ORIGINAL ARTICLE

Non-invasive transcriptomic analysis using mRNAs in skin surface lipids obtained from children with mild-to-moderate atopic dermatitis

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Abstract

Background Specimens for analysing the molecular pathology of skin disease are generally obtained through invasive methods, such as biopsy. However, less burdensome methods are desirable for paediatric patients. We recently established a method that comprehensively analyses RNA present in sebum (skin surface lipid–RNAs: SSL-RNAs) using a next-generation sequencer. Using this method, biological information can be obtained from the skin in a completely non-invasive manner.

Objectives To verify the applicability of the SSL-RNA method for analysis of paediatric skin and analyse the molecular pathology of mild-to-moderate atopic dermatitis (AD) in children.

Methods We collected sebum specimens from the whole faces of 23 healthy children and 16 children with mild-tomoderate AD (eczema area and severity index (EASI) score: 5.9 ± 2.6) ranging in age from 6 months to 5 years, using an oil-blotting film. We then extracted SSL-RNAs from the samples and performed an AmpliSeg transcriptomic analysis.

Results The expressions of genes related to keratinization (*LCE*, *PSORS1C2*, *IVL* and *KRT17*), triglyceride synthesis and storage (*PLIN2*, *DGAT2* and *CIDEA*), wax synthesis (*FAR2*), ceramide synthesis (*GBA2*, *SMPD3* and *SPTLC3*), antimicrobial peptides (*DEFB1*) and intercellular adhesion (*CDSN*), all of which are related to the skin barrier, are lower in children with AD than in healthy children. The children with AD also have higher expression of *CCL17*, a Th2-cytokine and an increased Th2-immune response as demonstrated by a gene set variation analysis. Moreover, *KRT17* and *CCL17* expression levels are significantly correlated with the EASI score.

Conclusions Molecular changes associated with abnormal immune responses and the epidermal barrier in children with mild-to-moderate AD can be determined using the SSL-RNA method. This non-invasive method could therefore be a useful means for understanding the molecular pathology of paediatric AD.

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Conflict of interest

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Introduction

Atopic dermatitis (AD) is an inflammatory skin disease that develops in early infancy. The incidence of paediatric AD is as high as 20% worldwide.¹ AD is characterized by pruritic eczema with repeated exacerbations and remissions and is greatly influenced by diet and environment.^{2,3} AD may also cause sleep disorders and affect growth and development.⁴⁻⁶ In addition, AD is associated with subsequent allergic march.⁷⁻¹⁰ Recently, it has been reported that early aggressive application of topical corticosteroids to shorten the duration of eczema in infants with AD is associated with a decrease in the subsequent development of food allergies.¹¹ Therefore, AD detection and appropriate therapeutic interventions in the early stage are important. It is equally important to accurately evaluate and understand the molecular pathology of AD to diagnose AD appropriately in the early stage and generate an effective treatment plan.

The methods for collecting specimens to analyse the molecular pathology of the skin include punch biopsy and tape stripping of the stratum corneum.¹²⁻¹⁶ Based on analysis of moderate-to-severe AD patients using specimens obtained by such methods, it has been reported that AD is characterized by increased Th2, Th17 and Th22 immune responses, as well as decreased skin barrier function.¹²⁻¹⁶ However, even the minimally invasive tape-stripping procedure can pose a certain degree of invasiveness and risk of skin damage (i.e. barrier disruption, epidermal proliferation and immune response),¹⁷ depending on the number of stratum corneum required. Although most children with AD have mild disease,¹⁸ little is known regarding the underlying molecular pathology. Thus, it is desirable to further expand and improve the knowledge of mild AD in children. However, collecting specimens from children with AD using invasive methods is difficult, and has served as a major hindrance to advancing the current understanding of paediatric AD pathology.

Previously, we discovered that analysable human mRNA is present in the sebum on the skin surface, and we designated it as skin surface lipid-RNAs (SSL-RNAs). Specifically, we collected sebum samples non-invasively using an oil-blotting film and established a method for comprehensively analysing SSL-RNAs by improving the AmpliSeq transcriptomic analysis method.¹⁹ We confirmed that SSL-RNAs mainly constitute RNA derived from the sebaceous glands, epidermis and hair follicles, and can be used for collecting gene expression data, particularly in the upper part of the skin. Moreover, we collected samples from adult AD patients and healthy adults using this method, through which we detected the pathological features of AD, i.e. increased immune responses and decreased barrier function, via comparative analysis. Thus, considering that this new method can be performed in a non-invasive manner, it can be readily applied for children with immature skin structure and function.^{20–23}

In the present study, we further verified the applicability of the SSL-RNA method for the analysis of paediatric skin by comparing SSL-RNA profiles between children with AD showing mild-to-moderate AD and healthy children. Furthermore, the molecular pathology of paediatric AD was analysed.

Materials and methods

Participants

Sixty-seven children (age 6 months to 5 years) delivered at full term with a birthweight > 2500 g in Tokyo, Japan, were enrolled in the study between June 2019 and July 2019. The participants were selected by dermatologists. Twenty-three children with no atopic predisposition who did not have eczema on any part of the body were selected as healthy children, and 16 children with AD who had eczema due to AD on the body were selected as children with AD. Children who had not used systemic or topical medicine (systemic medicine: immunomodulators, antimicrobials and antihistamines; topical medicine: corticosteroids, calcineurin inhibitors and crisaborole) since having a bath on the previous day were selected as study participants. The use of moisturizers (heparinoids, white petrolatum and commercially available moisturizers) was allowed. The disease severity of the children with AD was evaluated using the eczema area and severity index (EASI).

Ethics statement

The present study was approved by the Ethics Committee of Kao Corporation (approval number: T216-190415) and conducted with the written informed consent of the parents of the participants.

Collection of SSLs, RNA preparation and AmpliSeq transcriptomic analysis

The collection of SSLs and purification of SSL-RNAs were performed according to previous reports with slight modifications.^{19,24} In brief, sebum specimens were collected from whole faces of the study participants by wiping the skin surface for <1 min using a single sheet of oil-blotting film (5.0 × 8.0 cm; 3 M Japan, Tokyo, Japan) per participant. The film was then cut into small pieces and RNA was extracted using QIAzol reagent (QIAGEN, Hilden, Germany), followed by purification using the RNeasy Mini kit (QIAGEN). The obtained RNA was dissolved in 10 µL of nuclease-free water. Sequence library preparation, template preparation and sequencing were performed using the Ion AmpliSeq Transcriptome Human Gene Expression Kit, Ion Chef System and Ion S5 XL System (Thermo Fisher Scientific, Waltham, MA, USA), respectively, by following our previously described modified protocols.^{19,24}

Sample quality control, data analysis and statistics

Data analyses were performed according to previous reports.^{19,24} The normalization and statistical analysis of the AmpliSeq transcriptomic data were performed using R language. Read count data were obtained using the Ion Torrent Suite Software (Thermo Fisher Scientific). The R DESeq2 package (Bioconductor)²⁵ was used for the normalization and quality check of the read count data. Samples in which the proportion of genes detected among the 20 802 target genes analysed in the Ion AmpliSeq Transcriptome Human Gene Expression kit was <20% were excluded from analysis. Only genes for which one or more reads were detected in ≥90% of the samples were normalized by the size factor. Principal component analysis (PCA) was performed using genes with the top 500 largest variances after adjustment by variance-stabilizing transformation. Differentially expressed genes (DEGs) between the healthy children and those with AD were analysed using the likelihood ratio test, and those with a false discovery rate (FDR) were adjusted by the Benjamini-Hochberg method below the threshold value of 0.05. For the gene ontology (GO) analysis of genes obtained by differential expression analysis, the Database for Annotation, Visualisation and Integrated Discovery^{26,27} (DAVID v6.8) was used. To evaluate the balance among T helper cells, gene set variation analysis (GSVA) was performed using various T helper cell-related gene sets reported previously.^{15,28,29} Enrichment scores for each gene set in healthy children and those with AD were calculated. Comparisons between the healthy children and the children with AD were performed using the Student's t-tests. To account for multiplicity, the FDR adjusted by the Benjamini-Hochberg method was calculated. Spearman's correlation analysis was performed to assess the relationship between EASI score and gene expression levels, using normalized expression levels.

Results

Study participants

Twenty-three healthy children and sixteen children with AD were enrolled in this study (Table 1). No differences in age or sex were observed between the healthy children and those with AD (age: P = 0.74, Mann–Whitney U test; sex: P = 0.69, Fisher's

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	Healthy (<i>n</i> = 23)	Atopic dermatitis (n = 16)
Age (months)		
$\text{Mean}\pm\text{SD}$	$\textbf{21.9} \pm \textbf{14.4}$	$\textbf{21.9} \pm \textbf{18.0}$
Median (range)	18.5 (7–69)	16.5 (6–63)
Sex		
Female	10	8
Male	13	8
Clinical severity score		
EASI		
$\text{Mean} \pm \text{SD}$	0	5.9 ± 2.6
Median (range)	0	5.75 (3.1–13.2)

EASI, eczema area and severity index.

exact test). Based on the EASI scores, 13 children with AD had mild disease and 3 had moderate disease. The mean EASI score of the 16 children with AD was 5.9 ± 2.6 , indicating that the study population mainly consisted of children with mild AD.

Comparison of SSL-RNA profiles between healthy children and children with AD

As in the case of healthy children, sebum specimens were collected from whole faces of children with AD, who showed localized eczema lesions, and were used for analysis. The library from 1 of the 23 children did not meet the concentration criterion for sequencing^{19,24} and was not sequenced. The mean proportion of the genes detected in the 22 healthy children was 36%. Samples that did not meet the criterion for gene detection^{19,24}, which was 20%, were excluded from the analysis resulting in the samples from 20 healthy children being included in subsequent normalization and data analysis. The mean proportion of the genes detected in the 16 children with AD was 46%. All samples met the sample quality control criterion and were used for subsequent normalization and data analysis.

Transcriptomic analysis of SSL-RNAs was performed, and 3217 genes that met the sample quality control criterion were selected for further analysis. PCA of genes with the top 500 largest variances confirmed relative separation between the samples collected from healthy children and those with AD (Fig. 1a). Differential expression analysis between the healthy children and the children with AD revealed 15 upregulated genes and 247 downregulated genes in the children with AD (Fig. 1b). GO analysis of the biological functions assigned to these genes using DAVID showed that among the 247 downregulated genes in the children with AD, 'keratinization', 'keratinocyte differentiation' and 'lipid metabolic process' were significantly enriched (Fig. 1c). Among the DEGs, late cornified envelope genes (LCE1B and LCE2A) and genes encoding psoriasis susceptibility 1 candidate 2 (PSORS1C2), involucrin (IVL) and keratin 17 (KRT17), which are involved in keratinization,^{14,30,31} defension beta 1 (DEFB1), which is an antimicrobial peptide,³² and corneodesmosin (CDSN),^{33,34} which is involved in intercellular adhesion, were downregulated in the children with AD (Fig. 2). Additionally, perilipin 2 (PLIN2),35 diacylglycerol Oacyltransferase 2 (DGAT2)³⁶ and cell death-inducing DFFA-like effector A (CIDEA),³⁷ which are involved in triglyceride synthesis or storage, fatty acyl-CoA reductase 2 (FAR2),³⁸ which is involved in wax synthesis, as well as glucosylceramidase beta 2 (GBA2),³⁹ sphingomyelin phosphodiesterase 3 (SMPD3),³⁹ serine palmitoyl transferase long-chain base subunit 3 (SPTLC3)³⁹ and abhydrolase domain containing 5 (ABHD5),⁴⁰ which are involved in ceramide synthesis, were also downregulated in the children with AD (Fig. 3).

In contrast, for the 15 upregulated genes in the children with AD, no significant GO terms were detected. Nevertheless, genes encoding thymus and activation-regulated chemokine (*TARC*/

(a)

10 PC-2(10.5%) Log₁₀(FDR) 2 🔴 HL AD -20 20 Ó Ż 4 -2 Log₂(Fold Change) PC-1(31.5%) (c) keratinization(GO:0031424) pentide cross-linking(GO:0018149) keratinocyte differentiation(GO:0030216) lipid metabolic process(GO:0006629) mitophagy(GO:0000422) 5.0 0.0 4 0 1 0 2.0 3.0 FDR(-Log10)

(b)

Figure 1 Characterization of SSL-RNAs profiles in healthy children and children with AD. (a) A principal component analysis based on transcriptional data of healthy children (n = 20) and children with AD (n = 16). (b) Volcano plot of differentially expressed genes between healthy children (n = 20) and children with AD (n = 16). Upregulated genes in children with AD show red spots and downregulated genes in children with AD show blue spots. (Benjamini and Hochberg's false discovery rate (FDR) < 0.05). (c) GO terms of biological process (DAVID, category: GOTERM_BP_DIRECT) in downregulated genes (FDR <0.05). HL, healthy; AD, atopic dermatitis.

CCL17) – a member of the Th2 chemokine family known as a blood biomarker for AD –, were upregulated in children with $AD^{41,42}$ (Fig. 2).

Helper T-cell responses in the children with AD

To analyse abnormal immune balance among T helper cells, enrichment scores were calculated through GSVA using T helper cell gene sets that were used in previous reports^{15,28,29} (Fig. 4). On one hand, the enrichment score for Th2-related genes was significantly higher in the children with AD than in the healthy children. On the other hand, no significant differences were observed in the Th1-, Th17- or Th22-related genes between the two groups.

Genes correlated with EASI score

To identify genes impacted by disease severity, we calculated Spearman's correlation coefficients and found that the expression of *KRT17* and *CCL17* was significantly correlated with EASI score in mild-to-moderate AD (Fig. 5).

Discussion

In the present study, we performed a molecular pathology analysis of children with mild-to-moderate AD using a comprehensive analysis method of the RNA in SSLs that could be collected non-invasively. This investigation showed that the SSL-RNA method is applicable to children. In the children with AD, the expression of Th2-type immune response-related genes was higher, while that of keratinization-, lipid synthesis/ metabolism- and antimicrobial peptide-related genes was lower. Moreover, the expression levels of certain genes were found to be correlated with EASI score. These findings indicate that the SSL-RNA method is a useful method for understanding the molecular pathology of paediatric AD.

In paediatric patients with moderate-to-severe AD, increased Th17- and Th22-type, as well as Th2-type, immune responses have been reported.^{12–15} In the present study, the score for Th2related genes following GSVA was higher, and the expression of CCL17 was significantly higher in children with AD compared to healthy children, suggesting an increased Th2-type immune response. However, there were few immune-related genes that showed significant differences in expression in the children with AD compared with that in the healthy children, and the GSVA analysis results did not indicate increased Th17- or Th22-type immune responses. Several factors are responsible for these discrepancies: First, the subjects in the present study were children with mild-to-moderate AD whose disease severity was lower than that reported in previous studies. Therefore, in children with mild-to-moderate AD, Th2-type immune responses may dominate or increase earlier than Th17- and Th22-type immune responses. Second, the specimens in the present study differed from those used in the previous reports. In previous studies, specimens were obtained by punch biopsy or tape stripping of the stratum corneum, mainly from the lesional site in the extremities, while we collected specimens from the whole face. The fact that the whole faces had both lesional and non-lesional sites may have influenced the results. SSL-RNAs mainly contain information derived from sebaceous glands, epidermis and hair follicles.¹⁹ Therefore, the source/structure of the information output of SSL-RNAs is not identical to that of the stratum corneum obtained by tape stripping or biopsy samples containing blood and all layers of the skin. This may have influenced our results.

In terms of AD pathology, impairment of skin barrier function is as important as the immune/inflammatory responses.^{12,43} The epidermal barrier consists of the tight junction barrier, comprising intercellular adhesion, and the stratum corneum barrier based on sebum/intercellular lipids, which are formed during epidermal keratinization. The SSL-RNA analysis broadly captured the differential expression of genes related to the stratum corneum barrier, including genes involved in triglyceride and ceramide synthesis, which is more prominent than increased immune responses. This may be related to the characteristics of the SSL-RNA method. That is, since SSLs mainly contain RNA derived from sebaceous glands and the epidermis,¹⁹ it is presumed that the expression of genes related to sebum and stratum



Figure 2 Differentially expressed genes related to keratinization, antimicrobial peptide, cell adhesion and Th2 immune response between healthy children and children with AD. Boxes represent median \pm interquartile range (IQR), and whiskers represent 1st and 3rd quartile 1.5 * IQR. FDR values (Benjamini–Hochberg adjusted *P*-values) were calculated using the likelihood ratio test between healthy children (*n* = 20) and children with AD (*n* = 16). *FDR <0.05, **FDR <0.01. HL, healthy; AD, atopic dermatitis; FDR, false discovery rate.

corneum lipids was particularly easy to detect. This also suggests the importance of the stratum corneum barrier function in the development and progression of AD in children.

The SSL-RNA analysis captured abnormal lipid synthesis/ metabolism in children with mild-to-moderate AD. The downregulation of genes involved in the synthesis and accumulation of triglycerides and wax ester (*PLIN2*,³⁵ *DGAT2*,³⁶ *CIDEA*³⁷ and *FAR2*³⁸) were observed in children with AD, suggesting that the amount and composition of sebum may differ from those in healthy children. Similar differential expression of these genes was detected by SSL-RNA analysis of adult AD.¹⁹ Considering that in adult patients with AD, a decrease in the amount of sebum^{44,45} and changes in the composition of sebum⁴⁶ have been reported, it is conceivable that the differential expression of these genes detected in the SSL-RNA analysis suggests changes in the sebum of children with mild-to-moderate AD at the molecular level.

Epidermal ceramides also play an important role in epidermal barrier function, and the amount and composition of epidermal

ceramides are greatly altered in patients with AD resulting in abnormal barrier function.^{47,48} Among the ceramide-related genes downregulated in children with AD, *ABHD*^{40,49} and *DGAT2*⁵⁰ are involved in the synthesis of acylceramides, which are ceramide species important for epidermal barrier function,⁵¹ indicating changes in ceramide composition and abnormal barrier function in children with mild-to-moderate AD. Indeed, in paediatric patients with AD, quantitative and qualitative changes in epidermal ceramides have been reported.⁴⁷ The abnormal ceramide synthesis/metabolism suggested by SSL-RNA analysis confirms the changes in ceramide levels in children with AD at the gene expression level and supports the importance of ceramide care for children with AD.^{52,53}

The fact that the expression of keratinization-related genes (*LCE* family, *IVL*, *PSORS1C2* and *KRT17*) and *CDSN* was lower in the children with AD suggests abnormal epidermal differentiation, abnormal formation of the cornified envelope and decreased intercellular adhesiveness. These findings are mostly consistent with those of previous studies in paediatric patients



Figure 3 Differentially expressed genes related to lipid synthesis between healthy children and children with AD. Boxes represent median \pm interquartile range (IQR), and whiskers represent 1st and 3rd quartile 1.5 * IQR. FDR values (Benjamini–Hochberg adjusted *P*-values) were calculated using the likelihood ratio test between healthy children (n = 20) and children with AD (n = 16). *FDR <0.05, **FDR <0.01. HL, healthy; AD, atopic dermatitis; FDR, false discovery rate.

with moderate-to-severe AD or adult patients with mild AD.^{12–15,54} Since the expression levels of these genes correlate with transepidermal water loss, an indicator of skin barrier

function,¹⁵ we presume that children with mild-to-moderate AD have similar structural weakening of the stratum corneum and decreased barrier function.



Figure 4 Mean enrichment scores of T helper cells in healthy children and children with AD. Each dot represents the GSVA enrichment score in each sample. Solid lines represent the mean \pm SD. FDR values (Benjamini–Hochberg adjusted *P*-values) were calculated using the Student's *t*-tests between healthy children (n = 20) and children with AD (n = 16). ** FDR <0.01. HL, healthy; AD, atopic dermatitis; FDR, false discovery rate.



Figure 5 Scatter plots of EASI score and gene expression among HL children and children with AD. Spearman correlation coefficient was calculated by using EASI score of children with AD and gene expression level of *KRT17* and *CCL17*. Approximate line was drawn using data of children with AD. HL (Healthy children; n = 20) and children with AD (atopic dermatitis; n = 16).

Defensin beta 1 is a regulatory factor of skin bacterial flora produced by epidermal cells^{32,55} and is known to have antimicrobial activity against *Staphylococcus aureus*, a species of bacteria on the skin surface involved in the development of AD.⁵⁶ The expression of *DEFB1* is lower in patients with atopic dermatitis⁵⁷ and is thought to be associated with susceptibility to bacteria and viruses in patients with AD.⁵⁷ The observation that the expression of *DEFB1* is already decreased in the children with mild AD suggests the possible involvement of changes in bacterial flora due to a weakened antimicrobial barrier in the pathophysiology of AD at the early stage in children.

The most prominent characteristic of the SSL-RNA method is the simple and non-invasive collection of specimens. Furthermore, specimens can be collected from a varied group of subjects (irrespective of age or disease severity), including neonates and children with eczema, and from various skin regions.¹⁹ This method also allows facile repeated collection of specimens. Therefore, this analysis method could be particularly useful for continuous molecular monitoring of skin diseases in children from the early stages of disease. However, local sampling is required for gene expression analysis in the local skin, hence, the application range of this method might be limited compared with that using tape-stripped stratum corneum.¹⁴ To further clarify the usefulness of the SSL-RNA method, it is necessary to verify the possibility of application to local skin analysis. Our future work will, therefore, focus on the collection and analysis of SSLs from both non-lesional and lesional skin areas to allow for further elucidation of the characteristics of children with mild AD.

In conclusion, we applied the SSL-RNA method to children with mild-to-moderate AD and obtained findings that support the pathophysiology of AD at the molecular level. This SSL-RNA method may be a promising means for understanding the pathophysiology of paediatric skin diseases.

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The participants in this manuscript have given written informed consent to publication.

Data availability statement

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

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