Effects of Intensity-modulated Radiotherapy on Human Oral Microflora

Zi-Yang SHAO¹, Zi-Sheng TANG², Chao YAN³, Yun-Tao JIANG², Rui MA², Zheng LIU² and Zheng-Wei HUANG²*

PCR-DGGE/Bacterial communities/Radiotherapy/Hyposalivation.

This study aimed to evaluate changes in the biodiversity of the oral microflora of patients with head and neck cancer treated with postoperative intensity-modulated radiotherapy (IMRT) or conventional radiotherapy (CRT). Pooled dental plaque samples were collected during the radiation treatment from patients receiving IMRT (n = 13) and CRT (n = 12). Denaturing gradient gel electrophoresis (DGGE) was used to analyze the temporal variation of these plaque samples. The stimulated and unstimulated salivary flow rates were also compared between IMRT and CRT patients. Reductions in the severity of hyposalivation were observed in IMRT patients compared with CRT patients. We also observed that the temporal stability of the oral ecosystem was significantly higher in the IMRT group (69.96 ± 7.82%) than in the CRT group (51.98 ± 10.45%) (P < 0.05). The findings of the present study suggest that IMRT is more conducive to maintaining the relative stability of the oral ecosystem than CRT.

INTRODUCTION

The head and neck region of the human body is a compact space where many critical organs are densely assembled. Irradiation is a major modality used for treatment of cancers in these regions. During treatment, ionizing irradiation can also affect the salivary glands and dentition leading to many deleterious side effects such as hypo-salivation, mucositis, xerostomia and radiation caries.¹ Most of these side effects are related to ecological changes in oral microbiota.²⁻⁶ The pronounced reduction in salivary flow rate, altered dietary habits and direct effects of ionizing irradiation on the microbiota all contribute to an imbalance in the oral microecosystem,⁷,⁸ which in turn lead to poor health status.

It is now known that intensity-modulated radiotherapy (IMRT) for head and neck cancers can deliver higher radiation therapy doses to the target volumes, while sparing critical structures like the parotid glands and submandibular glands. Thus, the incidence and severity of such side effects is reduced after IMRT compared with conventional radiotherapy (CRT).⁹ To our knowledge, the ecological shift of oral microbiota during IMRT or CRT has not been reported, even though the microbiota has an important role in the maintenance of oral health.

The cultivation technique has been used to examine changes in the composition of oral microflora in patients after radiotherapy in the head and neck area.⁵,¹⁰,¹¹ However, only a small fraction of microorganisms in the oral cavity are cultivable,¹² the development of molecular technology has allowed the identification of microorganisms and the study of microbial diversity at the genetic level. Recently, culture-independent approaches using the sequence variability of the 16S rRNA have been developed that could be used to unravel the dynamic profile of oral microbiota during radiation therapy.¹³

The purpose of this study was to test the hypothesis that parallel to a reduction in the severity of the hypo-salivation, IMRT can maintain the stability of the oral ecosystem during radiation therapy. In the current study, we investigated the salivary flow rates and the molecular characteristics of the microbial diversity in the dental plaque of patients receiving IMRT and CRT. Our findings may contribute to more effective preventive oral care programs in the future.

*Corresponding author: Phone: +86-21-2377-1699, Fax: +86-21-6313-5412, E-mail: huangzhengwei@shsmu.edu.cn

¹Division of Radiation Oncology, Department of Oral and Maxillofacial Surgery, Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine. No. 639 Zhizaoju Road, Shanghai 200011, P. R. China; ²Department of Endodontics, Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology. No. 639 Zhizaoju Road, Shanghai 200011, P. R. China; ³Department of Oncology, the Affiliated Hospital of Medical College, Qingdao University. No. 308 Ningxia Road, Qingdao 266003, P. R. China. doi:10.1269/jrr.11085
MATERIALS AND METHODS

Subjects’ enrollment
This study was approved by the Ethics committee of Shanghai Jiaotong University, Shanghai Research Institute of Stomatology. Potential study subjects were identified from a group of cancer patients who would receive radiation therapy. The inclusion/exclusion criteria are listed in Table 1. From February 2008 to December 2010, all subjects gave their voluntary written consent to take part in the study. Before radiation therapy, an oral health examination was performed. If necessary, carious lesions were restored, endodontic treatment performed and doubtful teeth extracted. The patients were given oral hygiene instruction, but no special fluoride remedy was added. All clinical measurements and sample collections were performed between 0800 h and 1100 h, consistently by the same member of the research group. Twenty-five subjects (13 IMRT and 12 CRT) who completed their radiotherapy were enrolled in this study.

IMRT and CRT protocols
Patients were placed in the supine position on a commercial thermoplastic mask (head-neck masks for CRT patients and head-shoulder masks for IMRT patients) attached to a carbon-fiber laminate base plate. CT images were acquired using a CT simulator. The transverse images represented a carbon-fiber laminate base plate. CT images were acquired using a CT simulator. The transverse images represented 2.5 mm thick slices from 5 cm superior to the skull base to the clavicle heads. The acquired images were directly transferred to a XiO treatment planning system (version 4.33.02, Computerized Medical Systems, St. Louis, MO, USA). CT- based 3-dimensional treatment planning was used for the four patients treated with CRT. The primary field was irradiated through lateral parallel-opposed portals with 6 MV photons, 1.8–2.0 Gy/30 fractions, 5 times weekly. The parotid and submandibular glands were directly adjacent to the target volume and could not be spared. During IMRT, irradiation was delivered by a linear accelerator with an integrated multileaf collimator (MLC; Elekta, Crawley, UK) using a step-and-shoot technique with 6-MV photons. Application of the IMRT technique has been previously described in detail by Wang et al.15 In all IMRT patients, the planning goal was to achieve a mean dose ≤ 26 Gy to at least one parotid gland.

Measurement of salivary flow rates
The salivary flow rates from each of the major glands were measured before, midterm (at 3-week intervals), and after radiotherapy using a suction cup attached to the orifice of the parotid duct and from the submandibular/sublingual glands by gentle suction using a micropipette from Wharton’s duct orifices, as described previously by Ship et al.15 Unstimulated salivary flow rates were measured first, followed by measurement of salivary flow rates stimulated by the application of 2% citric acid on the dorsum of the tongue.

Microbial sampling
For each subject, during the 6 weeks of radiotherapy, microbial samples were collected at 7-day intervals using the method of Li et al.16 With a slight modification. In brief, the subjects thoroughly rinsed his/her mouth with 10 ml sterile distilled water for 20 seconds. After the sample sites were dried and isolated with cotton rolls, a sterile Gracey curette was used to collect a pooled plaque sample from the buccogingival surfaces of the upper first molar. The collected plaque sample was released from the curette by agitation in 300 μl of TE buffer (10 mM Tris-Cl [pH 7.5] and 1 mM EDTA). The microbial samples were immediately transported on ice to the laboratory. There are 175 samples in total (7 samples for each subject) collected for the genetic fingerprinting study.

DNA extraction and PCR assay
The plaque samples were lysed in a Mini-Beadbeater-16 (Biospec Products, Bartlesville, OK, USA) according to the manufacturer’s directions. The total genomic DNA was obtained from the lysate using a Bacterial Genomic DNA Extraction Kit (Tiangen, Beijing, China).17 DNA concentrations of the samples were calculated by measuring A260 and the quality was estimated by the A260/A280 ratio. PCR primers V3f (5’-CGC CGC CCG CCG CCC CCC CCC GCG CCC CCG CCG CCC CCG CCC CCT ACG GGA GGC AGC AG-3’), the GC clamp region is underlined) and V3r (5’-ATT ACC GCG GCT GCT GG-3’) were used to amplify
the V3 region of the 16S rRNA gene. Each reaction mixture contained 5 μl of 10 × PCR buffer (100 mM Tris-HCl [pH 9], 15 mM MgCl₂, 500 mM KCl, 0.1% [w/v] gelatin, 1% [v/v] Triton X-100), 0.2 mmol deoxynucleotide triphosphate, 1U of HotstarTaq DNA polymerase (Qiagen, Hamburg, Germany), 0.25 mmol of each forward and reverse primer, 50 ng template cDNA, and enough sterile MilliQ water to bring the final volume to 50 μl. The PCR program used was as follows: 5 minutes of initial denaturation at 95°C, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (60°C for 30 seconds) and extension (72°C for 30 seconds) with a final extension of 10 minutes at 72°C.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE analysis was performed using the CBS system (C.B.S. Scientific Co., Del Mar, CA, USA). Electrophoresis was carried out on a 1 mm thick gel (16 cm × 16 cm) that contained 6% (w/v) polyacrylamide in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA; pH 7.4). A gradient of 40–70% denaturant was used to separate the PCR fragments: 100% denaturant was defined as 7 M urea and 40% (v/v) deionized formamide. The gels were run at 80 V for 16 hours at 60°C and silver stained as described by Jiang et al. The digitized gel images were analyzed using gel analysis software of GeneTools (version 4.01, Syngene Ltd., Beacon House, Cambridge, UK). The software was used to detect bands by normalizing against the total intensity data for each lane. Bands were detected in each lane and matched using a tolerance of 0.5%. A similarity matrix was constructed using Dice’s similarity coefficient. This is defined as \[ \frac{2j}{(a + b)} \times 100\% \] where \( j \) is the number of bands in common between two lanes and \( a + b \) is the total band number of both lanes.

Analysis of statistical significance was carried out using the SPSS 13.0 program (SPSS, Inc., Chicago, IL, USA). The one-way-ANOVA test was used to compare averages of the Dice coefficient between the IMRT and CRT groups. The significance level was set at \( P < 0.05 \).

RESULTS AND DISCUSSION

Irradiation induced a pronounced hyposalivation

When head and neck cancer patients receive radiotherapy, the major salivary glands are generally exposed to the radiation field. Due to the high radiosensitivity of serous cells, immediate and profound serous cell death occurs that is accompanied by inflammatory cell infiltration into the salivary glands. This will lead to a rapid and predictable reduction in saliva production. In the current study, both IMRT and CRT subjects suffered from hyposalivation. As shown in Fig. 1, their stimulated and unstimulated salivary flow rates were decreased. However, the severity of this side effect was different between the groups.

For the unstimulated saliva (Fig. 1A), the Δ salivary flow rates (salivary flow rate – pre-RT salivary flow rate) did not differ significantly between the IMRT (1.68 ± 0.72 ml/min) and CRT (1.95 ± 0.53 ml/min) groups at the midterm of radiotherapy (post-RT). * indicates the Δ salivary flow rates (salivary flow rate – pre-RT salivary flow rate) was significantly lower in IMRT subjects than in CRT ones (\( P < 0.05 \)).
Effects of IMRT on Human Oral Microflora

radiotherapy ($P > 0.05$). After radiotherapy, the change in salivary flow rate was significantly lower in the IMRT ($1.68 \pm 0.68 \text{ ml/min}$) group than the CRT ($3.47 \pm 0.56 \text{ ml/min}$) group ($P < 0.05$).

The stimulated salivary flow rate (Fig. 1B), an indicator of salivary gland function, was inhibited during radiation treatment. At the midpoint of radiotherapy, a significant difference in the $\Delta$ salivary flow rate was observed between the IMRT ($2.28 \pm 1.08 \text{ ml/min}$) and CRT ($4.24 \pm 0.58 \text{ ml/min}$) groups ($P < 0.05$). After radiotherapy, the change in salivary flow rate of IMRT subjects ($2.48 \pm 0.99 \text{ ml/min}$) was significantly lower than in CRT subjects ($5.74 \pm 0.47 \text{ ml/min}$) ($P < 0.05$).

Dosimetric advantages of IMRT over CRT have been confirmed by numerous studies.$^{20,21}$ Taking into consideration that IMRT delivers sharp dose radiation therapy gradients as well as higher radiation therapy doses to the target volumes, critical structures can be spared by assuring limited organ motion with correct immobilization.$^6$ In the present study, we found that due to the high sensitivity of salivary glands to irradiation, all patients suffered from hyposalivation. However, a significantly decreased salivary flow rate was observed in CRT subjects than IMRT subjects. The decreased salivary flow rate may also aggravate the imbalance of the oral ecosystem in these patients.$^3,11,22$

The plaque microbiota displayed low community constancy

This study used PCR-DGGE to evaluate the temporal variation of the plaque profile during radiotherapy. PCR-DGGE is one of the molecular fingerprinting methods targeting of the hypervariable regions of 16S rDNA that were capable of surveying entire bacterial communities without cultivation and displaying the overall pattern of bacteria diversity directly on one gel.$^{13}$ We monitored the temporal variation in the microbiota composition of irradiated subjects. An estimate of community constancy was determined as the average similarity of DGGE profiles of plaque samples obtained every week for each individual. All samples showed changes in community constancy over time. There were no identical DGGE fingerprints, indicating that the microbiota changed during the course of the study. Figure 2 shows two examples of DGGE profiles obtained over time in individuals from the IMRT and CRT groups respectively.

The degree of change over time varied between the IMRT and CRT subjects (Fig. 3). The average similarity for the IMRT group was $69.96 \pm 7.82\%$ compared to $51.98 \pm 10.45\%$ for the CRT group. Comparisons between the different treatment groups indicated that temporal stability of microflora was significantly higher in the IMRT group than the CRT group ($P < 0.05$). To our knowledge, there is no report on the similarity of dental plaque microflora among individuals. However, the research of Rasiah et al.$^{23}$ on saliva microflora may provide an indirect compare. They report...

Fig. 2. Examples of temporal variation in the plaque communities in samples (subject I-H from IMRT group and subject C-S from CRT group) analyzed with PCR-DGGE. Samples were collected every week during radiotherapy (0 stands for before RT). The side bars indicate the site of the excised bands.

Fig. 3. Box and whiskers graph of similarity coefficients calculated for the DGGE profiles of plaque communities. The box extends from the 25th percentile to the 75th percentile, with a line at the median (the 50th percentile). The whiskers extend above and below the box to show the highest and lowest values. There are significant differences at $P < 0.05$ between the IMRT and CRT samples.
ed that the overall similarity between saliva samples from the same individual at different time points remained stable (approximately 80% concordance), while the microflora between individuals displayed greater variability (approximately 52% concordance). Here, we found that during CRT, the temporal variations of the same subject were even closer to the inter-individual variations, indicating that the oral eubiosis of the subjects had been profoundly disturbed. During IMRT, the temporal variations were closer to the healthy subjects’ temporal variations, and thus their ecological profiles remained relatively stable.

Sequence analysis of the excised bands

Dental plaque harbors a highly diverse resident community of microorganisms. To date, there are more than 700 taxa have been identified from oral microbiota. From the report of Keijser, it is estimated that the richness of total bacterial communities ranged from 18,922 to 26,202 phylogenotypes in plaque. This complex microflora is kept in a eubiosis that is required to maintain oral health. Traditionally, a shift in the microflora has been studied using cultivation techniques. However, there are still many bacteria that cannot be cultured.

Our understanding of biological variation in the plaque microflora is central to exploring many aspects of radiotherapy and the oral ecosystem. The DGGE profiles of the twenty-five subjects receiving radiotherapy were compared, and those bands with an obvious tendency of increased intensity were excised from the gels (examples are shown in Fig. 2). Ninety-two bands were excised (50 from the IMRT group and 42 from the CRT group), after which the DNA was extracted, reamplified and cloned. An average of 5 clones for each band (a total of 458 clones) were picked and sequenced. From the 458 clones examined, 39 genera were identified (Fig. 4). Unidentified bacteria accounted for 9.82% of the sequenced clones.

From the sequence analysis, we found that the increases in bacterial populations in different patients show no significance, i.e. different subjects had different bacteria genera that increased in their abundance. There is no specific favorite for the shift of plaque microbiota during irradiation among individuals. This finding is inconsistent with some other researches, it was reported that oral microflora will change specifically with hyposalivation, several caries related bacteria like lactobacilli and non Mutans Streptococcus will gain their abundances. However, those researches focused on bacteria communities several months after completed radiotherapy instead of during the irradiation like this one. Our findings indicated the irradiation as a disequilibrium (dysbiosis) process. The eubiosis of the mouth system was profoundly disrupted during this process. Only after the therapy, without the further damage from the irradiation, a new ecosystem might be established then. Several specific bacteria groups may be favored consequently, which will bring to a high prevalence of caries for subjects after radiotherapy. Further studies are needed to clarify when the “window” period is for those virulent bacteria established their roles in the new eco-environment.

![Fig. 4. Genera identified in the subjects during radiotherapy, with an increase in their abundance.](image-url)
In summary, we show that IMRT reduces the severity of hyposalivation compared to CRT. We also demonstrate that PCR-based 16S rRNA gene DGGE is a valuable tool for evaluation of the ecological shift of oral microbiota. This study shows that the ecological shift of oral microbiota is more pronounced during CRT than IMRT. Finally, the findings of this study can be used as a basis for more effective oral care programs for patients receiving radiotherapy.

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REFERENCES