

Appendix S1

MATERIAL AND METHODS

Patient selection

This study included patients with burn scars who visited Hangang Sacred Heart Hospital, Hallym University, Seoul, Korea. Inclusion criteria were burn patients who were 7 years or older at the time of examination. Exclusion criteria were (i) pre-existing chronic systemic or dermatologic diseases, which are known to frequently cause pruritus, (ii) concurrent systemic medications affecting pruritus symptoms, such as antihistamines, morphine or systemic steroids, (iii) concurrent psychotic disease, (iv) pregnancy, (v) patients younger than 7 years of age, and (vi) patients who were unable to give detailed information about their pruritus. The study protocol was approved by the Institutional Review Board of Hangang Sacred Heart Hospital. Informed consent was obtained from each patient.

Pruritus and other abnormal sensations in burn scars. All patients were asked whether they felt pruritus on their burn scars. The patients were divided into 2 groups: those with pruritus (Group A) and those without (Group B). The patients of Group A were asked to describe the severity of pruritus using a 10-point Visual Analogue Scale (VAS): 10, the worst itching sensation and 0, no pruritus.

Scar assessment (subjective). The burn scars were assessed using the Patient Scar Assessment Scale (PSAS) and the Observer Scar Assessment Scale (OSAS). The PSAS consists of pain, pruritus, colour, stiffness, thickness and irregularity (0–10) as assessed by the patient, with a total score of 0–60. The OSAS consists of vascularity, pigmentation, thickness, relief and pliability (0–10) as assessed by the physician, with a total score of 0–50. In patients under 12 years old, caregivers helped the children to describe their symptoms.

Scar assessment (objective). We measured the percentage of total body surface involved with burns (TBSA) classified using the 'Rule of Nine', in which body regions are grouped as a multiple of 9% of the total to provide an easy estimation of the extent of the surface area of the burns. The burn scars were assessed by various non-invasive methods, including measurement of (i) transepidermal water loss (TEWL) (Tewameter[®], TM 300, Courage-Khazaka, Cologne, Germany); (ii) melanin index and erythema index (colourimetry, Mexameter[®] MX 18, Courage-Khazaka, Cologne, Germany); (iii) sebum excretion (Sebumeter[®], SM 815, Courage-Khazaka, Cologne, Germany); and (iv) thickness (Ultrasonography; 128 BW[®], Medison, Seoul, Korea). The measurements were made after 30 min of rest in a sealed room with a sustained room temperature of 20°C–25°C and relative humidity of 40%–60%. The physicians made the measurements 3 times and the mean values of the 3 measurements were recorded as the results.

Histopathological analysis. Skin samples (6 mm punch biopsy) were obtained from 2 different sites: one from a burn scar and the other from normal skin. We obtained normal tissues from the inguinal area, which is non-sun-exposed skin, in order to gather consistently normal tissues from all the patients. All tissues were placed in 10% neutral buffered formalin for 18 h and were then processed for paraffin embedding with Paraplast (Sigma-Aldrich, St. Louis, MO, USA). Serial 5 µm thick tissue sections were processed for routine histology. In Group A, the specimens were obtained from the most pruritic areas. By haematoxylin eosin staining, a single pathologist and a single dermatologist examined epidermal thickness and mononuclear infiltration (0: none; 1: mild; 2: moderate; 3: severe) as measured using a microscope (Plan-Apo, Nikon, Tokyo, Japan) with a SPOT FLEXTM digital camera (FX1520, SPOT Flex Shifting Pixel Color Mosaic, Diagnostic Instruments, Inc., Sterling Height, MI, US) in a blind manner. To evaluate the density and thickness of collagen bundles and elastic fibers, the samples were stained with Masson's trichrome and Verhoeff's elastic. The density and thickness in Groups A and B were scored as binary variables compared to normal skin

(dense/not dense for the density and thick/thin for the thickness). Toluidine blue staining was performed for mast cells. Substance P (SP) antibody RabMab (Epitomics, Burlingame, CA, USA) and anti-calcitonin gene-related peptide (CGRP) antibody (Abcam, Cambridge, MA, USA) were used for staining itch mediators as assessed by the 2 above-mentioned physicians (0: none; 1: mild; 2: moderate; 3: severe). Disagreements between the 2 evaluators were resolved through a consensus conference. The rate of initial concurrences was >90% in our study.

Immunohistochemistry. Tissues were incubated with antibodies for TRPV1 (Abnova, Taipei city, Taiwan), TRPV3 (Acris antibodies, Herford, Germany), TRPV4 (Abcam, Cambridge, MA, USA) and TRPA1 (Abnova, Taipei city, Taiwan). As negative controls, the primary antibodies were omitted. Controls for specificity included (i) staining TRPV sections as described above but omitting the primary antibody, which resulted in no detectable labelling; (ii) incubation with a single primary antibody followed by the appropriate secondary antibody, to ensure that the labelling pattern for each substance in the double-stained sections was similar to that observed in the single-labelled section; (iii) incubation with a single primary antibody, followed by a mixture of 2 secondary antibodies to test the species specificity of the secondary antibodies. Semi-quantitative scoring of stained slides was done using software.

An image analysis technique was employed for quantitative scoring of the stained slides with bright field imaging and colour deconvolution (12); (i) Bright field images were obtained using the image capture system consisting of a light microscope (Nikon NIS-BR, Nikon, Japan) and a CCD colour camera (Nikon DS-Ri1, Nikon, Japan) connected to a desktop computer. (ii) A colour deconvolution technique was used to separate the colour mixture of haematoxylin and DAB and also to obtain optical densities of the antibody stained with DAB. (iii) A method of thresholding was employed to segment the IHC expression of TRPV1, TRPV3, TRPV4, and TRPA1 effectively. (iv) Epidermal and dermal regions were marked interactively using Adobe Photoshop and then saved as masking images. To obtain a pair of robust and consistent stain vectors from a large number of stained slides, an NMF (non-negative matrix factorisation) technique (13) was applied to the composite image of all the stained slides with the initial estimates given in (12). This image analysis technique was implemented using Mathworks Matlab where an efficient NMF implementation is available.

RNA extraction and qRT-PCR. After grinding tissues through liquid nitrogen, RNA (ratio > 1.8) was extracted using an Easy-Spin Total RNA Extraction kit (Intron, 17221). A total of 1 µg RNA and 100 pmol of oligo (dT) primer (Bioneer, N-7053), were incubated at 70°C for 5 min and reverse transcription was performed using reverse transcription pre-mix (Bioneer, K-2012) together with 50 ng of cDNA. For the qRT-PCR, a reaction mix was prepared containing TaqMan Mastermix (Applied Biosystems), TaqMan probe (Hs_TRPV1_1_FAM QuantiFast Probe Assay, TRPV1; Hs_TRPV3_1_FAM QuantiFast Probe Assay, TRPV3; Hs_TRPV4_1_FAM QuantiFast Probe Assay, TRPV4; Hs_TRPA1_1_FAM QuantiFast Probe Assay, TRPA1; Hs00243225_m1, TAC1; and Hs99999909_m1, HPRT1, Applied Biosystems). The master mix together with cDNA was then aliquoted on a 96-well plate. Data was collected using a Light-Cycler480II (Roche) with the following conditions: 55 cycles of 95° for 5 s, 60° for 30 s, and 72° for 1 s.

Statistical analysis

All statistical analyses were conducted using SPSS 12.0 for Windows (SPSS Korea, Inc, Seoul, Korea). Statistical comparisons of the normal skin and the burn scars were made using the paired *t*-test or McNemar's test. Statistical comparisons between Groups A and B were made using the Pearson chi-square, Fisher's exact test, linear by linear association, the Spearman coefficient, the Mann-Whitney *U*-test and ANCOVA and the Pearson correlation coefficient.