Changes in oral microbiota after the initiation of chemotherapy in patients with hematopoietic tumors

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Abstract

Background

Recently, the gut microbiota has been shown to play an important role in the response and resistance to chemotherapy. Although there is much knowledge about chemotherapy-induced changes in the gut microbiota, chemotherapy-associated changes in the oral microbiota remain unclear. Herein, we aimed to evaluate the changes in oral microbiota associated with the initiation of chemotherapy in patients with malignant hematopoietic tumors.

Methods

Oral samples were collected before and 8–20 days after the start of chemotherapy from 50 patients with malignant hematopoietic tumors who were starting chemotherapy for the first time. The 16S ribosomal RNA gene sequencing of bacterial DNA extracted from oral samples was performed to compare the oral microbiota before and after the initiation of chemotherapy.

Results

The richness or evenness of diversity in the ‘after start of chemotherapy’ group decreased significantly, compared with the ‘before start of chemotherapy’ group (alpha-diversity; observed operational taxonomic units (OTUs) index, $p < 0.001$; and Shannon’s index, $p < 0.001$). The overall salivary microbiota structure between the pre- and post-chemotherapy groups differed significantly (beta-diversity; unweighted UniFrac distances, $p = 0.001$; and weighted UniFrac distances, $p = 0.003$). Linear discriminant analysis effect size analysis demonstrated an increased abundance of species of certain genera, such as *Staphylococcus*, and decreased abundance of species of some genera, such as *Streptococcus* and *Neisseria*, in the ‘after-chemotherapy’ group, compared with those in the ‘before-chemotherapy’ group. The amounts and trends of change in the oral microbiota before and after the start of chemotherapy differed among the subjects. Of the 25 bacterial genera whose prevalence changed significantly before and after the start of chemotherapy, the proportion of oral commensals such as *Streptococcus* and *Neisseria* decreased in many subjects. In contrast, *Staphylococcus* and *Pseudomonas* were detected only in a few subjects, but their relative abundance increased significantly after the start of chemotherapy.

Conclusions

The oral microbiota of patients with hematopoietic tumors changed markedly after the initiation of chemotherapy. Our findings are expected to aid the elucidation of the pathogenesis of oral mucositis, which is an adverse event of chemotherapy, and the development of treatment methods for this condition.

Background

On the surface of our bodies, there are far more microorganisms than the number of cells that constitute our bodies [1]; these microorganisms represent the human microbiota. Recent advances in microbial research have revealed that symbiotic relationships between commensal microbiota and the host are involved in various diseases and health conditions. Since imbalance of the gut microbiota caused by cancer therapy and chemotherapy plays an important role in the response to treatment and susceptibility to side effects [2, 3], the microbiota of the gastrointestinal tract influences the efficiency of treatment of malignancies [4, 5]. In addition, it has recently been shown that the gut microbiota may be able to predict the risk of systemic infections after chemotherapy for hematopoietic tumors [6]. The microbiota in the oral cavity, which represent the second largest population of commensal microorganisms after the gut, may also influence the therapeutic effects of chemotherapy; however, chemotherapy-induced changes in the oral microbiota are not fully understood.

Approximately 700 species of bacteria have been identified in the oral cavity, and the number of bacteria per gram of plaque wet weight is 10^{11} , which is comparable to the number of bacteria in 1 g of feces. If plaque control was poor, the number of bacteria increased further. Specific oral bacterial species are associated with systemic diseases, such as atherosclerosis and various malignancies [7, 8]. In recent years, it has been reported that the overall bacterial composition, including symbiotic and pathogenic bacteria in the oral cavity, i.e., the oral microbiota, is closely related not only to oral diseases, but also to systemic diseases such as diabetes, atherosclerosis, and malignancies such as pancreatic and colon cancer [9, 10]. We have established a method for assessing oral microbiota [11] and used this method to clarify the relationship between oral microbiota and atherosclerotic diseases [12] and type 2 diabetes mellitus [13]. Since the oral microbiota helps maintain homeostasis by preventing foreign pathogens from entering the body, disruption of the oral microbiota may lead to the colonization of pathogens and may be closely related to systemic conditions.

Chemotherapy for malignant tumors has long been known to alter the oral bacterial environment [14]. However, there is a lack of studies using sensitive, high-throughput methods to characterize the oral microbiota during chemotherapy. Recent advances in next-generation sequencing (NGS) and bioinformatics technologies, which easily collect and analyze a large amount of sequence data, hundreds to thousands of times larger than conventional methods, have made it possible to comprehensively evaluate the composition of microorganisms, including those that have not been detected by conventional culture methods or specific evaluation methods for specific bacteria. The oral environment of patients with hematopoietic malignancies is significantly altered by chemotherapy, causing adverse events such as oral dryness and oral mucositis, which are clinical problems associated with the use of chemotherapy [15]. Since the oral microbiota may be associated with these adverse events [16, 17], it is necessary to elucidate the changes in the oral microbiota caused by chemotherapy in patients with hematopoietic tumors in detail.

In the present study, we aimed to comprehensively evaluate the changes in oral microbiota associated with the start of chemotherapy in patients with hematopoietic malignancies via the metagenomic analysis of 16S ribosomal RNA (16S rRNA) using NGS.

Materials And Methods

Participants

The present study was conducted in accordance with the Declaration of Helsinki and its latest amendments and was approved by the Ethics Committee of Osaka Medical and Pharmaceutical University (approval no. 2145) and Chugoku Central Hospital of the Mutual Aid Association of Public School Teachers (approval no. 1905-02). Written informed consent was obtained from all participants.

The study participants included 50 patients with hematopoietic tumors, including 14 patients with acute myelogenous leukemia, 24 patients with malignant lymphoma, 3 patients with acute lymphocytic leukemia, 7 patients with multiple myeloma, and 2 patients with myelodysplastic syndromes, starting chemotherapy for the first time among patients who were diagnosed at the Department of Hematology of Chugoku Central, referred from the Department of Dental Surgery for Perioperative Oral Function Management between June 2019 and July 2020. Table 1 shows the baseline characteristics of the study population.

Table 1
Baseline characteristics of the study population (n = 50)

Variables	
Characteristics	
Age (years, median (range))	70 (16–86)
Sex (female / male)	17 / 33
Underlying disease	
Acute myelogenous leukemia	14
Malignant lymphoma	24
Acute lymphocytic leukemia	3
Multiple Myeloma	7
Myelodysplastic syndromes	2
Oral condition	
Number of teeth (median (range))	22 (0–32)
Denture wearing (%)	32.0
Sevier periodontitis (%)	14.0
Oral mucositis after chemotherapy (%)	10.0

Oral Sample Collection And Oral Examination

Oral samples were collected from the participants before the start of chemotherapy and 8 to 20 days after the start of chemotherapy using the oral rinse method, which we have previously reported [11]. Briefly, the participants were instructed to gargle 5 mL of isotonic sodium chloride solution (Fuso Pharmaceutical, Osaka, Japan) for 10 s and then spit the solution into sterile tubes. All samples were immediately frozen. After collecting the oral samples, the oral conditions were examined. After the start of chemotherapy, 5 of 50 subjects had oral mucositis grade 2 or higher, as assessed based on the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

Dna Extraction, 16s Rrna Sequencing, And Taxonomic Classification

DNA extraction, 16S rRNA sequencing, and taxonomic classification were performed as described previously [11], with some modifications. Briefly, the oral samples were homogenized with glass beads

using a homogenizer (Disruptor Genie, Scientific Industries, Bohemia, NY, USA). Bacterial genomic DNA from the homogenized samples was extracted using GENE PREP STAR PI-480 (Kurabo Industries Ltd., Osaka, Japan), according to the manufacturer's instructions. The V1–V2 region of the 16S rRNA gene was amplified, and each library was prepared in accordance with the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). DNA was sequenced for 500 cycles using MiSeq Reagent Kit v2 (Illumina). An average of 36,211 sequence reads with 250-bp paired ends were denoised and de-replicated, and amplicon sequence variants were counted using the DADA2 module in Quantitative Insights into Microbial Ecology 2 (QIIME2) version 2020.2. After quality filtering, 3,032,209 sequences were obtained, with a mean of 30,322 sequences per sample (min: 14,401; max: 60,742). A rarefaction minimum depth cut-off was chosen at 10,000, after which all samples were retained for downstream analysis. Taxonomies were assigned to the final sequences using the Silva 132 reference database.

Statistical analysis

Statistical analysis was performed by modifying some of the methods used in our previous study [12]. The within-subject alpha diversity of bacterial communities was assessed using Shannon's index and the observed operational taxonomic unit (OTU) index. The Kruskal–Wallis test was used for comparisons between the groups. Between-subject beta diversity was assessed based on the Bray–Curtis dissimilarity and unweighted and weighted UniFrac distance metrics [18]. To visualize global differences in the microbiota structure determined by UniFrac analysis, we performed principal coordinate analysis (PCoA). The significance of compositional differences between the groups was assessed using permutational multivariate analysis of variance (PERMANOVA). QIIME2 software was used for these analyses.

The linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used to detect differentially abundant genera among the two groups [19]. All analyses were performed with the α parameter of the LEfSe for pairwise tests being set to 0.05, and the threshold of the logarithmic score for LDA analysis was set to 3.0. Changes in oral microbiota before and after the start of chemotherapy were evaluated based on the Aitchison distance. The changes in each bacterial genus before and after treatment were evaluated by corresponding analysis of variance (RM ANOVA). R ver. 4.0.3 (2020-10-10) was used for these analyses.

Results

Microbiota composition

Salivary bacteria with a relative abundance of at least 0.1% in both groups were classified into 12 phyla, 19 classes, 38 orders, 74 families, and 128 genera. The predominant bacteria (> 1% of the total sequences in either group) at the phylum level were Firmicutes and Bacteroidetes, followed by Actinobacteria, Proteobacteria, and Fusobacteria, comprising 95.79% and 95.91% of the salivary microbiota in the before and after starting chemotherapy groups, respectively (Fig. 1A). At the genus level, the before group was

characterized by 111 genera, of which 12 were absent from the after group, whereas the after group included 114 genera, of which 13 were absent from the before group. A total of 37 genera were present in at least 50% of the subjects in both groups, with 22 genera being common to both groups. The predominant bacteria at the genus level (> 10% of the total sequences in either group) included *Streptococcus*, with mean relative abundances of 18.43% and 24.61%, respectively, and *Prevotella* 7, with mean relative abundances of 12.34% and 10.43%, in the before and after groups, respectively. The 20 most abundant genera in the before and after groups accounted for 86.57% and 86.64% of the total abundance of the genera, respectively (Fig. 1B).

Differences in diversity before and after the start of chemotherapy

Analysis of alpha diversity showed that the abundance and evenness of oral bacteria decreased significantly in the post-chemotherapy group, compared to the pre-chemotherapy group (OTU index, $p < 0.001$; Shannon index, $p < 0.001$; Fig. 2A). PCoA with unweighted and weighted UniFrac distances based on UniFrac analysis showing beta diversity demonstrated that the oral microbiota differed between the pre- and post-chemotherapy groups, although there were differences between individuals. This difference was confirmed by PERMANOVA (unweighted UniFrac distance, $p = 0.001$; weighted UniFrac distance, $p = 0.003$; Fig. 2B, C).

Oral bacteria with different abundance before and after the start of chemotherapy

Oral bacteria with different prevalence levels before and after the start of chemotherapy were identified by LEfSe analysis. The cladogram represented the taxa that differed significantly between the two groups in a taxonomic hierarchy from phylum to genus (Fig. 3A). At the phylum level, Proteobacteria was significantly more common in the pre-chemotherapy group, and Firmicutes was more common in the post-chemotherapy group (Fig. 3B). At the genus level, *Streptococcus* and *Neisseria* were more common in the pre-chemotherapy group, while *Staphylococcus* and *Rothia* were more common in the post-chemotherapy group (Fig. 3C). These results indicate that there are significantly different bacteria in the oral cavity before and after the initiation of chemotherapy.

Changes in the oral microbiota of each subject before and after the start of chemotherapy

The amount of change in the abundance of oral microbiota before and after the start of chemotherapy was evaluated for each subject by PCoA based on the Aitchison distance. The results showed that the amounts and the trends of change in the oral microbiota were not constant at the genus and species levels (Fig. 4).

Changes in the abundance of specific bacteria before and after the start of chemotherapy

Twenty-five bacterial genera showed significant changes in their abundance before and after the start of chemotherapy (RM ANOVA test, $p < 0.01$). Among these genera, some genera, such as *Streptococcus*, *Neisseria*, *Fretibacterium*, and *Kingella*, showed a decrease in relative abundance in most subjects (Fig. 5A), while some genera, such as *Filifactor*, *Aggregatibacter*, and uncultured *Eubacterium* E1-K9, were

detected in only a few subjects, but their relative abundances decreased after the start of chemotherapy (Fig. 5B). The relative abundances of some genera, such as *Staphylococcus* and *Pseudomonas*, which were only detected in some subjects, increased significantly after the start of chemotherapy (Fig. 5C).

Discussion

In the present study, we characterized the changes in the oral microbiota associated with the initiation of chemotherapy for hematopoietic malignancies by 16S rRNA metagenomic analysis. The overall diversity of the oral microbiota decreased after chemotherapy, and the bacterial composition changed after the start of chemotherapy. A decrease in the diversity of the oral microbiota due to chemotherapy for hematopoietic malignancies has been reported in children with leukemia [20] and in patients undergoing autologous hematopoietic stem cell transplantation [21], which is consistent with the results of our study. Furthermore, recent reports have shown that changes in the diversity of not only the gut microbiota [22], but also the oral microbiota, during chemotherapy for leukemia are associated with the risk of infection during treatment [23, 24]. These facts suggest that a more comprehensive study of changes in the oral microbiota during chemotherapy in patients with hematopoietic tumors may help predict the risk of infection, which represents an adverse event during chemotherapy.

The bacterial genera whose abundance decreased significantly with the initiation of chemotherapy in most of the subjects in this study were *Streptococcus* and *Neisseria*, and many bacteria belonging to these genera were the predominant oral commensals. The abundance of *Staphylococcus* and *Pseudomonas*, which has been reported to increase with chemotherapy in many studies [14], was also found to increase in this study. These genera are noteworthy because they are considered pathogenic genera with a low proportion of commensal presence; pathogens from these genera include *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are associated with opportunistic infections. However, to focus on specific bacterial species within the large changes in the oral microbiota associated with chemotherapy, it is necessary to use other methods of analysis, such as metagenomic shotgun sequencing.

Chemotherapy for patients with hematopoietic malignancies causes oral-related adverse events such as oral mucositis, cheilitis, xerostomia, and taste disorders [25, 26]. Oral mucositis can cause severe pain, eating disorders, and sleep disturbances, which may affect the completion of chemotherapy [27]. However, the causes and pathogenesis of oral mucositis remain unclear [15]. Microbiota have been reported to play an important role in chemotherapy-induced mucosal damage in the digestive tract [28, 29]. The characteristics of the oral microbiota involved in chemotherapy-induced oral mucositis have recently been reported [17, 30]. In the present study, five of 50 patients had oral mucositis 8–20 days after starting chemotherapy, and we could not detect any characteristic changes in the oral microbiota in these five patients. The characteristics of the oral microbiota associated with the incidence and severity of oral mucositis, and the causes of its occurrence, require further studies.

Because this study aimed to evaluate changes in the oral microbiota after the initiation of chemotherapy in a wide range of conditions, including patients with hematopoietic malignancies who received usual oral care treatment rather than a single disease, there are limitations associated with the discussion of our study's results. In the future, further evaluation of the effects of chemotherapy on the oral microbiota will be possible by studying local and systemic factors, as well as factors such as the type of medication, in a more homogeneous cohort.

In conclusion, we characterized the changes in the oral microbiota associated with the initiation of chemotherapy in patients with hematopoietic tumors. The results of this study suggest that focusing on the oral microbiota is crucial for understanding the pathogenesis of chemotherapy-associated adverse events such as oral mucositis. These findings will contribute notably towards the prediction, prevention, and amelioration of adverse events in cancer patients that are undergoing chemotherapy.

Declarations

Ethical approval: All procedures conducted in studies involving human participants were performed in accordance with the ethical standards of the Ethics Committee of Osaka Medical College, approval no. 2145, Chugoku Central Hospital of the Mutual Aid Association of Public School Teachers, approval no. 1905-02, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no conflict of interest.

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Author Contributions: All authors contributed to the conception and design of the study. Material preparation, data collection, and analysis were performed by Michi Omori, Nahoko Kato-Kogoe, Shoichi Sakaguchi, Eri Komori, Kazuya Inoue, Kayoko Yamamoto, Tomoyoshi Hayase, and Tomoyuki Tano. The first draft of the manuscript was written by Eri Komori, and all authors have commented on the previous versions of the manuscript. All authors have read and approved the final manuscript.

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Figures

A Phylum levels

B Genus levels

Fig. 1

Figure 1

Taxonomic composition of oral microbiota before and after the start of chemotherapy Vertical bar plot showing the relative abundance of bacterial phyla (a) and genera (b). (a) Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria were the five most abundant phyla in all subjects, comprising 95.79% and 95.91% of the total bacterial communities before and after starting chemotherapy, respectively. (b) Bar plot of the 20 most abundant genera before and after starting chemotherapy.

Fig. 2

Figure 2

Alpha and beta diversity before and after the start of chemotherapy (a) Alpha diversity, observed operational taxonomic unit (OTU) index, and Shannon's index in the before (green) and after (red) starting chemotherapy groups, are shown as box-and-whisker plots. p values, as calculated by Kruskal-Wallis test, are shown. Beta diversity: unweighted UniFrac distances (b) and weighted UniFrac distances (c) are shown. Principal coordinate analysis (PCoA) plots and box plots for the before chemotherapy (green) and after (red) starting chemotherapy groups. The boxplots represent the UniFrac distances before and after starting chemotherapy groups, relative to the before group. p values, as calculated by PERMANOVA, are shown.

Histogram of the linear discriminant analysis (LDA) scores for differentially abundant bacterial taxa at phylum level (b) and genus level (c) between the before (green) and after (red) starting chemotherapy groups. LDA scores ≥ 3.0 are shown.

A Genus levels

B Species levels

Fig. 4

Figure 4

Changes in the oral microbiota of each subject before and after the start of chemotherapy Principal coordinate analysis (PCoA) plot of Aitchison distance at the genus level (a) and species level (b). PCoA

plots showing paired-sample changes in the microbiota composition before and after starting chemotherapy. Each color represents an individual participant, with the before starting chemotherapy sample (green) linked to the after starting chemotherapy sample (red) by a line.

Fig. 5

Figure 5

Changes in the abundance of specific bacteria before and after the start of chemotherapy (a) Changes in four representative bacterial genera whose abundances changed significantly before and after the start

of chemotherapy are shown. p values for RM ANOVA are shown. (b) Changes in three representative bacterial genera that are only detected in some subjects but whose abundances decreased significantly after the start of chemotherapy are shown. p values for RM ANOVA are shown. (c) Changes in two representative bacterial genera that are only detected in a few subjects but whose abundances increased significantly after the start of chemotherapy are shown. p values for RM ANOVA are shown.