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Changes in Oral Microbiota in Patients Receiving Radical Concurrent Chemoradiotherapy for The Head and Neck Squamous Cell Carcinoma

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Radiotherapy (RT) is a mainstay in the treatment of head and neck squamous cell carcinoma (HNSCC). For locally advanced HCSCC, concurrent chemoradiotherapy (CCRT) benefits HCSCC patients in terms of better survival and loco-regional control. In this study, we evaluated changes in oral microbiota in patients, who received CCRT for head and neck cancer. Oral rinsed samples were weekly collected before and during CCRT and at 4 weeks following treatment from HNSCC patients, who had received 70 Gy of radiation delivered to the primary sites for over 7 weeks and concurrent chemotherapy. Oral microbiota changes in three patients were analyzed by next-generation sequencing using 16S rRNA 454 pyrosequencing. On an average, 15,000 partial 16S rRNA gene sequences were obtained from each sample. All sequences fell into 11 different bacterial phyla. During early CCRT, the microbial diversity gradually decreased. In a patient, who did not receive any antibiotics during the CCRT, Firmicutes and Proteobacteria were the most abundant phylum. During the early CCRT, proteobacteria gradually decreased while Firmicutes increased. During the late CCRT, firmicutes gradually decreased while Bacteroides and Fusobacteria increased. In all the patients, yellow complex showed a gradual decrease, while orange and red complex showed a gradual increase during the CCRT. At 4 weeks after CCRT, the recovery of oral microbiota diversity was limited. During CCRT, there was a gradual increase in major periodontopathogens in association with the deterioration of the oral hygiene. Henceforth, it is proposed that understanding oral microbiota shift should provide better information for the development of effective oral care programs for patients receiving CCRT for HNSCC.

Key words: Pyrosequencing, Oral microbiota, Head and neck squamous cell carcinoma, Concurrent chemoradiotherapy

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Introduction

Head and neck cancer, over 4,000 new cases predicted in 2014 in Korea [1]. Radiotherapy (RT) is a mainstay in the treatment of head and neck squamous cell carcinoma (HNSCC).

Oral complications that may occur in patients receiving radiotherapy include mucositis, caries, and candidiasis [2, 3]. For locally advanced HCSCC, it was shown that concurrent chemoradiotherapy (CCRT) benefits HCSCC patients in terms of better survival and loco-regional control [4]. However, CCRT for head and neck sites significantly increase severe oral mucositis compared with RT alone. About 40 to 80% of patients undergoing CCRT for HNSCC experience mucositis of grade 3 or higher [5-7]. Although the usage of intensity-modulated radiotherapy (IMRT) reduces the risk of severe xerostomia [8-10], radiation-induced mucositis still remains a major acute toxicity which hampers the delivery of CCRT.

Oral microbiota is an important determinant of oral conditions. The composition of oral flora is determined by dynamic interactions among the oral microorganisms themselves, the host tissues, and the mechanical flushing action and antimicrobial activity of saliva [11]. Any clinical conditions involving oral cavity might be expected to accompany corresponding shifts in oral microbiota. RT in head and neck sites can drastically alter oral environment by disrupting mucosal integrity and reducing saliva secretion [12-15]. Thus, radiation-induced milieu of oral cavity may lead to altered microbial population during RT. However, the role of oral microbiota in pathogenesis of radiation-induced stomatitis is not well established.

Oral microbiota harbors a highly diverse resident community of microorganisms. Varying degrees of overlap of oral microbiota of healthy individuals have been revealed by recent studies of oral microbiome [16]. The conventional culture-based or biochemical methods can identify anticipated bacterial taxa, but lack the capacity to detect non-cultivable microorganisms and the possibility to address hitherto unknown taxa. Modern molecular methods for identifying bacterial taxa have made it possible to assess a bacterial community with a reduction in bias experienced in culture-based methods [17], and furthermore, a massively parallel DNA sequencing technique, 454 pyrosequencing, has now greatly increased the capacity to detect bacteria of low abundance [18, 19]. Although several studies reported oral microbial shift during radiation therapy, no clear pattern regarding the changes in the oral bacterial community can be discerned from the literature, most likely due to the limited number of studies published.

In this study, we employed 16S rRNA gene 454 pyrosequencing to illustrate the diversity and relative abundance of oral microbiota in each selected individual head and neck patients treated with CCRT. The oral microbiota were assessed before and during the CCRT in an attempt to follow the dynamics of the oral microbiota.

Materials and Methods

Patients

Patients were eligible for this study if they received definitive CCRT for a newly diagnosed, non-metastatic squamous cell carcinoma in the HNSCC. Other eligibility criteria were age of 18 to 80 years, the use of CCRT, and at least 50% of the oral mucosa receiving a dose of 50 Gy or higher. Patients were ineligible if they received antibiotics for an oral infection within 2 weeks of the start of CCRT. Patients with any oral mucosal defects from a previous surgery were ineligible. The study was approved by the institutional review board (Dongnam Institute of Radiological and Medical Sciences IRB, D-1106-004-001), and all subjects submitted informed consent forms.

Concurrent chemoradiotherapy

All study subjects underwent pre-radiotherapy oral and dental evaluation, and any risk factors of radiation complications were corrected before the start of CCRT. For simulation, patients were immobilized by a commercial thermoplastic face-neck-shoulder immobilized device. Treatment planning computed tomography (CT) scan was obtained with 2.5-mm thickness from the orbit to the carina. The acquired images were transferred to Monaco treatment planning system (Elekta, Stockholm, Sweden). CT images were reviewed, and clinical target volumes (CTVs) and normal structures were determined by one radiation oncologist. CTVs were defined according to documented or potential tumor burdens. Gross tumor CTV was defined a volume containing gross tumor and adjacent 5-mm tissue. Volumes at risk of subclinical metastases (surgical bed or lymph node groups) were defined as high-risk or low-risk CTVs according to estimated probability of tumor involvement. Planning target volumes (PTVs) were generated by adding 3-mm margins around CTVs to address daily setup variations. Treatment plans were generated by inverse planning algorithms to deliver prescription doses to CTVs while respecting radiation tolerance of normal organs. During 7 weeks, patients received once daily fractions of radiotherapy only on weekdays. Briefly, gross tumor CTV received 70 Gy delivered over 33 to 35 fractions. Microscopic or subclinical CTV received 50 to 60

Table 1. Patient characteristics of Receiving CCRT

Patient No.	Age/Sex	Primary site	TNM stage	Prescription dose	Chemotherapy	Stomatitis
Pt 1	61/M	Tonsil	cT4N2M0 (IVA)	70 Gy/33 Fx's	CDDP 40mg , six times every week	Grade 2/6 wk
Pt 2	45/M	Tonsil	cT2N2M0 (IVA)	70 Gy/35 Fx's	CDDP 100mg/m^2 , three times every 3weeks	Grade 4/ 3wk
Pt 3	50/M	Tonsil	cT2N2bM0 (IVA)	70 Gy/33 Fx's	CDDP 40mg/m^2, six times every week	Grade 4/ 3 wk

Stomatitis grade was scored according to Common Toxicity Criteria of Adverse Events version 4.0.
Abbreviation: Fx, fraction; CDDP, cisplatin.

Gy according to physician's discretion. Simultaneous integrated boost techniques were used to deliver differential prescription doses in one fraction of treatment. All patients received IMRT using volumetric modulated arc therapy. Concurrent chemotherapy was composed of weekly (40mg/m^2) or triweekly (100mg/m^2) intravenous cisplatin (dose mg/m^2) during RT. Cisplatin administration is described in Table 1.

Microbial sampling

Oral rinsed samples were obtained from patients prior to, during and at 4 weeks after CCRT. During CCRT, samples were obtained every 7 days. The patients were guided to thoroughly rinse the oral cavity with 15 ml sterile distilled water for 30 seconds. The samples were stored at -70°C before DNA extraction.

DNA extraction

The rinsed samples were centrifuged for 10 min at 3,000 rpm and the pellets were retrieved for DNA extraction. The total genomic DNA was prepared by using a Bacterial Genomic DNA extraction kit (Qiagen, Limburg, Netherlands). DNA concentration of the samples was determined by Nanodrop (Thermo Science, Wilmington, DE, USA). DNA samples were stored at -20°C until use.

Pyrosequencing

Polymerase chain reaction (PCR) amplification of the 16S rDNA hypervariable V1–V3 region was carried out using the forward primer 27F and reverse primer 518R, and pyrosequencing was performed with Roche 454 GS-FLX by Macrogen (Seoul, Korea). The primer sequences and 8-bp barcode were removed. The sequences that were less than 200 bp, contained ambiguous bases or homopolymeric stretches, or checked as chimeric artifacts were discarded. The qualified sequences were submitted to the SILVA database (SILVA; http://www.arbsilva.de) for taxonomic analysis. R program was applied to generate rarefaction curves.

Results

Patient selection for pyrosequencing analysis

Between Jul. 2011 and Aug. 2012, a total of 16 patients were enrolled to the study. The planned sample collection was completed in 9 patients. The rinsed oral samples were analyzed by PCR for the relative amount of periodontopathogens [20]. Three patients with relatively low amount of red complexes were selected for the pyrosequencing study (Table 1). History of antibiotic treatment during the sample collection is summarized in the Table 2.

Table 2. Antibiotics treatment history

	Antibiotics	Treatment period	Reason for antibiotic treatment
Pt 1	None		
Pt 2	Cefazolin	Weeks 3 to 4 during CCRT	Percutaneous endoscopic gastrostomy
Pt 3	cefazolin	Weeks 2 to 3 during CCRT	Percutaneous endoscopic gastrostomy
	Methylol Cephalexin Lysinate (MexesinR)	Weeks 3 during CCRT	
	Cefotaxime	Weeks 4 to 5 during CCRT	Fever
	Levofloxacin	1 month after of CCRT	Pneumonia

Overall sequence data

Of total 567,846 obtained sequences, 519,961 (91.57%) passed quality control. The mean number of sequences per sample at each time point was 14,267 ± 3,261 (range, 9,966 to 21,009). The average read length was 417 ± 84 bps. The richness of bacterial species within rinsed samples at each time point was estimated by rarefaction curves (Fig. 1A). The curve representing the early phase (weeks 1 to 3) of CCRT showed a steeper slope compared with that of the later period (weeks 5 to 7) and 1 month following the completion of CCRT (Fig. 1A). For a given number of sampled sequences (e.g., 10,000 sequences; Fig 1B), the number of total bacteria species gradually decreased during the early phase of CCRT.

Composition of the bacterial community

After eliminating unidentified sequences, 8 predominant phyla were commonly found in the oral microbiota (Figure 2). The predominant phyla include *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Cyanobacteria*, and *Tenericutes*. To understand the change within a person, each patient's microbiota composition was respectively analyzed.

First patient did not receive any antibiotics during the full radiation cycles and follow-up period. At the phylum level, the two most prevalent phyla before CCRT were *Firmicutes* and *Proteobacteria*. During the early CCRT (e.g., week 1,2,3), *Proteobacteria* gradually decreased while *Firmicutes* increased. During late CCRT (e.g., week 4,5,6), Firmicutes gradually decreased while *Bactereoidetes* and *Fusobacteria* increased (Fig 2A). At the genus level, during early CCRT, *Streptococcus*, which consists the majority of *Firmicutes*, gradually increased, while *Neisseria*, which consists the majority of *Proteobacteria*,

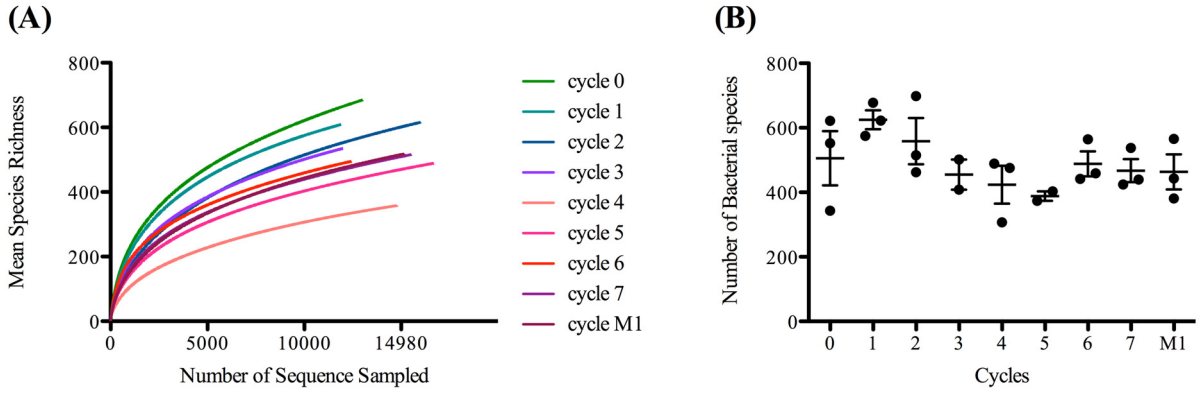

Fig. 1. Characterization of all time points rarefaction curves and the correlation analyses between the number of species and CCRT cycles. (A) Rarefaction curves were used to estimate richness during the radiation treatment. (B) For a given number of sequences sampled (e.g., 10,000 sequences), there was a negative correlation between the number of bacteria species and radiation cycles.

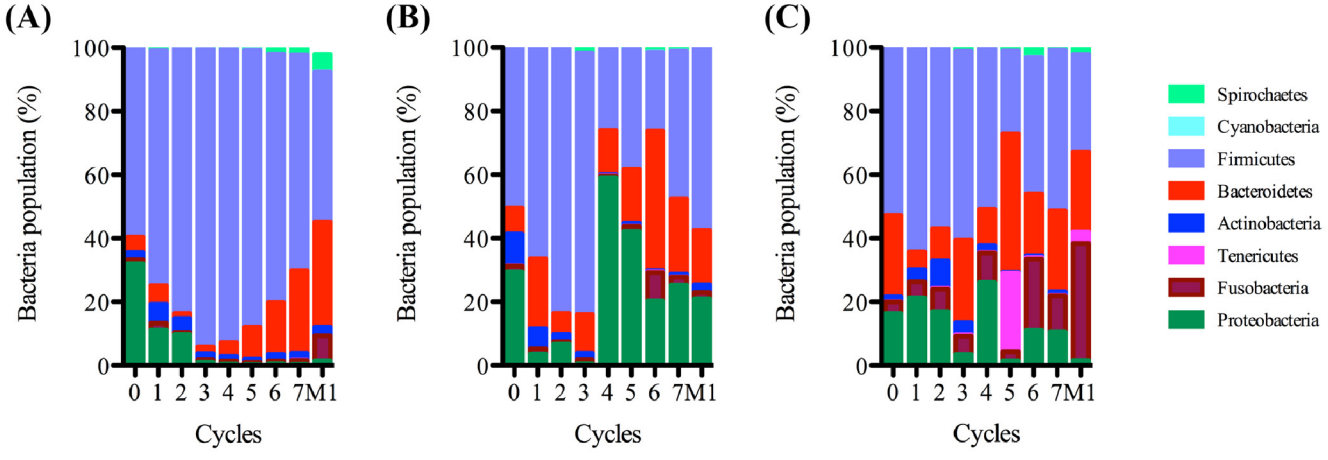

Fig. 2. Relative abundance of predominant phyla of each patient during CCRT cycles. Members of *Actinobacteria, Bacteroidetes, Firmicutes*, and *Proteobacteria* were found at all time points in all subjects and comprised the core microbiome.

gradually decreased. During late CCRT, among *Firmicutes*, *Veillonella* gradually increased while *Streptococcus* and *Gemella* decreased. During late CCRT, *Prevotella*, *Porphyromonas* and *Xylanibacter*, which belongs to *Bacteroidetes*, also showed gradual increase (Fig. 3A).

Second patient had received cefazolin for percutaneous enteral gastrostomy (PEG) during 3rd and 4th week of CCRT. At the phylum level, the two most prevalent phyla before CCRT were *Firmicutes* and *Proteobacteria*. *Actinobacteria* and *Bacteroidetes* also constituted substantially proportion of the oral microbiota. During the early CCRT, *Proteobacteria* and *Actinobacteria* gradually decreased while *Firmicutes* increased. On the 3rd week, microbiota change was observed as the patient was treated with cefazolin. *Firmicutes* drammatically decreased while *Proteobacteria* dominated the microbiota population. During late CCRT, *Firmicutes* gradually increased and *Proteobacteria* decreased (Fig 2B). *Bacteroidetes* consisted substantial portion of the microbiota population through out the treatment. At the genus level, during early CCRT, *Streptococcus* and *Gemella* gradually increased, while *Neisseria*, *Actinomyces* and *Rothia* gradually decreased. After the cefazolin treatment, *Neisseria* constituted the majority of the oral microbiota while *Streptococcus* decreased. During late CCRT, competition between *Neisseria* and *Streptococcus* was repeated and *Neisseria* gradually decreased while *Streptococcus* gradually increased. Among *Bacteroidetes*, *Prevotella* and *Porphyromonas* showed gradual increase during late CCRT (Fig. 3B).

Third patient had received several antibiotics throughout the treatment. The patient had received various cephalosporine antibiotics during 2nd and 5th week for PEG and fever. At the phylum level, the three most prevalent phyla before CCRT were *Firmicutes, Bacteroidetes* and *Proteobacteria.* Since this patient had received antibiotics for prolonged period, the microbiota change was not as prominent as the previous two patients. An interesting finding could be the prominent increase of *Tenericutes* at 5th week and gradual increase of *Fusobacteria* throughout the treatment (Fig. 2C). At the genus level, *Prevotella* consisted more than 20% of the microbiota before CCRT. *Mycoplasma* infection was detected by the 5th week and *Fusobacterium* gradually increased throughout the CCRT (Fig. 3C).

Socransky *et al.* had classified the oral bacterial species into several complexes. The red complex is categorized together based on their association with severe forms of periodontal disease. Orange complex appeared closely related to the red complex [21]. At the species level, the microbiota change was analyzed according to the complex during CCRT. In all patients, orange and red complex showed gradual increase while yellow complex showed gradual decrease throughout the CCRT. Also, increase of orange complex preceding the red complex was noted in all the patients (Fig. 4).

Discussion

CCRT is the most widely used approach to locally advanced HCSCC. CCRT involves direct mucosal damage as well as major salivary glands being inevitably exposed to the radiation

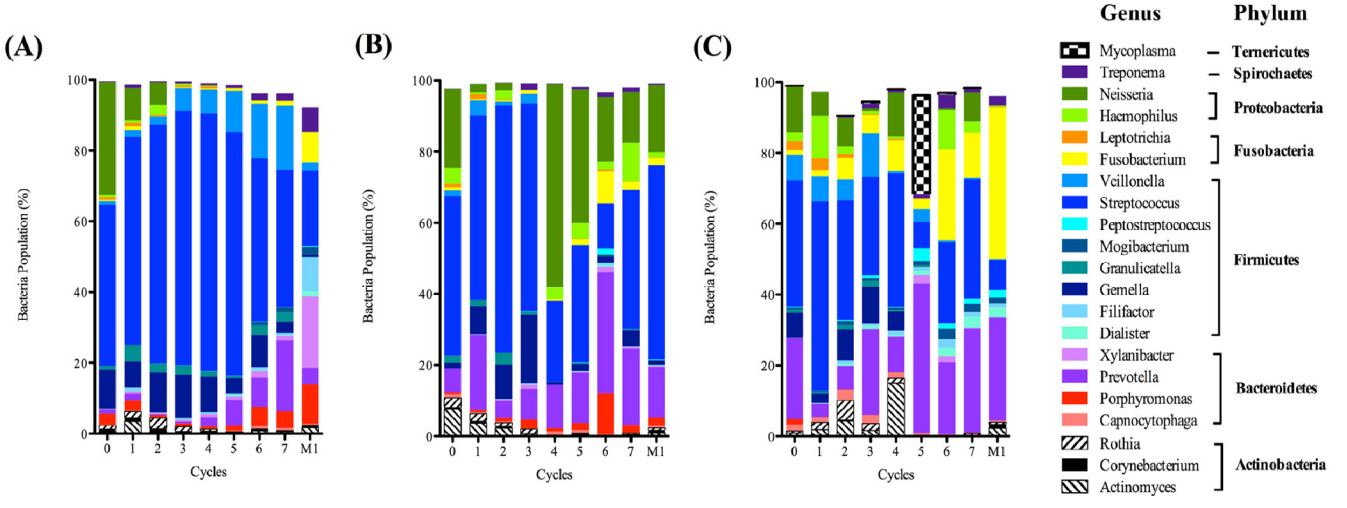

Fig. 3. Relative abundance of relatively abundant 21 genera of each patient during CCRT cycles. These 21 genera constituted more than 95 % of the total sequences.

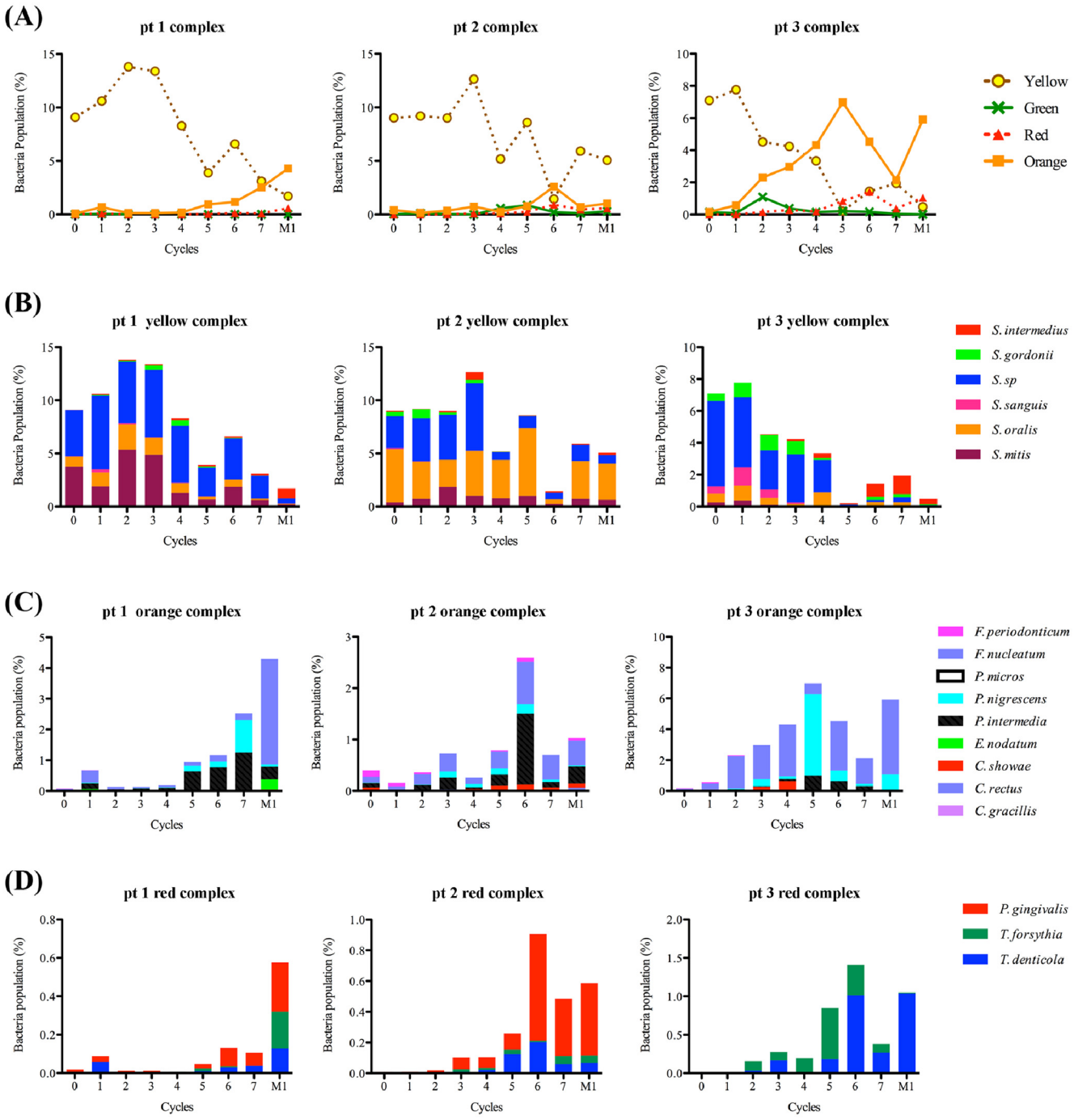

Fig. 4. Dynamic change of bacteria species according to complexes. (A) Overall change of yellow, green, red and orange complex in each patient during CCRT cycles. (B) Change of bacteria species included in yellow complex during CCRT cycles. Total population of yellow complex gradually decreases during CCRT cycles. (C) Change of bacteria species included in orange complex during CCRT cycles. Total population of yellow complex gradually increases during CCRT cycles. (D) Change of bacteria species included in red complex during CCRT cycles. Total population of yellow complex gradually increases during CCRT cycles.

field. As a result, CCRT causes hyposalivation [22] and produces a range of inflammatory mediators that lead to apoptosis and tissue injury which may also provide feedback loop that drives the destructive process forward [15]. As a result of mucosal damage and hyposalivation, an imbalance in the oral microbial ecosystem is inevitable.

In this study, we tried to illustrate detail microbial changes within each patient and further find some common features of oral microbiota change during CCRT. Oral microbiota within patients with oral cancer differ from healthy persons [20, 23]. Considering that CCRT-induced oral mucositis and periodontitis are both linked with continuing presence of systemic inflammation, a link between periodontitis and oral mucositis in patients treated with CCRT has been proposed [24]. Since, bacterial pathogens including *Porphyromonas gingivalis* are often found in oral cancers [25], we selected cancer patients with relatively low number of periodontopathogens. All the patients with relatively low periodontopathogens had tonsil squamous cancer and were treated with CCRT for 7 weeks.

The richness of bacterial species showed dramatic decrease at the early phase (week 1,2,3) of CCRT while it was quite stable at the late phase (week 5,6,7). This is consistent with other studies reporting negative correlation between radiation dosage and number of operational taxonomnic units (OTUs) [26]. While Hu *et al.* reported continuous decrease [26], our result showed decrease only in the early phase.

First patient who did not receive any antibiotics during the CCRT showed oral microbiota shift throughout and post CCRT. This result may represent the true oral microbiota change induced by CCRT compared to other results. At early CCRT, *Firmicutes* overwhelmed the *Proteobacteria* and by the 3rd week, *Firmicutes* was the most dominant oral microbiota. At the genus level, *Streptococcus* and *Gemella* showed a gradual increase while *Neisseria* showed a decrease. As a result of hyposalivation inducted by CCRT, production of antimicrobial factors such as lysozyme and peroxidase, should have been decreased. Lysozyme causes bacterial cell lysis by hydrolyzing the bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall [27]. The peroxidase catalyses the oxidation of salivary thiocyanate ions to antimicrobial compounds such as hypothiocyanite [28]. Thus, decrease in such antimicrobial factors in the saliva should have also influenced each bacterial growth to shift the balance between oral microbiota. Also, nutritional change induced by CCRT can influence bacterial competition for certain nutrient. In *in vitro* culture system, *S. salivarius* strains can inhibit the growth of *N. meningitidis* [29, 30]. At late phase of CCRT, *Veillonella* showed a gradual increase while *Gemella* and *Streptococcus* showed a decrease. *Veillonella* is well known for its lactate fermenting abilities. When *Veillonella* and *Streptococcus* is simultaneously given to a gnobiotic rat, a rapid succession of *Veillonella* occurs in the oral microbiota [31]. At late CCRT, *Prevotella, Fusobacterium, Porphyromonas,* and *Haemophilus* also gained significant proportion of the oral microbiota. Taken together, serial bacterial shift suggest that CCRT had greatly influenced the oral microbiota.

Second patient had received cefazolin, a 1st generation cephalosporin antibiotic, during the CCRT. Although overall oral microbiota structure was quite different from the first patient, similar bacterial shift was observed during the early CCRT period. However, when the patient received cefazolin, there was a dramatic shift in the oral microbiota. Since cefazolin more selectively target Gram-positive bacteria [32], *Firmicutes* including *Streptococci* and *Gemella* showed a dramatic decrease while *Neisseria* repopulated within the oral microbiota. Following the antibiotic cessation, the microbiota shift showed similar change as the early phase.

Following radiation therapy, periodontal parameters including gingival recession, clinical attachment level and plaque index were significantly increased after radiotherapy [33]. Leung *et al.* have reported that the risk of periodontal infection is also increased due to radiation therapy and a concomitant increased plaque accumulation and shift in oral micro flora [34]. Such shift of oral microbiome reflects the availability of nutrients derived from the degradation of host tissue and from bacterial cells destroyed by the host immune response [35]. When oral complexes were studied in the patients during CCRT, an interesting feature was commonly observed regardless of antibiotic treatment. Orange complex and red complex, which are well known to be involved in the initiation and progression of periodontal disease [21, 36], showed a gradual increase at the late CCRT period in all patients. The increase of red and orange complex could reflect the availability of nutrients derived from the host.

Oral microbial communities can remain relatively stable over time in healthy individuals [37, 38]. Our results show that dramatic physical and chemical shift in the oral cavity can impose significant changes in the composition of the oral microbiome during the CCRT.

Our study has several limitations. First, the number of patients analyzed in the study is little. Further study with more patients enrolled should be conducted. Second, although we have followed 1 month after the final CCRT, chronic changes should be further studied. Third, antibiotic treatments to treat complications during the CCRT had severe effect on the oral microbiota change. If possible, samples from patients with minimum antibiotic treatment would be recommended.

In spite of such limitations, our study clearly shows oral microbiota change in locally advanced HNSCC patients treated with CCRT. Based on this study, strong recommendation for dental treatment should be aimed at improving the oral hygiene status of patients through professional dental care. Patients should be constantly motivated toward maintaining a meticulous oral hygiene status before, during, and after CCRT to prevent and control the oral and periodontal sequelae.

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Conflict of interest

The authors have no financial conflicts of interest.

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