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Compositional and functional variations of oral microbiota associated with the mutational changes in oral cancer☆

Shun-Fa Yang[a,b], Hsien-Da Huang[c,d], Wen-Lang Fan[e], Yuh-Jyh Jong[c], Mu-Kuan Chen[f], Chien-Ning Huang[a,g], Chun-Yi Chuang[h,i], Yu-Lun Kuo[j], Wen-Hung Chung[e,k,l,m], Shih-Chi Su[e,m,*]

[a] Institute of Medicine, Chung Shan Medical University, Taichung 402, Taiwan
[b] Department of Medical Research, Chung Shan Medical University Hospital, Taichung 402, Taiwan
[c] Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 300, Taiwan
[d] Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsin-Chu 300, Taiwan
[e] Whole-Genome Research Core Laboratory of Human Diseases, Chang Gung Memorial Hospital, Keelung 204, Taiwan
[f] Department of Otorhinolaryngology-Head and Neck Surgery, Changhua Christian Hospital, Changhua 500, Taiwan
[g] Department of Internal Medicine, Division of Endocrinology and Metabolism, Chung Shan Medical University Hospital, Taichung 402, Taiwan
[h] School of Medicine, Chung Shan Medical University, Taichung 402, Taiwan
[i] Department of Otolaryngology, Chung Shan Medical University Hospital, Taichung 402, Taiwan
[j] Biotools, Co., Ltd, New Taipei City 221, Taiwan
[k] School of Medicine, College of Medicine, Chang Gung University 333, Taoyuan, Taiwan
[l] Chang Gung Immunology Consortium, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan
[m] Department of Dermatology, Drug Hypersensitivity Clinical and Research Center, Chang Gung Memorial Hospital, Linkou, 333, Taiwan

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ABSTRACT

Objectives: Both genetic and environmental factors are conceivably required to assess the prognosis of oral squamous cell carcinoma (OSCC), yet little is known regarding the relationship between oral microbiome and the mutational spectrum of OSCC.

Materials and methods: Here, we used 16S rRNA amplicon sequencing to study the composition of oral microorganisms in OSCC patients, whose cancer mutational profiles were previously defined by whole-exome sequencing, to evaluate the relationship between oral microbiome and the mutational changes in OSCC.

Results: Analyzing the contributions of the five mutational signatures extracted from the primary tumors revealed three groups of OSCC (mutational signature cluster, MSC1-3) that were significantly associated with demographic and clinical features. Taxonomic analysis of the predominant phyla in salivary samples showed variation in the relative abundance of *Firmicutes* and *Bacteroidetes* in the three MSC groups. In addition, significant differences in bacterial species richness (alpha diversity) and slight sample-to-sample dissimilarities in bacterial community structures (beta diversity) were noted among different MSC groups. Further, predicting the functional capabilities of microbial communities by reconstruction of unobserved states showed that many pathways related to cell motility were differentially enriched among the three MSC groups.

Conclusion: Collectively, these results indicate a potential association of oral microbiome with the mutational changes in OSCC.

Introduction

Oral cancer is a prevalent malignancy globally, with a huge majority (~90%) of cases being oral squamous cell carcinoma (OSCC) [1]. In spite of advances in etiological studies and therapeutic options, the mortality of OSCC has not improved substantially over the past decades [2]. It is known that OSCC is a multifactorial neoplasm in which genetic variants interact with environmental triggers in the predisposition to this disease [3]. Various genetic alterations that influence cell cycle, apoptosis, and DNA repair [4] alone or in combination with external risks, including human papillomavirus (HPV) infection and habitual exposure of carcinogens, such as tobacco and alcohol use and betel nut chewing [5] have been demonstrated to contribute to the etiology and pathogenesis of oral cancer. Moreover, other possible risks of oral

☆ Association of oral microbiota with OSCC genomics.

* Corresponding author at: Whole-Genome Research Core Laboratory of Human Diseases, Chang Gung Memorial Hospital, No. 200, Ln 208, Jijin 1stRd, Keelung 204, Taiwan.
E-mail address: ssu1@cgmh.org.tw (S.-C. Su).

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tumorigenesis comprise but not limited to poor oral hygiene, periodontitis [6], and chronic microbial infections and inflammation [7].

Alterations in oral microbiome may disturb the relationship between microorganisms and humans, potentially leading to disease. Increasing evidence has indicated a role for oral microbiota in OSCC through direct metabolism of carcinogens and inflammatory effects [8]. Earlier studies of oral microbiome using culture-dependent methods examined only small numbers of species and thus provided no consensus regarding cancer-associated changes in species abundance [9,10]. Strikingly, the wide spectrum of oral microbiota is consisting of more than 600 bacterial species, the majority of which, however, are uncultivated [11]. The advent of high-throughput sequencing technology has enabled the use of recently developed, culture-independent strategies for exploring the composition of microbial ecosystems in human health [12]. The culture-independent creature of this approach does not rule out species that are difficult to grow or currently uncultivated, and its open-ended and highly sensitive nature potentiates the discovery of new taxa of low abundance that are likely associated with oral cancer.

Recent studies using the small subunit ribosomal RNA (16S rRNA) gene sequence in culture-free settings have compared the microbial communities present in oral cancer versus anatomically matched normal tissues, OSCC biopsies versus deep-epithelium swabs from matched control subjects, and saliva samples from OSCC patients with different statuses of HPV positivity and treatment [13–15]. Although mostly inconsistent, a significant reduction in the abundance of Firmicutes (particularly *Streptococcus* and *Haemophillus*) was observed in oral cancer as compared to normal samples. It is conceivable that genetic changes and environmental factors are interrelated and required to assess the prognosis of OSCC. However, at present, little is known regarding the relationship between oral microbiome and the mutational changes in OSCC. Using whole-exome sequencing, we have previously characterized the mutational catalog of oral cancer and provided clues for a molecular-based classification of OSCC patients with prognostic and therapeutic implications [16]. In this study, we analyzed the composition of oral microorganisms in OSCC patients with different mutational profiles to evaluate the potential association between the microbial flora of oral cavity and genomic alterations of oral cancer.

Materials and Methods

Subject enrollment and sample collection

The subjects were recruited from 2014 to 2015, with the approval by the institutional review board of Chung Shan Medical University Hospital, Taichung, Taiwan. Patients with any history of diabetes mellitus or immune-related diseases were excluded. All participants were free of antibiotics therapy within three months and provided informed written consent at enrollment. Of 123 eligible cases, 103 participated in the study. Salivary samples of 39 male patients, whose exome had been previously sequenced [16] and who had been neither previously treated nor proven metastatic disease at the time of diagnosis, were collected within 1–3 weeks after disease diagnosis in this study. TNM staging, mutational profiling, and data on age, gender, alcohol drinking, betel quid chewing, and cigarette smoking were assessed and reported previously [16]. Non-stimulated saliva was obtained in the morning between 8 and 10 a.m. from each patient using a prior protocol [17]. In brief, OSCC patients were asked to refrain from eating, drinking, smoking, or oral hygiene procedures for at least one hour before sample collection. Saliva was allowed to accumulate in the floor of the mouth and the subject spat it out into the preweighed test tube every 60 s for a period of 10 min. Salivary samples in sterile Falcon tubes were vortexed, then aliquoted into sterile cryogenic tubes, and stored at −80 °C until analysis.

16S rRNA gene amplification and sequencing

Before DNA extraction, salivary samples were centrifuged at 14,000 rpm for 2 min to pellet the bacterial communities [18]. The pellet was re-suspended and incubated with proteinase K (Sigma-Aldrich) at 55 °C overnight. Bacterial genomic DNA was then isolated with a Qiamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions and quantified by a NanoPhotometer N50 (Implen). The variable region 4 (V4) of small subunit rRNA (16S rRNA) gene was PCR-amplified using the primer set described previously [19]. Gel electrophoresis of PCR products on 2% agarose gels was performed for quality control. Amplicons were purified using the AMPure XP PCR Purification Kit (Agencourt) and then quantified using a Qubit dsDNA HS Assay Kit (Qubit) on a Qubit 2.0 Fluorometer (Qubit). To construct a library, the Illumina sequencing adapters were ligated to the purified amplicons by a second-stage PCR using the TruSeq DNA LT Sample Preparation Kit (Illumina). Purified libraries were quantified, normalized, pooled, and applied for cluster generation and sequencing on a MiSeq instrument (Illumina).

Processing and analysis of sequence data

Paired-end reads were merged using FLASH v1.2.7 [20]. Quality filtering of reads was assessed using the QIIME 1.7 pipeline [21] and chimeric sequences were removed by UCHIME [22]. The processed sequencing reads (effective tags) were clustered into operational taxonomic units (OTU) at 97% sequence identity using the UPARSE [23], and taxonomy classification was assigned according to the information retrieved from the Greengenes database [24]. Any sequence with one-time occurrence (singletons) or detected in only one sample was filtered out, and samples with less than 10^4 effective tags were excluded from further analyses. To evaluate the phylogenetic relationship of different OTUs, alignment of multiple sequences was conducted using the PyNAST software v.1.2 [25] against the core-set dataset of the Greengenes database, and a phylogenetic tree was generated with the FastTree [26].

Prior to subsequent analysis of alpha and beta diversities, data regarding OTU abundance were rarefied to the minimum sequence depth to normalize the variations in sequence depth across samples. For estimating alpha diversity, species richness was evaluated by the Chao1 and abundance-based coverage estimators (ACE) indices. A rarefaction curve was generated by a random selection of certain amount of sequencing data from each sample for representing the number of the observed species, and a species accumulation curve was plotted by the occurrence rate of new OTUs (species) under continuous sampling. For evaluating beta diversity, the weighted and unweighted UniFrac parameters [27] were calculated by using the QIIME pipeline. Principal coordinate analysis (PCoA) was conducted using the weighted correlation network analysis (WGCNA), stat, and ggplot2 packages in R software by transforming a distance matrix of weighted or unweighted UniFrac parameters among samples into a new set of orthogonal axes.

Functional composition of metagenomes was predicted from 16S rRNA data by the PICRUSt software [28], the pipeline of which is composed of two workflows, gene content prediction and metagenome prediction. A table of gene copy numbers for each gene family in each sequenced bacterial and archaeal genome based on the IMG database [29] and a phylogenetic tree from the Greengenes database [24] were precomputed for gene content prediction. Subsequently, metagenome prediction was performed through multiplying the vector of gene counts for each OTU by the abundance of that OTU in each sample, and summed across all OTUs.

Statistical analysis

The significance of association between the clusters and the clinical data was tested using Fisher's exact test. The relative abundance of the

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