Non-invasive evaluation of subjective sensitive skin by transcriptomics using mRNA in skin surface lipids

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Abstract

Sensitive skin is a condition characterized by hypersensitivity to environmental stimuli, and its pathophysiology has not been fully elucidated. Questionnaires based on subjective symptoms, intervention tests, and measuring devices are used to diagnose sensitive skin; however, objective evaluation methods, including biomarkers, remain to be established. This study aimed to investigate the molecular profiles of self-reported sensitive skin, understand its pathophysiology, and explore its biomarkers. Here, we analyzed RNAs in skin surface lipids (SSL-RNAs), which can be obtained non-invasively by wiping the skin surface with an oil-blotting film, to compare the transcriptome profiles between questionnaire-based 'sensitive' (n = 11) and 'non-sensitive' (n = 10) skin participants. Exactly 417 differentially expressed genes in SSL-RNAs from individuals with sensitive skin were identified, of which C-C motif chemokine ligand 17 and interferon-γ pathways were elevated, while 50 olfactory receptor (OR) genes were downregulated. The expression of the detectable 101 OR genes was lower in individuals with sensitive skin compared to that in those with non-sensitive skin and was particularly associated with the subjective sensitivity among skin conditions. The receiver operating characteristic (ROC) curve demonstrated that the mean expression levels of OR genes in SSL-RNAs could discriminate subjective skin sensitivity with an area under the ROC curve of 0.836. SSL-RNA profiles suggest a mild inflammatory state in sensitive skin, and overall OR gene

expression could be a potential indicator for sensitive skin.

Keywords

Non-invasive techniques, Olfactory receptors, Sensitive skin, Skin surface lipids, Transcriptome

Introduction

Sensitive skin is characterized by hypersensitivity to environmental stimuli. It has been proposed as an independent syndrome[1,2] and can be often associated with other skin diseases, such as atopic dermatitis (AD), acne, rosacea, and psoriasis.[1–4] Itching, burning, and stinging are typical symptoms of sensitive skin, and its prevalence is reportedly around 25% worldwide.[5] The precise mechanisms of sensitive skin have not yet been elucidated, but some factors have been proposed, such as dermatosis, barrier disturbance, and sensory nerve dysfunction.[6] Additionally, lifestyle, as well as environmental and endogenous factors, are also related to sensitive skin; thus, sensitive skin is a condition that is caused by multiple factors.[6,7]

Several investigations on the prevalence and characteristics of sensitive skin have been reported based on subjective evaluation (self-reported sensitive skin) using a survey.[8] In addition to the integrative question items,[9–11] intervention tests, including stinging test, thermosensitivity test, itching response test, and repeated washing test have been used for diagnosing sensitive skin objectively.[6,12] Bioengineering measurements, such as corneometry, laser Doppler velocimetry, colorimetry, neurometry, and transepidermal water loss (TEWL) measurement are also used to evaluate sensitive skin.[6,12] Although sensitive skin can be evaluated to some extent using the above methods, it is difficult to

analyze and understand sensitive skin at the molecular level because of the difficulty in obtaining skin samples. Comprehensive molecular profiling of sensitive skin is poorly investigated, and the lack of objective evaluation methods based on these profiles has been a serious obstacle in understanding the pathophysiology of sensitive skin and in developing therapeutic methods.

To examine the molecular profile of the skin, invasive methods, such as punch biopsy, are required to collect skin samples. Recently, we have established a non-invasive, comprehensive method for analyzing human RNAs using skin surface lipids (SSL).[13] RNAs in SSL (SSL-RNAs) can be obtained non-invasively by wiping the skin surface with an oil-blotting film, and mainly include biological information derived from the sebaceous glands, epidermis, and hair follicles, thus, reflecting skin conditions. Furthermore, we demonstrated the potential application of this method for the characterization of skin diseases, such as AD.[13] In this study, we aimed to investigate the molecular profile of self-reported sensitive skin using SSL-RNAs, understand the pathophysiology of sensitive skin, and identify the relevant biomarkers.

Methods

Ethics statement

This study protocol complied with the Declaration of Helsinki and was approved by the Human Research Ethics Committee of Kao Corporation (792-20160829). Written informed consent was obtained from all participants.

Participants

Forty-two healthy female individuals (mean age, 38.5; range, 20–56 years; standard deviation, 11.5) were recruited, and the study was conducted in October 2016. No obvious skin disease or condition was confirmed by dermatologists before the commencement of the study. On the test day until the SSL sampling, all participants were restricted from removing the facial sebum by washing or using wipes.

SSL collection and facial skin evaluation

The sebum level of the cheek was measured using a Sebumeter (SM815, Integral, Tokyo, Japan), followed by SSL collection. SSL was collected by wiping the entire face (forehead, cheek, face line, nose, and chin) using an oil-blotting film (5.0 × 8.0 cm, 3M Japan, Tokyo, Japan), and the films were stored in RNase-free vials at -80°C until RNA preparation.[13] Conditioning for 20 min at 20 ± 2°C with a relative humidity of 50 ± 5% after facial

washing, the measurement of cheek skin parameters was performed for TEWL, capacitance, conductance, pH, and index of erythema and melanin using a Tewameter (TM300, Integral, Tokyo, Japan), Corneometer (CM825, Integral, Tokyo, Japan), Skicon (200Ex-USB, YAYOI CO., LTD., Tokyo, Japan), Skin-pH-meter (PH905, Integral, Tokyo, Japan), and Mexameter (MX18, Integral, Tokyo, Japan), respectively. A question-based 4-class scoring method for sensitive skin was reported previously,[4,8,14] and we used this evaluation method with modifications and additional questions. Briefly, participants were asked about each facial skin condition and were requested to rate how much they suffer from these conditions as follows: "1; strongly disagree," "2; disagree," "3; agree," or "4; strongly agree." The items consisted of oily skin, comedo, dryness, wrinkles, sagging, resiliency, spots, freckles, dullness, irregularity, clarity, brightness, glow, eye bags, pore, texture, redness, sweat, sensitivity, roughness, and swelling.

SSL-RNA preparation and AmpliSeq transcriptome analysis

The preparation of SSL-RNAs and sequencing libraries was performed according to a previously described protocol.[13] Briefly, the SSL-blotted oil-blotting film was cut into small pieces with a scalpel and homogenized using QIAzol reagent (QIAGEN, Hilden, Germany). The supernatant was collected, and an aqueous phase obtained from the

phenol/chloroform separation was used for subsequent SSL-RNA purification using the RNeasy Mini kit (QIAGEN). Sequencing libraries were prepared using 1.75 μl of purified SSL-RNAs and Ion AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific, Waltham, MA, USA) as described previously.[13] Fifty pM sequencing libraries were subjected to template preparation with the Ion Chef System and Ion 540 Chip Kit and sequenced using the Ion S5 XL System (Thermo Fisher Scientific) according to the manufacturer's instructions.

Statistics and data analysis

The AmpliSeq RNA plug-in of Ion Torrent Suite Software Plugins (Thermo Fisher Scientific) was used for the primary analysis of the sequencing data. The obtained raw read count data were subjected to statistical analysis and data analysis using R (3.6.1) and RStudio (1.1.442). Data cleaning and normalization of the raw read counts were performed in two steps using DESeq2 (Bioconductor).[15] First, tentative normalization was performed with DESeq2 regarding the genes with non-zero read counts in more than 90% of the samples, and samples with low median normalized read counts (lower than 7.0) and a high coefficient of variation (> 70%) were removed. Then, definitive normalization of raw read counts was performed in selected samples and the genes with

non-zero read counts in more than 90% of the samples using DESeq2. Differentially expressed genes (DEGs) were extracted via a likelihood ratio test using normalized count with Benjamini and Hochberg's false discovery rate (FDR) < 0.05, and fold change (FC) > 2.0, as a threshold value. Gene set variation analysis (GSVA, Bioconductor) was performed using normalized counts.[16] Gene ontology (GO) analysis of DEGs was performed using the database for annotation, visualization, and integrated discovery (DAVID) to extract GO terms of biological processes (Category: GOTERM_BP_DIRECT).[17,18] Mann-Whitney U test was performed with the wilcox_test function of the coin package in R, regarding age, skin measurements and scores, gene expression levels, and GSVA enrichment score to compare two groups. For correlation analysis, the corr.test function of the psych package in R was used to calculate Spearman's rank correlation coefficients (rho). The R packages used for the generation of plots were as follows: Tidyverse, reshape2, ggbeeswarm, ggpubr, psych, gplots pROC, ggsignif, and cowplot.

Results

Background of participants and experimental groups

We enrolled 42 healthy female participants but excluded four participants because they did not satisfy our SSL-RNA normalization criteria. We confirmed the uniformity of normalized expression profiles via generating a boxplot of normalized read counts in each sample (Figure S1). After data cleaning by removal of four samples, it was confirmed that the variability of the data, as assessed by the median and interquartile range, was suppressed and we used these 38 samples for subsequent analyses. The participants were asked about their awareness of facial skin sensitivity with the following phrase: “I suffer from sensitive skin” (n = 38). Participants who answered “strongly disagree” were assigned to the “non-sensitive skin” group (n = 10), and those who answered “agree” were assigned to the “sensitive skin” group (n =11) (Figure 1A). Regarding the 21 facial skin questions, the scores for comedo and roughness were significantly higher, and those for freckles were lower in the sensitive skin group (Table 1). The scores for comedo, freckles, sensitivity, and roughness were correlated with each other, indicating that these phenomena were associated with subjective skin sensitivity (Table S1). In contrast, there were no significant changes in facial skin measurements, such as sebum, hydration, and barrier function. Altogether, participants were divided into non-sensitive and sensitive

skin groups based on individual subjectivity rather than physiological conditions.

C-C motif chemokine ligand 17/thymus and activation-regulated chemokine (CCL17/TARC) is highly expressed in SSL-RNAs of individuals with subjective sensitive skin

In AmpliSeq transcriptome analysis using SSL-RNAs, the average number of reads was 7,150,995 for the non-sensitive skin group and 9,133,119 for the sensitive skin group; the average number of detected genes was 10,183 in the non-sensitive skin group and 11,309 in the sensitive skin group. After data cleaning and normalization, 3,381 genes were obtained and used in subsequent analyses. Differential expression analysis between non-sensitive and sensitive skin groups revealed 417 DEGs, of which 80 were upregulated and 337 were downregulated in the sensitive skin group (Figure 1B). Interestingly, upregulated genes included CCL17/TARC (Figure 1C). The expression of C-C motif chemokine ligand 22/macrophage-derived chemokine (CCL22/MDC), which is another Th2 type chemokine that binds to C-C motif chemokine receptor 4 (CCR4), also tended to be higher in SSL-RNAs of the patients in the sensitive skin group (Figure 1C). We examined not only Th2 type immune response but also processes related to Th1, Th17, Th22, and epidermal terminal differentiation and lipid using GSVA (Table S2).[19–21] There

were no significant differences in the GSVA scores between the two groups (Figure 1D), suggesting that the difference in CCL17/TARC levels between groups was associated with skin sensitivity and was distinct from the abnormal inflammation and epidermal differentiation.

DEGs and GO terms identified in SSL-RNA analysis between individuals with sensitive and non-sensitive skin

To explore the biological processes involving DEGs, we extracted GO terms using DAVID. GO analysis of 80 upregulated genes revealed “interferon-gamma-mediated signaling pathway (GO:0060333)” and “antigen processing and presentation of exogenous peptide antigen via major histocompatibility complex (MHC) class II (GO:0019886)” (Table 2). These GO terms included MHC, class II, DR alpha (HLA-DRA), MHC, class II, DP beta 1 (HLA-DPB1), MHC, class II, DQ alpha1 (HLA-DQA1), beta-2-microglobulin (B2M), IFI30 lysosomal thiol reductase (IFI30), and PML nuclear body scaffold. Interferon-gamma (IFN-γ) signaling induces CCL17/TARC and CCL22/MDC expression in keratinocytes,[22] suggesting that higher expression of CCL17/TARC and CCL22/MDC in SSL-RNAs of sensitive skin group was induced by the activation of IFN-γ pathways. Among the 337 downregulated genes, GO analysis

revealed: “detection of chemical stimulus involved in sensory perception of smell (GO:0050911)”, “G-protein coupled receptor signaling pathway (GO:0007186)”, “detection of chemical stimulus involved in sensory perception (GO:0050907)”, “sensory perception of smell (GO:0007608)”, “nucleosome assembly (GO:0006334)”, and “DNA replication-dependent nucleosome assembly (GO:0006335)” (Table 2). Notably, the top GO term in downregulated genes, “detection of chemical stimulus involved in sensory perception of smell (GO:0050911)”, showed considerably low FDR values (FDR = 7.16E-29) and included 43 olfactory receptor (OR) genes. Therefore, it was suggested that the downregulation of the series of OR genes in SSL-RNAs are associated with subjective sensitive skin.

The subjective skin sensitivity is negatively associated with OR gene expression levels

GO analysis suggested that downregulation of OR genes in SSL-RNAs was related to subjective sensitive skin. We then confirmed the expression of 382 OR genes that were detectable in our AmpliSeq analysis using SSL-RNAs and found that 101 OR genes were detectable in more than 90% of participants. Expressions of all 101 OR genes showed a decreasing trend in the sensitive skin group, of which 50 genes showed statistical

significance (FDR < 0.05, Figure 2A). We performed GSVA with a set of 382 detectable OR genes (OR gene set, Table S3), and the enrichment score of the OR gene set decreased in the sensitive skin group (Figure 2B). To analyze the relationship between the expression levels of the 101 OR genes and skin condition, we divided the participants into low or high groups based on each skin measurement and questionnaire (low: median and below in measurements or score 1 and 2 in the questionnaire, high: higher than the median in measurements or score 3 and 4 in the questionnaire), and compared OR expression levels between the two groups (Table S4). Overall, OR expression levels were downregulated in the group with high roughness and sebum, as well as sensitivity (Figure 2C, D). Of these, statistically significant decreases ($p < 0.05$) were observed in 15 genes related to roughness, 24 genes related to sebum, and 40 genes related to sensitivity, suggesting that the expression level of ORs was associated particularly with subjective sensitivity.

Global OR gene expression patterns help discriminate between sensitive and non-sensitive skin

We further tested the potency of the overall OR gene expression level for the discrimination of sensitive skin. The raw read counts detected using SSL-RNAs via

AmpliSeq were simply transformed to $\log_2$ (read per million counts + 1) as expression levels, and zero values were transformed to missing values. The mean expression level of 382 OR genes was used to discriminate sensitive skin, and the performance was evaluated using the area under the receiver operating characteristic (ROC) curve (AUC). The mean OR expression level could distinguish between sensitive and non-sensitive skin with an AUC of 0.836 at a cut-off value of 3.7 (Figure 3A, B). Furthermore, regression analysis of the mean OR expression level of all participants (n = 38) showed a negative correlation with subjective sensitive skin (Figure 3C). Taken together, these results indicated that the overall expression level of OR genes in SSL-RNAs could be an indicator of sensitive skin.

Discussion

In this study, we analyzed the gene expression profile of self-reported sensitive skin using non-invasively collected SSL-RNAs and revealed that genes related to immune and inflammatory responses, such as the IFN-γ pathway and CCL17/TRAC, were upregulated in individuals with sensitive skin. Furthermore, we found that the expression of a series of OR genes was downregulated in individuals with sensitive skin, which may be applied as an indicator to classify subjective skin sensitivity.

The top GO term for upregulated genes in individuals with sensitive skin was the "interferon-gamma-mediated signaling pathway", which plays an important role in the maintenance of tissue immune/inflammatory homeostasis. Among IFN-γ-related molecules, CCL17/TARC and CCL22/MDC are downstream molecules of the IFN-γ pathway,[22] and CCL17/TARC was significantly upregulated in individuals with sensitive skin. CCL17/TARC and CCL22/MDC are produced from various types of cells, including monocytes/macrophages, dendritic cells, and keratinocytes, and induce chemotaxis of T cells by interacting with the cell surface chemokine receptor CCR4, which is associated with inflammatory/allergic skin diseases.[22–24] Serum CCL17/TARC is a well-established severity marker for AD and is also increased in SSL-RNAs collected from patients with AD.[13,25,26] The prevalence of sensitive skin is higher in patients with AD than that in

healthy individuals, and thus, the pathophysiological relationship between sensitive skin and AD has been discussed.[2,6,9,27] Here, higher expression of CCLs was not concomitant with AD-related molecular changes, such as up-regulation of "Th1- and Th2-related" gene sets and downregulation of "epidermal terminal differentiation and lipid-related" gene sets. Therefore, increased CCL17/TARC expression in self-reported sensitive skin suggests that sensitive skin may be a mild inflammatory state distinct from AD, or a rather weak atopy-like state. The score of skin sensitivity was correlated with that of the comedo, which was consistent with a high frequency of simultaneous manifestations of sensitive skin and acne.[4,28] *Cutibacterium acnes* has a pivotal role in the pathogenesis of acne vulgaris and has been reported to stimulate the production of IFN-γ.[29] Thus, the enhancement of IFN-γ pathways in SSL-RNAs of individuals with sensitive skin may be associated with acne. So far, SSL-RNAs have been shown to reflect gene expression in the epidermis, sebaceous glands, and hair follicles,[13] but it is not clear whether they contain immune cell-derived information. However, considering that MHC class II genes have been identified as upregulated DEGs in individuals with sensitive skin, SSL-RNAs may contain information derived from immune cells such as macrophages and dendritic cells.

Our results provide the possibility that the expression level of ORs can be a common

indicator for skin sensitivity, regardless of skin condition. ORs are primarily expressed in the olfactory neurons of the nasal epithelium and detect odorants in the environment, whereas OR genes are also expressed ectopically in the skin, as well as in several tissues.[30,31] OR genes encode G-protein coupled receptors and comprise the largest gene family in humans, which includes approximately 400 functional OR genes and 600 non-functional OR pseudogenes.[30,32,33] Here, the most marked change observed in this SSL-RNA analysis was the decreased expression of the OR genes in individuals with sensitive skin, and the expression level was associated with the subjective sensitivity relative to other skin conditions. Moreover, we showed that sensitive skin and healthy skin can be distinguished by comparing the mean expression levels of OR genes. Several OR genes have roles in epidermal/keratinocyte proliferation, migration, regeneration, and cytokine production,[34–36] suggesting the possibility that downstream signaling of ORs causes sensitive skin through disturbance of epidermal homeostasis. We previously showed in SSL-RNA analysis between patients with AD and healthy individuals that the GO term "detection of chemical stimulus involved in sensory perception of smell (GO:0050911)" was obtained for downregulated DEGs in patients with AD.[13] Furthermore, it has been reported that downregulated genes in patients with AD are associated with OR gene-related pathways in RNA analysis, compared with healthy individuals, using tape-stripped

stratum corneum.[37] Interestingly, the observed changes in OR gene expression in sensitive skin were not specific, but overall. Such expressional changes in multiple OR genes have also been reported in AD, Alzheimer's disease, and cancer;[36–39] however, the transcriptional regulation and role of OR genes are largely unknown. Therefore, there is also a possibility that the lower expression of OR genes in SSL-RNAs collected from individuals with sensitive skin may be a consequence of sensitive skin rather than a cause. Considering the marked expression changes of OR genes in individuals with sensitive skin and their correlation with subjective indicators, the mean expression level of OR genes analyzed using SSL-RNAs may serve as an indicator for sensitive skin.

To the best of our knowledge, transcriptomic analysis in individuals with sensitive skin has been reported from two groups and reviewed by one group.[40–42] Two existing studies conducted transcriptome analysis using RNAs obtained from the whole skin via punch biopsy. Kim et al. conducted a comparative analysis of sensitive and non-sensitive skin using DNA microarrays and reported differential gene expression related to inflammatory and immune responses, muscle composition/contraction, and carbohydrate/lipid metabolism.[40] Yang et al. performed RNA-seq and reported differences in gene expression in "system development" related to keratinocyte differentiation and epidermal development, and in "protein binding and binding" related

to inflammation.[41] Although it is difficult to simply compare these three studies, including ours, because of the different methods of skin sampling and analysis, there was partial agreement on the differential expression of some processes, including innate immunity and inflammation. In contrast, regarding the ORs, for which a remarkable expression difference was observed in this study, no difference was observed in previous reports. ORs are highly expressed in the epidermis/stratum corneum relative to the dermis and subcutaneous tissue as shown using immunostaining and transcriptome analysis.[34,36] Skin samples obtained via punch biopsy contain RNAs derived not only from the skin (epidermis, dermis, and subcutaneous tissue) but also from muscles and nerves, whereas SSL-RNAs mainly contains RNAs derived from the epidermis, sebaceous glands, and hair follicles,[13] which may have a significant impact on the results. Thus, SSL-RNA analysis may be useful for understanding skin pathophysiology directly or indirectly, especially by obtaining gene expression information from the upper layers of the skin. Furthermore, unlike the invasive punch biopsy method, SSL-RNAs can be collected non-invasively, and thus can be easily and repeatedly collected for analysis. Therefore, the SSL-RNA analysis method is expected to be applied for the evaluation of various skin diseases, as well as sensitive skin.

There are some limitations to our study. This is the first study describing a

transcriptomic analysis of sensitive skin using SSL-RNAs which was conducted in a single institution. Furthermore, our analysis of sensitive skin was focused on subjective recognition of sensitivity in healthy individuals and was not evaluated via intervention tests. Therefore, it is necessary to verify the relationship between sensitive skin and SSL-RNA expression through intervention tests, as well as the accuracy of predicting sensitive skin using SSL-RNAs on a larger scale at multiple institutions.

In summary, the gene expression profile of individuals with subjective sensitive skin was investigated via non-invasive transcriptomic analysis using SSL-RNAs, which showed the possible involvement of the CCL17/TARC and IFN-γ pathways in the pathophysiology of sensitive skin. Furthermore, analysis of the global downregulation of OR genes could help identify sensitive skin. Our results provide novel insights for molecular-based diagnostic methods for sensitive skin and could be utilized to close the gap between subjective symptoms, objective measurements, and molecular mechanisms in clinical practice.

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Author contributions

UY and MT were involved in drafting the article and all authors contributed to revising the article critically for intellectual content. UY, IT, and MT contributed to the study conception and design. UY and IT contributed to the acquisition, analysis, and interpretation of data. MT, ON, and IS contributed to the interpretation of data. All authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflict of interest to declare.

Data availability statement

The data of this study are available from the corresponding author upon reasonable request.

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Table 1. Facial skin measurements and questionnaire results.

		Non-sensitive (n = 10, mean ± SD)	Sensitive (n = 11, mean ± SD)	*p*-value
Measurement	Sebum (μg/cm^2)	28.8 ± 22.3	41.2 ± 31.0	0.376
	Capacitance (A.U.)	46.3 ± 8.7	49.9 ± 10.3	0.251
	Conductance (μS)	132.1 ± 45.9	177.7 ± 59.0	0.137
	TEWL (g/h/m^2)	14.1 ± 3.5	15.4 ± 3.1	0.282
	Melanin index (A.U.)	152.8 ± 31.8	156.9 ± 31.3	0.500
	Erythema index (A.U.)	196.3 ± 52.6	211.7 ± 30.7	0.570
	pH	6.6 ± 0.3	6.6 ± 0.3	0.904
Questionnaire	Oily skin	2.5 ± 0.9	3.1 ± 0.7	0.161
	Comedo	1.8 ± 0.6	2.6 ± 0.8	0.023
	Dryness	2.8 ± 1.2	3.1 ± 0.8	0.697
	Wrinkle	2.6 ± 1.2	2.1 ± 1.2	0.377
	Sagging	2.7 ± 1.1	2.3 ± 1.1	0.440
	Resiliency	2.8 ± 1.1	2.1 ± 1.0	0.152
	Spots	3.4 ± 0.7	2.7 ± 1.3	0.334
	Freckles	3.3 ± 0.7	2.0 ± 1.0	0.009
	Dullness	3.1 ± 0.9	2.7 ± 1.1	0.535
	Irregularity	2.6 ± 1.3	2.7 ± 1.2	0.841
	Clarity	2.9 ± 1.0	2.9 ± 1.0	1.000
	Brightness	2.9 ± 1.0	2.7 ± 1.1	0.825
	Glow	2.9 ± 1.0	2.5 ± 0.9	0.481
	Eye bags	3.0 ± 1.0	2.8 ± 0.7	0.589
	Pore	2.9 ± 0.7	2.8 ± 1.0	0.967
	Texture	2.8 ± 1.1	2.8 ± 0.8	0.885
	Redness	2.3 ± 1.0	2.6 ± 1.0	0.502
	Sweat	2.2 ± 1.0	2.5 ± 1.1	0.644
	Sensitivity	1.0 ± 0	3.0 ± 0	N/A
	Roughness	1.1 ± 0.3	2.2 ± 0.8	0.001
	Swelling	1.6 ± 0.7	2.1 ± 1.0	0.337

Summary of facial skin measurements and questionnaires the mean ± SD is shown, and

p-values are calculated using the Mann-Whitney U test. N/A, not applicable; SD, Standard deviation.

Table 2. Significant GO terms in individuals with sensitive skin.

Regulation	Term	*p*-value	FDR
UP	GO:0060333~interferon-gamma-mediated signaling pathway	1.61E-5	1.13E-2
	GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	5.64E-5	1.98E-2
DOWN	GO:0050911~detection of chemical stimulus involved in sensory perception of smell	1.22E-31	7.16E-29
	GO:0007186~G-protein coupled receptor signaling pathway	3.93E-21	1.16E-18
	GO:0050907~detection of chemical stimulus involved in sensory perception	1.46E-14	2.86E-12
	GO:0007608~sensory perception of smell	4.28E-8	6.31E-6
	GO:0006334~nucleosome assembly	1.26E-6	1.48E-4
	GO:0006335~DNA replication-dependent nucleosome assembly	4.92E-4	4.83E-2

Extracted GO terms in 80 upregulated and 337 downregulated genes, respectively (FDR < 0.05). FDR, false discovery rate.

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Answer	Number	Subgroup	Age (Mean ± SD)
Strongly agree	0	N/A	N/A
Agree	11	Sensitive	34.2 ± 12.9
Disagree	17	N/A	N/A
Strongly disagree	10	Non-sensitive	44.3 ± 11.8

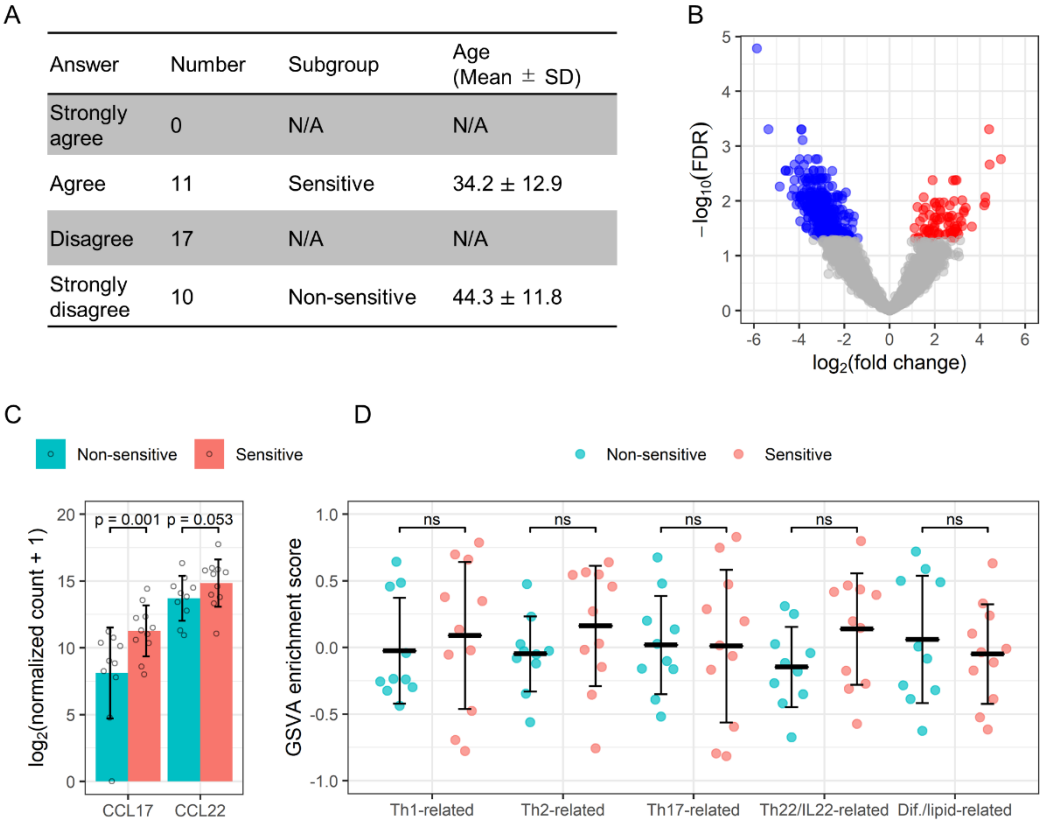

Figure 1. Differential expression of SSL-RNAs in individuals with sensitive skin.

(A) A questionnaire-based experimental grouping. Participants were asked their awareness of sensitive skin with the following phrase: “I suffer from sensitive skin” (n = 38). Participants who answered “Strongly disagree” were assigned to the ‘Non-sensitive’ group (n = 10), and those who answered “Agree” were assigned to the ‘Sensitive’ group (n =11). The *p*-value of the age difference between the non-sensitive and sensitive groups was 0.12 calculated using the Mann-Whitney U test. (B) Volcano plot of expression differences for 3,381 preprocessed genes. 337 genes are downregulated (blue) and 80 genes are upregulated (red) in the sensitive skin group (FDR < 0.05 and FC > 2.0). (C)

CCL17/TARC and CCL22/MDC expression levels in SSL-RNAs. Barplots and error bars represent the mean ± SD. Dots show expression levels in each sample. *p*-values were calculated using the likelihood ratio test. (D) GSVA of Th1, Th2, Th17, Th22/IL22, and epidermal terminal differentiation and lipid (Terminal dif./lipid) gene set between non-sensitive and sensitive skin groups. Each dot represents the GSVA enrichment score in each sample. Solid lines represent the mean ± SD. *p*-values were calculated using the Mann-Whitney U test, and $p \geq 0.05$ was indicated as not significant (ns). FDR, false discovery rate; FC, fold change; SD, standard deviation; GSVA, gene set variation analysis.

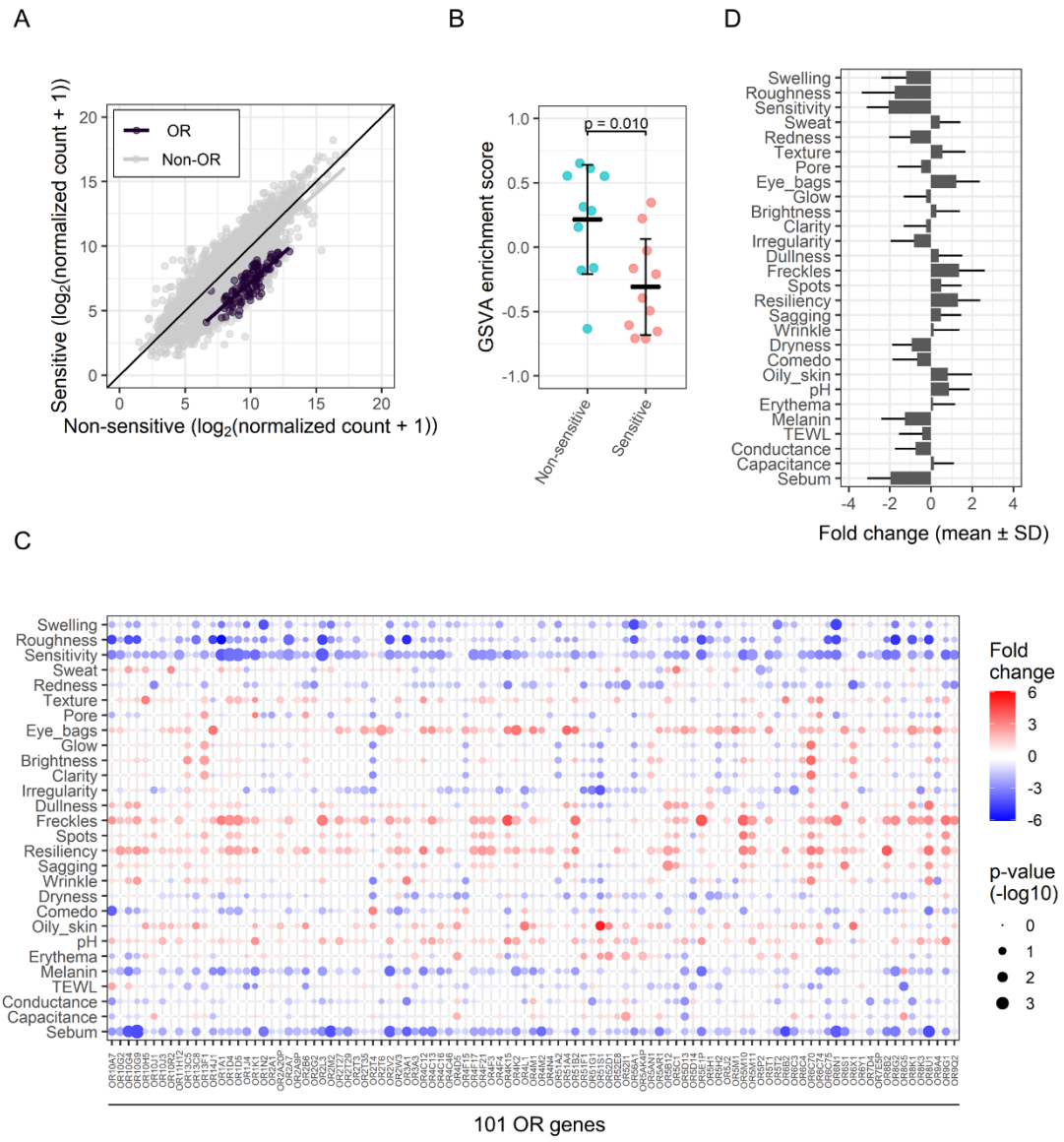

Figure 2. Global downregulation of OR gene expression in individuals with sensitive skin.

(A) Comparison of mean normalized expression levels between non-sensitive (n = 10) and sensitive groups (n = 11). OR genes are highlighted in dark purple (101 genes) and other genes are in gray (non-OR, 3,280 genes). Linear regression of OR and non-OR

genes are represented as dark purple and gray lines, respectively. (B) GSVA of the OR gene set between non-sensitive and sensitive groups. Each dot represents the GSVA enrichment score in each sample. Solid lines represent the mean ± SD. *p*-values were calculated using the Mann-Whitney U test. (C) Heatmap of 101 OR genes and skin conditions. Fold change and *p*-values were calculated using $\log_2$ (normalized count + 1) by comparing the low and high groups based on each skin measurement and questionnaire. The low group was used as the base for fold change and the Mann-Whitney U test was used to calculate *p*-values. (D) The mean fold change of 101 OR genes in each phenotype. Error bars represent SD. SD, standard deviation; GSVA, gene set variation analysis; OR, olfactory receptor.

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Predicted \ Actual	Cut-off = 3.7	Sensitive	Non-sensitive
	Sensitive	8 (0.727)	1 (0.100)
	Non-sensitive	3 (0.273)	9 (0.900)

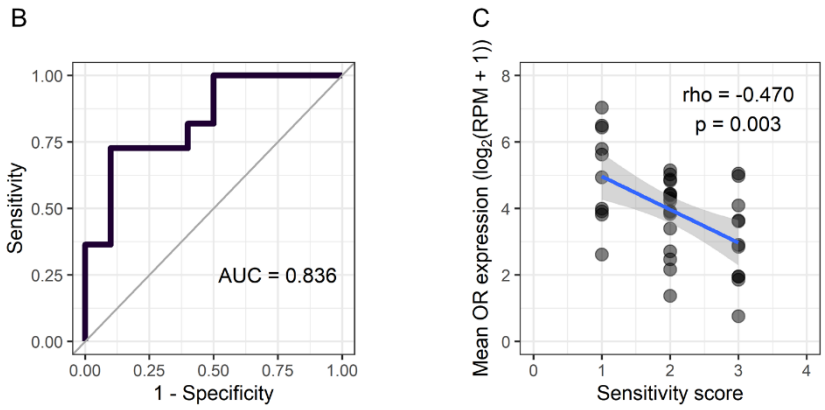

Figure 3. Discrimination of subjective sensitive skin using the mean expression level of OR genes in SSL-RNA samples.

(A) Confusion matrix of sensitive skin classification. Highest Youden's index = 0.63 at cut-off value = 3.7. (B) ROC curve and AUC of the discrimination model. (C) Regression model for the subjective sensitive skin score. Each plot represents each sample (n = 38), and linear regression with a 95% confidence interval is represented as a blue solid line with a gray band. RPM; Read per million counts; ROC, receiver operating characteristic; AUC, area under the curve.

Table S1. Correlation among facial skin evaluations.

rho (*p*-value)	Comedo	Freckles	Sensitivity	Roughness
Comedo	1	-0.546	0.529	0.635
	(0)	(0.013)	(0.014)	(0.002)
Freckles	-0.546	1	-0.596	-0.478
	(0.013)	(0)	(0.006)	(0.033)
Sensitivity	0.529	-0.596	1	0.713
	(0.014)	(0.006)	(0)	(< 0.001)
Roughness	0.635	-0.478	0.731	1
	(0.002)	(0.033)	(< 0.001)	(0)

The correlation matrix shows Spearman's correlation coefficients (rho) and *p*-values between comedo, freckles, sensitivity, and roughness.

Table S2. Gene sets for Th1, Th2, Th17, Th22/IL22, epidermal terminal differentiation, and lipids.

Th1-related	Th2-related	Th17-related	TH22/IL-22-related	Terminal Dif./Lipids-related		
IL12RB2	IL10	IL23R	FLG	GJB5	ANXA6	SCEL
IL1B	CCR4	CCL20	S100A9	GJB3	CDSN	ACOT2
STAT1	CCR5	CAMP	S100A8	ANXA9	PSORS1C2	DEGS2
CCR1	IL7R	CXCL1	S100A7	FLG	ELOVL5	CGNL1
CCR2	TSLP	CXCL3	S100P	LCE2D	FABP7	NR2F2
IL8	IL5	CXCL2	SERPINB1	LCE2B	AWAT1	CERS3
CXCL9	IL13	IL17A	AHR	LCE2C	CLN8	PPL
CXCL10	IL4	IL17F	CALML5	LCE2A	LPL	CDH11
CXCL11	IL9	CCR6	KRT1	LCE1F	FABP4	FA2H
IRF1	CCL26	LCN2	IL22	LCE1E	SPTLC1	CLDN7
IL12B	CCL24	IL23A	IL32	LCE1D	ELOVL3	ORMDL3
IFNGR1	IL33	STAT3	KRT10	LCE1C	GPAM	KRT23
IL2RA	STAT6	PI3	SERPINB4	LCE1B	FADS2	KRT33A
OASL	IL31			SOAT1	FADS1	KRT34
CCL5	IL4R			LPIN1	GAL	EVPL
CCL3	CCL22			CIDEC	DHCR7	CDH19
CCL4	CCL17			PPARG	DGAT2	SPTLC3
IFNGR2	CCL7			CLDN11	FAR2	ACER1
MX1	CCL13			CLDN1	KRT2	PNPLA3
	CCL18			ANXA5	KRT77	CLDN8
				HMGCS1	KRT79	

Each set is shown as corresponding to each column. Gene sets are from A.B. Pavel et al., 2021 (reference 21).

Table S3. Gene sets for OR genes.

OR10A2	OR13J1	OR2D2	OR4C12	OR51G2	OR5D13	OR6P1
OR10A3	OR14A16	OR2D3	OR4C13	OR51I1	OR5D14	OR6Q1
OR10A4	OR14C36	OR2F1	OR4C15	OR51I2	OR5D16	OR6S1
OR10A5	OR14I1	OR2F2	OR4C16	OR51L1	OR5D18	OR6T1
OR10A6	OR14J1	OR2G2	OR4C3	OR51M1	OR5E1P	OR6V1
OR10A7	OR1A1	OR2G3	OR4C46	OR51Q1	OR5F1	OR6W1P
OR10AD1	OR1A2	OR2G6	OR4C6	OR51S1	OR5H1	OR6X1
OR10AG1	OR1B1	OR2H1	OR4D1	OR51T1	OR5H14	OR6Y1
OR10C1	OR1C1	OR2H2	OR4D10	OR51V1	OR5H15	OR7A10
OR10G2	OR1D2	OR2J2	OR4D11	OR52A1	OR5H2	OR7A17
OR10G3	OR1D4	OR2J3	OR4D2	OR52A5	OR5H6	OR7A5
OR10G4	OR1D5	OR2K2	OR4D5	OR52B2	OR5I1	OR7C1
OR10G7	OR1E1	OR2L13	OR4D6	OR52B4	OR5J2	OR7C2
OR10G8	OR1E2	OR2L1P	OR4D9	OR52B6	OR5K1	OR7D4
OR10G9	OR1F1	OR2L2	OR4E2	OR52D1	OR5K2	OR7E24
OR10H1	OR1F2P	OR2L3	OR4F15	OR52E2	OR5K3	OR7E37P
OR10H2	OR1G1	OR2L8	OR4F17	OR52E4	OR5K4	OR7E5P
OR10H3	OR1I1	OR2M1P	OR4F21	OR52E6	OR5L1	OR7E91P
OR10H4	OR1J1	OR2M2	OR4F3	OR52E8	OR5L2	OR7G1
OR10H5	OR1J2	OR2M3	OR4F4	OR52H1	OR5M1	OR7G2
OR10J1	OR1J4	OR2M4	OR4F5	OR52I1	OR5M10	OR7G3
OR10J3	OR1K1	OR2M5	OR4F6	OR52I2	OR5M11	OR8A1
OR10J5	OR1L1	OR2M7	OR4K1	OR52J3	OR5M3	OR8B12
OR10K1	OR1L3	OR2S2	OR4K13	OR52K1	OR5M8	OR8B2
OR10K2	OR1L4	OR2T1	OR4K14	OR52K2	OR5M9	OR8B3
OR10P1	OR1L6	OR2T10	OR4K15	OR52L1	OR5P2	OR8B4
OR10Q1	OR1L8	OR2T11	OR4K17	OR52M1	OR5P3	OR8B8
OR10R2	OR1M1	OR2T12	OR4K2	OR52N1	OR5R1	OR8D1
OR10S1	OR1N1	OR2T2	OR4K5	OR52N2	OR5T1	OR8D2
OR10T2	OR1N2	OR2T27	OR4L1	OR52N4	OR5T2	OR8D4
OR10V1	OR1Q1	OR2T29	OR4M1	OR52N5	OR5T3	OR8G1
OR10W1	OR1S1	OR2T3	OR4M2	OR52R1	OR5V1	OR8G2
OR10X1	OR1S2	OR2T33	OR4N2	OR52W1	OR5W2	OR8G5

OR10Z1	OR2A1	OR2T34	OR4N3P	OR56A1	OR6A2	OR8H1
OR11A1	OR2A12	OR2T35	OR4N4	OR56A3	OR6B1	OR8H2
OR11G2	OR2A14	OR2T4	OR4N5	OR56A4	OR6B2	OR8H3
OR11H1	OR2A2	OR2T5	OR4P4	OR56A5	OR6B3	OR8I2
OR11H12	OR2A20P	OR2T6	OR4Q3	OR56B1	OR6C1	OR8J1
OR11H2	OR2A25	OR2T8	OR4S1	OR56B4	OR6C2	OR8J3
OR11H4	OR2A4	OR2V2	OR4S2	OR5A1	OR6C3	OR8K1
OR11H6	OR2A42	OR2W1	OR4X1	OR5A2	OR6C4	OR8K3
OR11L1	OR2A5	OR2W3	OR4X2	OR5AC2	OR6C6	OR8K5
OR12D2	OR2A7	OR2W5	OR51A2	OR5AK2	OR6C65	OR8S1
OR12D3	OR2A9P	OR2Y1	OR51A4	OR5AK4P	OR6C68	OR8U1
OR13A1	OR2AE1	OR2Z1	OR51A7	OR5AN1	OR6C70	OR9A2
OR13C2	OR2AG1	OR3A1	OR51B2	OR5AP2	OR6C74	OR9A4
OR13C3	OR2AG2	OR3A2	OR51B4	OR5AR1	OR6C75	OR9G1
OR13C4	OR2AK2	OR3A3	OR51B5	OR5AS1	OR6C76	OR9G4
OR13C5	OR2AT4	OR3A4P	OR51B6	OR5AU1	OR6F1	OR9I1
OR13C8	OR2B11	OR4A15	OR51D1	OR5B12	OR6K2	OR9K2
OR13C9	OR2B2	OR4A16	OR51E1	OR5B17	OR6K3	OR9Q1
OR13D1	OR2B3	OR4A47	OR51E2	OR5B2	OR6K6	OR9Q2
OR13F1	OR2B6	OR4A5	OR51F1	OR5B21	OR6M1	
OR13G1	OR2C1	OR4B1	OR51F2	OR5B3	OR6N1	
OR13H1	OR2C3	OR4C11	OR51G1	OR5C1	OR6N2	

A set of 382 OR genes which are detectable in AmpliSeq human transcriptome.

Table S4. Grouping analysis for the comparison of OR expression levels.

	Low (mean ± SD)	High (mean ± SD)
Sebum	11 (13.8 ± 11.4)	10 (58.9 ± 20.6)
Capacitance	11 (40.8 ± 7.7)	10 (56.2 ± 3.2)
Conductance	11 (111.1 ± 29.3)	10 (205.3 ± 37.8)
TEWL	11 (12.0 ± 1.1)	10 (17.8 ± 2.1)
Melanin	12 (132.0 ± 20.1)	9 (180.2 ± 20.9)
Erythema	11 (171.9 ± 29.5)	10 (240.1 ± 23.1)
pH	11 (6.4 ± 0.3)	10 (6.8 ± 0.1)
	Low (score1, score2)	High (score3, score4)
Oily_skin	6 (2, 4)	15 (11, 4)
Comedo	13 (4, 9)	8 (7, 1)
Dryness	7 (2, 5)	14 (6, 8)
Wrinkle	11 (8, 3)	10 (5, 5)
Sagging	10 (6, 4)	11 (6, 5)
Resiliency	10 (6, 4)	11 (7, 4)
Spots	6 (3, 3)	15 (5, 10)
Freckles	10 (4, 6)	10 (4, 6)
Dullness	10 (1, 9)	11 (2, 9)
Irregularity	8 (6, 2)	12 (5, 7)
Clarity	8 (2, 6)	13 (5, 8)
Brightness	9 (3, 6)	12 (4, 8)
Glow	8 (3, 5)	13 (8, 5)
Eye_bags	7 (1, 6)	14 (8, 6)
Pore	6 (2, 4)	15 (10, 5)
Texture	8 (2, 6)	13 (7, 6)
Redness	9 (5, 4)	12 (9, 3)
Sweat	11 (6, 5)	10 (7, 3)
Sensitivity	10 (10, 0)	11 (11, 0)
Roughness	18 (11, 7)	3 (2, 1)
Swelling	16 (9, 7)	5 (4, 1)

The composition of the low and high groups was used to compare the OR expression levels for each measurement and questionnaire item. SD, Standard deviation.

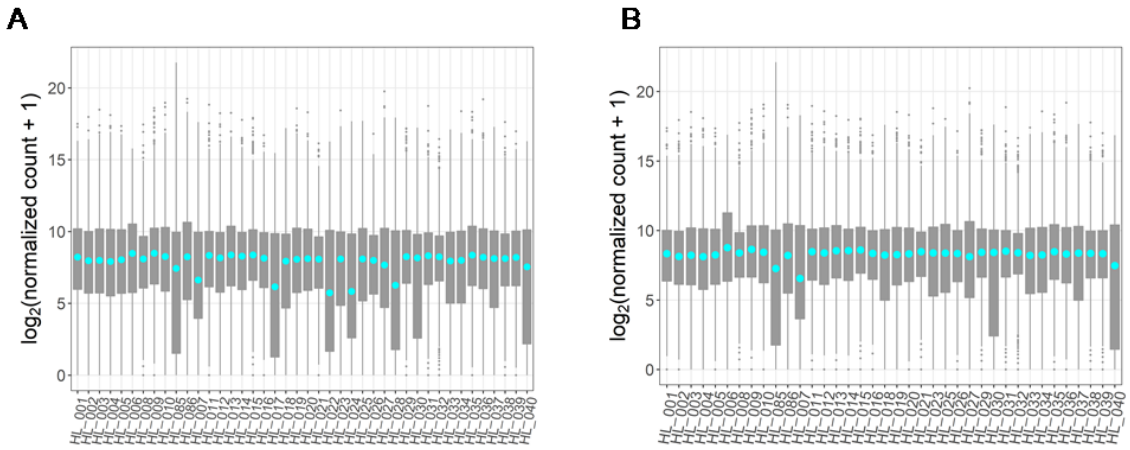

Figure S1. Effect of data cleaning and normalization on the distribution of read counts.

Boxplots of normalized read count (A) before (n = 42) and (B) after data cleaning (n = 38). Boxes represent the median ± interquartile range, and whiskers represent the 1.5-fold interquartile range. The median is shown as cyan dots.