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CASE REPORT

Analysis of subgingival microbiota in monozygotic twins with different severity and progression risk of periodontitis

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Abstract

The study aims to reveal the composition of subgingival bacteria in monozygotic twins with discordant in severity and progression risk of periodontitis. Microbiome analysis indicated that most bacteria were heritable but differed in their abundance and immune response. The dysbiotic bacteria can be considered as risk markers for periodontitis progression.

KEYWORDS

disease progression, dysbiosis, environmental factors, microbiome, monozygotic twins, periodontitis

1 | INTRODUCTION

Monozygotic twinning is a rare event that occurs in 1 per 250 live births in Japan. This type of twin formation originates with one zygote that undergoes independent mitotic divisions. The two embryos that result develop independently and are birthed together.[1] Twins are useful for investigating the effects of genetic and environmental factors on disease etiology since they are considered genetically identical. Previous studies that examined systemic diseases in monozygotic twins have indicated that genetic factors are not the major causes of chronic diseases, such

as prominent cancers, cardiovascular diseases, neurologic diseases, lung diseases, and autoimmune diseases.[2]

Periodontitis is a chronic inflammatory disease caused by oral polymicrobial infection. The disease severity is influenced by genetic and environmental factors. These risk factors can change the composition of the microbiota, resulting in disruption of the normal balance between oral commensal flora and the host innate immune system, which is termed dysbiosis.[3,4] Heritability estimates of periodontitis in twins were approximately 35%–45%, but these rates were lower for older twins.[5,6] The discordance for periodontitis between twins is significantly influenced by non-shared environmental factors, such as differences in oral microbiomes, smoking habits, body mass index (BMI), prosthetic treatment, and the mental health of each sibling.[7–9] However, the intricate microbial pathogenesis of periodontitis remains unclear.

Younger twins raised together are less impacted by exposure to epigenetic modification and non-shared environmental factors. Recently, next-generation sequencing (NGS)-based studies have indicated that certain bacterial species are inherited. However, environmental and behavioral factors are influential in determining the composition of supragingival plaque microbiomes in young twins.[10,11] Therefore, NGS analysis of the subgingival microbiome in young adult twins displaying differences in periodontal severity, albeit rare, may be valuable to further characterize the microbial and environmental factors for progressing periodontitis.

In this study, we examined three young adult siblings raised together, including a pair of twins. One of the twins developed mild periodontitis, while the other twin and their elder brother had severe periodontitis. The purposes of the study were to reveal dysbiotic bacteria associated with the severity and risk of progression of periodontitis in the siblings, particularly in the twin, and to assess the link between the microbiome and clinical classification of periodontitis. Comprehensive microbial analysis using NGS clarified the differences in microbiome profiles and estimated the potential microbial risk markers that could be used to track the progression of periodontitis.

2 | CASE PRESENTATION AND STUDY METHODS

2.1 | Patients

Japanese female monozygotic twins (#1 and #2; 36 years old) and their older brother (39 years old) were enrolled in this study. They were referred to the Department of Periodontics and Endodontics in Okayama University Hospital for treatment of periodontitis characterized by multifocality gingival swelling. All three individuals were assessed in medical interviews. None of the patients had received any periodontal therapy in the preceding 6 months, or any systemic or local antibiotics in the preceding 3 months. Both the twins had smoked approximately 10 cigarettes per day for the last 18 years. Their brother had never smoked. Notably, twin #2 previously smoked more than 10 cigarettes per day until she had her first child at the age of 23. Their father had suffered from severe periodontitis since his 40s. The twins had been raised together until they were 17 years old but were separated after each twin married. Both twins have three children without any sign of systemic disease, whereas the brother is single. Twin #1's medical history includes scleritis, Hashimoto's thyroiditis, and dysthyroid ophthalmopathy. She has taken prednisolone for the last 5 years. Twin #2 and the brother do not have systemic disease. The BMI of all three is slightly elevated, and they are classified as "pre-obese" according to the World Health Organization criteria.

2.2 | Clinical oral examinations

Each patient received a full-mouth periodontal examination, including the examination of dental X-rays. The periodontal inflamed surface area (PISA)[12] was calculated using periodontal pocket depth (PD) and bleeding on probing (BOP) measurements at six sites per tooth. PISA reflects the surface area of the bleeding pocket epithelium (mm^2). The ratio of bone loss (%) was determined from the measured distance between the cement-enamel junction and the bone crest to the whole root length that measures the cement-enamel junction to the root apex. The bone loss was measured from available radiographs using a Schei ruler.[13] Tooth mobility was assessed by the Miller mobility index.[14]

Full mouth periodontal examination (Table 1) and intra-oral and X-ray images (twin #1, Figure 1A; twin #2, Figure 1B; brother, Figure S1) revealed abundant plaque accumulation and plaque-induced gingival inflammation. The inflammation was relatively mild in twin #1 (PISA = 575.5 mm^2) and severe in twin #2 (2842.8 mm^2) and in the older brother (1192.7 mm^2). Advanced alveolar bone loss was observed in twin #2 (46.6% bone loss) and in the brother (31.0%). The brother used removable partial dentures. In contrast, twin #1 displayed mild horizontal bone loss (22.3%), except for one fractured tooth. The number of teeth with mobility (classification 2 and 3) was significantly higher in twin #2 (31.0%) compared with that in twin #1 (3.8%) and the brother (10.5%). Enamel hypoplasia was observed on the lower incisors of the twins.

TABLE 1 Clinical data for periodontal diagnosis and location of sampling sites

	Age (years)	Full mouth							Sample sites		
		Total teeth (*n*)	Mean PD (mm)	4–6 mm PD (%)	PD>6 mm (%)	BOP (%)	PISA (mm^2)	Bone loss (%)	Bone loss/ Age	Diagnosis (Stage/Grade)	Site	PD (mm)	Bone loss (%)
Twin #1	36	26	2.6	16.0	1.3	37.2	575.5	22.3	0.62	II/B	14	5	10
											19	6	50
Twin #2	36	29	5.3	46.0	33.3	65.5	2842.8	46.6	1.29	IV/C	13	6	50
											31	7	60
Brother	39	19	4.1	12.3	34.2	72.8	1192.7	31.0	0.79	IV/B	10	7	50
											19	5	40

Note: Diagnosis (Stage and Grade) was defined based on the 2018 classification of periodontal diseases. Sample sites were numbered 1–32 according to the Universal Numbering System.

Abbreviations: BOP, bleeding on probing; PD, periodontal pocket depth; PISA, periodontal inflamed surface area.

2.3 | Periodontal disease classification and diagnosis

According to the 2018 classification of periodontal diseases,[15] the stage and grade of periodontitis were classified. Owing to severe bone loss and the need for complex rehabilitation, twin #2 and the brother were classified as Stage IV. Based on the ratio between percentage bone loss over age (twin #1, 0.62; twin #2, 1.29; brother, 0.79), twin #2 was classified as Grade C. As summarized in Table 1 and Table S1, twin #1 was categorized as localized Stage II/Grade B, twin #2 as generalized Stage IV/Grade C, and the older brother as generalized Stage IV/Grade B.

2.4 | Detection of serum IgG antibody titers against periodontal bacteria

Peripheral blood was collected to measure serum IgG antibody titers against periodontal bacteria. The humoral immune response to multiple bacterial species, including *Aggregatibacter actinomycetemcomitans* (Y4, ATCC29523, and SUNY67), *Capnocytophaga ochracea S3*, *Eikenella corrodens* (FDC1073), *Fusobacterium nucleatum* (ATCC25586), *Prevotella intermedia* (ATCC 33563 and ATCC25611), *Porphyromonas gingivalis* (FDC381 and SU63), *Treponema denticola* (ATCC35405), *Campylobacter rectus* (ATCC33238), and *Tannerella forsythia* (ATCC43037), were assessed using an enzyme-linked immunosorbent assay (ELISA) as described previously.[16] Following the formula for clinical use, the mean ±2 standard deviation for the controls, based on the dataset of IgG titers to each pathogen from 10 healthy individuals, was defined as a standard value of 1.[17]

2.5 | Extraction of periodontal bacterial DNA and microbiome analysis

Based on clinical assessments, two non-adjacent diseased teeth with deep PD (ranging from 5 to 7 mm) with BOP were selected for subgingival plaque collection from each patient. After ensuring the absence of supragingival plaque deposits, the sites were isolated from the saliva using cotton rolls and then air-dried. Subgingival plaque samples were collected from the deepest periodontal pockets by inserting three sterile paper points for 30 s in each pocket. The collected samples were pooled into one sample for each patient.[18] Bacterial DNA was extracted from the subgingival plaque using InstaGene™ Matrix (Bio-Rad Laboratories).

An amplicon library was constructed from each bacterial DNA sample *via* PCR-based amplification using

FIGURE 1 Oral photographs and dental X-ray radiographs of the twins at their first visit. Oral photographs (upper) and dental X-ray radiographs (lower) of twin #1 with mild periodontitis (A) and twin #2 with severe periodontitis (B) are shown. Enamel hypoplasia was concordantly observed on the lower incisors in both patients

TaKaRa Ex Taq™ HS (Takara Bio) and specific primers targeting the hypervariable regions V3–V4 of bacterial 16S rRNA genes. Illumina adapter overhang nucleotide sequences were added to the gene sequences. PCR was performed according to the manufacturers' protocol (Illumina). NGS was conducted using the MiSeq® system (Illumina) at the Oral Microbiome Center (Taniguchi Dental Clinic, Kagawa, Japan). Sequence analysis was performed using Quantitative Insights into Microbial Ecology (QIIME) software according to the tutorial at http://qiime.org.[19] The obtained sequence was compared to the expanded Human Oral Microbiome Database (*e*HOMD-14.51; http://www.homd.org/). Principal component analysis (PCoA) and clustering analysis were performed using R statistical software (The R Foundation; www.r-project.org).

2.6 | Ethical consideration

Sera and subgingival bacteria samples are routinely collected for periodontal treatment on the patients to evaluate the infectious status in each treatment phase. The rest of the samples, obtained at the first visit of each patient, were used for this study. The study was approved by the Ethics Committee at Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (No. 1701–010). The study complied with the Helsinki Declaration. Each participant signed an informed consent form.

3 | RESULTS

3.1 | Evaluation of immune response against periodontal bacteria in patients

The three patients had high serum titers of IgG antibody against *P. gingivalis*. The titers were highest in the brother and relatively higher in twin #2 compared with twin #1 (Figure 2). The findings corresponded to the amount of *P. gingivalis* quantified by real-time PCR analysis (data not shown). The twins displayed similar patterns of IgG titers, with, that is, high titers against *P. gingivalis* and low titers against *A. actinomycetemcomitans*, *C. ochracea*, *E. corrodens*, *F. nucleatum*, *P. intermedia*, and *T. denticola*. Notably, Twin #2 had high titers selectively against *T. forsythia* and *C. rectus*.

3.2 | Comparison of subgingival microbiome

To evaluate the relationship between subgingival microbiota and the status of periodontitis, the relative abundance of bacteria among the twins and the brother was

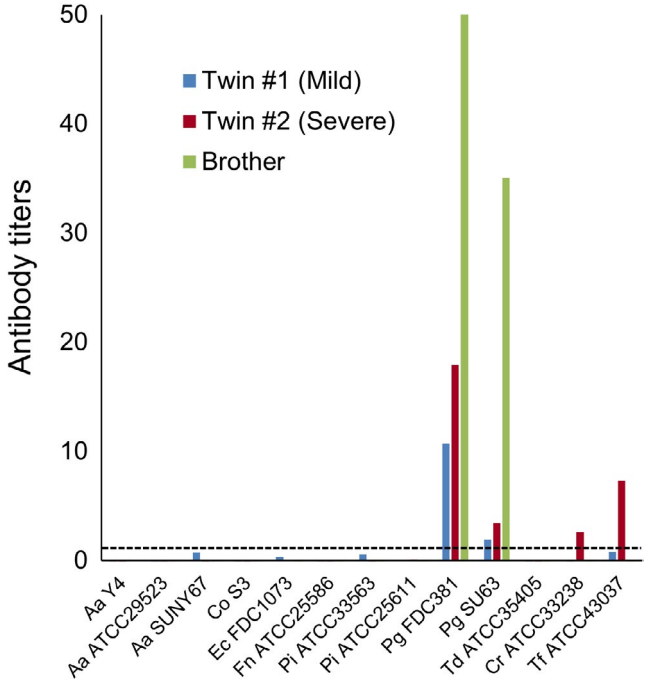

FIGURE 2 Serum IgG antibody titers to periodontal bacteria. Serum IgG antibody titers against 13 types of periodontal bacteria (*x*-axis) were measured using ELISA. The bacteria are as follows: *Aggregatibacter actinomycetemcomitans* (Aa), *Capnocytophaga ochracea* (Co), *Eikenella corrodens* (Ec), *Fusobacterium nucleatum* (Fn), *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg), *Treponema denticola* (Td), *Campylobacter rectus* (Cr), and *Tannerella forsythia* (Tf). The *y*-axis indicates the normalized titer against healthy controls without periodontitis. The dotted line indicates the standard value (=1.0). IgG titers from twin #1 (mild periodontitis; blue), twin #2 (severe periodontitis; red), and the brother (severe periodontitis; green) are shown

compared (Figure 3). In twin #2, a higher percentage of the genera *Treponema* (14.6%; the mean value of two sampling sites) and *Fretibacterium* (14.5%) were observed compared to the genera from twin #1 and the brother. The genus *Tannerella* was not highly abundant but was higher in twin #2 compared to that in twin #1. In contrast, a lower percentage of *Fusobacterium* (13.8%) was observed in twin #2 compared with that from twin #1 (44.7%) and the brother (33.9%). The genus *Porphyromonas* was also lower (2.8%) in twin #2.

3.3 | Inter- and intra-patient diversity of subgingival microbiome and clustering based on grading of periodontitis

To compare the phylogenetic distances among the six plaque samples from each patient, UniFrac-based PCoA (Figure 4A) and hierarchical clustering (Figure 4B) were performed. Based on the unweighted UniFrac analysis (i.e., comparison of microbial community memberships), twin #1 and twin #2 formed a cluster, suggesting that many similar bacterial species were likely inherited. Based on the weighted UniFrac analysis (i.e., quantitative comparison of microbial community structures), twin #1 and the brother formed a distinct cluster and were separated from twin #2. These clusters could be classified as Grades B and C, respectively, in accordance with the periodontal diagnoses.[14] Therefore, the relative abundance of bacteria was compared between the periodontitis grades to assess the risk of periodontal progression based on microbiome compositions (Figure 4C). In Grade B (moderate risk for periodontal disease progression), the genera *Fusobacterium* and *Prevotella* were dominant. In Grade C (high risk for rapid progression), the relative abundance of the aforementioned genera decreased, but the genera *Treponema*, *Fretibacterium*, and *Tannerella* became more dominant. These data indicate microbial dysbiosis in Grade C. Levels of the genera *Porphyromonas* and *Campylobacter* were relatively low (approximately 2%–6%) in both Grades B and C.

4 | DISCUSSION

Our findings from all three patients emphasize the importance of the interplay between the dysbiotic subgingival microbiome and the humoral immune response in determining the status of periodontitis. Notably, high titers against *T. forsythia* and *C. rectus* may, in part, explain the pathophysiology of the aggravated periodontitis in twin #2. These bacteria can synergistically progress periodontitis, along with other periodontal pathogens.[20] Elevated serum IgG titers against *P. gingivalis* indicate the presence of strong antibody response to this bacterium in all patients. The titer was relatively higher in twin #2 compared with that in twin #1. Although only present at a low frequency, *P. gingivalis* is capable of inducing dysbiosis and changing both the amount and composition of the oral microbiome by immune subversion, thereby acting as "keystone pathogen".[4] Some non-shared environmental factors, such as the degree of smoking in twins#2, may have induced the strain and virulence diversity within the *P. gingivalis* population, resulting in the severe dysbiosis in twin #2. Twin #1 had developed only mild periodontitis and had taken prednisolone for the last 5 years. This synthetic corticosteroid is well known for its anti-inflammatory and immunosuppressant properties. Although earlier studies showed that topical application of corticosteroids to inflamed gingiva of patients with periodontitis resulted in a reduction of inflammation, systemic or topical corticosteroids have little impact on the periodontium, disease severity, and risk of progression.[21,22] However, corticosteroids

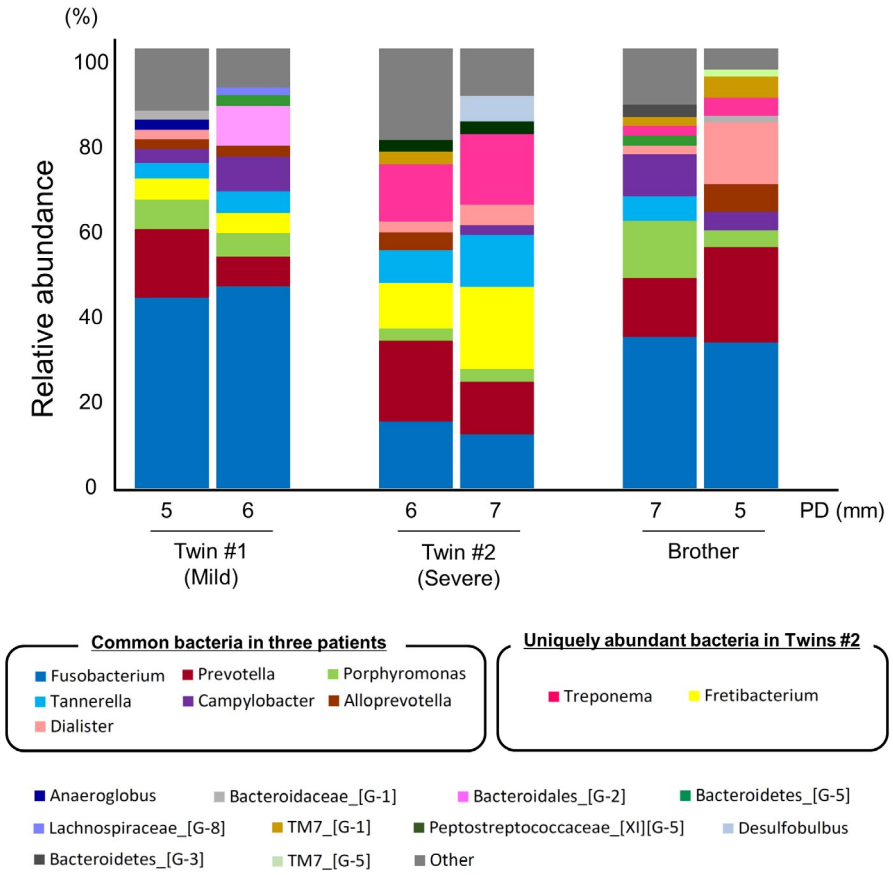

FIGURE 3 Relative abundance of microbiota at the genus level in subgingival plaque from individual periodontal pocket. The members of the top 10 genera are shown as indicated by the colored bars. Each color represents a different genus of bacteria (as indicated in the legend key). The *y*-axis indicates the relative abundance of bacterial genera identified in the periodontal pocket from each patient. Individual pocket depth (PD; mm) is shown on the *x*-axis. Three genera (*Treponema*, pink; *Fretibacterium*, yellow; *Tannerella*, light blue) were elevated in twin #2

may have altered the composition of subgingival microbiota in twin #1.[23] A longitudinal investigation is needed to assess this speculation.

Microbiome analyses on samples from the three patients indicated that a higher percentage of the genera *Treponema* in the phyla *Spirochetes* and *Fretibacterium* in the phyla *Synergistetes* were observed in twin #2, compared with those in twin #1 and the brother. Previous microbiome analyses of severe and progressive periodontitis also indicated that *Spirochetes* and *Synergistetes* are more abundant in this disease.[24,25] The most well-isolated and well-studied oral Spirochete is *T. denticola*, a member of the red-complex bacteria. Since *P. gingivalis* and *T. denticola* exhibit symbiosis and synergistic virulence in subgingival plaques,[20,26] *T. denticola* may have been essential in the immunological pathogenesis of severe periodontitis in twin #2. However, IgG titers to *T. denticola* remained in the normal range, suggesting that the *P. gingivalis*-induced immune subversion to the dysbiotic microbiota may be a crucial pathological event for disease progression in twin #2.

Members of the phyla *Synergistetes* have rarely been detected in healthy subjects. However, they have frequently been detected in subgingival plaques in periodontitis patients.[27] Recent studies reported the association of genera *Fretibacterium* in the phyla *Synergistetes* with severe periodontitis; these bacteria were significantly decreased following periodontal treatment.[28,29] Therefore, *Fretibacterium* could be a diagnostic bacterial biomarker when screening for severe periodontitis.[30]

The unweighted UniFrac analysis, which considers only whether the species are present or absent, showed that twins #1 and #2 belonged to the same cluster. It is intriguing that most bacterial species are still heritable and present as periodontal pathogens in twins. However, the similarities in the abundance of the twins' microbiota did not persist into adulthood. As they have lived apart after marrying, the changes in environmental factors in their different living environments may have affected the composition of their microbiomes. On the one hand, the weighted UniFrac analysis showed that twin #2 was separated from a cluster comprising twin #1 and the brother. Genus-level diversity clearly indicated that the genera *Treponema* and *Fretibacterium* corresponded to overt dysbiosis and may have been crucial in the rapid progression of periodontitis in twin #2 with Grade C.

It is generally accepted that genetic factors play an important role in the disease susceptibility of aggressive periodontitis, which is characterized by young age at onset, rapid progression in otherwise healthy individuals, and familial aggregation,[31] However, the diagnostic criteria for aggressive periodontitis are uncertain, and information on the microbial characteristics of affected individuals is also limited. Therefore, aggressive periodontitis is no longer considered a different disease entity. Rather, it is currently

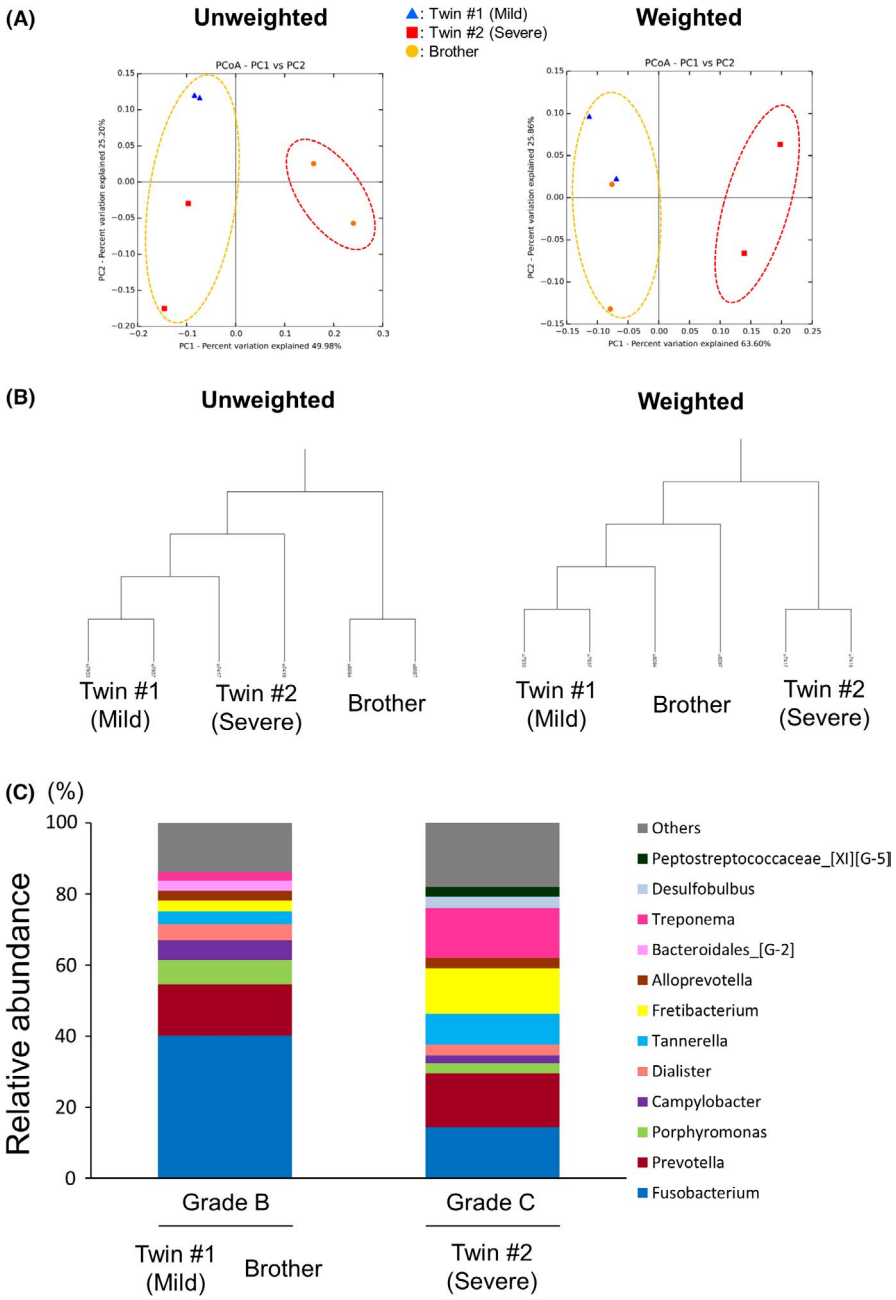

FIGURE 4 Comparison of microbial diversities and diagnostic grading for periodontitis. (A) Principal coordinate analysis (PCoA) of unweighted (left) and weighted (right) UniFrac distances of samples (PC1 vs. PC2). The PCoA results from each sample are tagged as follows: blue triangle: twin #1 (mild); red square: twin #2 (severe); orange circle: brother (severe). (B) Phylogenetic tree dendrogram of the hierarchical clustering, based on unweighted (left) and, weighted (right) UniFrac analysis, illustrates microbiota abundance similarities amongtwin #1 (mild), twin #2 (severe), and brother (severe). (C) The members of the top 10 genera are shown by colored bars. Each color represents a different genus of bacteria, as indicated in the legend key. The *y*-axis indicates the relative abundance of bacterial genera identified in the periodontal pockets from the patients with Grade B (twin #1 and brother) or Grade C (twin #2). Two genera (*Treponema* and *Fretibacterium*) were highly abundant in Grade C compared to Grade B

classified as periodontitis Grade C.[15,32,33] It is conceivable that twin #2 has aggressive periodontitis based on the onset of severe periodontitis (Table S1), even though aspects of her lifestyle, such as smoking, substantially affected the pathogenesis of this disease. Collectively, our data emphasize that complex microbial interactions influenced by genetic and environmental factors may induce the early onset and rapid progression of periodontitis in young patients.

An obvious limitation of this study is that we have investigated the sibling in only one family. Additionally, limited information was available from only two sites of infection within the periodontal pocket in each patient. Therefore, the findings are too limited to draw any definitive correlations between dysbiotic subgingival microbiota and periodontitis progression. Whether changes in the microbiota are a cause or consequence in periodontitis remains to be definitively proven. Future longitudinal and large-scale studies are necessary to identify dysbiotic bacteria that promote the progression of periodontitis and the mechanism underlying immune subversion to the bacteria.

5 | CONCLUSION

Research of young monozygotic twins with different periodontal disease severity will greatly contribute to

clarifying the microbial and environmental risk markers that influence the rapid progression of periodontitis. Components of the subgingival microbiome are mostly inheritable between monozygotic twins. However, each person's microbial abundance varies dramatically due to their non-shared environmental risk factors, which can lead to polymicrobial synergistic dysbiosis resulting in periodontitis. The most important findings in this case report are that the genera *Treponema* and *Fretibacterium* are dysbiotic bacteria associated with the rapid progression of periodontitis (Grade C) in one of the twins. Since monozygotic twins are considered genetically identical, the dysbiotic microbiota could be clinically applied as bacterial risk markers for the progression of periodontitis in the future.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interests.

AUTHOR CONTRIBUTION

TY designed the study, performed the initial examination and patient assessment, reviewed the available literatures, and drafted the manuscript. MT designed the study and performed NGS analysis. KM, YK, MK, and K Okubo involved in bacterial DNA extraction, data collection, and data analysis. KY and K Omori reviewed the literature and revised the drafted manuscript. ST commented on the design of the study and was responsible for editing the final manuscript. All authors read and approved the final manuscript.

CONSENT

Written informed consent was obtained from the patient to publish this report in accordance with the journal's patient consent policy.

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SUPPORTING INFORMATION

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