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


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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...
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 nature 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 normal tissues, OSCC biopsies versus deep-epithelium swabs from OSCC patients with matched control subjects, and saliva samples from OSCC patients with normal tissues, OSCC biopsies versus deep-epithelium swabs from OSCC patients with normal tissues, OSCC biopsies versus deep-epithelium swabs from OSCC patients with normal tissues, OSCC biopsies versus deep-epithelium swabs from OSCC patients with normal tissues, OSCC biopsies versus deep-epithelium swabs from OSCC patients with normal tissues, OSCC biopsies versus deep-epithelium swabs from OSCC patients with normal tissues.

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 protease K (Sigma-Aldrich) at 55 °C overnight. Bacterial genomic DNA was then isolated with a Qiaprep 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 with 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 107 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.1.1.2 [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 sequence 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

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 included 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 included in the study.

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 preweighted 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.

The signiﬁcance of association between the clusters and the clinical data was tested using Fisher’s exact test. The relative abundance of the
taxa present in each cluster was compared at the phylum and genus levels using Welch’s t test. Continuous variables, such as chao1 and ACE index, were analyzed using the Kruskal-Wallis test. Data were analyzed by using the SAS statistical software (Version 9.1, 2005; SAS Institute Inc., Cary, NC). Statistically significant biomarkers were evaluated by the linear discriminant analysis (LDA) of effect size (LEfSe) analysis [30], which employed the non-parametric factorial Kruskal-Wallis test, Wilcoxon rank-sum test and LDA to identify differentially abundant taxa between two metadata classes. High LDA scores reflect significantly higher abundance of certain taxa. All reported p values were two-tailed, and a p value of < 0.05 was considered significant.

Results

Characterization of study participants

The study cohort was composed of 39 OSCC patients, all of whom were male. The average age at disease onset was 53 (Table S1). Various anatomical sites were represented, including buccal mucosa (46.2%), tongue (20.5%), lip (15.4%), gingiva (10.3%), and others (7.6%). Of the anatomical sites represented, including buccal mucosa (46.2%), tongue (20.5%), lip (15.4%), gingiva (10.3%), and others (7.6%). Of these, the tongue was the most common site, followed by the lip and gingiva. Additionally, lymph node metastases occurred in a total of 26.6% of cases. Of the patients, 46.2% were from the buccal mucosa, 20.5% from the tongue, 15.4% from the lip, and 10.3% from the gingiva. The remaining patients were from other anatomical sites (7.6%).

Bacterial profiling by 16S rRNA gene sequencing

A total of 19,01,329 sequencing reads, ranging from 6781 to 1,77,645 per sample, was generated from the V4 hypervariable region of 16S rRNA gene. After strict quality and size filtering, eight samples were excluded from further analyses due to low read numbers, such as a total of 1050 OTUs was assigned (per sample range 283–583) (Table S2). Rarefaction curves show that a plateau of species richness (up to 400 OTUs) was achieved in approximately 16,000 reads per sample (Fig. S1). To control the sample heterogeneity, roughly 16,000 reads were used hereafter as the minimum sampling depth to evaluate the diversity. In addition, the shape of the species accumulation curve generated from our dataset reveals that the community was well sampled as the specimens we collected contained considerable information about total species richness (Fig. S2). Taxonomic analyses identified a predominance of phylum Firmicutes (37.7%); that is, 37.7% of the total number of sequences obtained, Bacteroidetes (20.3%), Proteobacteria (16.5%), and Actinobacteria (10.4%), with less frequent presence of Fusobacteria (6.8%) in the saliva of OSCC patients (Fig. S3). At the lower taxonomic level, the most abundant genera were Streptococcus (16.2%), Prevotella (13.4%), Neisseria (9.1%), and Veillonella (8.9%), with lower numbers of Rothia (5.4%) and Porphyromonas (4.2%).

Microbial composition and abundance categorized by the distributions of mutational signatures within OSCC

To explore the potential relationship between bacterial flora of oral cavity and mutational changes in oral cancer, we categorized OSCC patients based on the distributions of five mutational signatures extracted from the dataset of somatic variations in the primary tumors of our cohort [16]. 31 OSCC tumors were classified into three groups (mutational signature cluster, MSC1-3) and a singleton based on the intensity of the mutational signatures operative in each tumor using an unsupervised hierarchical clustering method (Fig. 1A). Tumors in the MSC3 group (n = 11) predominantly presented at the late stage (p = .006) and with a greater tumor size (p = .021) (Fig. 1B). The proportion of patients with a history of smoking in MSC3 was lower than that for the other groups (p = .021). The MSC1 group (n = 9) was characterized by frequent TP53 mutations (p = .021). In addition, the MSC2 group (n = 10) exhibited a late histological stage (p = .017) and was associated with recurrent mutations in key driver genes (FAT1, EZR1, and AXIN1) related to the Wnt pathway (p = .032). These findings suggest that molecular classification of OSCC according to the intensity of mutational patterns in oral cancer reflects clinicopathological parameters and etiological backgrounds.

Further analysis of bacterial composition and abundance categorized by the distributions of mutational signatures in OSCC revealed that the relative abundance of many taxa at the phylum and genus levels differed among the three MSC groups (Fig. 1C, Fig. 1D, and Fig. S4). Specifically, for phyla with a relative abundance higher than 1%, patients in the MSC2 group harbored higher levels of Firmicutes than did those in the other groups (Fig. 1C and Fig. S5). Significant differences in relative abundance of phylum Bacteroidetes and Synergistetes were observed between the MSC2 and MSC3 groups. At the lower taxonomic level, comparisons of dominant genera (> 1%) showed that Selenomonas and Rothia distinguish MSC2 from MSC1 and MSC3, respectively (Fig. 1D and Fig. S6). In addition, a significantly higher prevalence of genus Capnocytophaga was found in MSC3 than that in the other groups.

We also sought to predict the biomarker for each category by taking statistical significance and biological consistency into consideration using linear discriminant analysis (LDA) of effect size (LEfSe). Accordantly, we observed a profusion of Firmicutes in the saliva of the MSC2 over the MSC1 group (Fig. 2). On the contrary, samples in the MSC1 group had significant enrichment for phylum Actinobacteria, class Actinobacteria, and order Actinomycetales as compared with those in the MSC2 group.

Diversity of microbiota associated with molecular OSCC subgroups

In addition to changes in relative abundance of different taxa, we also evaluated alterations in microbial diversity among three molecular OSCC subgroups. Significant differences in bacterial species richness and evenness (alpha diversity) were detected between the MSC2 and MSC3 groups but not between subgroups assigned with regard to the mutation rate or the status of TP53 alterations (Fig. 3). Although not statistically significant, a marginal decrease in alpha diversity in the MSC1 group compared to the MSC2 was also observed. Moreover, analyses of sample-to-sample dissimilarities in bacterial community structures (beta diversity) demonstrated a slight discrimination among three MSC groups, especially between MSC2 and MSC3, but not between tumors positive and negative for TP53 mutations (Fig. 4).

Functional prediction of bacterial communities related to mutational changes in OSCC

To gain an insight into the functionality of oral microbiota in OSCC biology, we inferred the functional profile of microbial communities by phylogenetic reconstruction of unobserved states (PICRUSt) [28]. We found that many pathways relevant to cell motility were differentially enriched among the three MSC subgroups (Fig. 5A). Notably, as for MSC2 and MSC3, between which bacterial composition and diversity differed profoundly, the genetic markers with significant discriminative power in many pathways assigned to environmental information processing, including membrane transport (transporters, phosphotransferase system, and ABC transporters), signal transduction (two-component system), and signaling molecules and interaction (cellular antigens) were detected (Fig. 5B). Collectively, data shown in the present study indicate a potential association of oral microbiome with the mutational changes in OSCC.

Discussion

Disturbance in the relationship between microbiota and humans is known to be central to various physiological and pathological conditions. Recent studies have demonstrated associations of host genetics with the intestinal microbiome, revealing another level of host-microbe
In the present study, we showed changes in abundance and diversity of oral microorganisms among different OSCC subgroups clustered by the distribution of mutational patterns. We also unraveled that microbial gene pathways were differentially enriched in salivary samples of OSCC patients with distinct mutational profiles.

All cancers carry somatic mutations that were accumulated through multiple mutational processes. Different mutational processes give rise to unique combinations of mutation types, termed mutational signatures [33]. Our previous large-scale analysis of OSCC genomes detected five mutational signatures operative in our cohort [16]. Here, we showed that changes in composition and abundance of oral microbiota were associated with OSCC subgroups categorized by the distributions of mutational signatures. It is known that microorganisms and their products activate fibroblasts and immune cells to generate reactive oxygen species that elicit DNA damage in epithelial cells [34]. In addition, microbial genotoxins (cytolethal distending toxin, typhoid toxin, and colibactin) or metabolites (hydrogen sulfide, ammonia and fatty acids) may directly target to DNA and induce mutations. These suggest that oral microbiota, as an exogenous trigger, may be involved in certain mutational processes in oral tumorigenesis, ultimately contributing to differential intensities of mutational patterns in oral cancer. Notably, such host-microbe relationship we observed among different mutational signature clusters was not seen while tumors were classified with regard to the mutation rate and the status of TP53 alterations (Fig. 3 and 4), implying that the genetic alterations mediated by oral microbiota may be induced at a relatively even rate across samples and dispersed in various tumor suppressor genes and proto-oncogenes.

Consistent with other reports [13,14], the most predominant phyla of oral microbiota detected in the present study were Firmicutes (37.7%), Bacteroidetes (20.3%), Proteobacteria (16.5%), and Actinobacteria (10.4%). This is not in concordance with the finding from a recent study [15] where Fusobacteria was shown to be the most abundant taxa at the phylum level in OSCC biopsies. In addition to a potential regional impact derived from OSCC etiology, such difference in bacterial associations with oral cancer may be partly accounted for by the types of samples analyzed. Moreover, the relative abundance of many taxa was found to vary within the three OSCC subgroups (Fig. 2 and Fig. S4–6). Among these, Actinobacteria was identified as a biomarker for the MSC1 group by LEfSe in our dataset (Fig. 2). In a recent investigation on the oral mucosal and salivary microbial communities, members of Actinobacteria, especially the Rothia spp., have been found to be associated with Belchet’s syndrome, a multisystemic immune-related condition characterized by recurrent oral ulceration [35]. Additionally, significant differences in relative levels of phylum

Fig. 1. Taxonomic profiles, at the phyla and genus levels, of salivary samples from OSCC patients classified by the contributions of mutational signatures. (A) Five mutational signatures were previously deciphered in the OSCC cohort [16] and corresponding to the updated consensus signatures (#1, 5, 6, 7, and 13) [33]. Tumor classification (mutational signature cluster, MSC) based on the contributions of mutational signatures. The fraction and number of mutations attributed to each signature in each tumor, together with location of tumors, are represented by the colored bars below the dendrogram. (B) Clinicopathological and etiological characteristics associated with each MSC group. (C-D) The distribution of top 10 phyla (C) and top 10 genera (D) detected in the OSCC subgroups.
Fig. 2. Determination of bacterial biomarkers for each molecular subgroup. Taxa that best characterize each OSCC subgroups were identified by using linear discriminant analysis of effect size (LEfSe) on OTU tables (Upper panel). The relative abundance of each taxon across all samples is shown in the lower panel where the straight and dotted lines plot the means and medians of the relative abundance, respectively, in each subgroup.

Fig. 3. Comparisons of bacterial species richness in saliva of OSCC patients categorized by the distribution of mutational signatures (MSC), mutation rate, and status of TP53 alterations. Bacterial species richness was estimated by the Chao1 and ACE indexes. The box-plot shows the median, the 25th, and the 75th percentile in each molecular classification. The boundary between the high and low mutation rate group is the average of somatic mutations in our cohort (mean = 154), \( p < .05 \), Kruskal-Wallis test.
Synergistetes were observed between the MSC2 and MSC3 groups (Fig. S5). Association of salivary Synergistetes with periodontitis has been documented [36], as periodontitis is considered a risk factor for oral cancer [6]. Of note, Capnocytophaga species, whose levels in the MSC3 group were higher than that in the other groups (Fig. S6), were known to potentially cause bloodstream infections in patients with severe oropharyngeal mucositis or periodontal disease [37]. Intriguingly, although less abundant, salivary samples of the MSC3 group were more highly colonized with Bifidobacterium spp. compared to the MSC1 group (MSC3, 0.116%; MSC1, 0.027%; \( p = .029 \)), while oral administration of Bifidobacterium has been shown to promote antitumor immunity and facilitate the efficacy of immunotherapy with check-point inhibitors by altering the composition of intestinal microorganisms [38]. Our results together with findings from others implicate a usefulness of oral microbiota in not only characterization of OSCC etiology but also stratification of OSCC patients with differential therapeutic efficacy.

Previous investigations showed changes in alpha and beta diversity of bacterial communities between salivary specimens of OSCC patients and that from control subjects [13,15,39], whereas the bacterial diversity of tissue biopsy or swab samples between OSCC and controls remained similar [14,40]. Here, we observed alterations in bacterial diversity in saliva obtained from OSCC patients with distinct mutational profiles (Fig. 3 and 4), suggesting a better discriminative potential for saliva in analyzing OSCC-associated oral microbiota in comparison with other sample types. Nevertheless, difference in microbial diversity was not seen in saliva from OSCC patients categorized by mutation rate, the status of TP53 alterations, or the location of oral cancer (Fig. S7). In addition, several functional pathways related to cell motility were predicted to be differentially enriched among the three MSC subgroups (Fig. 5A). Bacterial flagella were recognized as key inflammatory structures [41], and defects in bacterial chemotaxis were shown to dampen the inflammation [42]. These restate a role of oral microbiota in regulating cancer-related inflammation and connect genetic variations of OSCC to oral flora-mediated immunity.

To explore the relationship between oral microorganisms and the genomic landscapes of OSCC, additional work is needed to address
several limitations of the present study. One issue is the limited sample size, which likely underestimated the potential associations of salivary microbiome with the alteration status of a given driver gene or pathway in oral cancer. In addition, hyposalivation often occurs in patients with oral diseases [43] and could shift the quality of saliva towards a more acidogenic microflora [44]. The association of changes in the oral microbiota with hyposalivation was shown to be related to the reason for the hyposalivation rather than to the magnitude of reduced salivary flow rate [45]. A relation between salivary flow and oral symptoms in patients with advanced terminal cancer has been observed [46]. Yet, salivary flow rate was not taken in consideration in the present study and its variations could be a potential caveat of the current study design. Also unavailable was the status of oral hygiene and periodontitis. Mounting evidence has indicated the potential association of OSCC risk with oral health parameters, such as tooth loss, cavity, gum bleeding, tartar deposits, and periodontitis [47–49]. These OSCC-associated oral conditions collectively perturb the finely-tuned equilibrium of the oral ecosystem and may be the additional confounding factors in our analysis. Another drawback is that the functional capacities of bacterial communities were inferred based on 16S rRNA gene sequencing. Further defining the network of diverse pathways employed in OSCC-associated oral microbiota will require a comprehensive analysis of whole metagenome in future investigations. Moreover, our data failed to define whether oral microbiome has altered during the development of OSCC or after the formation of tumors.

Overall, we showed that oral microbiota is compositionally and functionally associated with the mutational changes in oral cancer. Our study provides potential avenues for the understanding of oral tumorigenesis and the identification of OSCC patients who could likely benefit from individual treatment regimens through the integration of cancer genomics and metagenomics.

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The research sponsors were not involved in study design, the collection, analysis and interpretation of data, the writing of the report, and the decision to submit the article for publication.

Conflict of interest

None declared.
Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/joraloncology.2017.12.005.

Reference