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Author	村上, 慎之介(Murakami, Shinnosuke) 馬場, 藤貴(Baba, Fujitaka) Aw, Wanping(Fukuda, Shinji) 福田, 真嗣(Soga, Tomoyoshi) 曽我, 朋義(Fujishima, Hiroshi) 藤島, 浩(Tomita, Masaru) 冨田, 勝
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［研究論文］

Comprehensive Analysis of Microbes and Metabolites in Human Tear Fluids
ヒト涙液中の微生物および代謝物質の網羅的解析

Shinnosuke Murakami
Doctoral Program, Graduate School of Media and Governance, Keio University
村上 慎之介
慶應義塾大学大学院政策・メディア研究科後期博士課程

Fujitaka Baba
Staff, Institute for Advanced Biosciences, Keio University
馬場 藤貴
慶應義塾大学先端生命科学研究所所員

Wanping Aw
Project Researcher, Graduate School of Media and Governance, Keio University
ワンピン アウ
慶應義塾大学大学院政策・メディア研究科研究員

Shinji Fukuda
Project Associate Professor, Graduate School of Media and Governance, Keio University
福田 真嗣
慶應義塾大学大学院政策・メディア研究科特任准教授

Tomoyoshi Soga
Professor, Faculty of Environment and Information Studies, Keio University
曽我 朋義
慶應義塾大学環境情報学部教授

Hiroshi Fujishima
Professor, Department of Ophthalmology, Tsurumi University Dental Hospital
藤島 浩
鶴見大学歯学部附属病院眼科教授

Masaru Tomita
Professor, Faculty of Environment and Information Studies, Keio University
冨田 勝
慶應義塾大学環境情報学部教授

Abstract: Lacrimal fluids are important in protecting the eyes from environmental factors and also possess antimicrobial abilities. Although human tear fluid metabolites have been reported, the relationships between these metabolites and eye diseases have yet to be elucidated. In addition, the microbial composition of human tear fluids has not been investigated yet, even though numerous human microbiome analyses of various body sites have been reported. Therefore, microbiome and metabolome analyses of human tear fluids from healthy subjects and atopic keratoconjunctivitis (AKC) patients were conducted. The current study suggested that lactic acid bacteria in tear fluids might have a potential to prevent pathogenesis of AKC, whereas o-acetylcarnitine reduction and urea increment might be involved in AKC pathogenesis.

涙液は環境要因から眼を保護する役割や、抗菌作用を有することが知られている。ヒト涙液に含まれる代謝物組成はこれまでにも報告されているが、眼疾患との関連は不明である。近年、人体常在菌に関する研究が多数報告されているが、涙液中細菌叢に関する報告はほとんどない。本研究では、健常者とアトピー性角結膜炎（AKC）患者の涙液を用いて細菌叢および代謝物を網羅的に解析した。その結果、涙液中乳酸菌による AKC 発症予防の可能性や、o-アセチルカルニチンの減少および尿素の増加が AKC 発症に関与する可能性が示唆された。

Keywords: tear fluid, atopic keratoconjunctivitis, microbiome analysis, metabolome analysis, lactic acid bacteria
涙液、アトピー性角結膜炎、細菌叢解析、メタボローム解析、乳酸菌

1 Introduction

Recent studies have revealed that thousands of species of commensal microbes are colonized in and on the human body [1-3]. There are a huge number of commensal microbes that reside in the human body that have a profound influence on human physiology, immunology, and nutrition through host-microbial crosstalk [4-11]. Previously, there have been numerous reports on the microbiome analyses of various human body sites such as intestine, skin and vagina. However, microbiome analysis of human tear fluids has yet to be presented. Eyes are extremely important sensory organs for maintaining our quality of life. Nevertheless, the eyes are constantly exposed to

environmental factors such as microbes and possible allergens. It is well known that tear fluids play an important role in the protection of eyes from environmental factors by not only physically flushing them away but also possess antimicrobial abilities derived from lysozyme, lactoferrin, IgA and IgG [12]. According to previous research, 60 metabolites including amino acids, carnitines and nucleotides were detected from human tear fluid by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis [13]. These molecules are expected to be involved in the maintenance of physiological homeostasis of eyes. However, the exact relationships between these molecules and eye diseases are still currently unknown.

To date, it has been reported that *Staphylococcus, Propionibacterium* and *Corynebacterium* are present in the ocular microbiota that were observed by a culture-based method in human conjunctival scraping samples collected from cataract patients [14]. Furthermore, microbiome analysis of conjunctival swab samples collected from healthy volunteers showed that the ocular microbiota was comprised of 5 phyla and 59 genera, mainly comprising of *Pseudomonas, Bradyrhizobium* and *Propionibacterium* [15]. Although partial ocular microbiota compositions from conjunctival swabs have been observed, the exact microbiota compositions of tear fluids that are the main players for maintaining ocular surface homeostasis remain obscure.

Ocular allergic inflammation is a common problem amongst individuals suffering from allergies. A previous study has reported that 25% to 42% of atopic keratoconjunctivitis (AKC) patients have atopic dermatitis [16]. Allergic conjunctival disease is typically divided into three types: AKC, vernal keratoconjunctivitis (VKC) and seasonal allergic conjunctivitis (SAC) [17]. AKC is the most severe form and SAC is the least severe form of allergic conjunctival diseases. Common ocular clinical features of AKC, VKC and SAC include

redness, itching, and tearing. Two common pathological findings of AKC are conjunctival mast cell activation and eosinophil recruitment to the ocular surface. Histamine and leukotriene released from mast cells are observed to be up regulated in the tear fluid analysis of patients with these conditions [18, 19], suggesting that biochemical imbalances in amino acid and fatty acid metabolism may underlie the pathogenesis of these ocular allergic diseases.

In this study, in order to investigate the characteristics of microbial and metabolite profiles in tear fluids between normal eyes and eyes suffering from ocular allergic inflammation; and the relationships between microbiota or metabolites and eye diseases; we conducted microbiome and metabolome analysis of human tear fluids collected from healthy subjects and AKC patients.

2 Materials and Methods

2.1 Subjects and collection of tear fluids

This study was approved by the Ethics Committee of Keio University School of Medicine and Keio University Shonan Fujisawa Campus. All subjects were informed of the purpose of this study, and written consents were obtained from all subjects. For microbiome analysis, tear fluids were collected from 4 healthy subjects and 5 AKC patients. After tilting the face for lateral side, the suction tube or tip was placed on the corner of the outside of eye, and tears were collected. A minimum of 30 μl of tear fluids were collected from both eyes and stored at -20° C until further use. For metabolome analysis, tear fluids were collected from 5 healthy subjects and 10 AKC patients. Similarly, a minimum of 30 μl of tear fluids were collected from both eyes. Then, the tear fluids were immediately centrifuged at 10,000 rpm for 5 min at 4°C. After centrifugation, supernatants were transferred to new tubes and stored at -20°C until further use.

For all cases, AKC diagnosis was based on the criteria as described previously [20]. In the case of metabolome analysis, AKC patients were classified into 2 groups (severe or moderate). Severe AKC patients were defined as AKC complicated severe corneal lesions such as ulcers, while moderate AKC patients were defined as those who have AKC with mild or no corneal complications. Eye drops included 0.1% dexamethasone, 0.1% fluorometholone, 0.05% cyclosporine A or cromolyn sodium were used for treatment of the AKC patients but all patients did not receive systemic corticosteroid therapy. All AKC patients did not have any other systemic diseases.

2.2 DNA extraction

For DNA extraction from tear fluids, QIAamp DNA Stool Mini Kit (QIAGEN N.V., Limburg, Netherlands) was used. Initially, 1.4 ml of Buffer ASL (included QIAamp DNA Stool Mini Kit) and 0.1 g of 0.1 mm glass beads were added to tear fluids, and then horizontally vortexed for 5 minutes. Subsequent steps of DNA extraction were conducted according to the manufacturer's instructions.

2.3 PCR amplification and sequencing of 16S rRNA genes

The V3-V4 region of the 16S rRNA genes were amplified from the DNA isolated from the tear fluids using the bacterial universal primer set 341F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') [21]. PCR was performed with TaKaRa Ex Taq DNA polymerase (Takara Bio Inc., Shiga, Japan) and amplification proceeded with one denaturation step at 95 ° C for 5 min, followed by 30 cycles of 95° C for 30 s, 48° C for 30 s, and 72° C for 2 min, with a final extension step at 72° C for 5 min after which sequencing of 16S rRNA genes using Roche GS FLX Titanium platform was outsourced to Filgen, Inc. (Aichi, Japan).

2.4 Analysis of 16S rRNA gene sequences

The 16S rRNA gene sequences were analyzed using the RDP-Classifier and RDP-Seqmatch provided by the Ribosomal Database Project (RDP) [22] In advance, sequences whose lengths were less than 250 bp were removed as RDP-Classifier only accepts sequences that are over 250 bp long. All sequences were assigned genus level taxonomy using the RDP-Classifier. Species level taxonomies were also assigned using RDP-Seqmatch using the following parameters; Strain: type, Source: isolates, Size: ≥1200. Sequences whose homologies against known 16S rRNA gene sequences were less than 95% were filtered out for further analysis. The microbial profiles of tear fluids were compared with that of skin microbiota. The data of skin microbiota were obtained from a previous study conducted [23]. Coefficient of determinations (square of the Pearson correlation) between microbial compositions of tear (average of the microbial components collected from 5 healthy subjects) and that of each skin category were calculated. Orthogonal partial least squares discriminate analysis (OPLS-DA) on the tear microbial data was conducted with the SIMCA-P+ software v12.0 (Umetrics Inc., Umetrics AB, Umeå, Sweden). For the drawing of the autocorrelation map, Spearman's rank correlations between each microbial pairs were calculated by JMP (SAS Institute Inc., North Carolina, USA). Bacterial populations that dominated at least 1% in any samples were used for autocorrelation analysis. Network analysis of tear microbiota was conducted using Cytoscape v2.8.1 [24]. A force-directed layout algorithm was used to draw the network. Bacterial species that dominated at least 0.01% in any samples were used for network analysis.

2.5 Metabolome analysis

In order to extract metabolites from tear fluids, 900 µl of methanol including the internal standards (20 µM of methionine sulfone (Alfa Aesar, Massachusetts, USA) and 25 µM of 2-Morpholinoethanesulfonic acid (MES) (DOJINDO LABORATORIES, Kumamoto, Japan)) were added to the samples and then mixed with 400 µl Milli-Q water. Each 600 µl of the solutions were transferred to a centrifugal filter tube to remove protein and lipid molecules. The filtrate was centrifugally concentrated and dissolved in 120 µl of Milli-Q water containing reference compounds (200 µM of both 3-aminopyrrolidine (Sigma-Aldrich, Missouri, USA) and trimesic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan)) immediately before capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry (CE-TOFMS) analysis. The measurement of extracted metabolites in both positive and negative modes was performed by CE-TOFMS (Agilent Technologies, California, USA). The alignment of detected peaks was performed according to the m/z value and normalized migration time. Then, peak areas were normalized against those of the internal standards methionine sulfone and MES for cationic and anionic metabolites, respectively. Annotation tables were produced from measurement of standard compounds and were aligned with the datasets according to similar m/z value and normalized migration time. Relative concentrations of each metabolite were transformed to Z-score by subjects and demonstrated as heatmap using MeV (v4.8) [(25)]. Principal component analysis (PCA) and OPLS-DA on the tear metabolome data were conducted with the SIMCA-P+ software v12.0.

2.6 Statistical analysis

Statistical evaluation between two groups and normality of

the distribution of each data were investigated by Kolmogorov-Smirnov's test. When the data was determined to model normal distribution, P values were calculated by Student's t-test or Welch's t-test. Statistical analyses were performed by Microsoft Excel. All statements indicating significant differences show at least a 5% level of probability.

2.7 Nucleotide sequence accession number

The microbiome analysis data have been deposited at the DDBJ Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/) under accession number DRA003590.

3 Results

3.1 Microbiome analysis of human tear fluids collected from healthy subjects and AKC patients

In this study, microbial profiles of human tear fluids collected from 4 healthy subjects and 5 AKC patients were analyzed. 16S rRNA gene sequences collected from tear fluids were assigned to 462 strains. *Propionibacterium acnes* and *Leuconostoc citreum* occupied 30-50% of tear microbiota in both healthy subjects and AKC patients (Fig. 1A). Microbial profiles of tear fluids were comparable with that of skin (Fig. 1B-C). Microbial profile of tear fluid that excluded *Lactobacillales* was similar to the microbial profiles of sebaceous sites such as glabella and alar crease that are located around eyes.

To investigate the differences of microbial components between healthy subjects and AKC patients, PLS-DA was conducted (Fig. 2A-B). According to the S-plot, proportions of lactic acid bacteria such as *Lactococcus plantarum* and *L. citreum* were higher in healthy subjects. Subsequently, correlation analysis using proportions of 21 dominant microbes was performed. As a result, several lactic acid

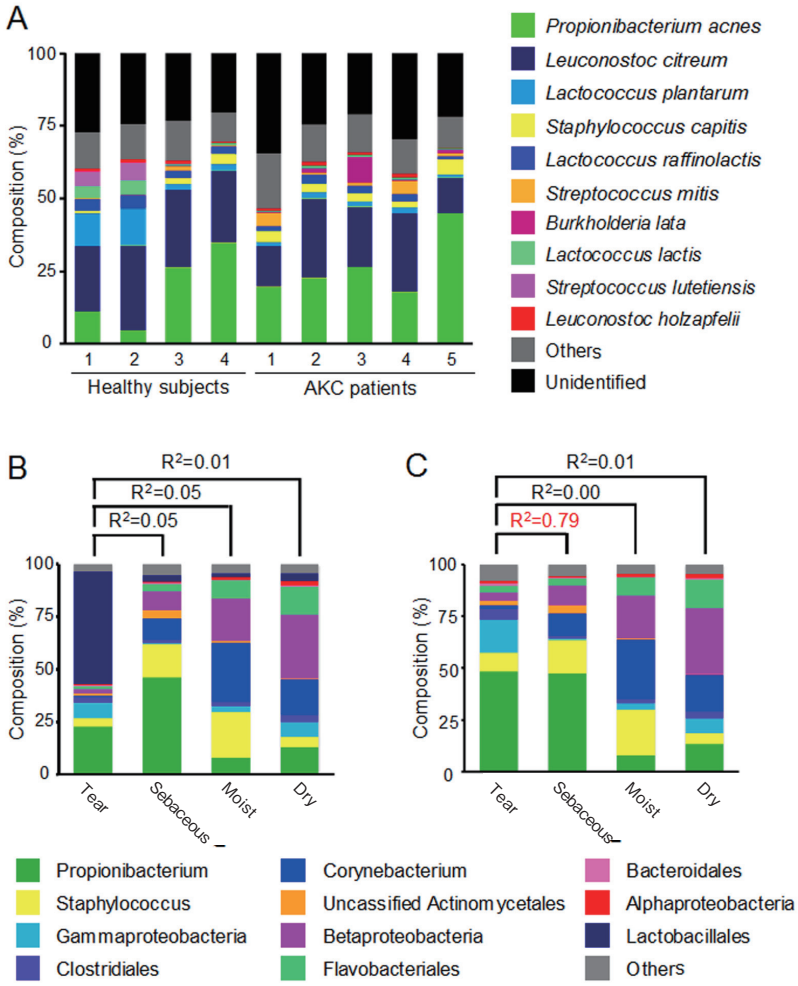

Figure 1　Comparisons of tear microbiota compositions between healthy subjects and AKC patients.

(A) Species level composition of tear microbiota of healthy subjects and AKC patients. (B-C) Microbial compositions of both human tear fluid and skin that were included (B) and excluded (C) *Lactobacillales*. The data of skin microbiota (sebaceous, moist and dry) were obtained from a previous study conducted [23]. Coefficient of determinations (square of the Pearson correlation) between microbial compositions of tear and that of each skin category were demonstrated.

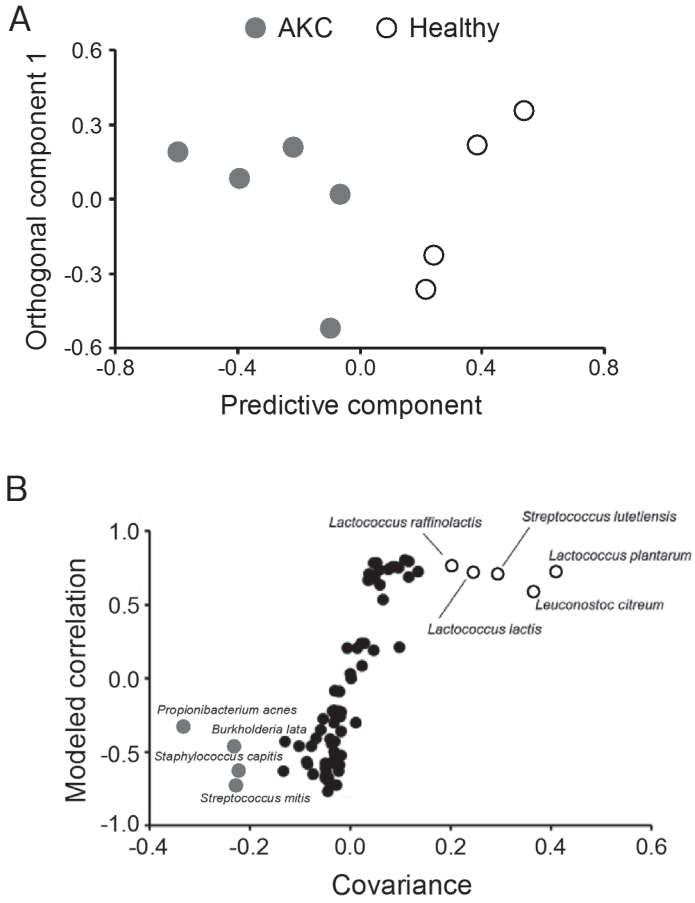

Figure 2 Determination of effective microbes to separate healthy subjects and AKC patients.

(A) Cross-validated score plots from OPLS-DA of tear microbiota collected from healthy subjects and AKC patients. (B) S-plot derived from the predictive component of OPLS-DA of tear microbiota collected from healthy subjects and AKC patients. The white or gray plots ($|\text{covariance}| > 0.2$) in the S-plot have high contribution and reliability for class separation between healthy subjects and AKC patients.

bacteria (E.g. *Lactococcus lactis* and *Lactococcus raffinolactis*) were positively correlated among each other and potential pathogens of various diseases such as *P. acnes, Corynebacterium tuberculostearicum, Staphylococcus aureus* and *Shigella flexneri* [26–29] were also positively correlated to each other. However lactic acid bacteria and potential pathogens were strongly, negatively correlated (Fig. 3A). Network analysis showed that lactic acid bacteria were well clustered and the cluster including potential pathogens such as *P. acnes* was also constructed; however *S. aureus* was not clustered together (Fig. 3B). Network analysis also revealed that lactic acid bacteria cluster and potential pathogens cluster were negatively correlated with each other, and this was consistent with correlation analysis.

3.2 Metabolome analysis of human tear fluids collected from healthy subjects and AKC patients

In the present study, metabolome profiles of human tear fluids were collected from 5 healthy subjects and 10 AKC patients. To compare the metabolome profiles derived from distinct severity of AKC, AKC patients were classified into 2 groups (severe or moderate). Representative pictures of eyes in healthy subject, moderate and severe patients are as depicted in Fig. 4A. A total number of 47 metabolites were detected from human tear fluid by using CE-TOFMS. Relative concentrations of each metabolite were transformed to Z-score by subjects and demonstrated as heatmap (Fig. 4A-B).

To investigate the differences of metabolites between healthy subjects and AKC patients, PLS-DA was conducted (Fig. 5A-B). According to the S-plot, the proportion of o-acetylcarnitine was higher in healthy subjects whereas that of urea was higher in AKC patients. In the tear fluids of AKC patients, concentration of

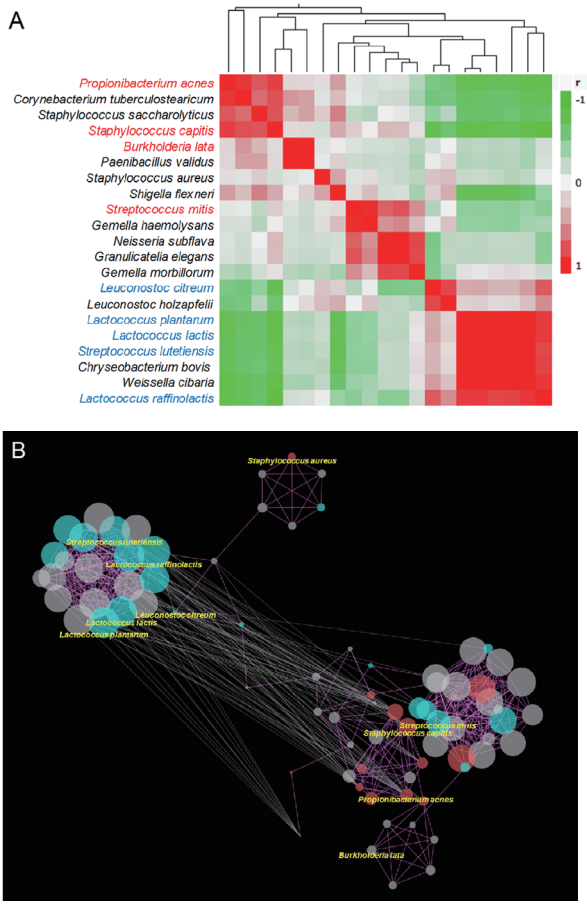

Figure 3 Correlations between each microbe observed in the current study.

(A) Autocorrelation map using bacterial populations that dominated at least 1% in any samples was demonstrated based on the Spearman's rank correlation. Bacterial species that highlighted as white or gray in Figure 2B were colored as blue (proportions were higher in healthy subjects) or red (proportions were higher in AKC patients), respectively. (B) Network of tear microbiota collected from healthy subjects and AKC patients was constructed based on the Spearman's rank correlation. Bacterial species that dominated at least 0.01% in any samples were used. Blue, red and white circles indicate lactic acid bacteria, potential pathogens of various diseases and others, respectively. Size of each circle corresponds to numbers of positively correlated pairs. Positive and negative correlations are shown as purple and white lines, respectively.

o-acetylcarnitine was significantly low and that of urea tended to be elevated (Fig. 5C). Subsequently, concentrations of 20 amino acids were used in PCA to investigate the differences in amino acids profiles of healthy subjects and AKC patients. Based on the PCA, it was found that healthy subjects and AKC patients were separated into 2 different clusters, whereas moderate and severe patients were not separated (Fig. 6A). Loading plot of the PCA demonstrated that aspartic acid was higher in healthy subjects, whereas leucine, phenylalanine, lysine, methionine, valine, tyrosine and alanine were higher in AKC patients, especially in severe patients (Fig. 6B).

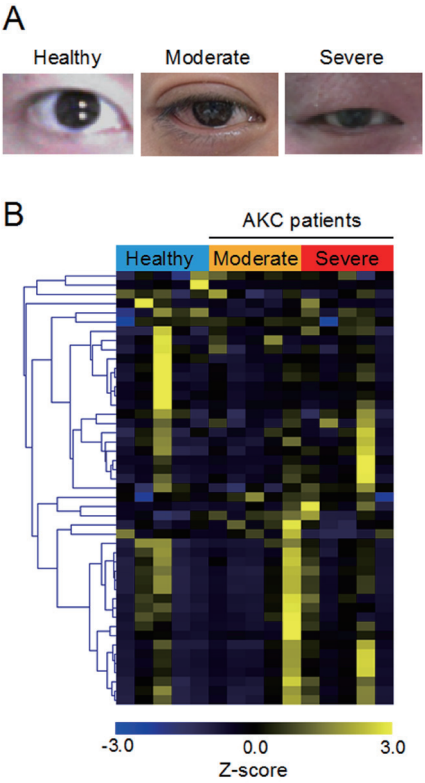

Figure 4　Comparisons of tear metabolites collected from healthy subjects and AKC patients.

Patients were classified into 2 groups based on with (severe) or without (moderate) complication. (A) Representative pictures of eyes in healthy subject, moderate and severe patients. (B) Heatmap profiles of individual metabolites in tear. 47 metabolites that detected at least two thirds of the samples were shown. Relative concentrations of each metabolite were transformed to Z-score by subjects.

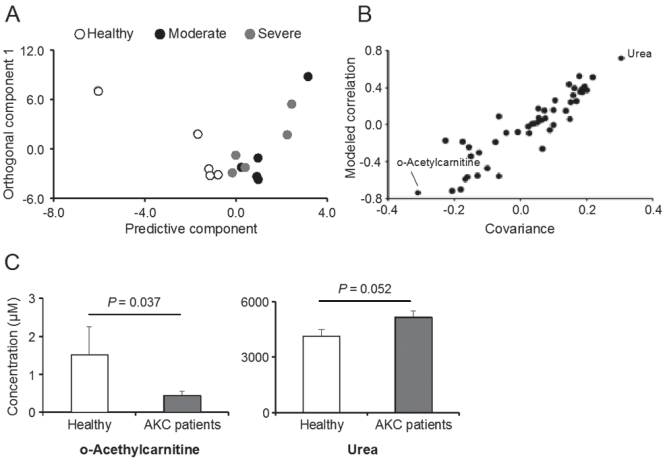

Figure 5 Determination of effective metabolites to separate healthy subjects and AKC patients.

(A) Cross-validated score plots from OPLS-DA of tear metabolites collected from healthy subjects and AKC patients. (B) S-plot derived from the predictive component of OPLS-DA of tear metabolites collected from healthy subjects and AKC patients. (C) Comparisons of o-Acetylcarnitine and Urea concentrations between healthy subjects and AKC patients. Data were expressed as mean ± standard deviation. *P* values were calculated using t-test.

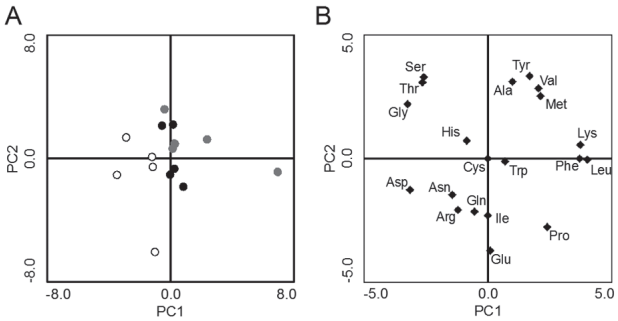

Figure 6 PCA on amino acids concentrations derived from metabolome analysis of tear fluids.

Amino acid concentrations were normalized by dividing by total concentrations of 20 amino acids included in same sample as previously reported [30]. PCA score plot is color-coded according to healthy subject, moderate and severe patients (A) and (B) demonstrates the loading plot of amino acid concentrations.

4 Discussion

In the present study, microbiome and metabolome analyses of human tear fluids collected from healthy subjects and AKC patients were conducted. Interestingly, the microbial profile of tear fluids that excluded *Lactobacillales* was similar to the microbial profile of sebaceous sites. Since sebaceous sites such as glabella and alar creases are located around eyes, tear microbiota might be constructed from skin microbiota located around the eyes. Additionally, the results also indicate that *Lactobacillales* species were unique in tear fluid as compared to skin microbiota. However, the origin of the *Lactobacillales* species in the current study is unknown. The *Lactobacillales* species were abundant in healthy subjects when compared with AKC patients according to the PLS-DA, and *Lactobacillales* species has been known to have preventive effects for various diseases in intestine and vagina [10, 31, 32]. In addition, autocorrelation analysis and network analysis have demonstrated a negative correlation between lactic acid bacteria and possible pathogens. Taken together, *Lactobacillales* species in tear fluids may have a potential to prevent pathogenesis of eye diseases.

Since the abundance of *P. acnes* was higher in AKC patients and *P. acnes* has been reported to be involved in postoperative endophthalmitis [26], this bacteria might be one of the possible pathogens of AKC. As *Propionibacterium* is presented as the highest population in sebaceous sites of skin, influx of the bacteria from the surrounding environment may be an initiation factor for eye diseases. On the other hand, *S. aureus* that has been reported to be a possible pathogen of several diseases such as atopic dermatitis [28], was not strongly correlated with other bacteria and this bacteria was also not higher in AKC patients. Although it has been reported that atopic dermatitis is a significant cause of ocular morbidity [17], *S. aureus* may not influence AKC pathogenesis.

Metabolome analysis of human tear fluids revealed that concentration of o-acetylcarnitine was significantly decreased and that of urea was tended to increase in AKC patients. O-acetylcarnitine has been known to be catalyzed from acyl-CoA and carnitine by carnitine o-acetyltransferase (CRAT) in humans [33]. Thus, CRAT activity and/or expression may be downregulated in AKC patients. Despite acetylcarnitine being beneficial in depression and Alzheimer's disease [34, 35], its role in other positive effects for human health is unclear. It was reported that urea concentrations in tear fluids and serum were not correlated although other components of tear fluids and serum has been known to be similar [36]. In addition, arginase, which converts L-arginine into urea and L-ornithine, has been reported to express in lacrimal gland, conjunctiva and cornea, thus urea is expected to be locally supplied from these tissues to the tear fluid [36]. According to the PCA of amino acid concentrations, arginine concentration was lower in AKC patients, it may be the result that arginine was converted to urea in AKC patients. Consequently, increment of urea concentration in AKC patients may be derived from increasing of activity and/or expression of arginase. Additionally, profiles of amino acid concentrations were different between healthy subjects and AKC patients. Acetylcarnitine, urea and amino acids were previously detected from tear fluid [13], and the current research also demonstrated that alterations of those metabolites could possibly be involved with AKC.

Finally, this present study showed novel features of microbial and metabolite compositions of human tear fluids and their variations in AKC patients when compared with healthy subjects. Since the current results of this study are not sufficient to completely understand the pathogenesis of AKC, further analyses are required to reveal the relationships between tear microbiota, metabolites and eye diseases.

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