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## Research Paper

# The potential effect of oral microbiota in the prediction of mucositis during radiotherapy for nasopharyngeal carcinoma

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## ABSTRACT

**Background:** Oral mucositis is probably the most debilitating complication that can arise in treating a patient with head and neck cancer. Little is known about the impacts of oral microbiota on the initiation and progression of mucositis.

**Methods:** Based on 16S rRNA gene sequencing, dynamic changes in oral bacterial profile as well as correlations between the severity of mucositis and bacterial shifts during radiotherapy were investigated.

**Findings:** Our results revealed that bacterial community structure altered progressively during radiation therapy, in parallel with a marked increase in the relative abundance of some Gram-negative bacteria. Patients who eventually developed severe mucositis harbored a significantly lower bacterial alpha diversity and higher abundance of *Actinobacillus* during the phase of erythema – patchy mucositis. Accordingly, a random forest model for predicting exacerbation of mucositis was generated, which achieved a high predictive accuracy (AUC) of 0.89.

**Interpretation:** Oral microbiota changes correlate with the progression and aggravation of radiotherapy-induced mucositis in patients with nasopharyngeal carcinoma. Microbiota-based strategies can be used for the early prediction and prevention of the incidence of severe mucositis during radiotherapy.

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## 1. Introduction

Oral/Oropharyngeal mucositis (OM) is probably the most common and debilitating complication that can occur during and shortly after radiotherapy and/or chemotherapy for patients with head-and-neck cancer (HNC). It is typically characterized by: erythema, edema, mucosal ulceration and pseudomembrane formation in the oral cavity and oropharynx. Patients with OM lesions frequently complain of mild to severe pain and pharyngeal dysphagia. Currently, there are no effective mucoprotective strategies for the management of OM. Severe mucositis can not only affect a patients' quality of life, but also increases the need for narcotic analgesics, total parenteral nutrition, interruption of cancer therapy, prolong hospitalization and increases the risk of local and systemic infection (Trotti et al., 2003; Villa and Sonis, 2015; Stokman et al., 2006).

Recent studies have suggested that the pathogenesis of mucositis involves a cascade of inflammatory events, which chronologically consist of five continuous overlapping phases: initiation, upregulation of inflammation, signaling and amplification, ulceration, and finally wound healing

(Sonis, 2004). Although it is hypothesized that genetic factors may correlate with an increased risk of developing mucositis (Venkatesh et al., 2014; Pratesi et al., 2011), the role of microorganisms in promoting inflammation and exacerbating mucositis still remains to be explored.

Using culture-based techniques, several Gram-positive/negative species of bacteria were isolated from the oral cavity of patients undergoing radiochemotherapy in the head and neck region (Napenas et al., 2007; Almstahl et al., 2008; Panghal et al., 2012). Their normal bacterial flora was significantly altered during the course of treatment, which leads naturally to the question as to whether changes in the oropharyngeal microflora may correlate with the onset and aggravation of OM. Unfortunately, there are so far no well-defined patterns with respect to these changes, and no consistent correlations were found between the presence of specific pathogens and the severity of OM. On the other hand, it is estimated that > 700 bacterial species have been detected in the oral cavity, of which >50% remain to be cultivated (Aas et al., 2005). However, using culture-independent high-throughput 16S rRNA gene sequencing, it has been shown that changes in oral microbiota during radiochemotherapy are far more complicated than previously thought (Vanhoecke et al., 2015; Hu et al., 2013). Nevertheless, and more importantly until now, there has been few analysis made to our knowledge on the correlation between the OM severity and the dynamic changes it may make in bacterial profiles throughout radiochemotherapy.

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Hence in this study, the OM severity was assessed and scored longitudinally in a cohort of patients with nasopharyngeal carcinoma (NPC) undergoing radiotherapy with or without chemotherapy, during which the mucosa of patients was inspected regularly and sampled eight times throughout their treatment. Based on 16S rRNA gene sequencing and bioinformatics analysis, we were able to investigate dynamic changes in oropharyngeal bacterial profile and their associated correlation between the severity of mucositis and the bacterial shifts which had occurred.

## 2. Materials and Methods

### 2.1. Sample Collection

This prospective cohort study was conducted in newly diagnosed NPC patients undergoing three-dimensional conformal radiation therapy at South China's Nanfang Hospital between May 2012 and August 2013. Ethical approval was granted by the Ethics Committee of China's Southern Medical University (SMU). The devised study protocols were explained to each patient and family members, and those who agreed to participate signed an informed consent form. The patients, as well as their relatives, who presented with poor oral hygiene and/or had developed severe forms of periodontal diseases were excluded from this study; and subjects with prior head-and-neck radiation or who had taken antibiotics within 2 weeks before the study commenced, were also not enrolled in the study. A total of 41 NPC patients (27men; 14 women; age range 22–75 years; average age 47.2) were recruited to take part in this study and their medically healthy relatives matched by sex and age were served as controls.

Before radiation treatment began, all patients received a full-mouth clinical examination and oral hygiene instruction. Surgical procedures were carried out in patients with caries, pulpal diseases and gingivitis, including professional dental cleaning, filling, endodontic treatment, and extraction of non-restorable teeth. For all recruited patients, radiotherapy was administered 5 times per week at 2 Gy per fraction in a total dose of 60–70 Gy within 6–7 weeks, in which 17 patients underwent radiotherapy alone, two patients received induction chemotherapy followed by radiotherapy, and the remainder, some 23 patients were given concurrent chemoradiotherapy followed by adjuvant chemotherapy.

In all patients, oral and oropharyngeal mucositis were scored clinically by the same radiation therapist once every 3 days, according to the Radiation Therapy Oncology Group (RTOG) criteria. To trace dynamic changes of bacterial community through the entire course, a series of microbial samples were regularly taken from 19 of these patients prior to irradiation, after their 5th, 10th, 15th, 20th, 25th, 30th and 35th irradiation, corresponding to 10, 20, 30, 40, 50, 60 and 70 Gy respectively. Samples were collected in the patients presenting with OM by swabbing over mucosal lesions in the retropharyngeal wall with disposable medical sterile swabs, whereas in patients without clinical signs of mucositis, or in the control group, retropharyngeal mucosa was sampled. All samples were frozen immediately at  $-20^{\circ}\text{C}$  and then stored at  $-80^{\circ}\text{C}$  until analysis.

### 2.2. DNA Extraction, 16S rRNA Gene Amplification and Sequencing

Bacterial genomic DNA was extracted from mucosal samples using the DNA MAGNETICS and EXTRACT kit (Shenzhen BioEAsy Biotechnologies Co., Ltd., China), according to the manufacturer's instruction as previously validated (Peng et al., 2013). The barcoded V4F-GAGTGCCAGCMGCCGCGTAA and V4R-GGACTACHVGGGTWTCTAAT primers were used to amplify bacterial 16S rRNA V4 fragments. The PCR cycle conditions were:  $94^{\circ}\text{C}$  for 3 min, followed by 30 cycles of  $94^{\circ}\text{C}$  for 30 s,  $57^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s, and a final extension at  $72^{\circ}\text{C}$  for 5 min. The PCR products were purified (QIAquick PCR Purification Kit, Qiagen), and sequenced using the 100-bp paired-end strategy on the Illumina HiSeq 2000 according to the manufacturer's instructions. The raw sequence data for 16S rRNA gene sequencing

data sets is available from the European Bioinformatics Institute (<http://www.ebi.ac.uk/>) at accession number PRJEB15392.

### 2.3. Bioinformatics and Statistic Analysis

The Illumina sequencing quality report indicates that the quality of sequences is relatively high before PE80bp (pair-end 80 bp), while there is a sharp decrease after that. As a result, we trimmed the raw sequences to 80 bp for each end. Sequences were then de-multiplexed, barcode-primer trimmed and filtered if they contain ambiguous bases or mismatches in the primer regions according to BIPES protocol (Zhou et al., 2011). We then used 30Ns to concatenate the two single-ended sequences for the following analyses sequences, because our PE sequences are not able to span the V4 regions of the 16S rRNA gene. All the tools we have used in this study have been validated to be able to handle gapped sequences. UCHIME (implemented in USEARCH, version 6.1) was used to remove chimeras using the de novo mode with default parameters (Edgar et al., 2011). We normalized all samples at the level of 3000 sequences to avoid any uneven sequencing effort between samples. USEARCH was then used to do de novo clustering for the sequences using the default parameters, with the identity cutoff set to 0.97. Multiple alignments of representative sequences were performed using PyNAST. The Green genes core set (Version: 13.8) was used as the template file (Lozupone et al., 2011). The RDP classifier was used to classify these representative sequences into specific taxa using the default database (Wang et al., 2007). PD whole tree and Shannon index were applied to evaluate alpha diversity and the UniFrac distance was used to analyze the beta-diversity (Lozupone and Knight, 2005). All of the analyses, from chimera checking to alpha and beta diversity calculation, were performed using QIIME (1.8.0) (Caporaso et al., 2010). Statistical analysis of the relative abundance of the genera and the diversity indices and estimators were performed using R (v3.2.2). We used LEfSe (linear discriminant analysis effect size) to determine differential features between groups (Segata et al., 2011). LEfSe is an algorithm for high-dimensional biomarker discovery as it identifies genomic features characterizing differences between two or more biological conditions. LEfSe determines the features most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect size (Segata et al., 2011). The threshold on the logarithmic LDA score for discriminative features was set to 2.0. Random Forests models were trained by "randomForest" package with default parameters (ntree = 5000, mtry = square root of p, where p is the number of input features) in R using OTU data. The performance of the model was assessed with a ten-fold cross-validation approach and measured by area under the ROC.

## 3. Results

### 3.1. The Oropharyngeal Microbiota of NPC Patients are Different to that of Healthy Individuals

In this study, retropharyngeal mucosal samples were taken from patients before the commencement of their radiation treatment and medically healthy controls. Demographic characteristics and health-related lifestyle factors of our NPC patients and control subjects are summarized in Table 1. At baseline, both groups were similar in terms of age, gender,

**Table 1**  
Baseline characteristics of nasopharyngeal carcinoma patients and control subjects.

	NPC (n = 41)	CON (n = 49)	P value
Age (years, mean $\pm$ SD)	47.0 $\pm$ 12.9	48.3 $\pm$ 11.7	0.51
Gender, male, n (%)	27 (65.9)	37 (75.5)	0.35
Smoking, n (%)	14 (34.1)	9 (18.4)	0.14
Drinking, n (%)	9 (22.0)	11 (22.4)	0.99

Percentages are shown in parentheses as appropriate.  
NPC, nasopharyngeal carcinoma; CON, controls.

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