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Effect of Tacrolimus on Skin Microbiome in Atopic Dermatitis

A Short running head: Tacrolimus and atopic dermatitis skin microbiome

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Abstract

**Background:** Atopic dermatitis (AD) is a common allergic skin disease in which genetic and environmental factors influence the development of skin barrier and immune system dysfunction. Recently, evidence has emerged to support the notion that skin microbial flora can modulate development and exacerbation of this disease. Our study is the first to characterise the skin microbiome in Thai patients with atopic dermatitis before and after 4-week monotherapy with tacrolimus.

**Methods:** Swab samples from skin lesions at volar forearm of 9 patients with atopic dermatitis and normal skin samples of 12 healthy subjects were collected. The skin microbiome was characterized using 16S ribosomal RNA gene sequencing.

**Results:** The diversity of skin microbes is significantly different between the control and AD subjects. Lower prevalence of Actinobacteria and Gammaproteobacteria, but higher prevalence of Firmicutes was observed in the AD group. A significant increase in *Staphylococcus* spp. but decrease in several commensals such as *Coryebacterium* spp. and *Dermacoccus* spp. was detected in AD compared to healthy subjects. After treatment with tacrolimus, the skin microbiota composition of AD individuals was comparable to the control group.

**Conclusion:** Our unique study in Thai patients provides unequivocal proof of the positive impact tacrolimus has on skin microbiome in AD.

**Keywords:** atopic dermatitis, skin microbiome, tacrolimus
1. Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease, the prevalence of which varies from 5-30% worldwide, and it is clear that incidence of AD has increased recently in industrial countries\(^1,2\). The disease is chronic and often heralds other atopic diseases such as asthma and allergic rhinitis.\(^3\) Therefore, AD has become one of the most burdensome skin diseases amongst people of all ages and ethnic backgrounds. The disease is characterised by a dysfunctional skin barrier and associated immune response leading to chronic eczematous skin eruptions. Both genetic and environmental inputs play roles in the development and maintenance of the disease. In particular, several factors have been found to promote AD including exposure to irritant substances, and recently the advance in metagenomics coupled next generation sequencing has specifically identified dysbiosis of skin microbiome as being a major factor. Excessive hygiene associated with urban lifestyle may lead to altered microbial skin contact especially in early life, which results in dysbiosis and immune dysregulation in AD.

Recent research has revealed the role of dysbiosis of the skin microbiome in pathogenesis of AD. A reduction in antimicrobial peptides, defects of epidermal barrier and dysregulation of the adaptive immune response results in a corresponding increase in skin colonization by *Staphylococcus aureus*, which leads to a loss of skin bacterial diversity and increases in specific IgE antibodies against bacterial toxins in the patients’ serum.\(^4\) Meta-analysis reports estimated that pool prevalence of *S. aureus* colonization in AD skin lesion was 70% and the prevalence of colonization correlated with disease severity.\(^5\) Furthermore, *S. aureus* has been reported to facilitate skin inflammation and barrier dysfunction via several mechanisms.\(^6-9\) Beside *the* colonization of *S. aureus* in AD skin lesion\(^s\) dysbiosis of *the* skin microbiome via reduction of commensal microbes such as *Staphylococcus epidermidis*, *Propionibacterium* spp. and *Corynebacterium* spp. has been evident in AD. In normal life, *S.*
*epidemidis* could inhibit rare colonization and biofilm formation by *S. aureus* and augment human beta-defensin (HBD) expression by human keratinocyte via toll-like receptor 2 (TLR2) signalling\(^{10,11}\). Propionibacterium and Corynebacterium can diminish *S. aureus* infection via porphyrin metabolism.\(^{12}\)

Several therapeutic approaches exist for AD and these can act by specifically by restoring the skin barrier, diminishing skin inflammation and reversing dysbiosis of skin microbiome. Topical corticosteroids have been used alone or in combination with topical antibiotics due to their cost-effectiveness. Nowadays, topical caucineurin inhibitors (TCIs) have been recommended as a maintenance therapy as they are low risk of triggering adverse events. To date, there is a paucity of information on the effect of TCIs on skin microbiome in AD and our study seeks to address this. Our aim is to report the findings of a comprehensive comparison of the healthy and AD skin microbiome following the introduction of tacrolimus. We report for the first time that the anti-inflammatory effect of tacrolimus is sufficient to restore the skin barrier leading to reversed dysbiosis of the skin microbiota in a Thai cohort with AD.

2. Methods

2.1 Patients and healthy controls

Nine patients diagnosed with atopic dermatitis according to Hanifin and Rajka criteria at King Chulalongkorn Memorial Hospital (4 males, 5 females) and 12 normal subjects (4 male, 8 females) were enrolled in the study. The severity of AD was classified according to the Scoring of Atopic Dermatitis (SCORAD), Eczema Area and Severity Index (EASI) and Investigators’ Global Assessment (IGA). Patients with other chronic inflammatory skin diseases were
excluded from the study. All patients were free from systemic skin therapies for at least 4 weeks, systemic antibiotics for at least 6 months or topical skin therapies and topical antiseptics for at least 2 weeks prior to sample collection. Patients were allowed to use only mild liquid soap and 10% urea cream for 2 weeks and avoid all washing 24 hours prior to sampling. The study was approved by the ethical committee of the King Chulalongkorn University. All participants provided informed consent. The demographic as well as the severity scales of AD (before and after tacrofilum treatment) data are shown in Table 1. For abbreviation, ‘D’ denotes disease, ‘Bf’ or ‘Before’ and ‘Af’ or ‘After’ denote AD-before and AD-after treatment, and the number in the middle denotes individual patient in random order. Similarly, ‘C’ denotes control followed by the number that denotes individual normal volunteer in random order.

2.2 DNA extraction

Samples were collected by rubbing the skin using a sterile cotton tipped applicators and transferred into microcentrifuge with 200 µl of ST solution (0.15 MNaCl with 0.1% Tween 20). Then, samples were centrifuged at 10,000g for 5 minutes, and supernatant was removed. The sample pellet was kept at -80 °C. Total genomic DNA was extracted from the pellet by GenElute bacterial genomic DNA kit (Sigma). Finally, genomic DNA was kept at -80°C.

2.3 16S rRNA gene library preparation and next generation sequencing

Universal prokaryote primers (5'-GTGCCAGCMGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for 16S rRNA gene V3-V4 with the 5' Illumina adapter and 3' Golay barcode sequences were used as previously described. To prevent PCR stochastic bias, the template quantity and quality was adequate, and a minimum of three independent PCR reactions were performed per sample. Paired-end sequencing, 2 × 150 was performed using
Illumina MiSeq platform (Illumina, San Diego, CA, USA) following the manufacturer’s protocols at Chulalongkorn Medical Research Center (Bangkok, Thailand).

2.4 Quality screening, taxon classifications and community comparison

All nucleic acid sequences in this study were deposited at the NCBI Sequence Read Archive (SRA) database (accession number SRP155450). The raw sequences (FASTQ files) were categorized individuals based on the 5′ barcode sequences. The sequences were processed following mothur’s MiSeq Standard Operating Procedures. The pre-processing steps included removal of (i) short read lengths of ≤ 100 nucleotides (excluding the primer and adaptor sequences), (ii) long homopolymers of ≥ 8 nucleotides, (iii) ambiguous nucleotides and (iv) chimera. Passing sequences were aligned to Greengenes to remove contaminate sequences such as mitochondria and chloroplast. The clean sequences were classified to the operational taxonomic unit (OTU) using the Ribosomal Database Project (RDP) Classifier. A minimum bootstrap confidence score of 80 % was used as a cutoff for taxonomic assignment. Genus and specie of OTU (GLOTU and SLOTU) were followed the phylotype-based methods. Good's coverage index to estimate the data coverage of a community, and the alpha diversity by number of OTUs, Shannon and Chao bacterial community richness, were computed using mothur. Data normalization was performed to normalize the varying sequencing depth among individuals. The relative abundance of bacterial genera was visualized as Heatmap using R statistics package. Venn diagram, and the beta diversity by Morisita-Horn community dissimilarity index and non-metric multidimensional scaling (NMDS) based on Morisita-Horn dissimilarity indices, along the analysis of molecular variance (AMOVA) and a homogeneity of molecular variance (HOMOVA) statistics to determine significant differences between or among the structures of the comparing communities (p-value < 0.05), were also computed using mothur. AMOVA determines whether the diversity is greater than their pooled
diversity, while HOMOVA determines whether the diversity in each is significant different. In addition, differentially abundant genus detection by Metastats and the linear discriminant analysis effect size (LEfSe) to find biomarkers between two or more groups from relative abundances were performed using mother.

2.6 Correlation analyses
Spearman correlation to evaluate the order and the directions of the species that drive the microbiota structures, and Pearson correlation to evaluate the direction and p-value statistics of the clinical data on the AD severity scales (Table 1: SCORAD, EASI and IGA) against the microbiota, were performed using mothur. The results were visualized by R package ggplot2 (https://cran.r-project.org/package=ggplot2).

3. Results
3.1 Skin microbiota in AD compared to healthy control
AD patients aged between 16-39 years and healthy subjects aged between 23-54 years, participated in this study. The demographic data of the participants are summarized in Table 1. All patients with AD reported significant improvement of all clinical scores (SCORAD, EASI and IGA) after 4-week monotherapy with Tacrolimus.

The 16S rRNA gene sequencing yielded an average of 146,922 clean sequences for OTU classification (Supplemental Table 1), and thus yielded high Good’s coverage indices of 98.45-99.93% (avg. 99.26%) at genus level (Supplemental Table 2). The number of GLOTUs vary from 6 to 162; hence, the diversity assessment within each microbiota was assessed (OTUs, Chao and Shannon) and the the variance box plot analysis showed that the species richness (Chao) and species richness and evenness (Shannon) were relatively high for the healthy controls than the AD groups (especially the Bf group). Several samples in the Bf group
had poor diversity (low number of OTUs, Chao and Shannon) (Figure 1 and Supplemental Table 2).

Taxonomic profiles demonstrated the diversity that might differ among the control and the AD: phylum Actinobacteria (class Actinobacteria) was relatively high in control followed by Proteobacteria (class Gammaproteobacteria), whereas phylum Firmicutes (class Bacilli) was generally higher in the AD, in particular the Bf group (Figures 2A and 2B). In detail, the Af group showed closer relative abundances of Actinobacteria by increasing from the matched Bf subjects, the moderate abundances of Firmicutes from the matched Bf subjects where a few were with minute and many with over high abundance, and likewise for the Proteobacteria. The number of the overlapping OTUs between the control and Af group was thereby greater than that between the control and Bf group (Figure 2C: Control-Bf overlapped 71.62%, Control-Af overlapped 81.66%). In continuation, the NMDS was constructed to visualize the relative dissimilarity among the microbiota structures, and the control and the disease groups were discrete, although the D8 data were an exception showing close to C11, C12 and C7, in orderly. When analysis without the D8 showed even more prominent the community structure difference between the control and the disease groups with the AMOVA statistic of \( p < 0.001 \) (Figure 3A and B).

3.2 Effect of Tacrolimus on skin microbiome in AD

To determine the microbiota structural differences within the disease group, before and after Tacrolimus treatment, AMOVA and HOMOVA statistical analyses among the three groups (Control, Bf and Af) were computed and both demonstrated significant differences of 0.003 and 0.04, respectively. Additionally, the statistical difference between the Control-Bf (AMOVA \( p = 0.003 \)) was suggested greater than between the Control-Af (AMOVA \( p = 0.15 \)). This is supported by the NMDS illustration in Figures 3C and D, particularly in Figure 3D
where D8 data were exempted. Nevertheless, the $p$-value statistic between the Bf and Af groups remained non-significant (AMOVA $p = 0.164$).

Metastats analysis highlighted species that were differentially statistically different between the comparing groups. Consistently, compared to the control group, there were a fewer number of species differences in the Af than the Bf groups (Supplemental Table 3). Supplemental Table 3A describes the species whose presence or absence might be associated with AD, Supplemental Table 3B describes the species that remained different even after the treatment, and Supplemental Table 3C highlighted the species that might be associated with the positive effect of Tacrolimus, for example the increases of *Dermacoccus*, *Pseudomonas*, *Corynebacterium*, *Proteus*, *Micrococcus luteus*, and *Lactococcus* in AF group. This effect of Tacrolimus caused the Bf community to become close to the Control.

3.3 Association of bacterial species and severity of AD

Spearman correlation analysis allowed determination of the associated direction of the certain bacteria species to the microbiota structures representing control and disease groups, given that the Af microbiota were found scatter around the middle between the Bf and the Control (Figure 4). Many taxa (such as *Dermacoccus* and *Corynebacterium*) were associated with the Control, and as well the Af since the communities of the Af, as displayed by the positions of the green dots, are closer to the Control. For *S. epidermidis* and *Staphylococcus lugdunensis*, both shared the directions for the majority of Af (5/7 samples equal 71.43%) and half of the Bf (4/8 samples equal 50%). Moreover, the association with AD severity scales were analyzed. The AD severity scales vectors were found scattered around the Bf and Af groups, and no significant correlation could be depicted between the AD indicators and the Bf groups ($p$-values of Scorad = 0.26, EASI = 0.59, IGA = 0.78). In parallel, sex and age factors were considered. AMOVA analysis between the male and female microbiota reported no
statistical difference \((p > 0.05)\). Pearson correlation analysis against age showed the vector direction of the microbiota among control samples, however with insignificant \(p\)-value \((p > 0.05)\) (Supplemental Figure 1).

As statistically differentially abundant species were observed, LEfSe analysis for species biomarker was performed to identify the species that separate the control from the AD (Figure 5: blue bar), and on the other hand the species that signature the disease groups (Bf and Af) (Figure 5: green and red bars). A total of 29 taxa were pointed as biomarkers for the control for the AD, and included Corynebacterium with the highest LDA scores followed by Acitnomycetales and Micrococcaceae. 3 taxa were pointed biomarkers for the Af, and Staphylococcus has the highest LDA score.\(^{22}\) 1 genus (Veillonella) was pinpointed for the Bf biomarker, with minor LDA score.

4. Discussion

Skin microbes participate in innate defense of the skin by several mechanisms. Restricted cutaneous microbial diversity and colonization of pathogenic bacteria are crucial biologic characteristics that drive in atopic dermatitis. As expected, we found that the bacterial diversity was relatively higher in the healthy controls than the AD groups and correlation analysis determined the associated direction of the certain bacteria species to the microbiota structures representing control and disease groups. Several previous reports from various countries demonstrated decreased prevalence of Actinobacteria and Gammaproteobacteria as well as increase colonization of \(S. \text{aureus}\) and \(S. \text{epidermidis}\) in AD and the involved site.\(^{22}\) In addition, allergy-defensive action of these commensals and allergy-provocation of \(S. \text{aureus}\) related to AD has been observed.\(^{23,24}\) Our data is unique in the fact that it highlights lower prevalence of phylum Actinobacteria (class Actinobacteria) and Proteobacteria (class Gammaproteobacteria),
but higher prevalence of phylum Firmicutes (class Bacilli) in the AD group. Interestingly, this study revealed significant increases in *Staphylococcus* spp. but decrease in several commensals (such as *Corynebacterium* spp., *Dermacoccus* spp. and *Lactobacillus* spp) in the AD. This finding is consistent with the recent metagenome analysis of skin microbiome in Singapore (similar tropical status to Thailand), which demonstrated that *Dermacoccus* spp. are also significantly diminished in patients with AD. The similarity of findings in both studies underpin the concept that dysbiosis of skin microbiome is one of important features of AD in the Thai population. Nevertheless, we could not demonstrate any correlation among skin microbiome and disease severity (either SCORAD, EASI and IGA) probably because of the limited number of patients.

Several therapies for AD aim to reduce the bacterial load leading to attenuated inflammation, restored skin barrier and reversed dysbiosis of skin microbiome. Tacrolimus, a TCI, has been widely used as an effective and safe treatment in AD. To the best of our knowledge, the effect of TCIs on skin microbiome has never before been reported. We discovered that after treatment with tacrolimus, the skin microbiota structure of AD returned to be comparable to control group. Furthermore, the fewer number of species differences in the Af group than the Bf group when compared to control. This finding reflected that tacrolimus could reverse some dysbiosis in AD. Nonetheless, there are some remaining species that may still persist to promote AD after treatment with tacrolimus. These species may require additional treatment either to equilirerate those species to the relative abundances representing the control subjects.

Tacrolimus can restore the skin barrier by several mechanisms. It acts as an immunosuppressive agent by inhibiting the activation of T cells and suppressing cytokines production by them. Additionally, tacrolimus has been reported to alleviate pruritus by suppressing
sensory nerve activation. Therefore, it is possible that the influence of tacrolimus in restitution of the skin microbiome might be a consequence of its anti-inflammatory effect and potential to restore the skin barrier.

To date, various methods have been used for skin microbiome analysis. Our study analyzed skin microbiome in AD using 16S rRNA gene sequence. It should be noted that the power of species and genus classification is in part limited by the partial 16S rRNA gene sequence. For future experiments of this nature, the unclassified and classified isolates of interest might be full-length sequenced to confirm the species annotation.

In conclusion, this study for the first time characterizes the skin microbiota in healthy and patients with AD in Thailand (a tropical country). Several mechanisms of tacrolimus efficacy in treatment of AD have been suggested. This study is the first original research study to describe the effect of tacrolimus on the skin microbiome in AD, and it may further influence the use of tacrolimus as a strategy to alleviate AD in the future.

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References


