Site-Specific Microarray Evaluation of Spontaneous Dermatitis in Flaky Tail Mice


TO THE EDITOR

Flaky tail mice (FTM) with mutations in the Flg and Tmem79 genes are known to spontaneously develop atopic dermatitis (AD)-like dermatitis with age. We have already reported that the spontaneous development of dermatitis exhibits site specificity; for example, the face, ears, and neck of old FTM develop severe dermatitis spontaneously, whereas the dorsal flank skin remains normal (Sakai et al., 2015).

Although some studies have revealed the importance of IL-1, thymic stromal...
lymphopoietin, and IL-17 for the development of dermatitis in FTM (Ewald et al., 2017; Kezic et al., 2012; Moniaga et al., 2013), the site specificity seen in FTM was not a focus of the studies, and the detailed mechanisms of site-specific emergence of dermatitis remain unclear. Here, we analyzed nonlesional and lesional skin in FTM using a microarray to investigate the pathogenesis of site-specific dermatitis.

Among the four sites—flanks of young FTM, necks of young FTM, flanks of old FTM, and necks of old FTM—visible dermatitis developed only on the necks of old FTM, as evaluated clinically and histologically. Increases in transepidermal water loss and stratum corneum pH and decreases in stratum corneum hydration were observed in the necks of old FTM compared with the flanks of old FTM, whereas transepidermal water loss in the necks of young FTM did not increase (Supplementary Figure S1). Whole skin from the flanks and necks of old FTM were microarrayed, and we carried out clustering analysis to visualize the differences in gene expression. In the heatmap of all genes, the difference between nonlesional and lesional skin was not clear, because the expression pattern of each mouse varied (Figure 1). Next, we created heatmaps in which the predominant genes related to AD were shown along with P-values and expression ratios (neck/flank) (Figure 2). The upregulation of TARC coincided with the emergence of dermatitis. It has been shown previously that the expression of T helper type 2 cytokines such as IL-4, -5, and -13 is very low in FTM, and that IL-17A is upregulated in lesional skin (Ewald et al., 2017). Interestingly, the expression of FLG was significantly upregulated, whereas the expression of FLG-2 was downregulated in the necks of old FTM (Figure 2). These results were also confirmed through reverse transcriptase-PCR (Supplementary Figure S2). Flg2 is located next to Flg on the chromosome, and the FLG-2 protein is known as an S100 fused-type protein in the epidermal differentiation complex. A recent study showed that FLG-2 is responsible for part of skin barrier function in addition to FLG (Pendaries et al., 2015), and that mutations in FLG2 are involved in the development of human AD (Margolis et al., 2014). The expression of both genes is known to be downregulated ordinarily by cutaneous inflammation, such as eczematous lesions, or by the effects of some cytokines, including T helper type 2 and T helper type 17, independent of mutation status (Broccardo et al., 2011; Gutowska-Owsiak et al., 2012; Howell et al., 2008), in contrast with the results of FLG in this study. On the other hand, a similar expression pattern of FLG and FLG-2 to that observed in our results was found in the dermatitis related to abnormal lipid metabolism induced by an essential fatty acid-deficient diet (Hansmann et al., 2012). Therefore, although the detailed mechanisms remain obscure, we speculate that abnormal lipid metabolism might be deeply involved in the site-specific emergence or maintenance of dermatitis on the necks of old FTM. This is supported by Kyoto Encyclopedia of Genes and Genomes pathway analysis using DAVID to investigate the pathogenic differences between the flank and neck, which found that some pathways involved in lipid metabolism had the highest statistical difference in old FTM (Supplementary Figure S3a), suggesting that the abnormality of lipid metabolism might be involved in the emergence or maintenance of the site-specific dermatitis on the necks of old FTM. This supports the previous indications that lipid metabolism might be deeply involved in the site-specific dermatitis on the necks of old FTM. In addition, alterations in lipid organization were also reported to lead to the upregulation of involucrin (Ekanayake-Mudiyanseelage et al., 1998), which was therefore also upregulated in the necks of old FTM (Figure 2).

These findings could be useful for exploring therapeutic targets. In particular, it is notable that the peroxisome proliferator-activated receptor (PPAR) signaling pathway, which is also related to lipid metabolism, was detected as a second pathway in the Kyoto Encyclopedia of Genes and Genomes pathway analysis of old FTM.

**Figure 1. Heatmap of differential expression of all genes in the flank and neck of old FTM.** Gene expression in the nonlesional flank and lesional neck of old FTM were measured through microarray, and a heatmap was generated as described in Supplementary Materials and Methods. The intensities of all genes assessed in this study are accessible through the Gene Expression Omnibus Series, Accession Number GSE124434 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE). The difference between the flank and the neck is not clear, because the expression pattern in each mouse varies. FTM, flaky tail mice.
In that pathway, the expression of several genes, including PPAR-\(\alpha\), was downregulated (Supplementary Figures S3b, S4, and S5). Accordingly, a decrease in PPAR-\(\alpha\) expression has been detected in AD, and PPAR-\(\alpha\)-deficient mice exhibited more severe hapten-induced AD-like dermatitis than wild-type mice (Staumont-Salle` et al., 2008). In addition, some studies have shown that agonists of PPAR-\(\alpha\) can normalize skin lipid ratio and improve cutaneous inflammation (Hatano et al., 2010; Wallmeyer et al., 2015), suggesting that a decrease in PPAR-\(\alpha\) might be a promising therapeutic target in the treatment of AD. Meanwhile, the expression pattern of PPARs, that is, downregulation of PPAR-\(\alpha\) and PPAR-\(\gamma\), but not of PPAR-\(\beta/\delta\), in the necks of old FTM seems to be consistent with what would be induced by permeability barrier abrogation in mice (Adachi et al., 2013); this suggests that permeability barrier abnormalities, possibly related to abnormal lipid metabolism, might be a key mechanism in the emergence or maintenance of site-specific dermatitis in the necks of old FTM.

This study is only an analysis of gene expressions, so further studies are required for proving detailed mechanisms, although site-specific evaluation using microarrays in individuals with the same genetic background could provide new insight into the pathogenesis and therapeutic targets of several skin diseases showing site specificity. Other limitations of the present study are that we used FTM with mutations in Flg and Tmem79 and analyzed the skin of only old FTM after dermatitis fully completed. The gene mutations in FTM may affect the results of the microarray. The process of dermatitis formation could not be focused in this study. In practice, it would be difficult to focus on the precise state of pre-onset dermatitis in vivo. In addition, dermatitis on the necks of old FTM never improves in lifetime spontaneously, and the dorsal flank never developed dermatitis in our laboratory observations.

Animals
Female FTM (Jackson Laboratory, Bar Harbor, ME) were used at 4 weeks old and 33–37 weeks old. Whole skin including dermis and fat of flank and neck in each mouse were sampled. All experiments with mice were approved by the Ethics of Animal Experimentation Committee of Oita University.

Data availability statement
Datasets related to this article can be found at https://doi.org/10.17632/v5th4rr68j.1, hosted at Mendeley Data (Sakai, 2019). The microarray data are accessible through the Gene Expression Omnibus Series, Accession Number GSE124434 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: TS; Data Curation: TS; Formal Analysis: TS; Investigation: TS, CA, YM, TY, HMH; Project Administration: TS; Supervision: YH; Validation: TS; Visualization: TS; Writing - Original Draft Preparation: TS; Writing - Review and Editing: YH

Supplementary Material

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.04.024.

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Physiological assessments
Transepidermal water loss, stratum corneum hydration, and stratum corneum surface pH were measured with the Tewameter TM300 (Courage & Khazaka, Cologne, Germany), the Corneometer CM825 (Courage & Khazaka), and a skin pH meter (PH 905; Courage & Khazaka), respectively, as described previously (Sakai et al., 2014).

Quantitative assessment of skin morphology
Skin samples from flank or neck were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4-μm sections were stained with hematoxylin and eosin. The thickness of the epidermis was measured with the scaled ocular lens of a light microscope.

Total RNA isolation
The total RNA was isolated from whole skin of each individual animal using TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified using SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer’s instructions. RNA samples were quantified by an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the quality was confirmed with a Experion System (Bio-Rad Laboratories, Hercules, CA).

Gene expression microarrays
The cRNA was amplified, labeled using a Low Input Quick Amp Labeling Kit, and hybridized to a SurePrint G3 Mouse Gene Expression Microarray 8×60K v2 according to the manufacturer’s instructions (Agilent, Santa Clara, CA). All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software, version 9.5.1.1. We generated a heat map using MeV software (Saeed et al., 2003) and a hierarchical clustering method to sort the genes. The intensities of all genes in this study are accessible through the Gene Expression Omnibus Series, Accession Number GSE124434 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE).

Data analysis and filter criteria
The raw signal intensities of samples from old FTM and Flags for each probe were calculated from hybridization intensities (gProcessedSignal), and spot information (glsSaturated, etc.), according to the procedures recommended by Agilent. Additionally, the raw signal intensities of all samples were normalized by quantile algorithm with ‘preprocessCore’ library package [P] on Bioconductor software [B]. Probes that call ‘P’ flag in at least one sample were selected, excluding lincRNA probes. We then applied Linear Models for Microarray Analysis (limma) package (Smyth et al., 2005) of Bioconductor software, and obtained 3065 genes. The criteria were that limma p-value < 0.05 and absolute log-fold-change (logFC) > 1 between dermatitis (+) and (-) samples. Total RNA isolation, microarray, and data analysis were performed in Cell Innovator, Co, Ltd, Japan.

Pathway analysis
Genes with significantly different amounts of expression that met the criteria described previously were imported into the DAVID v6.8 annotation tool (https://david.ncifcrf.gov), and Kyoto Encyclopedia of Genes and Genomes pathway analysis was carried out. The detected pathways and P-values are shown in Supplementary Figure S3. The gene map in the peroxisome proliferator-activated receptor signaling pathway was generated with KEGG Mapper (https://www.kegg.jp/kegg/tool/map_pathway1.html).

Real-time PCR
Reverse transcription of total RNA was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. Complementary DNA products were amplified on a LightCycler 480 System (Roche Diagnostics GmbH), as described previously (Sakai et al., 2014). Product specificity was evaluated by melting curve analysis, and relative gene expression was calculated by comparative Ct method. Relative mRNA expression levels were normalized with the housekeeping gene β-actin. The mouse primers used for real-time PCR are shown in Supplementary Table S1.

Statistical analysis
Physiological assessments and results of reverse transcriptase—PCR were analyzed by two-tailed Student’s t-test or Welch’s t-test and the results presented as means ± standard error of the mean of the number of experiments. A P-value < 0.05 was considered evidence of statistical significance.

SUPPLEMENTARY REFERENCES
Supplementary Figure S1. Clinicopathological and physiological findings of FTM. Apparent dermatitis developed only on the necks of old FTM among the four sites; flanks of young FTM, necks of young FTM, flanks of old FTM, and necks of old FTM were evaluated (a) clinically and (b) histologically. Increases in TEWL and SC pH and decreases in SC hydration were observed in the necks of old FTM compared with the flanks of old FTM, whereas (c) TEWL in the necks of young FTM did not increase. FTM, flaky tail mice; NS, not significant; SC, stratum corneum; TEWL, transepidermal water loss.

Supplementary Figure S2. Gene expression of Flg and Flg2. Expression of each gene was measured by (a) microarray or (b, c) RT-PCR. Analysis by both microarray and RT-PCR shows that the expression of Flg is significantly upregulated, whereas the expression of Flg2 is downregulated in the necks of old FTM. FTM, flaky tail mice; RT-PCR, reverse transcriptase–PCR.
Supplementary Figure S3. Results of KEGG pathway analysis with DAVID. KEGG pathway analysis was performed with DAVID as shown in Supplementary Materials and Methods. Some of the most statistically significant differences in old FTM were pathways involved in lipid metabolisms such as metabolic pathways and the PPAR signaling pathway. (a) The results of top 30 are shown. (b) Differences in gene expression in the neck compared with the flank of old FTM in the KEGG PPAR signaling pathway are shown (the blue panel indicates downregulated genes and the red panel indicates upregulated genes). Several genes in the pathway, including PPAR-α were downregulated. FTM, flaky tail mice; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPAR, peroxisome proliferator-activated receptor.
Supplementary Figure S4. Heatmap with the genes in KEGG PPAR signaling pathway. The expression ratios (neck/flank) and \( P \)-values were calculated as shown in Supplementary Materials and Methods. The results were ordered by neck/flank ratio. Several genes in the pathway were downregulated on the necks of old FTM. FTM, flaky tail mice; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPAR, peroxisome proliferator-activated receptor.
Supplementary Figure S5. Gene expressions in KEGG PPAR signaling pathway. Some gene expressions in KEGG PPAR signaling pathway were confirmed by RT-PCR. PPAR-α and PPAR-γ, but not PPAR-δ, were downregulated in the necks of old FTM. Several other genes were also downregulated. $N = 12$. FTM, flaky tail mice; KEGG, Kyoto Encyclopedia of Genes and Genomes; NS, not significant; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase–PCR.

Supplementary Table S1. Sequences of PCR Primers

<table>
<thead>
<tr>
<th>Mouse Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Flg</td>
<td>GAACCTCTGGAAGGACAACCTACA</td>
<td>TGTCATCGTGTCACAGATCAC</td>
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<td>Flg2</td>
<td>CTATCTCATTCTTCTTTGTTGA</td>
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<tr>
<td>Ppara</td>
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<td>AGGCCCTTCCACGACATAC</td>
</tr>
<tr>
<td>Ppard</td>
<td>ATGAAGACAAACCCACGGTAAAG</td>
<td>TCCTGTGCTGGTCGTTGAC</td>
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<tr>
<td>Pparg</td>
<td>GAAGCTCAAGAAACCAAGTAGTAC</td>
<td>TCACTGCTTCTTGTCATGAC</td>
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<tr>
<td>Fabp4</td>
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<td>CACTTCACACTCTTTCATGAC</td>
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<tr>
<td>Scd3</td>
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<td>GGGGCACCCACTCTCATAC</td>
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<tr>
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</tr>
<tr>
<td>Actb</td>
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