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Sunitinib decreases the expression of KRT6A and SERPINB1 in 3D

human epidermal models

Ayaka Yoshida^{a,b}, Kazuhiro Yamamoto^{a,*}, Takahiro Ishida^c, Tomohiro Omura^a, Tomoo Itoh^b, Chikako Nishigori^d, Toshiyasu Sakane^e, Ikuko Yano^a

^a Department of Pharmacy, Kobe University Hospital, 7-5-2 Kusunoki-cho, Chuo-ku,

Kobe 650-0017, Japan

^b Department of Diagnostic Pathology, Kobe University Graduate School of Medicine,

7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan.

^c R&D Department, Momotani Juntanken LTD, 1-4-1, Uemachi, Chuo-ku, Osaka, 540-

0005, Japan

^d Division of Dermatology, Department of Internal Related, Kobe University Graduate

School of Medicine, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan.

^e Department of Pharmaceutical Technology, Kobe Pharmaceutical University, 4-19-1

Motoyamakitamachi, Higashinada-ku, Kobe 658-8558, Japan

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2 *Corresponding author: Kazuhiro Yamamoto, Ph.D.

3 Department of Pharmacy, Kobe University Hospital, 7-5-2 Kusunoki-cho, Chuo-ku,

4 Kobe 650-0017, Japan

5 Tel: +81 78 382 6659

6 Mail: yamakz@med.kobe-u.ac.jp

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Abstract

Hand-foot skin reaction (HFSR) is a common side effect caused by several tyrosine kinase inhibitors, including sunitinib. However, the nature of the cornifying factors related to the molecular biological mechanisms underlying HFSR remains poorly understood. We used human keratinocyte models to investigate the key cornifying factors for dermatological and biological abnormalities induced by sunitinib. On the basis of the results of microarray analysis using the three-dimensional (3D) human epidermal model, keratin (KRT)6A, serine protease inhibitor (SERPIN)B1, KRT5 and SERPIN Kazal-type 6 were selected as candidate genes related to HFSR. Sunitinib treatment significantly decreased the expression of SERPINB1 and KRT6A in the immunohistochemical staining of the 3D epidermal model. In PSVK1 cells, but not in normal human epidermal keratinocyte cells, both of which are human normal keratinocyte cell lines, sunitinib decreased the expression of KRT6A with a concomitant decrease in levels of phosphorylated extracellular signal-regulated kinases (ERK)1/2 and phosphorylated p38 mitogen-activated protein kinase (MAPK). Inhibitors of the ERK and p38 MAPK signal pathways also significantly decreased KRT6A expression. Sunitinib-induced decrease in

KRT6A expression was suppressed by the inhibition of glycogen synthase kinase-3 β by enhancing ERK1/2 and p38 MAPK phosphorylation. Thus, sunitinib reduces the expression of KRT6A and SERPINB1 by inhibiting the ERK1/2 and p38 MAPK signaling pathways in the skin model. These changes in expression contribute to the pathology of HFSR.

Keywords

Hand–foot skin reaction, sunitinib, KRT6A, SERPINB1, 3D epidermal model

1 **Introduction**

2 Small-molecule tyrosine kinase inhibitors (TKIs) of angiogenic signals are
3 integral to current cancer chemotherapy. Considerable evidence supports the efficacy of
4 angiogenic inhibitors against various types of cancer.^[1,2] However, anti-angiogenic
5 therapy produces characteristic side effects. Hand–foot skin reaction (HFSR) is a
6 common side effect of several TKIs. HFSR causes hyperkeratosis on the palm or sole,
7 causing pain and resulting in functional problems such as difficulty in walking and
8 grasping. The quality of life (QOL) of the patients is therefore notably reduced.^[3,4]
9 Because the occurrence of HFSR is correlated with a favorable outcome of TKI therapy,
10 the management of HFSR is a key issue for successful TKI therapy.^[5] Increasing our
11 understanding of the mechanisms of HFSR is essential for the development of effective
12 prophylaxis, which will provide improvement of both the patients' QOL and favorable
13 prognoses.^[6]

14 Sunitinib is one of many TKIs targeting vascular endothelial growth factor
15 receptor (VEGFR) types 1–3, platelet-derived growth factor receptor (PDGFR) α and β ,
16 c-Kit, FMS-like tyrosine kinase-3, and colony-stimulating factor-1 receptor. Sunitinib has

1 been used as a first-line therapy for renal cell carcinoma (RCC).^[7] The frequency of
2 sunitinib-induced HFSR is approximately 30% in RCC patients when appropriate
3 prevention using moisturizing heparinoid- or urea-containing ointment is used.^[8] Patients
4 who develop severe HFSR are treated with corticosteroid-containing ointment, despite a
5 lack of evidence regarding the efficacy of topical corticosteroid therapy for HFSR.^[9,10]
6 To establish effective preventive and therapeutic strategies for HFSR, it is necessary to
7 clarify the molecular mechanisms underlying the progression of the conditions. In
8 sunitinib-induced HFSR, the granular and spinous layers of the epidermis appear thin,
9 and the basal layer is bloated in the epidermis of the palmar and plantar regions because
10 of hyperkeratosis associated with proliferation inhibition.^[11] However, the molecular
11 mechanisms underlying this cornification are not well understood.

12 In this study, we used human keratinocyte models to investigate the key
13 cornifying factors underlying the dermatological and biological abnormalities induced by
14 sunitinib.

16 **Materials and Methods**

Chemicals and antibodies

Sunitinib was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). U0126, a selective inhibitor of mitogen-activated protein kinase (MAPK)1/2 that is an upstream factor of the extracellular signal-regulated kinases (ERK)1/2, was obtained from Cell Signaling Technology (Danvers, MA, USA). SB203580, a selective p38 MAPK signaling inhibitor that blocks phosphorylation of MAPK-activated protein kinase (MAPKAPK)2, was purchased from FUJIFILM Wako Chemical Co. (Tokyo, Japan). SB216763, a selective glycogen synthase kinase (GSK)-3 β inhibitor, was purchased from Abcam (Cambridge, UK).

Anti-serine protease inhibitor (SERPIN) B1 and anti-KRT5 antibodies were purchased from Abcam. Anti-phosphorylated-p44/42 MAPK (ERK1/2) at threonine (Thr) 202/tyrosine (Tyr) 204, anti-ERK1/2, anti-phosphorylated-p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, anti-phosphorylated-MAPKAPK2 and anti- β -catenin antibodies, and anti-rabbit horseradish peroxidase (HRP) conjugate immunoglobulin G (IgG) were purchased from Cell Signaling Technology. Anti-SERPIN Kazal-type (SPINK) 6, anti-KRT6A, and anti- β -actin antibodies were purchased from Sigma-Aldrich Chemical.

Cells and cell culture

The three-dimensional (3D) human epidermal model (EPI-200) was purchased from KURABO Industries Ltd. (Osaka, Japan). It was incubated using an EPI-100 maintaining medium (EPI-100MM, KURABO). Normal human epidermal keratinocyte (NHEK) cells were also purchased from KURABO. These NHEK cells were maintained in HuMedia-KG2 (KURABO) supplemented with the HuMedia-KG supplement kit (KURABO) and were sub-cultured with 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA). PSVK1 cells, which are normal human keratinocytes immortalized by the pSV40 gene, were acquired from the JCRB Cell Bank (Osaka, Japan). These cells were maintained in EpiLife (Thermo Fisher Scientific; Waltham, MA, USA) supplemented with HuMedia-KG supplement kits and then sub-cultured with 0.125% trypsin and 0.01% EDTA. Thereafter, the cells were seeded into culture flasks and grown in a humidified atmosphere of 5% CO₂–95% air at 37 °C.

Drug treatment of monolayer cells

Monolayer cells were preincubated for 24 h before sunitinib treatment.

Following preincubation, culture medium was replaced by media with or without sunitinib and a specific inhibitor for 48 h, and incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. These samples were analyzed by western blotting and immunofluorescence imaging.

Drug treatment of 3D epidermal model

The 3D epidermal model was preincubated for 24 h before sunitinib treatment. Following preincubation, the medium under the dermal side of the cell layer was replaced by media with or without 1 µM of sunitinib for 120 h, a maximum tolerable time for treatment by dedicated medium, in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Dulbecco's phosphate-buffered saline (D-PBS) was placed on the epidermal side of the 3D epidermal model to prevent from promotion of cornification induced by surface dryness for the duration of drug treatment. The concentration of sunitinib used in the experiments was decided by referring to the previous report on the distribution and elimination rates of sunitinib in rat skin tissue and the transition of plasma sunitinib concentrations in humans.^[12] These samples were analyzed using microarrays and immunohistochemical staining.

Microarray analysis

Following the drug treatment, the 3D epidermal model was washed with D-PBS and then rapidly frozen in liquid nitrogen. RNA extraction using TRIzol[®] reagent and microarray analysis with GeneChip[®] Human Gene 2.0 ST Array (Affymetrix; Santa Clara, CA, USA) were performed by KURABO. Candidate genes were selected using a three-step extraction process. The data purification and preselection step were performed using the following exclusion criteria: genes with no symbol name; genes with less than 1.5-fold change; or genes with less than 100 signal intensity in control sample. The primary selection step was performed using Gene Ontology (GO) Biological Process Terms. Genes annotated with the terms listed in Figure 1A were the subjects for secondary selection. The secondary selection step was performed using the following selection criteria: genes with absolute values of logarithm of fold change to base 2 in the highest 10 of the candidate genes and genes reported to be related to abnormal keratinization.

Immunohistochemical staining and Hematoxylin and eosin (H&E) staining

After drug treatment, the 3D epidermal model was washed with D-PBS and fixed by immersion in a fixing solution consisting of Superfix[®] (KURABO) and 10% formalin buffer in the ratio of 2:1. After fixation, the 3D epidermal model was embedded in paraffin and then sliced. H&E staining was performed using this paraffin sections. For immunohistochemical staining section, deparaffinization, antigen activation treatment, and endogenous enzyme removal were performed and, after blocking, the samples were immersed in the primary antibody overnight at 4 °C. The sections were then immersed in a secondary antibody diluent for 30 min at room temperature and developed using 3,3'-diaminobenzidine-4HCl. Stained images were acquired using an all-in-one fluorescence microscope (KEYENCE; BZ-X700). The process from paraffin embedding to immunohistochemical staining was carried out by Kyodo Byori, Inc. (Kobe, Japan). Protein expression levels were evaluated using the stained area ratio, which is defined as the ratio of the area stained by each protein antibody to that of the whole area of the 3D epidermal model.

Western blot analysis

Proteins in the total cell lysate were extracted from cells treated with a 0.7% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate-based cell lysis buffer in addition to 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/mL leupeptin. These proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred onto a nitrocellulose blotting membrane (Amersham™ Protran™ NC 0.45; GE Healthcare). The blots were blocked in a wash buffer solution (10 mM of Tris, pH 7.5; 150 mM of NaCl; 0.05% Tween 20) containing 10% goat serum. The membranes were soaked overnight at 4 °C in a wash buffer containing specific primary antibodies, followed by incubation with HRP-conjugated secondary antibodies for 1 h. Antibody-bound proteins were visualized by treating the membrane with Amersham™ ECL prime (GE Healthcare). Blot images were acquired using ChemiStage CC-16 (KURABO). Wherever indicated, the membranes were stripped and re-probed with different primary antibodies. The intensities of the protein bands in the densitometric assay were determined using the ImageJ software from National Institutes of Health.^[13]

Immunofluorescence imaging

PSVK1 cells treated with sunitinib and/or SB216763 were fixed with 4% paraformaldehyde at room temperature for 15 min, blocked with 5% BSA, incubated with an anti- β -catenin antibody, and then incubated with FITC-conjugated anti-rabbit IgG (Santa Cruz). The nuclei were stained with Hoechst 33342 and visualized with the IN Cell Analyzer 2200 (GE Healthcare). Image acquisition was configured to yield at least 1,000 cells per replicate well. β -catenin nuclear entry was determined by measuring the integrated nucleus/cytoplasm intensity ratio, using the nuclear translocation analysis module of the dedicated analysis software IN Cell Investigator (GE Healthcare).

Statistical analysis

Data are expressed as means $\pm$ standard deviation. The statistical significance of the difference in mean values between the two groups was analyzed using Student's *t*-tests. Differences between three or more groups were analyzed using one-way analysis of variance, followed by the Dunnett's test. $P < 0.05$ (two-tailed) was considered to be statistically significant.

Results

Gene and protein expression in 3D epidermal models treated with sunitinib

The results of the microarray analysis are shown in Figures 1A and 1B. A total of 173 genes were identified as having an increase or decrease of more than 1.5-fold compared with controls. As a result of the primary selection step, 20 candidate genes were selected. After the full selection process, four genes were selected as candidates for further experiments.

H&E and immunohistochemical staining image and the intensity of each cornifying-related factor in the 3D epidermal model are depicted in Figures 1C and 1D. The granular and spinous layers in the sunitinib treatment model appeared thinner, and the basal layer were more bloated than those in control. SERPINB1 staining was observed only in the granular layer region. KRT6A and KRT5 staining was observed throughout the entire epidermis. The KRT6A and SERPINB1 stained area ratio of the control was significantly decreased in the 3D epidermal model treated with sunitinib, whereas there was no change in the KRT5 stained area ratio (Figure 1D). SPINK6 staining was not

1 detected in the 3D epidermal model. Although the thickness was different between the
2 control and sunitinib-treated cells, there was no significant differences of whole tissue
3 area between these cells. Therefore, we compared the mean stained area ratio directly.

5 *Effects of sunitinib on cornification-related factors in a monolayer cell culture*

6 The changes in the expressions of the cornifying-related factors in a monolayer
7 model are shown in Figure 2. No differences were found in the expression of any
8 cornifying-related factors in the NHEK cells treated with sunitinib (Figure 2A). The
9 expression of KRT6A was significantly decreased in PSVK1 cells by concentrations of
10 sunitinib over 1 μ M, and there were no changes in expression of KRT5, SERPINB1, or
11 SPINK6 (Figure 2B).

13 *Effects of the inhibition of ERK1/2 phosphorylation and the p38 MAPK signaling* 14 *pathway on sunitinib-induced changes of KRT6A expression in PSVK1 cells*

15 The expression levels of cornifying factors in PSVK1 cells treated with
16 sunitinib and MAPK inhibitors are shown Figure 3. Sunitinib inhibited the

phosphorylation of ERK1/2 and p38 MAPK at an incubation time of 48 h. Treatment of U0126 with or without sunitinib induced significant decreases in KRT6A expression and phosphorylated ERK1/2 and p38 MAPK in PSVK1 cells (Figure 3A). Treatment of SB203580 with or without sunitinib also induced significant decreases in KRT6A expression and phosphorylated ERK1/2 and phosphorylated MAPKAPK-2, a downstream target of the p38 MAPK pathway (Figure 3B).

Effects of GSK-3 β inhibitor on sunitinib-induced changes in KRT6A expression in PSVK1 cells

The changes in KRT6A expression and related signal transduction induced by treatment of SB216763 with or without sunitinib are shown in Figure 4A. Sunitinib-induced decreases in KRT6A expression and phosphorylated ERK1/2 and p38 MAPK expression were strongly suppressed by co-treatment with SB216763. SB216763 significantly enhanced nuclear translocation of β -catenin in PSVK1 cells (Figure 4B). Sunitinib alone did not significantly affect the nuclear translocation of β -catenin.

Discussion

1 In this study, we demonstrated that, in a 3D epidermal model, sunitinib
2 decreased the levels of cornifying factors related to normal keratinization. Our previous
3 study suggested that an 3D epidermal model treated with multiple TKIs is applicable to
4 the study of HFSR *in vitro* model using pathology images.^[14] These changes in expression
5 might be regulated by the MAPK signaling pathway, which is associated with the
6 functional mechanism of sunitinib.^[15-16] This is the first report to identify sunitinib-
7 induced molecular changes in cornifying factors in human keratinocytes in a 3D
8 epidermal model.

9 Serpins are a family of proteins that inhibit serine protease and are major
10 proteins involved in inflammatory response and regulation of the activity of apoptosis-
11 related factors.^[17] Clade B serpins include the causative gene of Nagashima-type
12 palmoplantar keratosis, which is a condition with strong similarities to HFSR.^[18-22] Our
13 results suggest that SERPINB1 may be an important protein contributing to the
14 development of HFSR. However, the sunitinib-induced changes in SERPINB1
15 expression in the 3D epidermal models were not replicated in monolayer human normal
16 keratinocyte cell lines. Because SERPINB1 is localized to the granular layer (Figure

1C),^[23] its changes in expression may not be due to the direct action of sunitinib but to an indirect action related to the higher-order structure of 3D epidermal models. The 3D epidermal models consist of keratinocytes in various states of differentiation. The expression of some cornifying factors, including serpins, depends on the degree of differentiation of the cells.^[24–27] NHEK and PSVK1 cells are maintained in an undifferentiated state. We consider that changes in sunitinib-induced SERPINB1 expression may be observed in differentiated keratinocytes near the stratum corneum, but further experiments are needed to investigate this hypothesis.

SPINK is a family of proteins that inhibit serine proteases. They are also important in skin barrier functions.^[28,29] KRT5 forms part of the cytoskeleton in the basal layer of the epidermis and has a major role in cell differentiation.^[30,31] Neither SPINK6 nor KRT5 showed any changes in expression in either the 3D epidermal models or the monolayer cell cultures, indicating that they are unlikely to be involved in keratinized abnormalities induced by sunitinib.

KRT6A, which is localized to the basal and supra-basal layer, is a key player in the protection against skin barrier injury, regulating the normal proliferation of

1 keratinocytes, cell adhesion, migration, and inflammation of keratinocytes.^[32–36]
2 Pachyonychia congenita including plantar keratoderma, which has a strikingly similar
3 HFSR pathology, is a genetic disease caused by heterozygous mutations of *KRT6A*
4 gene.^[37–39] These mutations reported to induce missense, nonsense, and small deletion of
5 *KRT6A* mRNA.^[40] The amount of KRT6A with normal function or activity in the tissue
6 with heterozygous mutations is expected to be almost half. The present study found that
7 KRT6A was significantly decreased to be approximately 70% of control in a monolayer
8 cell line and 3D epidermal models under sunitinib treatment. We consider that the
9 decreases in KRT6A and SERPINB1 observed in this study can cause biological and
10 pathological changes in epidermis. Moreover, we hypothesized that the decrease of
11 KRT6A causes inhibition of normal proliferation and promotes keratosis by sunitinib. The
12 monolayer keratinocytes in this study were undifferentiated and showed a profile close to
13 that of cells in the basal layer of the 3D epidermal model. Therefore, decreases in KRT6A
14 levels may be caused by the direct action of sunitinib on the basal layer of keratinocytes.
15 KRT6A has been reported to be expressed in the suprabasal layers *in vivo*.^[33] By contrast,
16 the previous study using an artificially constructed 3D epidermal model indicated that

1 KRT6 expresses in the whole epidermis.^[41] The expression region of KRT6A in our *in*
2 *vitro* study was similar to that in the previous study. *In vitro* 3D epidermal model may be
3 unable to completely reproduce the *in vivo* epidermal profile such as KRT6A expression
4 region.

5 A proposed schematic of the regulation of KRT6A expression mediated by the
6 ERK, p38 MAPK, and β -catenin pathways in keratinocytes is shown in Figure 4C.
7 KRT6A transcription is regulated by the activation of the ERK and p38 MAPK
8 pathways.^[33,42] Our previous study reported that sunitinib temporarily increases the
9 phosphorylation of ERK1/2 and p38 MAPK in epidermal keratinocytes after exposure for
10 2 h.^[43] We confirmed that the temporal activation of ERK1/2 or p38 MAPK causes
11 sunitinib-induced decrease in KRT6A expression. As a result, treatment with sunitinib for
12 48 h induced a reduction in the expression of KRT6A and phosphorylated ERK1/2 and
13 p38 MAPK. Decreases in KRT6A were also caused by selective inhibitors of the ERK
14 and p38 MAPK pathways. Inhibition of VEGFR and PDGFR is the main functional effect
15 of sunitinib. Both ERK1/2 and p38 MAPK are signal receptors downstream from these
16 growth factor receptors. Sunitinib therefore suppresses the phosphorylation of ERK1/2

1 and p38 MAPK with long-term exposure. The ERK pathway generally regulates cell
2 growth and differentiation, whereas the p38 MAPK pathway is generally a stress mediator,
3 acting by regulating apoptosis.^[44] KRT6A expression is enhanced as a response to
4 hyperproliferation and wound stress, and these conditions are signaled by the ERK and
5 p38 MAPK signal pathways, respectively.^[33] We cannot clarify whether the ERK or the
6 p38 MAPK pathway contributes more to the sunitinib-induced decrease in KRT6A
7 expression in our study because of the specificity of inhibitors and cross-talk in signal
8 transduction; it may be affected by both the ERK and p38 MAPK pathways. Moreover,
9 the decrease of KRT6A mediating the ERK or the p38 MAPK pathway may be a response
10 to the mechanistic effects of sunitinib.^[15-16] Some reports suggest the anti-tumor effects
11 of sunitinib on KRT6A-enhanced tumors such as oral and lung squamous cell
12 carcinoma.^[45-46] They have the potential to associate with sunitinib-induced decrease of
13 KRT6A mediating MAPK pathway.

14 Our study showed that the decrease in KRT6A expression was suppressed by
15 the GSK-3 β inhibitor (SB216763). The GSK-3 β inhibitor is well known to promote
16 nuclear translocation of β -catenin and to enhance ERK1/2 phosphorylation.^[35-37] The

1 suppression of the decrease in KRT6A expression by GSK-3 β inhibitor supports our
2 hypothesis that sunitinib-induced decrease in KRT6A expression is caused by the
3 suppression of ERK1/2 and p38 MAPK signal activity. However, β -catenin activity
4 cannot contribute to the mechanism of sunitinib-induced KRT6A expression change,
5 because sunitinib did not affect β -catenin nuclear translocation. In this study, it was not
6 possible to clarify whether ERK1/2 and p38 MAPK activity or β -catenin nuclear
7 translocation contributed significantly to the constitutive regulation of KRT6A in
8 keratinocytes.

9 Our recent clinical study found that patients developing HFSR have the
10 genetic polymorphism rs4796793 in the signal transducer and activator of transcription
11 3 (*STAT3*) gene.^[47] The study showed that patients with the C/C genotype of rs4796793
12 were likely to develop HFSR, and patients with the G/G genotype were not.^[47] NHEK
13 cells that showed no change in KRT6A expression had the G/G genotype rs4796793,
14 whereas PSVK1 cells that showed changes in KRT6A expression when treated with
15 sunitinib had the C/C genotype (data not shown). STAT3 is reported to regulate the
16 transcription of KRT6A in cooperation with ERK1/2 and p38 MAPK.^[33] Further

experiments are necessary to clarify the association between *STAT3* polymorphisms and sunitinib-induced changes in KRT6A and the activity of the ERK and p38 MAPK pathways.

In conclusion, sunitinib induced decreases in KRT6A and SERPINB1 expression levels in a 3D human epidermal model. The changes in expression of KRT6A were mediated by inhibition of the activities of the ERK1/2 and p38 MAPK pathways. Changes in expression of KRT6A and SERPINB1 appear to contribute to the pathology of HFSR induced by sunitinib.

Author Contributions

A.Y. and K.Y. performed the experiments and analyzed the data, drafted the initial manuscript, and reviewed and revised the manuscript. K.Y. designed and supervised the research. T.I. analyzed the data and reviewed pathological interpretation of data. T.O. and T.S. reviewed data analysis. C.N. reviewed the dermatological interpretation of data. I.Y. supervised the research and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

1

2 **Conflicts of Interest**

3 The authors have no potential conflicts of interest to disclose.

4

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9

10 **Data Availability Statement**

11 The data that support the findings of this study are available from the corresponding
12 author upon reasonable request.

13

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13 **Figure Legends**

14 **Figure 1: Gene and protein expression in 3D epidermal models with sunitinib** 15 **treatment**

16 (A) Schema for the selection of candidate genes and the results of microarray analysis.
17 GO Biological Process Terms were used in the primary selection step. Microarray

analysis was performed using total RNA extracted from 3D epidermal models treated with or without 1 μ M sunitinib at the basal layer side for 120 h. (B) Fold change of 20 candidate genes selected by the primary selection step. (C) Immunohistochemical images of 3D epidermal model incubated with or without 1 μ M sunitinib. 3D epidermal models were stained by H&E or specific antibodies against each protein in brown. The scale bar represents 50 μ m and applies to all images. (D) Each bar represents the mean of the relative stained area ratio $\pm$ S.D. ($n = 7$, $*P < 0.05$). The relative stained area ratio was defined as the ratio of the area stained by each protein antibody to the whole area of the 3D epidermal model against the control value. N.D.: Not detected.

Figure 2: Effects of sunitinib on protein expression in monolayer keratinocyte cell lines

(A) NHEK cells and (B) PSVK1 cells were incubated in a medium with the indicated concentration of sunitinib for 48 h. Proteins were evaluated using a western blotting assay with specific antibodies. Quantitative analyses of western blots were performed by determining the intensities of protein bands for the densitometric assay. Each bar

represents the mean of the relative expression ratio $\pm$ S.D. (n = 3, * P < 0.05 vs. control).

Figure 3: Effects of inhibition of ERK1/2 and p38 MAPK signaling on the expression of cornifying factors in PSVK1 cells

PSVK1 cells were incubated in a medium with 1 μ M sunitinib and (A) 10 μ M U0126 or (B) 10 μ M SB203580 for 48 h. Protein and phosphorylation levels were evaluated using a western blotting assay with specific antibodies. Quantitative analyses of western blots were performed by determining the intensities of protein bands for the densitometric assay. Each bar represents the mean of relative expression ratio $\pm$ S.D. (n = 3, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control).

Figure 4: Effects of GSK-3 β inhibitor on the expression of cornifying factors in PSVK1 cells

(A) PSVK1 cells were incubated in a medium with 1 μ M sunitinib and 10 μ M SB216763 for 48 h. Protein and phosphorylation levels were evaluated using a western blotting assay with specific antibodies. Quantitative analyses of western blots were

1 performed by determining the intensities of protein bands for the densitometric assay.
2 Each bar represents the mean of the relative expression ratio on the basis of sunitinib
3 (+) and SB216763 (–) $\pm$ S.D. (n = 3, $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs. sunitinib
4 treatment). (B) Immunofluorescent images of PSVK1 cells. PSVK1 cells were
5 incubated in a medium with 1 μ M sunitinib and 10 μ M SB216763 for 48 h and then
6 fixed and incubated with an anti- β -catenin antibody, followed by incubation with FITC-
7 conjugated anti-rabbit IgG (green) and Hoechst33342 (blue). Nuclear translocation of β -
8 catenin was determined using cell population analysis by determining the integrated
9 nucleus/cytoplasm intensity ratio. Each bar represents the mean of the relative β -catenin
10 nuclear translocation ratio $\pm$ S.D. (n = 3, $\dagger P < 0.05$, $\dagger\dagger\dagger P < 0.001$ vs. sunitinib
11 treatment). (C) A proposed schematic of sunitinib and various signal inhibitor-induced
12 KRT6A, ERK1/2, p38 MAPK, and β -catenin pathways in keratinocytes.

Figure 1

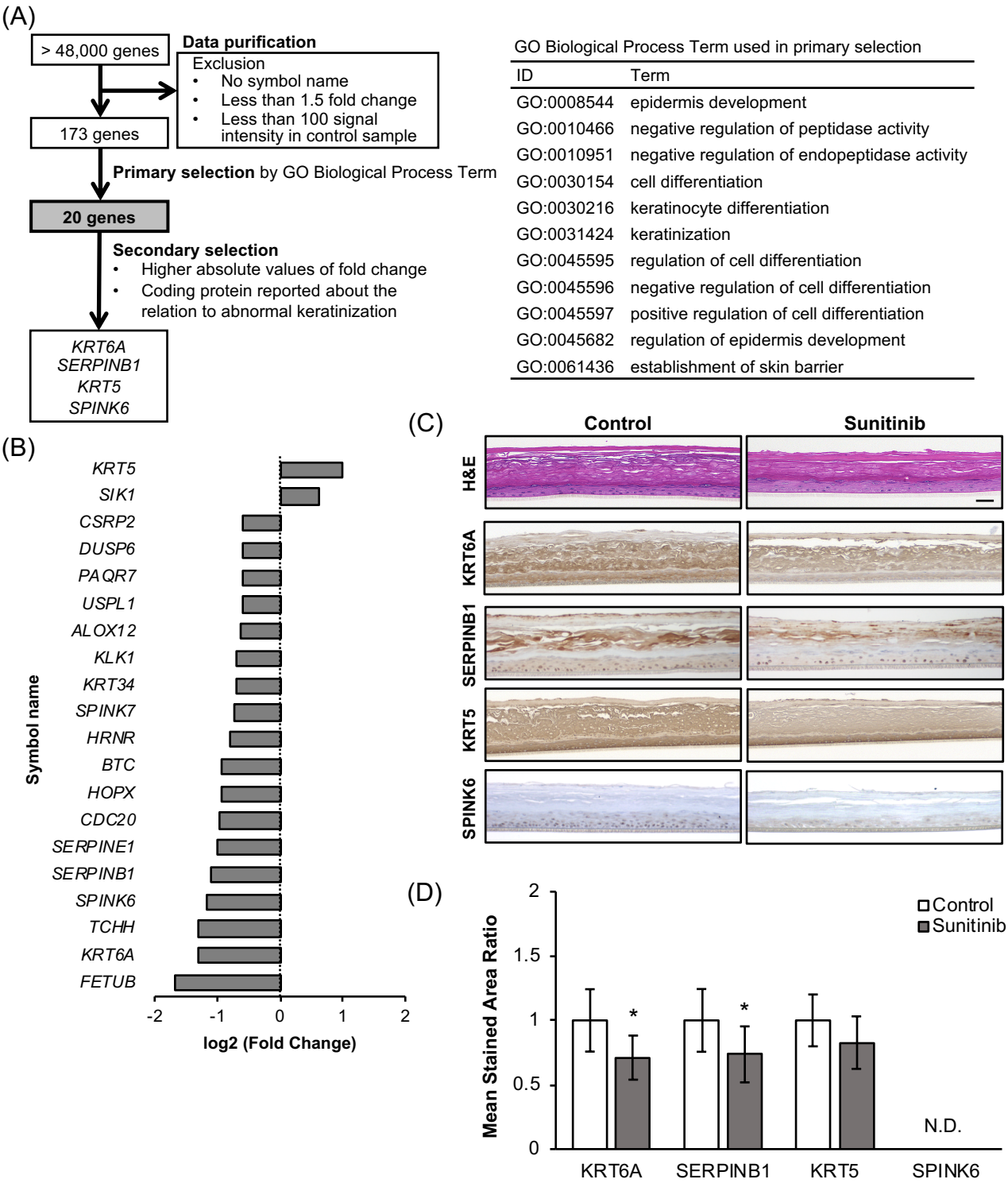


Figure 2

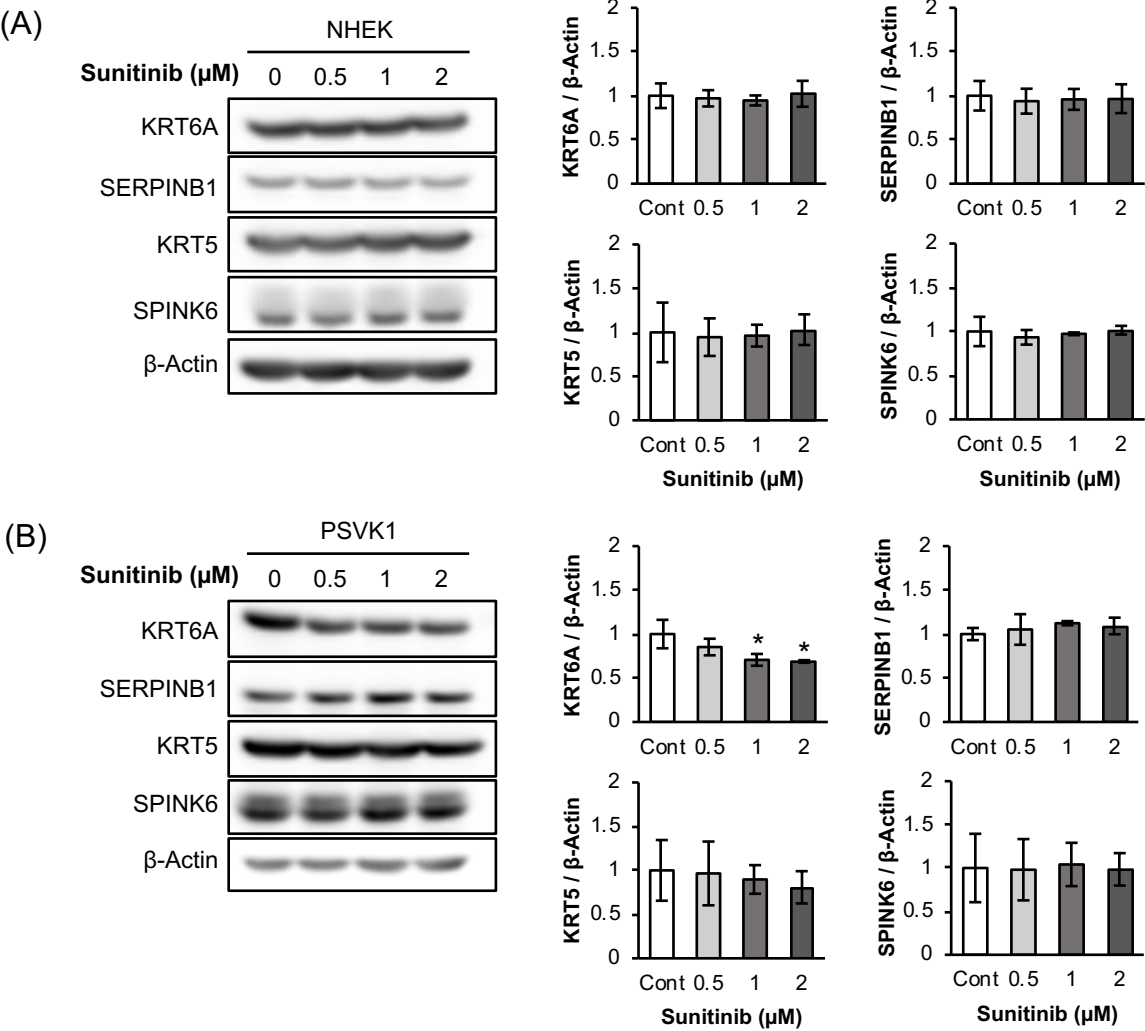


Figure 3

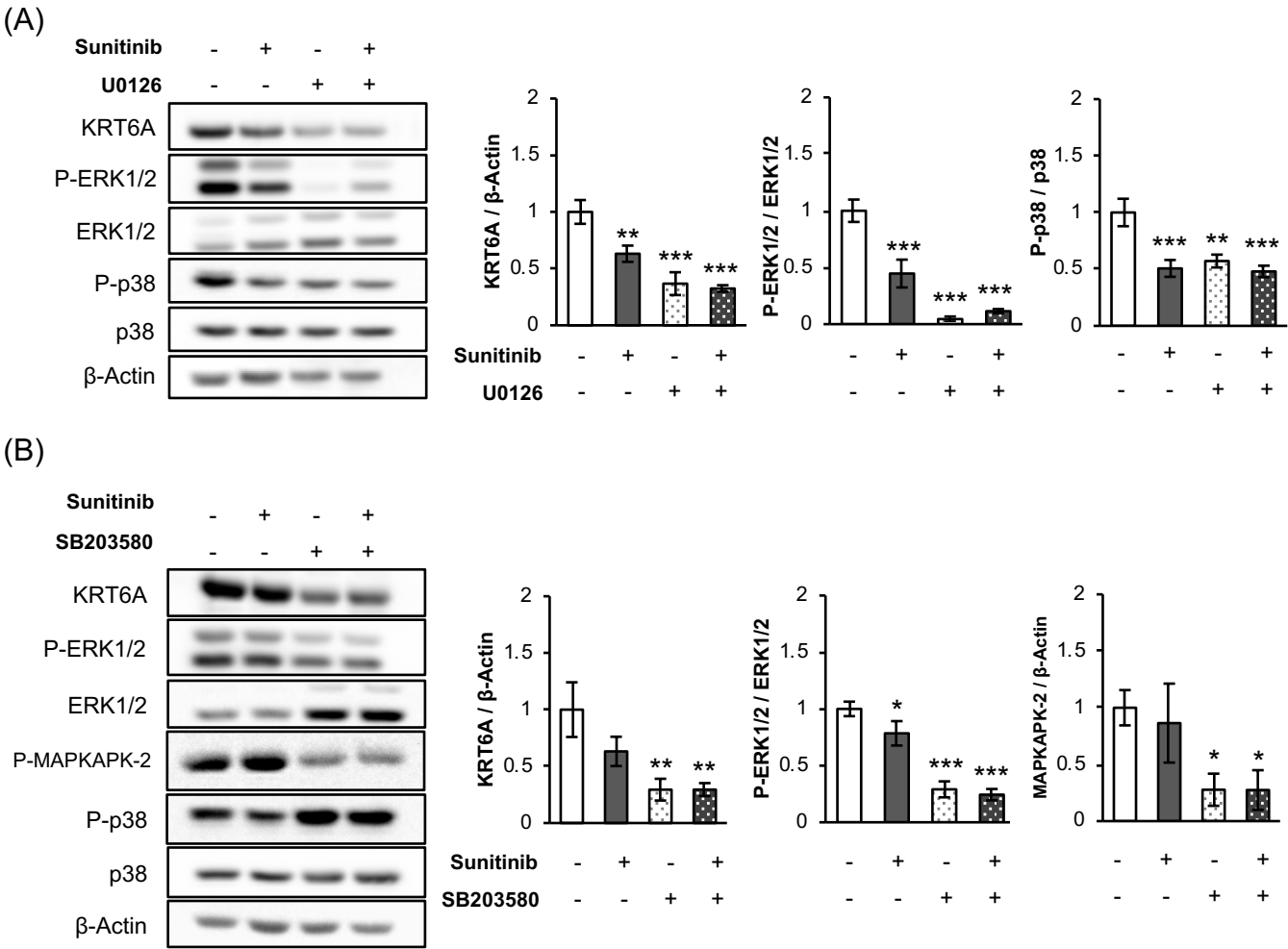


Figure 4

