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DOI: 10.1159/000496968**Characterization of the Blood Microbiota in Korean Females with Rosacea**Yeojun Yun^a Han-Na Kim^a Yoosoo Chang^{b-d} Yunho Lee^e
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Bacteria · Blood · Microbiota · Metagenome · Rosacea

Rosacea is a chronic, intractable skin disease that is generally accompanied by vascular dilation and subsequent inflammation of the face [1]. Rosacea has been reported to occur among people of diverse races throughout the world, and its prevalence varies widely from less than 1% to 20% according to the study country. The gender distribution is slightly female-predominant [2]. The symptoms of rosacea comprise a complex combination of erythema, papulopustules, telangiectases, or ocular redness. Rosacea may be a proxy for systemic disease, rather than a mere skin disorder, as it has been linked with metabolic, cardiovascular, autoimmune, and chronic kidney diseases [3, 4]. However, most of the relevant data are from epidemiologic studies without basic experimental details. Thus, further evidence must be sought to support the hypothesis that rosacea is associated with systemic inflammation and systemic diseases.

The analysis of 16S ribosomal RNA (rRNA) fragments from the human microbiome is emerging as a novel tool for understanding the pathogenesis of human disease. Metagenomic approaches for investigating microbial genomes are being used to determine the potential roles of the microbiotas of the gut, skin, blood, and other human derivatives in chronic inflammatory diseases [5–7]. Of

note, a new insight was made into the pathologic significance of the blood microbiota that had been overlooked in conventional culture-based research. By identifying identical bacterial gene sequences at two different sites, Huang et al. [8] determined that *Helicobacter pylori* exists in the peripheral blood as well as in the gastric mucosa of patients with duodenal ulcers.

Rosacea is associated not only with various gastrointestinal diseases, but also with gut dysbiosis [9–11]. Indeed, our previous cross-sectional study demonstrated that the gut microbiota was altered in rosacea subjects [11]. Given the aberrant composition of the gut microbiota in rosacea subjects and the putative role of the blood microbiota in comorbid diseases, there may be a connection between the gut, blood, and skin inflammation.

The objective of this study was to detect differences in the blood microbiota between Korean female rosacea subjects and rosacea-free controls.

In 2014, this study was performed to assess the differences in the blood microbiota between rosacea and rosacea-free subjects. We selected age- and body mass index-matched groups of rosacea patients and control subjects from a total of 282 female subjects. Rosacea patients were identified based on a history of rosacea or a definite manifestation of rosacea. Individuals with a history of antibiotic use within 6 weeks or probiotic use within 4 weeks, a history of diabetes mellitus, or a low glomerular filtration rate (<60 mL/min) were excluded ($n = 23$). Ultimately, 10 subjects with rosacea and 30 controls were included.

The blood samples were collected by peripheral vein puncture in sterile conditions after cleansing skin with an alcohol swab. One purple-top (EDTA) vacutainer containing whole blood was collected from each subject and immediately stored at -4°C . On blood collecting, genomic DNA was extracted from whole blood samples with the use of a commercial isolation kit (G-DEX IIb Genomic DNA Extraction Kit for Blood; iNtRON Biotechnology, Korea) according to the manufacturer's protocols. The buffy coat consisting mainly of leucocytes was used for DNA extraction from blood samples. Variable V3 and V4 regions of the 16S rRNA gene were amplified with the universal primers 341F (5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3') and 805R (5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G GA CTA CHV GGG TAT CTA ATC C 3'), with each primer modified to contain a unique 8-nt barcode index by combination with the Nextera XT DNA Library Preparation kit (Illumina, San Diego, CA, USA). The PCR reactions contained 5 ng/ μL DNA template, 2 $\times$ KAPA HiFi Hot-Start Ready Mix (KAPA Biosystems, Wilmington, MA, USA), and 2 pmol of each primer. The reaction conditions consisted of an initial incubation at 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension incubation at 72°C for 5 min. After PCR clean-up and index PCR, sequencing was performed on the Illumina MiSeq platform according to the manufacturer's specifications [12, 13].

Fig. 1. Diversity assessment of the blood microbiota in rosacea. **a** The alpha diversity between rosacea patients and rosacea-free controls, measured as Faith's phylogenetic diversity (Faith's PD), was of marginal significance ($p = 0.09$). **b** The beta diversity between rosacea patients and controls was calculated with weighted (left) ($p = 0.01$) and unweighted (right) ($p = 0.07$) UniFrac metrics. Accordingly, rosacea patients' microbiotas were more similar to those of other rosacea patients than to those of controls.

The DADA2 pipeline [14] within the QIIME2 package (<https://qiime2.org>) [15] was used to filter low-quality and chimera errors and generate unique sequence variants. Because the "operational taxonomic units (OTUs)" resulting from DADA2 are created through the grouping of unique sequences, these are the equivalent of 100% OTUs, and are generally referred to as sequence variants. QIIME2 was used to construct the FeatureTable, which is the equivalent of the BIOM table and the representative sequence file. The sequencing depth ranged from 6,554 to 136,763 reads per sample (total frequency = 1,415,559) and 9,705 features. For diversity analysis, we rarefied the data to 5,000 sequences per sample. The sequences were mapped at 99% sequence identity to an optimized version of the GreenGenes database (version 13.8) containing the V3-V4 region to determine taxonomies.

Exploratory and differential microbial composition analyses were conducted in QIIME2, version 2017.10. We used Shannon's diversity index, which aims to measure diversity by accounting for evenness and richness [16]: evenness, which measures community evenness, and the "observed OTUs," which reflect the actual number of different taxa observed in a sample. Additionally, alpha diversity was measured with a phylogenetic diversity measurement, Faith's phylogenetic diversity, often referred to as PD [17]. The Kruskal-Wallis test was used to estimate the differences between the two groups. Unweighted and weighted UniFrac distances [18] were used as a phylogenetic method to determine the dissimilarity between the communities in terms of their membership and structure. A permutational ANOVA (PERMANOVA) pairwise test for the distance matrix was performed via 999 Monte Carlo permutations and false discovery rate multiple correction. Analysis of composition of microbiomes (ANCOM) in QIIME2 was applied to evaluate the differential abundance of bacterial taxa between the sample groups [19].

The rosacea cases and controls were matched for age and BMI and were all females. The erythematotelangiectatic subtype was the most frequent, followed by papulopustular rosacea. In alpha diversity analysis, there was a marginally significant difference ($p = 0.09$) between the rosacea and control groups when Faith's phylogenetic

diversity was used, whereas other alpha diversity indexes (observed OTUs, Shannon's diversity index, and evenness) did not differ between the two groups (Fig. 1a; online suppl. Fig. S1; see www.karger.com/doi/10.1159/000496968 for all online suppl. material). In terms of beta diversity distances, the overall blood microbial communities of rosacea patients were distinguishable from those of rosacea-free controls, according to weighted ($p = 0.01$) and unweighted ($p = 0.07$) UniFrac metrics (Fig. 1b). Additionally, on the principal coordinates plot, the blood microbiota from the two groups clustered separately to some extent, especially when delineated by weighted UniFrac metrics (online suppl. Fig. S1).

To analyze the differential abundance of blood microbiota between rosacea patients and controls, we conducted ANCOM. Two bacterial taxa at the family level and 14 bacterial taxa at the genus level differed in abundance between rosacea patients and controls. For the identification of the differential abundance of blood microbiota, the W value is used to evaluate the null hypothesis of two groups having no significant difference in ANCOM. In detail, the higher the W value of a species, the more significantly the species differs across groups [19]. At the family level, it was found that Chromatiaceae (Gammaproteobacteria class, Alteromonadales order, Chromatiaceae family) and Fusobacteriaceae (Fusobacteriia class, Fusobacteriales order, Fusobacteriaceae family) were significantly elevated in rosacea patients (W value >100) (Fig. 2; online suppl. Fig. S2). Regarding blood microbes with high W values (>100) at the genus level, *Rheinheimera* (Gammaproteobacteria class, Alteromonadales order, Chromatiaceae family) was the most significantly enriched in rosacea subjects. The other blood microbiota identified at the genus level were *Sphingobium*, *Tissierellaceae* family unknown genus, *Paracoccus*, *Rhodovulum*, *Marinobacter*, *Chthoniobacteraceae* family unknown genus, *Methanobacterium*, *Armatimonadaceae* family unknown genus, *Clostridiaceae* family unknown genus, *Fusobacterium*, and *Citrobacter* (W value <30) (online suppl. Fig. S2). The significant taxa that rejected the null hypothesis in terms of their differential abundance between the rosacea and control groups in ANCOM are shown in Table 1.

Fig. 2. Relative abundance of blood bacterial taxa at the family level in subjects with or without rosacea. The Chromatiaceae family (a) and Fusobacteriaceae family (b) were enriched in blood samples from rosacea patients.

Table 1. Abundance of blood bacterial taxa at the family level and the genus level in rosacea patients versus controls based on the analysis of composition of microbiomes (ANCOM)

a ANCOM results at the family level						
Kingdom	Phylum	Class	Order	Family		W value
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Chromatiaceae		126
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae		109
b ANCOM results at the genus level						
Kingdom	Phylum	Class	Order	Family	Genus	W value
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Chromatiaceae	<i>Rheinheimera</i>	203
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingobium</i>	26
Bacteria	Firmicutes	Clostridia	Clostridiales	Tissierellaceae		24
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Paracoccus</i>	19
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	<i>Marinobacter</i>	11
Bacteria	Verrucomicrobia	Spartobacteria	Chthoniobacterales	Chthoniobacteraceae		11
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Rhodovulum</i>	9
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanobacterium</i>	7
Bacteria	Armatimonadetes	Armatimonadia	Armatimonadales	Armatimonadaceae		5
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		5
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	<i>Fusobacterium</i>	5
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Citrobacter</i>	5

We found that rosacea was related to an aberrant blood microbiota composition. Also, the blood microbiota of rosacea patients was distinct from and more diverse than that of controls. Fusobacteriaceae and Chromatiaceae were prevalent at the family level, and at the genus level, *Rheinheimera* was the most significantly abundant in rosacea patients' blood. To the best of our knowledge, ours is the first study using a metagenomics approach to detect alterations in the blood microbiota of rosacea patients.

There has been increasing interest in *Fusobacterium* residing in the colon because *Fusobacterium* acts as an immunologic trigger in inflammatory diseases. It is known that *Fusobacterium*, which is Gram-negative bacteria, is indigenous to the oral cavity and can be readily found in the colon under inflamed conditions [20]. In a study where *Fusobacterium* and relevant inflammatory markers

were investigated in subjects with colorectal adenoma, interleukin (IL)-10 and TNF- α were elevated in the colonic mucosa in proportion to the abundance of *Fusobacterium* in the colon, and IL-6 and IL-17 levels exhibited a statistically insignificant positive correlation with the abundance of *Fusobacterium*. Growing inflammation associated with *Fusobacterium* colonization seems to lead to the development of colorectal adenoma. As such, *Fusobacterium* may contribute to colorectal carcinogenesis [21]; indeed, *Fusobacterium* was enriched in colorectal cancer tissue compared with normal colonic tissue [22]. Whether *Fusobacterium* in the bowel can translocate into the bloodstream is uncertain so far. However, given that *Fusobacterium varium*, which inhabits the enteral mucosa, was highly detected in colon tissue samples from patients with active ulcerative colitis (UC), along with increasing serum antibody lev-

els of *F. varium*, a link between UC and *Fusobacterium* remains plausible [23]. The association between rosacea and UC has been well established by previous studies, based on the increased prevalence of UC in rosacea patients and the shared genetic background between rosacea and inflammatory bowel disease [10, 24]. In this regard, *Fusobacterium* may account for blood dysbiosis as well as enteral inflammation accompanied with rosacea, and thus may be a link between rosacea and UC.

Chromatiaceae, which is Gram-negative purple sulfur bacteria, inhabits aquatic anaerobic environments and requires sulfur compounds for anoxygenic photosynthesis [25, 26]. *Rheinheimera*, which belongs to Chromatiaceae, is a genus of oxidase- and catalase-positive, aerobic bacteria that is distributed in water, soil, and plants [27]. The pathophysiological roles of these bacteria in humans have not yet been elucidated. Nevertheless, it is presumable that alterations in the composition of the candidate blood microbiota stimulate inflammatory mechanisms, since the outer membrane of Gram-negative bacteria is composed of lipopolysaccharide. Lipopolysaccharide elicits innate immune responses via Toll-like receptor 4 and is associated with various chronic inflammatory diseases, including atherosclerosis, arthritis, and cancer [28].

We previously observed that changes in the gut microbiota were associated with rosacea. In the intestine, Peptococcaceae family unknown genus and *Methanobrevibacter* were reduced and *Acidaminococcus* and *Megasphaera* were elevated in rosacea subjects compared to rosacea-free individuals, even after adjustment for covariates [11]. Contrary to what was anticipated, the gut bacteria linked to rosacea differed from the blood bacteria that were altered in rosacea subjects in this study. As indicated in a previous study investigating blood and gut dysbiosis in patients with liver failure, bacterial profiles can differ between the blood and the gut despite the correlation between the two [7]. Moreover, differences in the study design and rosacea subjects may have influenced the study results.

For now, only a few investigations into blood microbial genomes in dermatologic diseases have been conducted. In a case-control study of hidradenitis suppurativa, there was no different bacterial composition in peripheral blood between affected and healthy individuals. Therefore, it was concluded that bacteremia does not seem to contribute to systemic symptoms in hidradenitis suppurativa patients [29]. However, the study has several limitations, including the small sample size, and thus warrants further study.

Our study had several limitations: the small sample size and the uncertain cause-effect relationship between certain bacteria and rosacea.

To conclude, our study suggests that changes in the blood microbiota are associated with rosacea. Although these results warrant validation in experiment-based and/or larger longitudinal studies, our use of a novel technology has broadened our perspective on the pathogenesis of rosacea and may contribute to future diagnostic and therapeutic efforts.

Key Message

Changes in the blood microbiota are associated with rosacea in Korean females.

Acknowledgments

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The data sets used and/or analyzed during the current study are available from the Clinical and Omics data archives (CODA) in the Korea National Institute of Health (KNIH). The raw 16S rRNA gene sequencing data sets (fastq files), and age and gender information per sample are available in the public repository, CODA (Clinical and Omics data archives, <http://coda.nih.go.kr/coda/frt/index.do>, accession number: R000635) at the KNIH.

Statement of Ethics

This study protocol was approved by the Institutional Review Board at Kangbuk Samsung Hospital (IRB number: 2013-01-245-12). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Disclosure Statement

The authors have no conflict of interest to declare.

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Author Contributions

Yeonjun Yun, Han-Na Kim, and Jae-Hui Nam analyzed the data and wrote the manuscript. Jae-Hui Nam proposed the study and approved the final paper. Yunho Lee and Won-Serk Kim assisted with data acquisition and reviewed the article. Hocheol Shin, Yoosoo Chang, and Seungho Ryu designed the original form of the study and collected the data. Hyung-Lae Kim aided in the interpretation of the data.

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