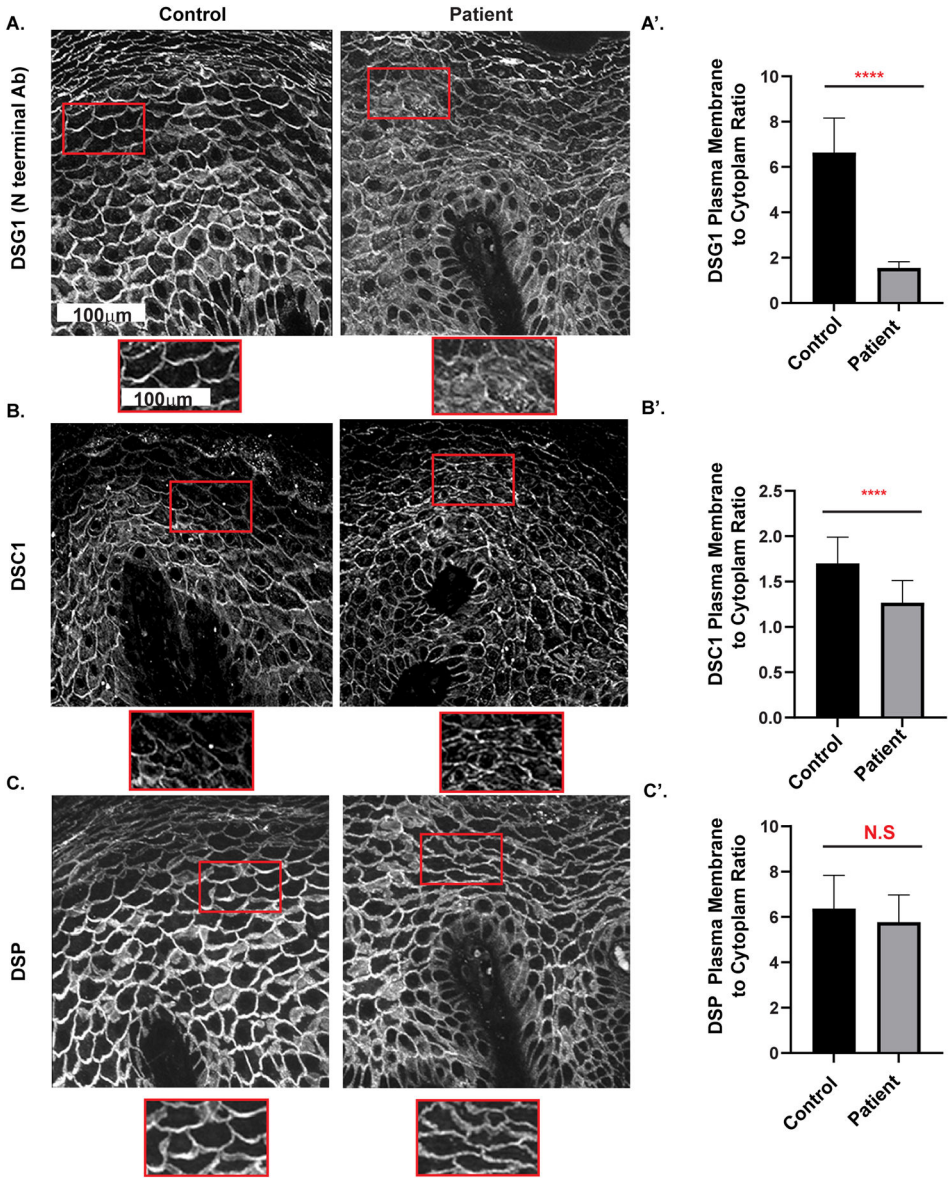

Figure 2. Mis-localization of DSG1 and DSC1 in a patient with NPPK.

A, B, C. Immunofluorescence staining of DSG1, DSC1 and DSP in lesional plantar skin sections from a patient with NPPK, caused by a homozygous mutation in *SERPINB7* c.796C>T (scale bars = 100 μm. Scale bars for all images and insets are identical to those presented in panel A). A', B', C'. Quantification of plasma membrane to cytoplasmic ratio of DSG1, DSC1 and DSP staining from panels A, B, C. (n = at least 30 borders from 5 different regions per sample. Error bars represent mean ± SD.; ****p<0.0001, N.S-not significant, by two-tailed Student's t test).

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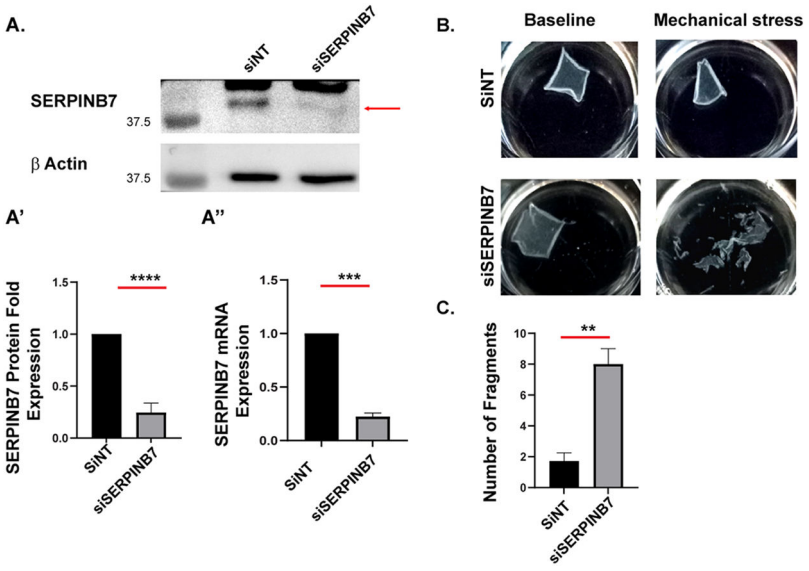

Figure 3. Loss of SERPINB7 results in reduced intercellular adhesion.

A. Immunoblot of SERPINB7 in NHEKs transfected with siNT or siSERPINB7 oligonucleotides 72h after calcium switch (β actin = loading control). A'. Quantification of fold changes in SERPINB7 protein expression presented in A (n = 6. Error bars = mean ± SD, by two-tailed Student's t test, ****-p<0.0001). A". SERPINB7 mRNA expression in siNT or siSERPINB7 transfected NHEKs. GAPDH gene expression was used as housekeeping gene (n = 3. Error bars = mean ± SD, by two-tailed Student's t test, ***-p=0.0007). B. An increase in monolayer fragments following mechanical stress is indicative of decreased cell-cell adhesive function. C. The number of cell sheet fragments was counted and graphed (n = 3. Error bars represent mean ± SD. **p = 0.003 by two-tailed Student's t test).

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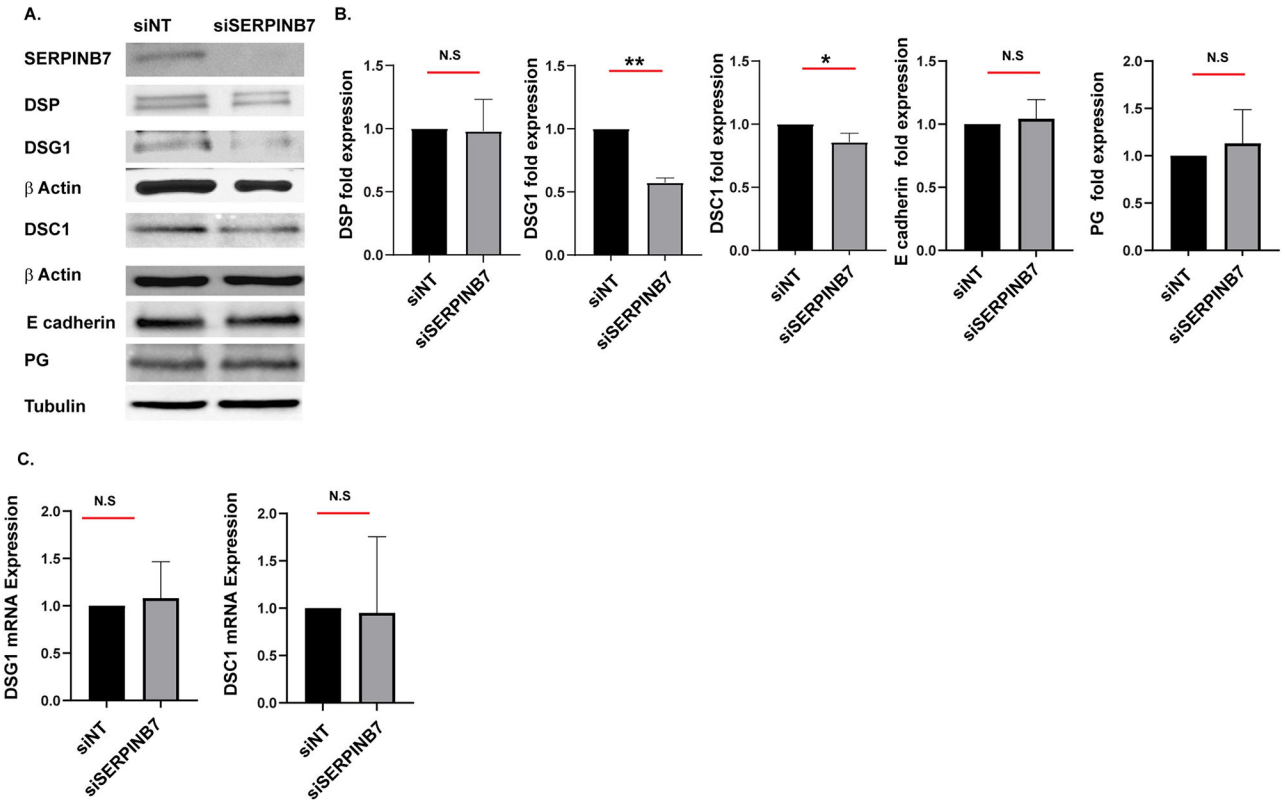

Figure 4. Reduced expression of DSG1 and DSC1 in SERPINB7-silenced NHEKs.

A. Immunoblot of DSP, DSG1, DSC1, E cadherin and PG in NHEKs transfected with siNT or siSERPINB7 oligonucleotides 72h after calcium switch (β actin and Tubulin = loading control). B. Quantification of fold changes in DSP, DSG1, DSC1, E cadherin and PG protein expression presented in A. (n=3. Error bars = mean ± SD. **-p=0.0025, *-p=0.04, by two-tailed Student's t test, N.S= not significant. C. DSG1 and DSC1 gene expression in siNT or siSERPINB7 transfected NHEKs (n = 3. Error bars = mean ± SD, by two-tailed Student's t test, N.S= not significant). C. DSG1 and DSC1 mRNA expression in siNT or siSERPINB7 transfected NHEKs. GAPDH was used as housekeeping gene (n = 3. Error bars = mean ± SD, by two-tailed Student's t test, N.S. = not significant).